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Elora FOURNIER

**Fate of microplastics in human digestive *in vitro* environment
and study of the dialogue between
epithelium, microbiota and mucus**

Defended on October 13th, 2022

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FATE OF MICROPLASTICS IN HUMAN DIGESTIVE *IN VITRO*
ENVIRONMENT AND STUDY OF THE DIALOGUE BETWEEN
EPITHELIUM, MICROBIOTA AND MUCUS



Cover illustration

Rudy Duca & Elora Fournier

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PhD thesis, Université Clermont-Auvergne

Abstract

Over the last half-century, plastic production has exploded resulting in environmental pollution. Accumulated plastics can be degraded into smaller pieces known as microplastics (MPs) found everywhere, and especially in the food chain. MPs have been detected in human feces and colon biopsies attesting of their transit through the gastro-intestinal tract (GIT). However, to date, little is known about the fate and potential effects of MPs in the human digestive sphere, particularly in the infant at-risk population. During their GIT travel, MPs can interact with physico-chemical digestive parameters, as well as gut microbiota, intestinal epithelium and its covering mucus layer. This joint PhD between MEDIS (Clermont-Ferrand) and Toxalim (Toulouse) aimed to investigate the fate of virgin MPs of polyethylene (PE), the most manufactured plastic polymer worldwide, in the adult and infant gut. This was studied using an original *in vitro* approach combining a human colon model and intestinal cells in culture.

This doctoral research started with evaluating the impact of various stool conservative methods (48-h freezing -80°C, 48-h freezing -80°C with glycerol or lyophilization with maltodextrin/trehalose) on gut microbiota composition and activity in the Mucosal Artificial Colon (M-ARCOL), set-up under adult conditions. We showed that inoculating with raw frozen stool (48-h at -80°C) was the best option among those tested compared to fresh stools. Next, the M-ARCOL model was adapted to the specific toddler conditions in a new configuration termed Tm-ARCOL. This adaptation allowed to reproduce *in vitro* the main particularities associated to infant microbiota, such as lower bacterial diversity and higher abundance of *Bifidobacteriaceae*, together with higher concentrations of acetate and lower amounts of propionate and butyrate, as compared to adults. Then, we assessed the effects of a chronic ingestion of PE MPs on adult and infant microbiota using M-ARCOL and Tm-ARCOL, respectively, as well as the indirect impact of gut microbe metabolites after PE MP exposure on the intestinal barrier simulated by a co-culture of Caco-2 and mucus-secreting HT29-MTX cells. Interestingly, the effect of PE MP exposure on the gut microbiota was donor-dependent and resulted in an increase in potential pathobionts, like *Enterobacteriaceae*, regardless of age conditions. Regarding the activity of the gut microbiota, we showed for the first time that PE MP exposure was associated with a significant decrease in butyrate production in infant conditions, while skatole production increased significantly in adults. In contrast, no significant impact of PE MPs on the intestinal barrier, as mediated by changes in gut microbial metabolites, was evidenced.

This PhD work provided pioneering and significant insights into the interactions of PE MPs with the adult and infant human gut microbiota and the intestinal barrier, filling some of the gaps in knowledge on MP behavior in the human GIT. This work contributes to a better understanding of MP health risk assessment for public policies. In the near future, our *in vitro* approach could be used for further investigations on more representative MP types (forms, sizes, aged and/or contaminated particles), in healthy but also disturbed situations including an altered gut barrier and dysbiotic microbiota (e.g. obesity, inflammatory bowel syndrome or inflammatory bowel diseases).

Résumé

Au cours des 50 dernières années, la production plastique a fortement augmenté, conduisant à une pollution environnementale massive. Les plastiques accumulés peuvent être dégradés en particules de plus petites tailles appelées microplastiques (MPs) et retrouvées tout au long de la chaîne alimentaire. Chez l'Homme, les MPs ont été détectés dans les selles et le côlon, attestant de leur transit le long du tractus gastro-intestinal (TGI). Cependant, à ce jour, peu de travaux ont évalué le devenir et les effets potentiels des MPs dans la sphère digestive humaine, en particulier chez les enfants. Au cours de leur transit gastro-intestinal, les MPs peuvent interagir avec les processus physico-chimiques liés à la digestion, le microbiote intestinal, l'épithélium intestinal, ou encore le mucus. Cette thèse, menée conjointement entre MEDIS (Clermont-Ferrand) et Toxalim (Toulouse), visait à étudier le devenir de MPs vierges de polyéthylène (PE), polymère plastique le plus fabriqué au monde, dans l'environnement digestif de l'adulte et de l'enfant. Cette étude a été réalisée grâce à une approche *in vitro* originale, combinant un modèle de côlon humain et des cellules intestinales en culture.

Ces travaux de thèse ont tout d'abord évalué l'impact de différentes méthodes de conservation des selles (congélation à $-80^{\circ}\text{C}/48\text{h}$, congélation à $-80^{\circ}\text{C}/48\text{h}$ avec du glycérol ou lyophilisation maltodextrine/tréhalose) sur la composition et l'activité du microbiote intestinal dans le modèle Mucosal Artificial Colon (M-ARCOL), en conditions adultes. Nous avons montré que l'inoculation du bioréacteur avec des selles congelées brutes ($-80^{\circ}\text{C}/48\text{h}$) était la meilleure option parmi celles testées comparativement aux selles fraîches. Ensuite, le dispositif M-ARCOL a été adapté aux conditions spécifiques du côlon chez l'enfant dans une nouvelle configuration appelée Tm-ARCOL. Cette adaptation a permis de reproduire *in vitro* les principales particularités associées au microbiote des enfants, comme une diversité bactérienne plus faible et une abondance plus importante des *Bifidobacteriaceae*, ainsi que des concentrations plus élevées en acétate et plus faibles en propionate et butyrate, comparativement aux adultes. Nous avons ensuite évalué les effets d'une ingestion chronique de MPs de PE sur le microbiote de l'adulte et de l'enfant en utilisant respectivement les modèles M-ARCOL et Tm-ARCOL, ainsi que l'effet indirect des surnageants de fermentation (contenant les métabolites issus des microbiotes intestinaux) sur la barrière intestinale mimée par la mise en œuvre d'une co-culture de cellules Caco-2 et HT29-MTX productrices de mucus. Les effets de l'exposition aux MPs de PE sur le microbiote intestinal se sont révélés donneur-dépendants. Les MPs induisent une augmentation de souches potentiellement pathogènes, comme les *Enterobacteriaceae*, et ce indépendamment des conditions d'âge. Concernant l'activité du microbiote intestinal, nous avons montré pour la première fois que l'exposition aux MPs de PE était associée à une diminution significative de la production de butyrate chez les enfants, tandis que la production de scatole augmentait chez les adultes. En revanche, aucun impact significatif des MPs de PE sur la barrière intestinale, régi par les modifications des métabolites microbiens, n'a été mis en évidence.

Ce travail de thèse a fourni des informations pionnières et importantes sur les interactions des MPs de PE avec le microbiote intestinal humain adulte et enfant, mais aussi avec la barrière intestinale, permettant de mieux appréhender le comportement des MPs de PE dans le TGI humain. Ce travail s'inscrit dans une meilleure compréhension de l'évaluation du risque sanitaire des MPs en appui aux politiques publiques. Dans un futur proche, notre approche *in vitro* pourrait être mise à profit pour des études plus fines sur des MPs plus représentatifs de l'exposition environnementale (formes, tailles, particules vieilles et/ou contaminées) dans des situations saines ou physiopathologiques, notamment en cas de barrière intestinale altérée et de microbiote dysbiotique (par exemple, obésité, syndrome de l'intestin irritable ou maladies inflammatoires chroniques de l'intestin).

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Tous les hommes pensent que le bonheur se trouve au sommet de la montagne alors qu'il réside dans la façon de la gravir. (Confucius)

Résumé de la thèse

Du fait de ses nombreuses propriétés, le plastique a vu sa production augmenter de manière considérable depuis le début des années 1950 atteignant 367 millions en 2020. Le plastique est un matériau très résistant, ce qui a conduit à son accumulation dans l'environnement. Les déchets plastiques subissent alors une lente fragmentation libérant des particules plus petites pouvant être classées selon leur taille. Les microplastiques (MP) ont été définis comme des particules synthétiques solides ou matrices polymériques mesurant entre 1 μm et 5 mm avec une forme régulière ou irrégulière, insolubles dans l'eau et provenant d'une source primaire (*i.e.* fabriquées directement par les industriels pour des usages variés) ou secondaire (*i.e.* issues du vieillissement et de la fragmentation des déchets plastiques). Ces microparticules sont retrouvées dans les environnements marins, terrestres ou atmosphériques mais également dans la chaîne alimentaire. Même si leur ingestion par l'Homme est désormais avérée, leurs effets potentiels sur la santé humaine restent à ce jour largement méconnus. A peine mentionné il y a cinq ans, cet enjeu de santé publique est aujourd'hui un sujet très étudié par la communauté scientifique à l'échelle internationale. Des travaux récents ont également montré que les enfants, une population à risque, semblent davantage exposés aux MP que les adultes. Dans l'environnement digestif humain, les MP peuvent interagir lors de la digestion, outre les paramètres physico-chimiques, avec différents acteurs, et plus particulièrement le microbiote intestinal, le mucus et l'épithélium. Alors que de nombreuses études sont menées chez le rongeur, les connaissances basées sur des modèles *in vitro* reproduisant l'environnement digestif humain restent parcellaires. De plus, aucune étude n'a été menée chez l'enfant dont le tractus digestif et le microbiote intestinal sont immatures.

Dans ce contexte, ce travail de thèse a eu pour objectif d'explorer les conséquences d'une exposition orale chronique aux MP sur la sphère digestive et la barrière intestinale de l'adulte et de l'enfant (6 mois – 3 ans) en se focalisant sur le triptyque épithélium/mucus/microbiote. Nous avons choisi de travailler avec le polyéthylène (PE), l'un des polymères plastiques les plus produits au monde. L'étude a été menée dans le cadre d'un partenariat entre l'Unité Mixte de Recherche Microbiologie, Environnement Digestif et Santé (UMR 454 MEDIS, Université Clermont Auvergne, INRAE, Clermont-Ferrand, France) et TOXALIM, centre de recherche INRAE en toxicologie alimentaire (UMR 1331, Université de Toulouse, ENVT, INP-Purpan, UPS, Toulouse, France). L'unité MEDIS a pour objectif de mieux comprendre le rôle du microbiote intestinal humain et animal dans la santé et les maladies, tandis que l'unité

TOXALIM, *via* l'équipe Neuro-Gastroentérologie et Nutrition, s'intéresse à l'étude de la fonction barrière de l'intestin et sa modulation par les facteurs environnementaux. Le laboratoire MEDIS possède une expertise de plus de 25 ans dans l'utilisation de modèles *in vitro* simulant le tractus digestif humain et le laboratoire TOXALIM dans l'utilisation de modèles cellulaires intestinaux et de modèles *in vivo* intégrés. Les travaux de recherche conduits au cours de cette thèse ont été financés par une bourse du Ministère français de l'Enseignement Supérieur et de la Recherche attribuée à l'UCA, par un financement collaboratif INRAE (projet tri-départements INRAE/PLASTOX 2018-2020) et par l'Agence Nationale de la Recherche française (ANR-19-MRS2-0011 projet HuPlastiX).

Les objectifs de la thèse ont été les suivants :

- (i) Evaluer les effets de différentes méthodes de conservation des selles humaines sur la composition et l'activité du microbiote intestinal dans un modèle *in vitro* colique de l'adulte, M-ARCOL, afin de sélectionner la méthode la plus appropriée pour nos expérimentations ultérieures avec les MP de PE.
- (ii) Développer un modèle de l'environnement colique de l'enfant âgé de 6 mois à 3 ans (Tm-ARCOL) en adaptant le modèle M-ARCOL existant aux conditions digestives spécifiques de l'enfant (pH, temps de transit, alimentation).
- (iii) Evaluer les effets d'une exposition chronique aux MP de PE sur la structure et l'activité du microbiote intestinal humain à la fois de l'adulte et de l'enfant en utilisant les modèles M-ARCOL et Tm-ARCOL, respectivement.
- (iv) Evaluer les effets indirects, *via* les métabolites produits par le microbiote intestinal, d'une exposition aux MP de PE sur l'intégrité de la barrière épithéliale intestinale, la synthèse de mucines et l'inflammation intestinale. Pour aborder ces aspects, une co-culture de cellules épithéliales intestinales humaines, Caco-2 et HT29-MTX (cellules sécrétrices de mucus), a été exposée aux surnageants de fermentation issus des modèles M-ARCOL et Tm-ARCOL, avant et après exposition aux MP de PE.

Le manuscrit de thèse comprend tout d'abord une revue de la littérature (section I) organisée en trois grandes parties. La partie 1 est dédiée à la présentation de la physiologie digestive humaine de l'adulte et de l'enfant ainsi que des principaux paramètres digestifs abiotiques et microbiens. La partie 2 présente les systèmes de digestion artificielle simulant le tractus digestif de l'adulte et de l'enfant, disponibles à l'heure actuelle. Enfin, la partie 3

présente les connaissances relatives à la contamination des aliments et des boissons par les MP et donc les sources d'exposition chez l'Homme. Cette partie résume également les connaissances actuelles du devenir et des effets toxiques des MP sous leur forme vierge ou en présence de contaminants (chimiques ou microbiens) chez les mammifères. Un focus particulier est apporté sur les approches *in vitro* et l'utilisation de modèles de digestion artificielle. Dans une seconde section, les résultats expérimentaux sont décrits. Dans le chapitre 1, les effets de trois méthodes de conservation des selles humaines sur la structure et la fonction du microbiote intestinal dans le modèle M-ARCOL sont présentés. Ce travail a débuté au cours de mon stage de Master 2 à l'UMR MEDIS et a été poursuivi au cours de mon doctorat menant à la publication d'un article dans le journal *Applied Microbiology and Biotechnology*. Le chapitre 2 présente les résultats obtenus sur les effets d'une exposition chronique orale aux MP de PE sur le microbiote intestinal humain de l'adulte. Ces résultats ont été publiés dans le journal *Journal of Hazardous Materials*. Le chapitre 3 introduit le développement et la validation du modèle *in vitro* colique de l'enfant, appelé Tm-ARCOL dont le travail a fait l'objet d'une publication dans le journal *Applied Microbiology and Biotechnology*. Enfin le chapitre 4 rapporte les résultats, publiés dans le journal *Journal of Hazardous Materials*, obtenus sur l'impact d'une exposition chronique aux MP de PE sur cette population à risque que sont les enfants. Les chapitres 2 et 4 présentent également les résultats des expériences menées sur l'épithélium et le mucus *via* l'utilisation de co-cultures de cellules intestinales humaines, exposées aux surnageants de fermentation des modèles M-ARCOL et Tm-ARCOL. La troisième et dernière section est consacrée à une discussion générale et aux perspectives de ces travaux de thèse.

Résultats principaux

Les principaux résultats expérimentaux et les méthodes employées sont présentés selon quatre parties afin de répondre aux objectifs de la thèse mentionnés ci-dessus.

Chapitre I – Comparaison des méthodes de conservation des échantillons fécaux préservant le mieux la structure et l'activité du microbiote intestinal dans un modèle *in vitro* du côlon humain

Les modèles intestinaux *in vitro*, tels que le modèle M-ARCOL, constituent des alternatives aux essais *in vivo*, permettant des études mécanistiques visant à mieux comprendre le rôle du microbiote humain dans la santé et les maladies. L'utilisation de tels modèles implique de considérer des selles humaines. L'inoculation de modèles intestinaux *in vitro* avec des échantillons fécaux provenant de patients ou de donneurs sains vise à reproduire la complexité et la diversité de l'écosystème microbien rencontré dans le côlon humain, avec des différences évidentes dans la structure et l'activité microbiennes par rapport aux selles initiales en raison des transitions fèces-côlon et *in vivo/in vitro*. L'utilisation de selles fraîches n'étant pas toujours possible, la définition d'une méthode de conservation appropriée est cruciale pour éviter la perte de populations microbiennes clés tout en maintenant les activités de fermentation au sein du bioréacteur. Les méthodes les plus appropriées pour préserver les spécificités initiales du microbiote intestinal ont déjà été décrites. Cependant, les effets des méthodes de conservation sur la structure et l'activité microbiennes dans des modèles d'intestin *in vitro* ont été peu étudiés.

Notre étude visait donc à évaluer l'impact du stockage à court terme et de la cryoconservation sur la cinétique de colonisation du microbiote luminal ou mucosal dans le modèle M-ARCOL et ainsi de sélectionner l'option la plus appropriée pour nos expériences ultérieures sur l'impact de l'exposition orale aux MP. Les échantillons fécaux de deux volontaires sains ont été soumis à différents protocoles de conservation : congélation pendant 48 heures à -80°C (selles congelées brutes, RFS « raw frozen stool ») ou congélation pendant 48 heures à -80°C avec du glycérol comme agent de conservation (selles congelées, GFS « glycerol frozen stool »), ou lyophilisation avec une combinaison de maltodextrine/tréhalose (selles lyophilisées, LS « lyophilized stool ») par rapport aux échantillons fécaux frais (FS « fresh stool »). L'impact des méthodes de conservation a été évalué au travers de l'analyse de la composition du microbiote et de l'activité métabolique microbienne (production de gaz et d'acide gras à chaîne courte (AGCC)) tout au long du processus de fermentation après inoculation avec les selles RFS, GFS ou LS, comparativement aux selles fraîches à différents intervalles de temps.

Nos résultats ont montré que tous les traitements de conservation ont permis le maintien d'un microbiote complexe et fonctionnel pour les deux donneurs dans le modèle M-ARCOL.

De manière intéressante, les Archaea méthanogènes ne sont pas impactées par les traitements ni dans la phase luminale ni dans la phase mucosale. Néanmoins, l'application des méthodes de conservation semble limiter l'expansion de certaines populations dominantes normalement présentes dans la phase mucosale, laissant l'opportunité aux Protéobactéries (ex : *Enterobacteriaceae*) et aux Actinobactéries (principalement avec le protocole GFS) de coloniser cette niche écologique. Des différences dans le temps de stabilisation et/ou dans les niveaux finaux stabilisés pour chaque paramètre analysé par rapport au contrôle FS ont été observées, indiquant que la congélation des selles sans aucun conservateur (condition RFS) était la méthode la plus efficace, suivie de la lyophilisation puis de la congélation avec du glycérol. Cependant, d'autres paramètres, tels que les contraintes techniques, doivent être pris en compte pour déterminer la méthode la plus appropriée pour inoculer les bioréacteurs intestinaux *in vitro*.

Chapitre II - Microplastiques : que se passe-t-il dans le tractus digestif humain ? Première démonstration chez l'adulte à l'aide de modèles *in vitro* intestinaux

Comme introduit précédemment, la production de plastique a explosé depuis les années 1950 entraînant une pollution environnementale massive. Les plastiques accumulés peuvent être dégradés en petites particules comme les MP que l'on retrouve dans l'air, l'eau potable mais aussi dans la chaîne alimentaire. Les MP ont été détectés dans les selles humaines et des biopsies du côlon, attestant ainsi de leur transit dans l'intestin après une exposition orale. En outre, de précédentes études *in vivo* menées sur des modèles murins ont mis en évidence la modification de la composition du microbiote intestinal après une exposition aux MP. Alors que le tractus gastro-intestinal humain est l'une des premières portes d'entrée des MP, leur devenir et leurs effets potentiels dans la sphère digestive humaine sont, jusqu'à présent, largement méconnus. L'objectif de cette étude était donc d'étudier les interactions des MP de PE sous forme vierge avec le microbiote intestinal humain dans des conditions coliques représentatives de l'adulte et simulées par le modèle M-ARCOL. Ce modèle d'intestin *in vitro* a été inoculé avec des échantillons fécaux collectés auprès de quatre donneurs adultes sains différents et considérés à l'état frais, cette condition ayant été démontrée au chapitre I comme la méthode la plus optimale. L'impact d'une exposition quotidienne de deux semaines aux MP de PE (21 mg/jour) sur la composition et l'activité du microbiote intestinal humain a été étudié chez ces quatre volontaires

à la fois pour les compartiments luminaux et mucosaux. La composition du microbiote intestinal a été déterminée par des analyses de métabarcoding de l'ADNr 16S et les activités microbiennes ont été évaluées par l'analyse des gaz de fermentation, des AGCC, du volatolome et de la production de ligands AhR (Aryl hydrocarbon Receptor – récepteur d'aryl hydrocarbure). Enfin, les surnageants luminaux des fermentations dans le modèle M-ARCOL avant et après exposition aux MP de PE ont été mis en contact avec une co-culture de cellules épithéliales intestinales et de cellules à gobelet (goblet cell en anglais) sécrétrices de mucus (Caco-2 et HT29-MTX, respectivement) pour étudier l'impact indirect des MP *via* les métabolites microbiens sur la réponse inflammatoire, la synthèse de mucines et la fonction de barrière épithéliale.

Le microbiote fécal initial utilisé pour l'inoculation de M-ARCOL présentait de fortes variabilités interindividuelles, qui ont été maintenues tout au long de l'expérience, comme le montrent les profils des adultes 3 et 4. L'exposition aux MP de PE a été particulièrement marquée pour ces deux individus puisque des modifications nettes de la β -diversité ont été mises en évidence après traitement. Malgré cette variabilité interindividuelle, des effets spécifiques ont été associés au microbiote luminal et au microbiote mucosal. Alors qu'une augmentation des pathobiontes intestinaux *Dethiosulfovibrionaceae*, *Enterobacteriaceae*, et *Desulfovibrionaceae* a été observée après l'exposition aux MP de PE pour les microbiotes luminal et mucosal, une diminution des *Christensenellaceae* et *Akkermansiaceae*, deux populations bénéfiques pour la santé, a été constatée pour deux donneurs parmi les quatre dans le compartiment luminal. Il faut noter que certains de ces changements microbiens ont déjà été décrits dans le syndrome du côlon irritable et les maladies inflammatoires chroniques de l'intestin. De manière originale, notre étude est la première à examiner les effets de l'exposition aux MP de PE sur la production de gaz, de composés organiques volatiles (COV) et d'AGCC par le microbiote intestinal humain. Même si ces effets restent mineurs, les tendances observées, tout comme pour la composition du microbiote intestinal, semblent dépendre du donneur. Toutefois, l'exposition aux MP de PE a modifié de manière significative le profil de cinq COV avec, en particulier, une augmentation de la production d'indole, 3-méthyl (ou scatole). Il est intéressant de noter que la population de *Desulfovibrio* augmentée dans notre étude pourrait être impliquée dans la production de scatole et expliquer, au moins partiellement, la production accrue de ce dernier. Par ailleurs, les images de microscopie à balayage ont mis en évidence la présence de micro-organismes à la surface des MP de PE. Cependant, aucune modification majeure de la composition polymérique des particules n'a été mise en évidence par

spectroscopie Raman, après contact avec le microbiote intestinal, probablement en raison de la stabilité chimique de ce type de polymère. Enfin, aucun impact significatif des MP de PE, médié par les changements de métabolites microbiens, n'a été observé sur la barrière intestinale. Les conséquences de l'exposition aux MP sur les populations à risque comme les enfants (voir Chapitre IV) mais aussi les personnes âgées, ainsi que dans des conditions physiopathologiques, pourraient maintenant être évaluées.

Chapitre III - Un enfant n'est pas un adulte : développement d'un nouveau modèle *in vitro* du côlon du jeune enfant

Dans le Chapitre II, nous avons clairement démontré un effet, dépendant du donneur, de l'exposition orale aux MP de PE sur la composition et les fonctions du microbiote intestinal dans le modèle M-ARCOL simulant les conditions coliques de l'adulte. Cependant, le début de la vie est une période critique où l'écosystème et les fonctions de l'intestin sont en phase de maturation avec un impact potentiel sur la santé à l'âge adulte. Même si les données d'exposition aux MP chez les enfants sont largement méconnues, nous avons émis l'hypothèse d'un risque plus élevé pour la santé chez cette population à risque, en raison de la coexistence de nombreuses sources de contamination (cf chapitre suivant). Dans ce contexte, nous avons étudié l'impact d'une telle exposition sur le microbiote intestinal immature des enfants. Dans un premier temps, la mise en place d'un modèle colique *in vitro*, adapté à la physiologie digestive du jeune enfant et appelé Tm-ARCOL, était nécessaire. Pour cela, une analyse détaillée de la littérature a été réalisée afin d'adapter le modèle M-ARCOL avec des paramètres physico-chimiques (pH, temps de transit et composition des effluents iléaux) et microbiens spécifiques des enfants âgés de 6 mois à 3 ans.

Des échantillons fécaux provenant de cinq enfants en bas âge ($20 \pm 3,4$ mois) et de quatre adultes ($34 \pm 6,7$ ans) ont été utilisés pour inoculer les bioréacteurs et des fermentations continues ont été réalisées pendant 8 jours. La structure des microbiotes luminal et mucosal et les fonctions associées (gaz et AGCC) ont été déterminées. Des signatures microbiennes distinctes ont été obtenues entre les deux conditions *in vitro* (enfant vs adulte), avec des indices d' α -diversité plus faibles et des abondances plus élevées de populations microbiennes propres aux enfants (par exemple, *Bifidobacteriaceae* et *Enterobacteriaceae*) dans les conditions enfant par rapport aux conditions adulte. Conformément aux données *in vivo* de la littérature, le

méthane n'a été trouvé que dans les bioréacteurs en conditions adulte, tandis qu'un pourcentage plus élevé d'acétate mais des proportions plus faibles de propionate et de butyrate ont été mesurés chez les enfants par rapport aux adultes.

Ce nouveau modèle *in vitro* a ensuite été utilisé pour notre évaluation de l'impact des MP de PE sur le microbiote intestinal des enfants (voir Chapitre IV), mais il constituera également une plateforme performante pour les études mécanistiques du microbiome intestinal dans un contexte pédiatrique, à la fois dans le cadre d'études nutritionnelles (ex : nutriments, probiotiques, prébiotiques) ou liées à la santé (ex : médicaments, agents pathogènes entériques).

Chapitre IV - L'exposition aux MP de PE modifie le microbiote intestinal immature de l'enfant dans le modèle intestinal *in vitro* Tm-ARCOL

Le début de la vie est une période critique où l'écosystème et les fonctions intestinales se mettent en place, ce qui entraîne une plus grande sensibilité aux xénobiotiques par rapport aux adultes. Les études toxicologiques portant sur l'impact potentiel des MP sur la santé des enfants sont rares, contrairement à la littérature plus abondante chez les adultes et à nos propres résultats dans le modèle M-ARCOL, présentés au Chapitre II. Cependant, déjà dans l'utérus de la mère, le fœtus semble être exposé aux MP car ces derniers ont été détectés dans le placenta et le méconium. Après la naissance, les enfants peuvent également être exposés par l'inhalation et l'ingestion de poussière pendant les déambulations, mais aussi *via* l'utilisation de biberons, décrits comme étant une source de MP, de même que le lait maternel. Les tétines en caoutchouc de silicone représentent une source d'exposition supplémentaire car leur stérilisation à la vapeur entraîne la libération de MP. A ce jour, aucune étude ne s'est penchée sur les interactions bidirectionnelles entre les MP et le microbiote immature en particulier pendant la phase de développement de l'enfant. Dans ce contexte, nous avons évalué les conséquences d'une exposition orale aux MP de PE sous leur forme vierge sur le microbiote intestinal des enfants (de 6 mois à 3 ans, quatre donneurs différents) dans des conditions coliques simulées par le modèle Tm-ARCOL, présenté au Chapitre III. Selon la même approche que celle mise en œuvre pour les conditions adulte, les effets indirects des MP de PE *via* les métabolites microbiens après exposition ont été évalués sur la co-culture de cellules Caco-2 et HT29-MTX en présence des surnageants des échantillons luminaux prélevés dans le modèle Tm-ARCOL.

L'exposition aux MP de PE a induit des changements microbiens, notamment une augmentation de l' α -diversité et de l'abondance de pathobiontes potentiellement dangereux, tels que les *Dethiosulfovibrionaceae* et les *Enterobacteriaceae*. Ces changements ont été associés à une diminution de la production de butyrate et à des changements majeurs dans les profils de COV. En revanche, aucun impact significatif sur la barrière intestinale n'a été rapporté. Notre étude, la première dans le domaine, suggère que l'ingestion répétée de MP de PE peut induire des perturbations dans le microbiote intestinal du jeune enfant.

Discussion generale

Une étude originale au cœur des préoccupations d'aujourd'hui

La première originalité du travail de thèse repose sur l'utilisation du modèle colique *in vitro* M-ARCOL pour étudier l'impact des MP dans le tube digestif humain. Ce modèle avait été précédemment développé au sein de l'UMR MEDIS pour mimer les conditions coliques de l'adulte et a été adapté au cours de ce travail de thèse à l'environnement digestif spécifique de l'enfant (modèle Tm-ARCOL), une population à risque pour l'ingestion de xénobiotiques, y compris les MP. Pour la première fois dans un modèle *in vitro* parfaitement représentatif de la physiologie humaine, nous avons évalué l'impact d'une exposition répétée aux MP de PE, et non suite à l'administration d'une dose unique, sur le microbiote intestinal de l'adulte et de l'enfant, dans les compartiments à la fois luminaux et mucosaux. Nous avons également déterminé non seulement l'effet des MP sur la composition du microbiote intestinal mais aussi, pour la première fois, sur son activité en analysant les produits de fermentation, notamment les gaz et les AGCC, ainsi que les ligands AhR dérivés du métabolisme du tryptophane, et les COV. De plus, nous avons utilisé une approche originale combinant des modèles de digestion *in vitro* avec des cellules intestinales épithéliales et caliciformes en co-culture pour l'évaluation de l'impact des MP sur la barrière intestinale.

Une comparaison entre les adultes et une population à risque, les enfants

Pour la première fois, nous avons montré *in vitro* des effets différents de l'exposition aux MP de PE sur le microbiote intestinal des adultes et des enfants. Cependant, nous avons observé

pour ces deux populations une augmentation de pathobiontes intestinaux, tels que les *Enterobacteriaceae* et les *Dethiosulfovibrionaceae*. Chez l'adulte, la proportion de *Desulfovibrionaceae* a augmenté tandis que celle des *Christensenellaceae* et des *Akkermansiaceae*, deux familles bactériennes associées à une bonne santé digestive, a diminué. Ces changements bactériens ont été associés à une diminution de la production de butyrate et à des modifications importantes du profil des COV (26 COV différenciellement produits). Chez l'adulte, nous n'avons pas observé de modifications de la production de butyrate et nous avons plutôt révélé des différences au niveau des COV, principalement caractérisées par une augmentation drastique de l'indole 3-méthyle (ou scatole).

L'augmentation de la production de butyrate pendant l'enfance a été décrite comme une étape clé de la santé digestive et associée à la colonisation progressive de l'intestin par des espèces productrices de butyrate. Dans notre étude, nous avons observé une perte de *Lachnospiraceae* et de *Ruminococcaceae* (chez deux enfants sur quatre) dans le microbiote intestinal mucosal après exposition aux MP de PE, ce qui peut expliquer en partie la diminution observée de la production de butyrate. Chez les enfants âgés de moins d'un an, le microbiote intestinal héberge des familles bactériennes faiblement productrices de butyrate. Ce manque de redondance dans la fonction de production de butyrate peut rendre les enfants moins résilients aux stress environnementaux ciblant cette fonction, comme l'exposition aux MP. La diminution de la production de butyrate pendant cette période précoce de la vie, telle qu'induite dans la présente étude, pourrait entraîner à plus long terme des problèmes de santé intestinale. En ce qui concerne la scatole, la production de ce composé dérivé du tryptophane par le microbiote intestinal humain implique divers genres, espèces ou familles dont les *Desulfovibrionaceae*. Leur augmentation après exposition aux MP de PE observée chez les adultes, et non chez les enfants, pourrait expliquer la production accrue de scatole.

Comme nous l'avons mentionné précédemment, 26 COV ont été différenciellement produits après exposition aux MP de PE chez l'enfant. Plusieurs études ont tenté de caractériser et d'associer certains profils de COV fécaux ou respiratoires à des pathologies gastro-intestinales pédiatriques. En particulier, plusieurs COV exhalés, tels que le pentane et le 1-heptène également observés dans notre étude, ont été surproduits chez les enfants obèses ou en surpoids atteints de stéatose hépatique non alcoolique. Par ailleurs, des profils spécifiques de COV fécaux ont été mis en évidence dans des cas de septicémie néonatale à début tardif et d'entérocolite nécrosante chez les prématurés, mais leur identité précise n'a pas été rapportée à

ce jour. L'étude du volatolome n'en est qu'à ses prémices, en particulier chez les enfants, et doit être approfondie pour une meilleure compréhension de l'exposition aux MP.

La nécessité d'étudier d'autres populations à risque

L'exposition potentiellement plus élevée des enfants et les différences marquées entre les enfants et les adultes observées dans ce travail de thèse soulignent la nécessité d'étudier les effets des MP non seulement sur les individus en bonne santé, mais aussi sur d'autres populations à risque. A ce jour, aucune étude n'a examiné les conséquences de l'exposition aux MP sur un microbiote intestinal humain « perturbé ».

Au laboratoire MEDIS, le modèle M-ARCOL a été récemment adapté pour simuler les conditions spécifiques retrouvées dans le côlon des patients souffrant du syndrome de l'intestin irritable ou d'obésité. De même, le modèle Tm-ARCOL pourrait également être adapté pour simuler le microbiote intestinal des enfants en condition physiopathologique. Dans le cadre du projet Next-PLASTOX INRAE (qui fait suite au projet PLASTOX INRAE), TOXALIM étudie actuellement les conséquences d'une exposition orale chronique de 90 jours chez des souris adultes nourries avec un régime normal *vs* occidental. Les animaux sont traités *via* l'alimentation avec le même type de MP de PE que dans mon travail de thèse à une dose de 10 mg/kg poids corporel/jour, fixée d'après les premières estimations d'exposition humaine. Dans ce travail actuellement en cours, l'influence des MP de PE et/ou du régime alimentaire (pour révéler tout effet révélateur ou synergique) est déterminée à la fois sur l'homéostasie intestinale et sur les perturbations de l'axe intestin/foie.

Les microplastiques : des contaminants aux multiples facettes

Les MP sont des particules complexes, caractérisées par de nombreux paramètres, tels que le type de polymère, la taille, la forme, les propriétés de surface, la présence de contaminants adsorbés et/ou la formation d'une couronne biomoléculaire, par exemple après digestion. Cependant, pour des raisons de disponibilité et de facilité de manipulation, la plupart des études menées à ce jour ont utilisé des microbilles de polystyrène (PS) vierges, bien que ce type de polymère ne représente qu'environ 6 % de la production totale de plastique. En outre, la forme sphérique n'est pas entièrement représentative des formes ingérées, soulignant ainsi la nécessité d'évaluer les effets des MP « réels ». Si les MP vierges peuvent mimer une source directe d'exposition humaine, l'utilisation de particules « vieilles » est également nécessaire pour simuler l'exposition environnementale avec de fortes modifications des MP en termes

d'oxydation, de fragmentation, de dégradation et/ou de formation de « biocorona » ou couronne biomoléculaire.

L'exposition humaine aux MP est sans nul doute due à un mélange de diverses particules plastiques en termes de taille et/ou de distribution de taille, ce qui pourrait conduire à des impacts sanitaires différents de ceux déjà évalués *in vivo* et *in vitro* pour des particules de taille homogène. De plus, la forme des MP est un autre facteur important qui pourrait influencer leurs effets sur la santé humaine. D'autres études devront ainsi être menées par la suite sur d'autres formes de MP mais aussi avec des cocktails de différents types de polymère, tailles et/ou formes.

Alors que près de la moitié des études existantes ne caractérisent pas les MP utilisés et compte tenu des nombreux facteurs pouvant influencer les effets des MP chez l'Homme, il est crucial d'harmoniser les conditions d'administration des MP (par exemple mg/L ou particules/L), de même que de standardiser les méthodes d'évaluation de la toxicité. Pour cette raison, l'Autorité européenne de sécurité des aliments (EFSA) tente de fédérer actuellement l'ensemble des communautés scientifiques impliquées dans les méthodologies de détection et de quantification des MP, ainsi que dans l'évaluation des dangers et des risques pour la santé humaine.

Modifications des propriétés physico-chimiques des MP pendant la digestion humaine

Dans ce travail nous avons considéré des MP dans leur forme vierge. Or, il est important d'élucider les transformations physico-chimiques pouvant survenir au cours de leur transit dans le tractus digestif humain. Quelques études ont déjà exploré les modifications des MP pendant le processus de digestion humaine en utilisant des modèles *in vitro* simplifiés et n'ont pas rapporté d'altérations majeures des MP mais plutôt la formation d'une couronne biomoléculaire à la surface des particules. En utilisant la combinaison de modèles *in vitro* des parties haute et basse de l'intestin, une étude a récemment montré une amorphisation progressive de la surface des MP de polyéthylène téréphtalate (PET), avec un dépôt de matière organique. Sur la base de l'expertise de l'UMR MEDIS, des premières caractérisations, décrites ci-dessous, ont été effectuées pour suivre les transformations physico-chimiques des MP en utilisant le modèle TIM-1 (TNO gastroIntestinal tract Model 1), simulateur le plus complet du tractus gastro-intestinal humain supérieur en reproduisant les principaux paramètres physico-chimiques de

l'estomac et de l'intestin grêle. Par rapport aux modèles statiques, le TIM-1 permet également une meilleure évaluation de la bioaccessibilité des composés ingérés, plus proche de la situation observée *in vivo*. L'absorption des MP de plus petite taille et/ou de molécules (comme les contaminants chimiques associés aux MP) peut être suivie dans le TIM-1 grâce à une membrane de dialyse. En outre, le TIM-1 pourrait être utilisé pour étudier l'influence des matrices alimentaires (y compris les matrices riches en lipides) sur les transformations physico-chimiques des MP, la dégradation et/ou la bioaccessibilité des contaminants associés.

Un autre verrou critique dans le domaine est lié à l'absence de méthodes totalement fiables pour suivre le devenir des MP pendant la digestion humaine. En effet, la détection, la quantification et l'analyse physico-chimique des MP dans des milieux complexes, tels que les matrices biologiques et fluides digestifs, représentent encore à l'heure actuelle des défis analytiques majeurs. De nombreuses méthodes sont aujourd'hui testées ou en cours de développement mais, comme mentionné précédemment, leur utilisation n'est pas encore standardisée.

Digestion des MP de PE dans le modèle TIM-1 et analyses associées pour leur détection dans les fluides digestifs à l'aide d'instrumentations innovantes

Au cours de mon travail de thèse, en réponse aux verrous scientifiques et méthodologiques soulevés ci-dessus, nous avons mené des expériences dans le modèle TIM-1 pour suivre le devenir des MP de PE et identifier leurs potentielles modifications physico-chimiques au cours de la digestion humaine. Des MP de PE (21 mg), mis en suspension dans un verre d'eau de Volvic, ont été digérés *in vitro* en conditions adulte et enfant (deux essais par condition). Des échantillons de digestat après 1 heure de digestion gastrique ont été collectés, ainsi que des échantillons de digestat iléaux, après 4 heures d'un processus de digestion complet après passage dans les compartiments gastrique, duodéнал, jéjunal et iléal. Nous avons ensuite procédé à l'analyse des différents échantillons *via* l'utilisation, pour la première fois à notre connaissance, de deux méthodes analytiques impliquant différents collaborateurs aux niveaux français et européen.

Nous avons tout d'abord utilisé l'imagerie par fluorescence DUV (ultraviolet lointain) pour détecter les MP de PE après digestion humaine sur la ligne de lumière DISCO du synchrotron SOLEIL (Gif sur Yvette, France). Cet instrument repose sur une source de

rayonnement électromagnétique couvrant une large gamme d'énergies, couplée à un laboratoire de recherche dédié à l'analyse de la matière jusqu'à l'échelle atomique. Nous avons analysé la poudre initiale de MP de PE, les effluents gastriques et intestinaux de TIM-1 à 1 heure et 4 heures de digestion, respectivement, dans les conditions adulte et enfant. Cette technique d'imagerie non invasive nous a permis de mettre en évidence les MP de PE dans nos échantillons biologiques sans aucun marquage préalable. L'auto-fluorescence des MP de PE a été observée avec une excitation à 280 nm dans la poudre initiale, ainsi que dans les effluents gastriques en conditions adulte et enfant. Cependant, des problèmes techniques sont apparus, comme l'extinction de la fluorescence des MP dans certains échantillons (*i.e.* les effluents intestinaux), probablement due à l'interaction avec les composés biologiques. Nos résultats ont ainsi souligné (i) l'influence du milieu biologique environnant sur l'émission de fluorescence des MP et (ii) la nécessité d'une signature chimique plus spécifique des MP.

Pour pallier à ces limitations analytiques et au-delà du travail en cours avec M-H. Ropers du laboratoire Biopolymères Interactions Assemblages (UR1268 BIA, INRAE, Nantes, France) sur l'utilisation de méthodes spectroscopiques dans le cadre du projet PLASTOX INRAE, nous avons initié en 2021 une collaboration avec P.G. Gucciardi de l'Institut des Procédés Chimiques et Physiques (IPCF, CNR, Messine, Italie) pour détecter et identifier les MP de PE dans les digestats du modèle TIM-1 en utilisant une instrumentation de type « Raman Tweezer » (RT). D'un point de vue expérimental, cette instrumentation est un microscope inversé équipé d'un objectif à haute ouverture numérique, couplé à un spectromètre Raman. Le dispositif mis en œuvre dans nos expériences était un RT à faisceau unique dans lequel le même faisceau laser est utilisé pour le piégeage et l'excitation Raman. Nous avons ainsi appliqué avec succès l'instrumentation RT pour l'analyse des fluides digestifs humains exposés aux MP en conditions adulte, en permettant à la fois le piégeage optique et l'identification chimique des particules plastiques de PE de petite taille (d'environ 1-2 μm). Il est intéressant de noter que des agglomérats contenant des MP de PE ont été détectés après la phase gastrique de digestion. Par ailleurs, aucun MP de PE isolé n'a été observé. Après la phase intestinale de digestion, une désagglomération des MP de PE a été mise en évidence, ce qui peut être dû à l'augmentation du pH et/ou à l'influence de composés organiques (*i.e.* les acides biliaires). En condition enfant, les mêmes comportements ont été obtenus sur les effluents gastriques et intestinaux.

Les MP en tant que vecteurs de polluants chimiques, de métaux lourds et de microorganismes : l'hypothèse du « cheval de Troie »

Les MP sont des vecteurs de divers polluants chimiques, métaux lourds et microorganismes. Il est à noter par ailleurs que la capacité de sorption des MP vieillis est plus importante que celle des particules vierges. Comme mentionné ci-dessus, une étude a montré une amorphisation progressive de la surface des MP de PET pendant la digestion humaine *in vitro*, ce qui pourrait conduire à une modification de leur capacité de sorption vis-à-vis des polluants organiques. De plus, le pH influence les interactions électrostatiques entre les MP et les composés adsorbés. Ces résultats posent la question de leur désorption éventuelle pendant la digestion. Jusqu'à présent, les études *in vitro* sont rares et se limitent principalement à des modèles simples exposant les MP en présence de contaminants chimiques adsorbés (métaux lourds exclusivement à ce jour) à des fluides digestifs simulés. Une désorption élevée dans des conditions acides a été décrite dans le cas spécifique du chrome (Cr) dans un modèle de digestion statique *in vitro* humain, alors qu'aucune libération n'a été observée dans la phase orale. Il s'agit maintenant d'aborder cette problématique par le biais de systèmes *in vitro* dynamiques et multi-compartimentaux plus complexes.

Le projet PLASTOX INRAE a un axe de recherche spécifique sur ce thème des MP en tant que vecteurs de polluants chimiques. En collaboration avec l'unité INRAE QuaPA (J. Ratel et E. Engel), nous nous sommes intéressés aux polychlorobiphényles (PCB). Nos collaborateurs ont ainsi réussi à adsorber les PCB sur des MP de PE. La prochaine étape sera d'étudier le relargage des PCB dans les compartiments supérieurs du tube digestif pendant une digestion *in vitro* dans le système TIM-1. Ensuite, la libération et l'impact de ces PCB adsorbés sur les MP de PE seront évalués sur le microbiote intestinal des adultes et des enfants dans les modèles M-ARCOL et Tm-ARCOL, respectivement. Il serait également intéressant d'étudier ces effets sur des MP de PE préalablement vieillis, car le profil de sorption des PCB pourrait être différent.

Interaction du microbiote intestinal, un acteur clé de la santé humaine, avec les MP

Comme des échantillons fécaux sont utilisés pour inoculer les dispositifs M- et Tm-ARCOL, un point important à considérer est la contamination initiale des échantillons de selles par les MP. En collaboration avec T. Rocha-Santos et A. Silva (CESAM, Université d'Aveiro,

Portugal), des tests selon la méthode MP-VAT (« Microplastics Visual Analysis Tool ») sont en cours et n'ont pas pu être inclus dans ce manuscrit de thèse.

Par ailleurs, l'émergence des technologies à haut débit permet des études approfondies du microbiote intestinal humain. Les analyses basées sur le séquençage se sont traditionnellement focalisées sur l'appartenance à la communauté microbienne et le profilage de l'abondance taxonomique. Cependant, la capacité métabolique, ainsi que l'influence sur l'hôte, soit structurellement, soit fonctionnellement, restent moins caractérisées, même si le « qui est actif ? » est plus important que le « qui est là ? ». Les études décrivant l'impact des MP sur le microbiote intestinal (principalement chez le rongeur) se concentrent sur la modulation de la composition bactérienne, mais ne se focalisent jamais sur son activité. Notre étude est donc la première à examiner l'effet de l'exposition aux MP sur diverses fonctions caractéristiques de l'activité du microbiote intestinal humain. Nous avons ainsi mis en évidence plusieurs altérations de l'activité métabolique, qui pourraient faire l'objet d'études plus approfondies, comme par exemple les prédictions basées sur les données issues du séquençage du gène de l'ADNr 16S et des génomes proches référencés à l'aide de la base de données « Kyoto Encyclopedia of Genes and Genomes » (KEGG). Cependant, la principale limitation de ces méthodes est le manque d'information disponible dans les bases de données permettant de caractériser à l'heure actuelle seulement 10 à 50% des séquences totales. Une autre possibilité est l'utilisation de techniques omiques complémentaires, telles que la métatranscriptomique, la métaprotéomique et la métabolomique. La mise en place d'une approche multi-omique intégrée serait idéale pour caractériser finement l'activité du microbiote intestinal, notamment dans le contexte de l'exposition aux MP, mais un tel développement n'en est qu'à ses débuts et nécessitera une collaboration très étroite entre bioinformaticiens, biostatisticiens et biologistes.

Meilleure caractérisation de l'interaction MP/microbiote intestinal

De nombreux xénobiotiques ont été décrits dans la littérature comme altérant le microbiote intestinal à la fois dans sa composition et son activité métabolique, avec des conséquences potentiellement délétères pour la santé de l'hôte. Réciproquement, le microbiote intestinal peut directement métaboliser ces mêmes xénobiotiques au niveau du côlon par l'action de plusieurs enzymes bactériennes.

Nous avons montré au cours de ce travail de thèse que des microorganismes, issus du microbiote intestinal des adultes et des enfants, étaient capables de former des structures de type « biofilm » à la surface des MP. Cependant, l'identité de ces microorganismes et les

mécanismes par lesquels ils interagissent avec les MP n'ont pas été étudiés. Plusieurs méthodes, déjà appliquées à des échantillons environnementaux, pourraient alors être utilisées pour étudier la « plastisphère » intestinale. Il s'agira tout d'abord d'isoler les MP en filtrant les échantillons tout en incluant si nécessaire une étape de purification. L'association bactérienne avec les MP sera analysée par microscopie électronique à balayage ou par microscopie à fluorescence. Pour étudier la matrice du biofilm, en particulier les polysaccharides extracellulaires, l'utilisation de la microscopie confocale à balayage laser pourrait être envisagée. Le séquençage du gène de l'ADNr 16S et la métagénomique fonctionnelle pourront enfin être appliqués pour caractériser la composition de la plastisphère intestinale et son activité, respectivement.

Enfin, aucune étude n'a encore évalué la capacité du microbiote intestinal à dégrader les MP de PE. Des études récentes ont néanmoins montré que plusieurs bactéries identifiées dans les sols sont capables de dégrader les MP de PE. Ainsi, la capacité des micro-organismes, bactériens mais aussi fongiques, à dégrader les MP de PE dans l'intestin humain doit encore être caractérisée. En collaboration avec F. Lagarde, F. Amiard et M. Edely du laboratoire IMMM au Mans (France), nous avons analysé par spectroscopie Raman la surface des MP de PE après fermentation dans les modèles M- et Tm-ARCOL afin de décrypter les modifications physico-chimiques potentielles par le microbiote intestinal humain. Nos résultats n'indiquent aucune modification par rapport aux particules initiales.

Du microbiote colique au microbiote de l'intestin grêle

Les interactions entre le microbiote colique et les MP ont été peu étudiées jusqu'à présent chez l'Homme, la plupart des études reposant sur des échantillons fécaux. Cependant, le microbiote colique n'est pas le seul à interagir potentiellement avec les MP lors de leur transit dans le tube digestif humain, et il serait également très intéressant de caractériser les interrelations entre les MP et le microbiote des parties hautes du tractus digestif, en particulier celui de l'intestin grêle. En effet, les microbiotes du côlon et de l'intestin grêle présentent plusieurs différences, avec en particulier plus de bactéries anaérobies facultatives dans la partie haute de l'intestin, comme les *Enterobacteriaceae*. Dans ce travail de thèse, tant chez l'adulte que chez l'enfant, nous avons montré une augmentation de l'abondance des *Enterobacteriaceae* après une exposition aux MP de PE, ce qui renforce l'intérêt de caractériser les effets dans ce type de compartiment. Cependant, l'étude du microbiote de l'intestin grêle chez l'Homme est très difficile et nécessite l'utilisation de méthodes invasives, telles que des cathéters naso-duodénaux, le prélèvement d'échantillons de muqueuse iléale lors d'une coloscopie, d'une

résection intestinale, d'une transplantation de l'intestin grêle ou encore chez des victimes de mort subite.

Comme alternative pour étudier l'intestin grêle et le microbiote associé, l'UMR MEDIS travaille sur le développement de différents modèles *in vitro* reproduisant le compartiment iléal. Tout d'abord, le modèle M-ARCOL est en cours d'adaptation en appliquant des paramètres physico-chimiques spécifiques de l'iléon humain à un échantillon fécal. Ensuite, le modèle ESIN (Engineered Stomach and Small Intestine) est maintenant optimisé pour introduire le microbiote dans les compartiments jéjunal et iléal. Il est intéressant de noter que les modèles TIM-1 et ESIN ont été, ou seront à court terme, optimisés pour intégrer, comme dans le modèle M-ARCOL, un compartiment mucosal, pour une meilleure évaluation des interactions des MP avec le microbiote associé au mucus.

Modèles de culture cellulaire pour les études liées aux MP

De nombreuses études ont déjà évalué les effets directs des MP sur les cultures cellulaires par simple mise en contact. Cependant, en raison de leur faible masse volumique (inférieure à celle de l'eau), certains MP, comme ceux de PE, flottent en surface pendant la période d'incubation. Pour surmonter ce problème, une configuration originale a été décrite dans la littérature, consistant en un modèle inversé de culture cellulaire. Alors que l'exposition aux MP de PE n'a induit aucune cytotoxicité sur les cellules dans le modèle de culture cellulaire classique, dans la configuration inversée, avec les mêmes conditions de culture, une cytotoxicité a pu être observée.

À ce jour, une seule étude sur les MP a couplé un système de digestion *in vitro* simplifié à des cellules en culture. En ce qui concerne les modèles plus complexes, une autre étude a utilisé un système de digestion *in vitro* dynamique en trois étapes (phases orale, gastrique et intestinale) pour étudier les différences d'effet entre les MP de PS vierges ou digérés sur des cellules Caco-2. Dans notre cas, nous avons étudié l'impact indirect des métabolites microbiens (*i.e.* surnageants luminaux des modèles M- et Tm-ARCOL avant et après exposition aux MP de PE) sur une co-culture de cellules Caco-2 et HT29-MTX. Pour éviter des conditions cytotoxiques pour les cellules du fait de la présence de certains composés dans le milieu fermentaire (enzymes, sels biliaires), nous avons dû diluer les surnageants (dilution au 1/20^{ème}) et, de ce fait, il est fort probable que les composés bioactifs (*i.e.* le butyrate) étaient eux aussi présents en très faibles quantités, sans réelle influence dès lors sur la réponse cellulaire.

L'importance du mucus dans les modèles cellulaires *in vitro*

Nous avons observé une augmentation des *Enterobacteriaceae* dans le microbiote intestinal des adultes et des enfants après exposition aux MP de PE. Ces bactéries, ainsi que la plupart des agents pathogènes entériques, doivent franchir la barrière du mucus avant d'atteindre l'épithélium intestinal pour favoriser leur colonisation et leur persistance. Cette donnée souligne l'importance du mucus dans toute étude liée au microbiote intestinal et/ou aux xénobiotiques. Nous avons choisi de travailler avec une co-culture de cellules Caco-2 et HT29-MTX, plus représentative de la physiologie intestinale par rapport à une simple monoculture de cellules Caco-2. D'autres types cellulaires, comme les cellules T84 ou LS-174T pourraient être utilisés comme alternative. Récemment, un modèle humain *in vitro* de triple culture (cellules T84, LS-174T et THP-1) a été développé dans la littérature, incorporant également une couche de géluse-mucine isolée du tapis cellulaire par un filtre poreux pour mimer la couche de mucus interne perméable aux seuls ions et petites molécules. Cette innovation méthodologique permet de tester directement *in vitro* des échantillons coliques bruts sur le modèle de culture cellulaire sans aucune centrifugation ni dilution préalables et ainsi d'éviter tout biais dans la mise en œuvre expérimentale et l'interprétation des résultats. D'autres couplages plus complexes entre des modèles de digestion *in vitro* et des cultures cellulaires seraient également envisageables. Une approche pertinente pourrait être un apport continu de milieu digestif issu de modèles intestinaux *in vitro* sur des cultures cellulaires, comme cela a été fait avec le module Host-Microbiota Interaction (HMITM), principalement adapté pour être combiné avec le modèle SHIME mais avec une limite de 48 h de temps de contact pour des raisons de cytotoxicité.

Les modèles 3D pour étudier l'impact des MP sur la barrière intestinale

Des modèles 3D plus complexes pourraient être utilisés à l'avenir, tels que des modèles multicellulaires, déjà mis en œuvre mais de manière parcellaire pour l'étude des MP ou des organoïdes intestinaux, quant à eux non explorés à ce jour. De même, les systèmes HuMiX, Intestine-Chip et Colon-Chip, permettraient de considérer l'influence de l'environnement hydrodynamique (péristaltisme, flux intestinal), et également des microorganismes commensaux et du mucus, sur le devenir et la toxicité des MP dans l'intestin. Tous ces modèles *in vitro* pourraient être adaptés à une barrière intestinale altérée (hyperperméabilité, inflammation) pour mimer l'état physiopathologique observé chez certaines populations (obésité, pathologies digestives).

L'intestin et son interaction avec d'autres organes pour des études supplémentaires dans l'évaluation de la toxicité des MP

En plus des effets des MP sur la sphère intestinale étudiée dans ce travail de thèse, il a été démontré chez les mammifères ou avec des cultures cellulaires *in vitro* un impact sur différents organes (cerveau, cœur, poumon, ovaires et testicules, foie et reins), même si les mécanismes sous-jacents sont encore peu étudiés. Il serait donc important d'évaluer les effets systémiques en se concentrant sur la communication entre les organes. Des modèles de (co)culture cellulaire, impliquant des cellules intestinales, extra-digestives mais aussi immunitaires, pourraient être envisagés.

Un focus sur l'évaluation des risques liés aux MP

Mon travail de doctorat a débuté en Octobre 2019, ce qui correspond à l'explosion des recherches académiques, majoritairement sur des modèles animaux et à des doses élevées, pour une meilleure compréhension de l'exposition humaine aux MP et des risques sanitaires associés. Ces études nécessitent une expertise interdisciplinaire réunissant notamment des biologistes, des chimistes et des physiciens. La problématique des MP ne s'intègre pas facilement dans les cadres réglementaires traditionnels car leur extrême diversité, le manque de méthodes analytiques standardisées (*i.e.* détection et quantification des MP dans les aliments, l'air, l'eau potable et les matrices biologiques) entraînent un niveau élevé d'incertitude dans l'estimation à la fois de l'exposition et des dangers. Une autre source de complexité réside dans le risque de contamination lors des analyses de MP dans les emballages, les aliments, l'air, l'eau, les selles humaines, les tissus biologiques, mais également *via* le matériel de laboratoire qui est essentiellement composé de matières plastiques.

Ma recherche, pionnière dans le domaine de l'impact des MP chez l'Homme en s'intéressant à la fois aux conditions à l'adulte et l'enfant, a conduit à la production de 8 publications scientifiques (4 articles originaux, 3 articles de revue de synthèse et un chapitre d'ouvrage). Elle contribue à une meilleure évaluation des dangers pour la santé humaine, et combinée aux données d'exposition, favorisera le développement d'un cadre d'évaluation des risques liés aux MP chez l'Homme.

Table of content

Abstract.....	1
Résumé.....	2
Acknowledgments/Remerciements.....	4
Résumé de la thèse.....	35
Table of content.....	27
List of Figures and Tables.....	29
Abbreviations.....	35
Foreword	38
1. Context of the PhD.....	38
2. Research questions.....	40
3. Manuscript organization.....	41
4. Outline of the PhD.....	42
Section 1: Literature Review	43
Part 1 - The human gastro-intestinal tract: from birth to adulthood.....	44
1. Abiotic physiology of the human gut.....	44
2. Gastro-intestinal gut microbiota.....	60
Part 2 – <i>In vitro</i> models of the human gut.....	73
1. <i>In vitro</i> gut models.....	74
2. <i>In vitro</i> models of the intestinal barrier.....	104
Part 3 - Microplastics: From environmental pollution to threat for human health.....	111
1. From plastics to microplastics.....	112
2. MPs: vector of contaminants and microorganisms.....	115
3. Human oral exposure to MPs.....	119
4. Consequences of oral exposure to MPs on intestinal homeostasis impairment: a focus on virgin particles.....	126
Section 2: Results	138
Chapter I - Comparative methods for fecal sample storage to preserve gut microbial structure and function in an <i>in vitro</i> model of the human colon.....	139
Chapter II - Microplastics: what happens in the human digestive tract? First evidences in adults using <i>in vitro</i> gut models.....	155
Chapter III - A child is not an adult: development of a new <i>in vitro</i> model of the toddler colon.....	176

Chapter IV - Exposure to polyethylene microplastics alters immature gut microbiome in an infant <i>in vitro</i> gut model.....	199
Section 3: Discussion	216
1. An original study at the heart of today’s preoccupations.....	2179
2. A comparison between adults and an at-risk population, the infants.....	218
2.1 Infant and adult gut is not the same and therefore MP effects are different	218
2.2 The need for more studies of at-risk populations.....	220
3. Microplastics: a multifaceted contaminant	222
3.1 Various types, sizes and shapes of MPs.....	222
3.2 MPs are not in a virgin state in the environment.....	223
3.3 Modifications of MP physico-chemical properties during human digestion	224
3.4 MPs as vector of chemicals, heavy metals and microorganisms: the “Trojan horse” hypothesis.....	231
4. Interaction of gut microbiota, a key player in human health, with MPs	233
4.1 Fecal inoculum: A focus on this key component of <i>in vitro</i> fermentation.....	233
4.2 Further investigations of the impact of MPs on gut microbiota activity.....	235
4.3 Better characterization of the MP/gut microbiota interaction	238
4.4 From colonic to small intestinal microbiota.....	239
5. The intestinal barrier and its interactions with MPs.....	240
5.1 Cell culture models for MP-related studies: some innovation in the field.....	240
5.2 Coupling cell culture and <i>in vitro</i> gut model to study MPs	241
5.3 The importance of mucus in physiologically-relevant <i>in vitro</i> cell models.....	2435
5.4 Complex 3D models to investigate MP impact on the intestinal barrier	245
5.5 Gut and its crosstalk with other organs for further studies in MP toxicity assessments	246
6. The outcomes of my research: a focus on MP risk assessment.....	248
References	251
Appendix	3055
Appendix 1: Scientific Curriculum Vitae.....	3066
Appendix 2: Book chapter from <i>Handbook of Microplastics in the Environment</i>	3100
Appendix 3: Review in Journal of Hazardous Materials	336
Appendix 4: Review in Biotechnology Advances	31251
Appendix 5: Review in Cahiers de Nutrition et de Di�t�tique.....	31379

List of Figures and Tables

Foreword

Figure 0. Outline of the experimental work during the PhD

Section 1: Literature Review

Part 1 - The human gastro-intestinal tract: from birth to adulthood

Figure 1.1 Gastrointestinal tract of healthy adults: summary of the key physical and chemical processes

Figure 1.2 Gastro-intestinal development across childhood

Figure 1.3 Oral digestion development across childhood

Figure 1.4 Cell lining of the small intestine (A) and colon (B)

Figure 1.5 Mucus thickness along the human gastro-intestinal tract

Figure 1.6 Biogeography of the luminal microbial composition of GIT in a healthy adult

Figure 1.7 Changes in microbiota composition between luminal and mucosal compartments throughout the human gut

Figure 1.8 Factors shaping the neonatal microbiome and its evolution from birth to adulthood

Figure 1.9 Key functions of the human gut microbiota

Figure 1.10 Pathways that are responsible for the biosynthesis of the major microbial metabolites that result from carbohydrate fermentation and bacterial cross-feeding

Part 2 - *In vitro* models of the human gut

Figure 2.1 Schematic representation (A) and picture (B) of the TIM-1 system

Figure 2.2 Picture of the M-ARCOL model with its mucin-alginate bead module

Figure 2.3 Scheme of the HMI module for host-microbiota interaction studies in the GIT

Table 2.1 Main *in vitro* static mono-compartmental digestive models of the upper adult GIT

Table 2.2 Main characteristics of the current infant *in vitro* static models mimicking the upper GIT

Table 2.3 Main *in vitro* dynamic mono-compartmental digestive models of the upper adult GIT

Table 2.4 Main *in vitro* dynamic bi-compartmental digestive models of the upper adult GIT

Table 2.5 Main characteristics of the current infant *in vitro* dynamic mono- and bi-compartmental models mimicking the upper GIT

Table 2.6 Main *in vitro* dynamic multi-compartmental digestive models of the upper adult GIT

Table 2.7 Main characteristics of the current infant *in vitro* dynamic multi-compartmental models mimicking the upper GIT

Table 2.8 Main *in vitro* static mono-compartmental models of the lower adult GIT

Table 2.9 Main *in vitro* dynamic mono-compartmental digestive models of the lower adult GIT

Table 2.10 Main characteristics of the current infant *in vitro* dynamic mono-compartmental models mimicking the lower GIT

Table 2.11 Main *in vitro* dynamic multi-compartmental models of the lower adult GIT

Table 2.12 Main characteristics of the current infant *in vitro* dynamic multi-compartmental models mimicking the lower GIT

Table 2.13 Main whole gastrointestinal models of the adult GIT

Table 2.14 Main characteristics of the current infant *in vitro* complete gastro-intestinal models

Table 2.15 Summary of the different *in vitro* models used to simulate the human intestinal barrier

Part 3 - Microplastics: From environmental pollution to threat for human health

Figure 3.1 The “Life cycle” of plastics

Figure 3.2 MP sources and formation

Figure 3.3 MPs: vectors for contaminants and microorganisms

Figure 3.4 Human GIT exposure to MPs

Figure 3.5 Transport of MPs through intestinal cells

Figure 3.6 Possible influence of MPs on the intestinal barrier as reported by *in vivo* and *in vitro* studies

Section 2: Results

Chapter I - Comparative methods for fecal sample storage to preserve gut microbial structure and function in an *in vitro* model of the human colon

Fig. 1 Experimental design of the *in vitro* fermentation experiments in the M-ARCOL

Fig. 2 Experimental set-up and sampling procedure during M-ARCOL experiments

Fig. 3 Relative abundance of bacterial families retrieved in the initial stools and in the luminal medium and mucin beads from M-ARCOL

Fig. 4 Effect of different stool storage conditions on the Shannon diversity index values

Fig. 5 Effect of different stool storage conditions on qPCR enumeration of main bacterial groups

Fig. 6 Gas composition and production in M-ARCOL according to stool storage conditions

Fig. 7 SCFA composition and production in M-ARCOL according to stool storage conditions

Fig. S1 Effect of different stool storage conditions on community structure at the phylum level

Fig. S2 Redox potential values measured in the M-ARCOL

Table 1 Primers used for qPCR and 16S metabarcoding

Table 2 Scientific and technical comparative data on preservative methods applied to fecal samples

Chapter II - Microplastics: what happens in the human digestive tract? **First evidences in adults using *in vitro* gut models**

Fig. 1. Outline of *in vitro* fermentation in M-ARCOL and Caco-2/HT29-MTX cell culture experiments

Fig 2. Effect of PE MP repeated exposure in M-ARCOL on luminal and mucosal bacterial microbiota composition at the family level

Fig 3. Effect of PE MP repeated exposure in M-ARCOL on α - and β -diversity indexes of bacterial communities

Fig 4. Scanning electron microscopy observations of PE MPs/gut microbiota interactions in M-ARCOL and Raman spectroscopy analysis of PE MPs at the end of exposed period

Fig 5. Impact of PE MP repeated exposure on gas and short chain fatty acid production in M-ARCOL

Fig 6. Impact of PE MP repeated exposure in M-ARCOL on volatile organic compounds produced by the gut microbiota in the luminal compartment

Fig 7. Ability of luminal M-ARCOL supernatants to activate AhR after PE MP exposure

Fig 8. Impact of luminal supernatants from M-ARCOL before and after PE MP repeated exposure on intestinal barrier integrity using a Caco-2/HT29-MTX co-culture

Fig. S1 Characterization of PE MPs

Fig. S2 Variation over time of the total bacterial load and archaea sequence number in the luminal and mucosal compartments before and after PE MP exposure in M-ARCOL

Fig. S3 Characterization of adult faecal samples used for M-ARCOL inoculation

Fig. S4 Inter-individual variability in luminal and mucosal microbiota composition at the phylum level before and after PE MP exposure in M-ARCOL and in bacterial β -diversity during the stabilization period

Fig. S5 Inter-individual variability at the family level on the effect of PE MP repeated exposure in M-ARCOL on luminal and mucosal bacterial microbiota

Fig. S6 Inter-individual variability in gas and short chain fatty acid production before and after PE MP repeated exposure in M-ARCOL

Fig. S7 Impact of luminal M-ARCOL supernatants before and after PE MP repeated exposure on intestinal gene expression using a Caco-2/HT29-MTX co-culture

Table 1. Primers used for qPCR for bacterial load, Illumina sequencing for bacteria and archeal populations and RT-qPCR to monitor intestinal gene expression

Table 2. Volatile organic compounds in the volatolome of the fecal microbiota as significantly altered by the repeated exposure to PE MPs

Chapter III - A child is not an adult: development of a new *in vitro* model of the toddler colon

Fig. 1. Stool donor information, sampling, experimental design and parameters of *in vitro* fermentations when reproducing toddler vs adult colonic conditions in the M-ARCOL model

Fig. 2. Characterization of toddler and adult initial fecal samples

Fig. 3. Total bacteria and Archaea composition of the luminal and mucus-associated microbiota during toddler vs adult fermentations

Fig. 4. Bacterial composition of the luminal and mucus-associated microbiota during toddler vs adult fermentations at the family level

Fig. 5. α - and β -diversity indexes of luminal and mucus-associated bacterial populations during toddler vs adult fermentations

Fig. 6. Luminal microbial metabolic activity followed by gas production and redox potential measurement during toddler vs adult fermentations

Fig. 7. Luminal microbial metabolic activity followed by short chain fatty acid production during toddler vs adult fermentations

Fig. 8. Predicted functional metabolic pathways during adult and toddler fermentations

Fig. S1. Bacterial composition of the luminal and mucus-associated microbiota during toddler vs adult fermentations at the phylum and genus levels

Fig. S2. α - and β -diversity indexes of luminal and mucus-associated bacterial populations during toddler vs adult fermentations at the individual level

Fig. S3. Inter-individual luminal microbial activity as assessed by gas and SCFA production during toddler vs adult fermentations

Table 1. Comparison of Tm-ARCOL with previously developed toddler colonic *in vitro* models

Table 2. *In vitro-in vivo* comparisons of relative changes in microbiota structure and metabolic activity between toddler and adult

Chapter IV - Exposure to polyethylene microplastics alters immature gut microbiome in an infant *in vitro* gut model

Fig. 1. Outline of *in vitro* fermentations and cell culture experiments

Fig 2. Scanning electron microscopy observations of PE MPs/microbiota interactions in Tm-ARCOL and Raman spectroscopy analysis of PE MPs at the end of exposed period

Fig 3. Effect of PE MP repeated exposure on luminal and mucosal bacteria in Tm-ARCOL

Fig 4. Effect of PE MP chronic exposure on α - and β -diversity indexes of bacterial communities in Tm-ARCOL

Fig 5. Impact of PE MP chronic exposure on gas and short chain fatty acid production in Tm-ARCOL

Fig 6. Impact of PE MP chronic exposure on the capacity of Tm-ARCOL luminal supernatants to activate Aryl Hydrocarbon receptor

Fig 7. Impact of PE MP chronic exposure on volatile organic compounds in the luminal compartment of Tm-ARCOL

Fig 8. Impact of Tm-ARCOL luminal supernatants before and after PE MP chronic exposure on inflammation and intestinal barrier integrity using a Caco-2/HT29-MTX co-culture

Fig. S1 Characterization of toddler faecal samples used for Tm-ARCOL inoculation

Fig. S2 Inter-individual variability in luminal and mucosal microbiota composition at the phylum level and in bacterial β -diversity in Tm-ARCOL

Fig. S3 Inter-individual variability in the impact of PE MP chronic exposure on gas and short chain fatty acid production in Tm-ARCOL

Fig. S4 Impact of Tm-ARCOL luminal supernatants before and after PE MP chronic exposure on intestinal gene expression using a Caco-2/HT29-MTX co-culture

Table 1. Primers used for qPCR for bacterial load, Illumina sequencing for bacteria and Archaeal populations and RT-qPCR to monitor intestinal gene expression

Table 2. Volatile organic compounds significantly altered by chronic exposure to PE MPs in the Tm-ARCOL

Section 3: General discussion

Figure 4.1. The PE MP digestion process in the TIM-1 model under adult or infant conditions

Figure 4.2. The SOLEIL synchrotron facility and the DISCO beamline

Figure 4.3. Analysis at the DISCO beamline of the initial PE MP powder and 1-hour gastric effluent in TIM-1

Figure 4.4. Simplified schematic representation of Raman Tweezer (on the left) and optical images (on the upper right) of a nylon fiber and a spheroidal PP particle dispersed in seawater with Raman spectra (on the lower right) of the optically trapped particles (blue and red, respectively)

Figure 4.5. RT analysis of a 1-hour gastric (A) and 4-hour intestinal effluent (B) samples from adult digestion in TIM-1

Figure 4.6. Approach and analytical methods to study the gut plastisphere

Figure 4.7. A triple co-culture model, incorporating an agar-mucin layer directly in contact with the bacterial suspension

Figure 4.8. Main results of the PhD work on the impact of PE MPs on the human digestive environment in adult and infant conditions

Abbreviations

A

ABC: ATP-binding cassette transporter

AhR: aryl hydrocarbon receptor

AMP: antimicrobial peptides

ASV: amplicon sequence variant

B

BSA: bovine serum albumin

Bw: body weight

C

CarbSIF: bicarbonate based simulated intestinal fluid

CFU: colony forming units

CH₄: methane

CLSM: confocal laser scanning microscopy

CoMiniGut: Copenhagen MiniGut

CONT: control

CO₂: Carbon dioxide

Cr: chromium

CTDM: Continuous Transfer Digestion Model

CYP: cytochrome

D

DEHP: di-(2-ethylhexyl) phthalate

DGM: Dynamic Gastric Model

DHSI-II: “near real” *in vitro* human stomach and small intestine system

DIDGI: Dynamic Gastrointestinal Digester

DMEM: Dulbecco/Vogt modified Eagle's minimal essential medium

DSC: differential scanning calorimetry

DSS: dextran sodium sulfate

DUV: deep ultraviolet

E

EFSA: European Food Safety Authority

EU: European Union

ESIN: Engineered Stomach and small Intestinal

EXP: exposed

F

FCS: fetal calf serum

FITC: fluorescein-5-isothiocyanate

FS: fresh stool

FTIR: Fourier transform infrared

G

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

GC-MS: gas chromatography-mass spectrometry

GDS: Gastric Digestion Simulator

GFS: frozen stool

GIS1: mono-compartmented system of *in vitro* colonic simulation

GIT: gastrointestinal tract

H

HBSS: Hank's balanced salt solution

HCl: hydrochlorhydric acid

HCM: Human colonic model

HDPE: high-density polyethylene

HGS: human gastric simulator

HIEC: human fetal small intestine

HMDS: hexamethyldisilazane

HMI: Host Microbiota Interaction

HPLC: high-performance liquid chromatography

HRP: horseradish peroxidase

HuMix: Human Microbial Cross-talk

H₂: products including dihydrogen

H₂S: hydrogen sulfide

I

IBD: inflammatory bowel diseases

IBS: irritable bowel syndrome

IL: Interleukine

ITS: Insulin Transferrin Selenium

IViDiS: In Vitro Digestive System

K

KEGG: Kyoto Encyclopedia of Genes and Genomes

L

LDPE: low-density polyethylene

LS: lyophilized stool

LY: lucifer yellow

M

MAMPs: microbe-associated molecular patterns

MBRAs: minibioreactor arrays

MCPI: monocyte chemoattractant protein-1

MCT1: monocarboxylate transporter1

MEDIS: Microbiology, Digestive Environment and Health unit

MEM- α : minimum essential medium Eagle alpha modification

MiCoMo: mini colon model

MIDA: Model of an Infant Digestive Apparatus

MP: microplastic

MP-VAT: Microplastic Visual Analysis Tool

MUCX: mucin X glycoprotein

M-ARCOL: Mucosal Artificial Colon

N

NA: numerical aperture

NEAA: non-essential amino acids

NGN: Neuro-Gastroenterology and Nutrition team

NLR: NOD-like receptor

N₂: dinitrogen

O

OCLN: occluding

OTU: operational taxonomic unit

OT: optical tweezer

P

PA: polyamide

PAH: polycyclic aromatic hydrocarbons

PAMPs: pathogen-associated molecular patterns

Papp: apparent permeability coefficient

PBS: phosphate buffer saline

PCB: polychlorinated biphenyl

PCA: principal component analyses

PCOA: Principal coordinate analysis

PE: polyethylene

PET: polyethylene terephthalate

PFAS: perfluoroalkylated substance

PLGA: poly(lactic-co-glycolic acid

PolyFermS: Polyfermentor Intestinal Model

PP: polypropylene

PRR: pattern recognition receptors

PS: polystyrene

PTFE: polytetrafluoroethylene

PU: polyurethane

PVC: polyvinyl chloride

R

RDA: Distance-based redundancy analysis

RFS: raw freezing stool

RI: retention indices

ROS: reactive oxygen species

S

SCFA: short-chain fatty acid

SGF: Simulated Gastric Fluid

SGFsp: SGF without pepsin

SHIME = Simulator of the Human Intestinal Microbial Ecosystem

SIMGI: Multicompartmental Dynamic Model of the Gastrointestinal System

SPME: solid-phase microextraction

T

TEER: transepithelial electrical resistance

TGA: thermogravimetric analysis

TIM-1: TNO gastrointestinal system-1

TIM-agc: TIM- advanced gastric compartment

TJ: tight junction

TLR: toll like receptors

Tm-ARCOL: Toddler mucosal Artificial Colon

Toxalim: Research Center in Food Toxicology unit

TSI: Smallest Intestine

U

USP-III: United States Pharmacopeia apparatus III

V

VOC: volatile organic compound

Z

ZO-1: zonula ocludens

Foreword

1. Context of the PhD

Due to the diversified properties of plastic materials, plastic production has increased worldwide since the 50's reaching 367 million tons in 2020 (PlasticsEurope 2021). Plastic is highly resistant to degradation leading to its accumulation in the environment if not reused or recycled. In the environment, plastics undergo slow breaking down processes releasing various smaller plastic particles. Those plastics can be classified according to their size. Microplastics (MPs) may be defined as “any synthetic solid particles or polymeric matrices, with regular or irregular shape and with size ranging from 1 μm to 5 mm, of either primary –voluntarily manufactured- or secondary –released from plastic debris weathering- manufacturing origin, which are insoluble in water” (Frias and Nash 2019). Because of their small size, these MPs can spread in all environmental media from marine to ground water, lakes, sediments, soil and even in the atmosphere. Inevitably, MPs can enter into the human food chain, with largely unknown effects. Furthermore, these MPs can be vector for several chemical and microbial contaminants. However, to date, the fate and impact of virgin or contaminated MPs in the human gastro-intestinal tract after oral exposure remain poorly understood. Barely addressed five years ago, this critical issue is now the subject of growing attention from the global scientific community. In the human gut environment, MPs can interact with different parameters including physico-chemical conditions during digestion, as well as gut microbiota, intestinal epithelium and mucus. While increasing work has already been carried out in rodents, scarce studies have been performed in *in vitro* models simulating the human digestive environment.

The present PhD work aims to explore the consequences of oral exposure to MPs on the human digestive sphere and the intestinal barrier, seen from the angle of the epithelium/mucus/microbiota gut players. This PhD work was performed in the frame of a partnership between the UMR MEDIS (Microbiology, Digestive Environment and Health, Université Clermont Auvergne-INRAE, Clermont-Ferrand) and Toxalim (Research Center in Food Toxicology, UMR INRAE, Université de Toulouse, ENVT, INP-Purpan, UPS, Toulouse) in the Neuro-Gastroenterology and Nutrition (NGN) team. MEDIS laboratory, led by Dr M. Desvaux and Dr. S. Blanquet-Diot, aims to better understand the role of human and animal gut microbiota in health and diseases. The team benefits from more than 25 years of internationally

recognized expertise and knowledge in the field of *in vitro* human gut simulation. The laboratory has developed a technological platform around the human and animal digestive environment associating *in vitro* digestion and fermentation tools, cellular models of the human intestinal epithelium, and molecular tools of genomics and post-genomics. The NGN team, led by Dr V. Theodorou, is specialized in the study of the intestinal barrier function and its modulation by environmental factors (food contaminants and additives, psychological and/or nutritional stress). The team has in-depth experience in digestive physiology and pathophysiology by combining various approaches from *in vitro* cellular models to complex *in vivo* models in rodents. This collaboration gathers the expertise of MEDIS in the human gut simulation and the one from Toxalim in the dynamics of the intestinal barrier to fully appreciate the tripartite relationship between epithelium, mucus and gut microbiota under environmental disturbance with oral exposure to MPs.

This PhD work was funded by a fellowship from the French ‘Ministère de l’Enseignement Supérieur et de la Recherche’ attributed to Université Clermont Auvergne, by a collaborative INRAE project (PLASTOX 2018-2020) and by the French National Research Agency (ANR-19-MRS2-0011 HuPlastiX project).

Beyond UMR MEDIS and Toxalim, and thanks to INRAE and ANR funding, the project also gathered several academic collaborators who performed additional analysis on virgin MPs (Regis Vaudemon, Luxembourg Institute of Science and Technology –LIST, Belvaux, Luxembourg), on samples from digestion (Marie-Hélène Ropers, Laboratory ‘Biopolymères, Interactions Assemblages’ –BIA-INRAE, Nantes), but also on samples from fermentation models (Fabienne Lagarde, Laboratory ‘Institut des Molécules et Matériaux du Mans’ –IMMM, Le Mans; Jeremy Ratel and Erwan Engel, Laboratory ‘Qualité des Produits Animaux’ –QuaPA-INRAE, Saint-Gènes Champanelle).

2. Research questions

In this context, this PhD work was dedicated to unravel for the first time the fate and impact of MPs in the simulated human digestive tract (both under adult and toddler conditions) by using complementary *in vitro* models of the gut lumen and intestinal epithelium. We chose to work with the most manufactured plastic polymer worldwide, namely polyethylene (PE) (PlasticsEurope, 2021), for all the experiments performed in the present study.

The objectives of the PhD work were as follows:

- (i) Evaluation of various stool conservative methods on gut microbiota composition and metabolic activities in our *in vitro* colonic model, Mucosal Artificial Colon (M-ARCOL), in order to select the most suitable method for the following experiments with PE MPs.
- (ii) Development of the Toddler mucosal Artificial Colon (Tm-ARCOL) from the existing M-ARCOL model, under specific digestive infant conditions. This gut model integrates the main physico-chemical (pH, transit time, nutritional sources) but also microbial (luminal and mucus-associated microbiota) parameters of the human colon of infant (in the age range between 6 months and 3 years).
- (iii) Evaluation of the impact of chronic exposure to PE MPs on the structure/activity of the human intestinal microbiota of adults and infants and assessment of the influence of mucus environment, using respectively M-ARCOL and the newly developed Tm-ARCOL system.
- (iv) Evaluation of the impact of luminal samples from M-ARCOL and Tm-ARCOL exposed microbiota of adults and toddlers on epithelial barrier integrity, mucin synthesis and inflammation, using a co-culture of Caco-2 and mucus-secreting HT29-MTX cells as an *in vitro* model of human intestinal epithelium.

3. Manuscript organization

The first section is a literature review, which provides a complete overview of the current knowledge on human exposure to MPs with a focus on the human gastro-intestinal tract as portal of entry. In this first section, **Part 1** provides a detailed description of the human gastro-intestinal tract with a focus on the specificities of toddler digestion, redrafted from our published review in the *Biotechnology Advances* journal. In **Part 2**, the currently available artificial digestive systems are presented. Lastly, **Part 3** sheds light on MPs-contaminated food items and the subsequent various sources of exposure in human. This part also describes the current knowledge regarding the fate and toxicological effects of virgin or contaminated MPs after oral exposure in mammals but also with a special emphasis on *in vitro* approaches with, in particular, the use of artificial digestive systems. These data were extracted from our published review (*Journal of Hazardous Materials*) and book chapter (*Springer Nature*) and actualized accordingly.

The second section presents the results of each step of the experimental work. **Chapter 1** presents the impact of conservative methods on microbial structure and function in the M-ARCOL model. This work, begun during my master's degree internship in UMR MEDIS, was extended during my PhD and published in the *Applied Microbiology and Biotechnology* journal. **Chapter 2** deals with oral exposure to PE MPs (in their virgin form) and their impact on the human colonic microbiota of adults published in *Journal of Hazardous Materials*. **Chapter 3** introduces the development and validation of the toddler M-ARCOL colonic model (Tm-ARCOL). This part of the work has been published in the *Applied Microbiology and Biotechnology* journal. Based on this newly-developed model for toddlers, **Chapter 4** investigates the impact of PE MPs on this at-risk population (*Journal of Hazardous Materials*, published). Chapters 2 and 4 also zoom on the epithelium and mucus gut players and display the experiments carried out on human intestinal cell lines in co-culture, incubated with luminal supernatants from *in vitro* M-ARCOL and Tm-ARCOL gut models.

The third section is a general discussion and gives perspectives of the current work.

4. Outline of the PhD

The thesis work was distributed as follows: the first 24 months took place in Clermont-Ferrand, within the UMR MEDIS, where the *in vitro* assays in the M-ARCOL and Tm-ARCOL models were conducted, while the next 6 months were carried out in Toulouse within the Toxalim unit, to perform *in vitro* cell culture experiments. Then, the 6 last months of the PhD work were at UMR MEDIS to perform all bio-informatic and biostatistical analyses as well as manuscript writing (**Fig. 0**).

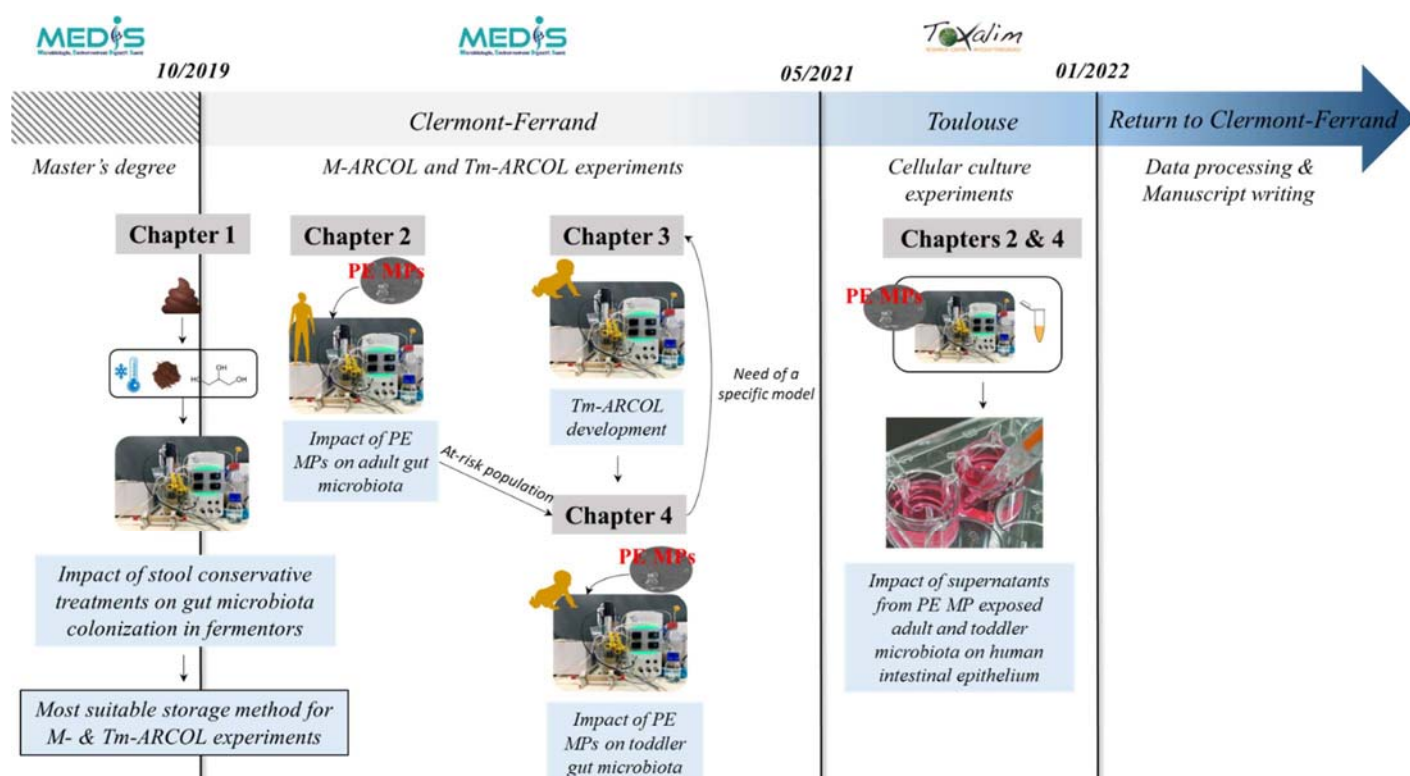


Figure 0. Outline of the experimental work during the PhD

The main stages of the experimental work are summarized in this figure. The different chapters correspond to chapters of the results section. From October 2019 to May 2021, experiments were performed at UMR MEDIS (Clermont-Ferrand), while from May 2021 to January 2022, experiments were conducted at Toxalim (Toulouse).

M-ARCOL: Mucosal Artificial Colon; MPs: microplastics; PE: polyethylene; Tm-ARCOL: Toddler mucosal Artificial Colon

Section 1: Literature Review

Part 1 - The human gastro-intestinal tract: from birth to adulthood

The following sections are dedicated to the definition of the human gut physiology. Focus will be successively brought on gastric and intestine parts of the human gastrointestinal tract (GIT), introducing their abiotic and microbial factors and epithelium characteristics. A specific focus on the infant digestion will be given throughout this part. Moreover, it is worth to note that some parts of this state of the art have been published in a review article in the journal *Biotechnology Advances* and redrafted / updated for the present section.

REVIEW: FOURNIER E, ROUSSEL C, DOMINICIS A, LEY D, PEYRON M-A, COLLADO V, MERCIER-BONIN M, LACROIX C, ALRIC M, VAN DE WIELE T, CHASSARD C, ETIENNE-MESMIN L, BLANQUET-DIOT S. *In vitro* models of gut digestion across childhood: current developments, challenges and future trends. *Biotechnol Adv.* 2022 Jan-Feb; 54:107796. doi: 10.1016/j.biotechadv.2021.107796.

1. Abiotic physiology of the human gut

The human digestion is a multi-step process including mechanical and chemical breakdown by which foods are converted into organic nutrients that can be absorbed and assimilated by the body. Thus, the digestive tract is formed by a group of hollow organs from the mouth to anus and ancillary solid organs (e.g. pancreas, liver and gallbladder) (**Fig. 1.1**).

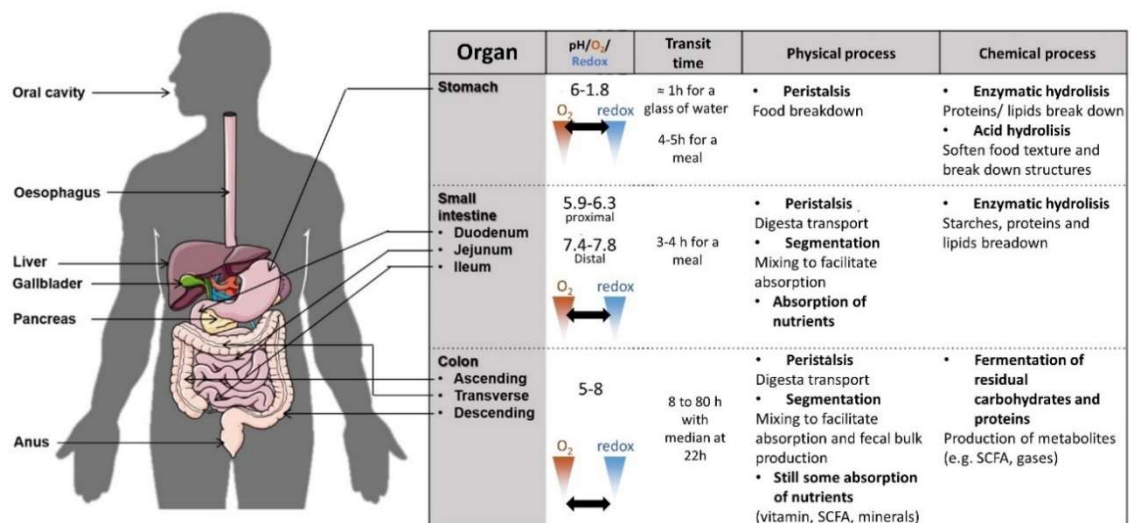


Figure 1.1 Gastrointestinal tract of healthy adults: summary of the key physical and chemical processes

SCFA: Short Chain Fatty Acids.

Modified from Bornhorst and Singh 2014.

These organs are involved in the ingestion, digestion and elimination of foods (<https://www.iffgd.org/>, consulted on 03/2021). The abiotic factors of the GI digestion of a healthy adult, starting from the stomach to the large intestine (colon) will be detailed in the following section. As aforementioned, the evolution of the abiotic factors of GI digestion from birth to adulthood will be described in each section of this part and are summarized in **Fig. 1.2**.

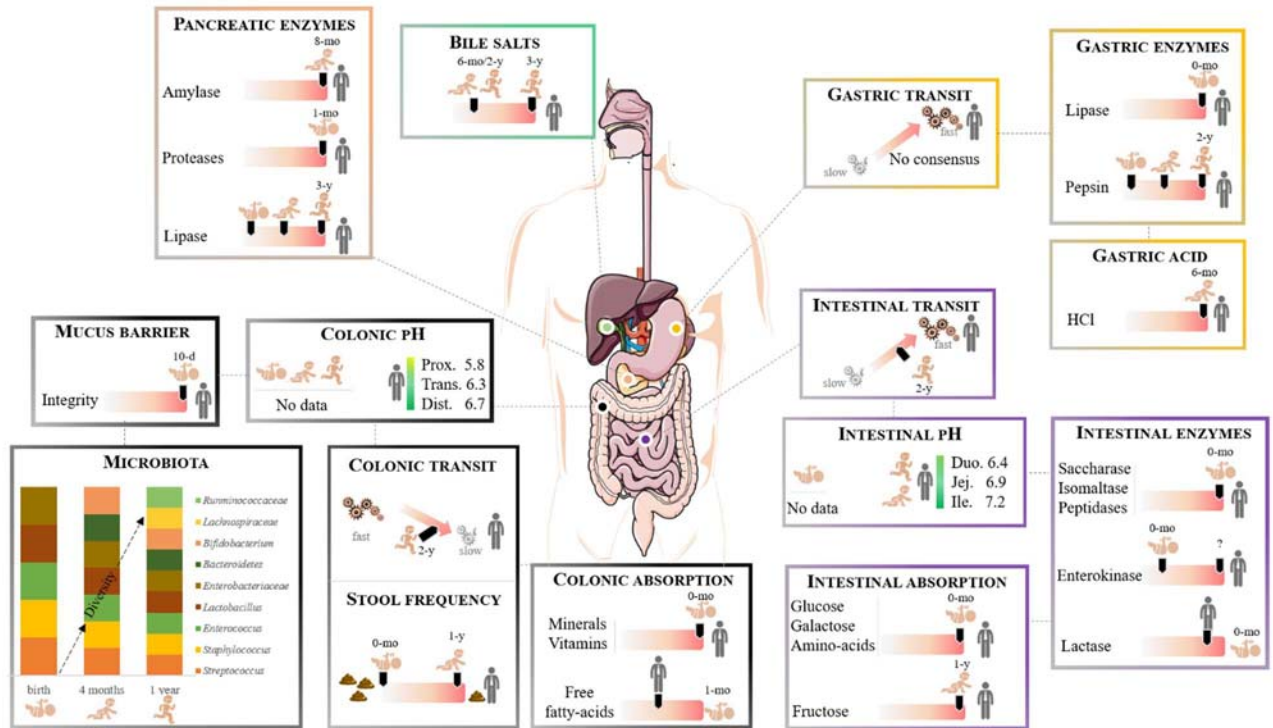


Figure 1.2 Gastro-intestinal development across childhood

Key parameters of the gastric, intestinal and colonic digestion/fermentation are summarized from birth up until 3 years of age. Lack of data are represented by “?”.

D: days; Dist: distal colon; Duo: duodenum; HCl: Hydrochloric acid; Jej: jejunum; Ile: ileum; Mo: months; Prox: proximal colon; Trans: transverse colon; Y: years.

Extracted from Fournier et al. 2021.

1.1 Oral phase

Often neglected, the first stage of digestion is the oral phase. In the mouth, chewing mixes the food with saliva and the mechanical process of digestion begins. Saliva contains digestive enzymes called amylase and lingual lipase, secreted by the salivary and serous glands on the tongue. These enzymes respectfully begin to degrade starch and lipids. These processes produce a bolus, which can be swallowed down through the esophagus to enter the stomach.

At birth, the orofacial and cervical anatomy is adapted to the sucking-swallowing function (Delaney and Arvedson 2008; Silva et al. 2016). During child development, the tongue gets

into a lower position as the pharynx grows, promoting the initiation of tongue's lateral movements and allowing mastication maturation (Le Révérend et al. 2014). Moreover, maxilla and mandible growths increase the oral cavity space to fracture larger pieces of food and supports increased bite and masticatory forces. The eruption of anterior primary teeth between the age of 6-8 and 18 months is an important step of oral function evolution. Around 3 years old, the complete occlusion contacts between primary molars allow the mandible stabilization (Le Révérend et al. 2014) (**Fig 1.3**). **Figure 1.3** summarizes the key parameters of the oral phase from birth up till 3 years of age.

It is also important to note that saliva undergoes manifold changes closely dependent on tooth eruption and food transition from milk to progressive solid food as evidences with changes in sodium, potassium and chloride quantities (Ben-Aryeh et al. 1984), changes in mucin secretion with a shift in predominance from MUC7 to MUC5B (Ruhl et al. 2005), as well as changes in α -amylase and lingual lipase (Fredrikzon et al. 1982; Ben-Aryeh et al. 1984). Saliva in babies under 1 month is highly viscous with a very low flow rate that increases with age to reach 0.37 mL/min in adults (Sinor et al. 2009). Saliva is also slightly more acidic at birth (pH 6) than in adults (pH 6.33) (Ben-Aryeh et al. 1984) (**Fig 1.3**).

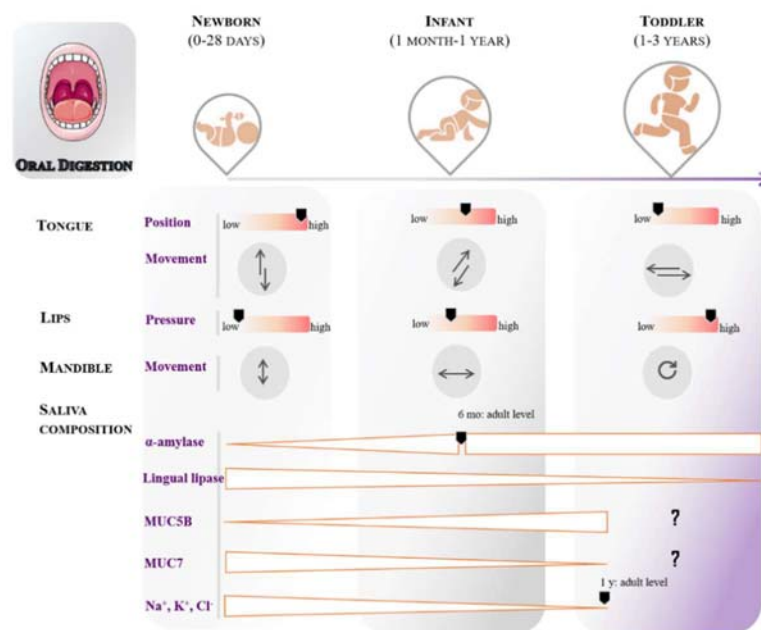


Figure 1.3 Oral digestion development across childhood

Key parameters of the oral phase are summarized according to the available literature data from birth up till 3 years of age. Lack of data are represented by “?”.

Mo: months; Y: years.

Extracted from Fournier et al. 2021.

1.2 Stomach

1.2.1 Anatomy and physiological parameters

The stomach is divided in three main anatomical regions: the fundus, the body and the antrum (Boland 2016). Because the upper part of the stomach (fundus) is highly distensible, the stomach can hold about one liter of food (Ferrua et al. 2011a). Two sphincters keep the contents of the stomach contained; the lower esophageal sphincter namely cardia at the junction of the esophagus and stomach, and the pylorus at the junction of the stomach with the duodenum. The stomach is not depleted in oxygen and is thus considered as an aerobic compartment. As explained below, the gastric pH depends on the digestion phase. The oxydo-reduction (redox) potential, which reflect the affinity of electron transfer to or from a chemical species in solution, is around +200mV (Obrenovich et al. 2020). Its temperature is steadily maintained at 37°C as in all subsequent GI segments (Koziolek et al. 2015) and will thus be no longer mentioned.

Globally, the stomach is about 25 cm length and 0.9 L volume (Ferrua et al. 2011b). After ingestion of a solid meal, the total gastric emptying could last around 4 to 5 h while the gastric emptying time for a simple glass of water will last only 13 min (Mudie et al. 2014). However, for a meal, gastric emptying time varies a lot between individuals (e.g. gender, ethnical origin, age-range) and according to meal consistency and composition (Boland 2016; Grimm et al. 2018). Gastric pH decreases gradually and continuously from 5 to 1.8 during adult digestion (Roussel et al. 2016a) but fluctuates greatly according to several factors (similar to those described for gastric emptying such as meal composition/consistency, gender, ethnical origin, age-range). In fasted state, gastric pH is considered to maintain around 2 while food intake tends to increase the pH before counteraction by HCl secretion (Barlow et al. 1994).

Only few data regarding infant stomach are available. At birth, the average stomach length is 7 cm (Osemlak et al. 1982) to finally reach 25 cm at around 23 years old (Begum et al. 2013). Gastric emptying time of newborns seems longer compared to adults despite the absence of consensus. In newborns, it extends from 10 to 100 min following milk consumption with a mean half-time emptying of 87 min (Signer 1975). Besides, formula-fed infants displayed a longer gastric emptying time compared to breast-fed infants (78 vs 48 min), due to a greater amount of casein in formula (Cavell 1979). Full-term infants have a mean pH of 3.5 (ranging from 2.0 to 6.1) before meal ingestion. This high pH range could be explained by a low acid production by infant parietal cells combined with the high buffering capacity of milk

(Dallas et al. 2012). Moreover, it is important to note that like in adults, gastric pH increases after food ingestion in child, but remains higher for a longer period (Hamosh 1996) (**Fig. 1.2**).

1.2.2 Digestive functions

Following ingestion of liquids, the average gastric emptying is around 13 minutes as evaluated by magnetic resonance (Mudie et al. 2014). On the contrary, after ingestion of a solid meal, the total gastric emptying lasts around 4 to 5 h, with considerable variations between individuals and according to the composition of the food ingested (Boland 2016; Grimm et al. 2018). Solid components remain in the stomach until they are small enough to be slowly released into the small intestine (Ramsay and Carr 2011). Also, it should be noted that this heterogeneity of the gastric emptying is conserved within one meal in which particles larger than 1–2 mm are the last ones to reach the duodenum (Siegel et al. 1988). Such aspect is important because the stomach is the last process in the GIT that has a mechanical function to break down particles. The proximal stomach is thought to act as a food reservoir, while the distal stomach is the main location of the physical breakdown of foods into particles resulting in chime formation. In fact, the peristaltic movements of the gastric wall act to crush and grind food particles (Schulze 2006).

The stomach secretes on a daily basis around 2-3 liters of gastric juice (e.g. mucus, acid, ions, enzymes, and intrinsic factors) (Johnson et al. 2012; Sherwood and Ectors 2015), actively participating in the digestion process. Hydrochlorhydric acid (HCl) secretion allows the gastric pH to stay acidic (Boland 2016). However, gastric pH is dependent of many parameters and especially the feeding status. In fasted individual, the gastric pH is considered to stay around 2, since food intake tends to elevate the pH, that in turn has to be counteracted by the HCl secretion (Barlow et al. 1994). Such acidic environment inhibits bacterial growth (Beasley et al. 2015) and allows the action of digestive proteases (Gore and Levine 2007). Among them, pepsin is an endopeptidase released in its inactive form pepsinogen and then converted into its active proteolytic form upon contact with HCl. Pepsin is then able of hydrolyzing peptide bonds (e.g. phenylalanine, tyrosine and leucine) of most proteins (Fruton 2002). The gastric lipase also takes part in the gastric digestion process and especially in lipids breakdown accounting for 10-30% of dietary triglyceride hydrolysis (Gallier and Singh 2012). As gastric acid pH would damage the stomach wall, mucus is constantly secreted by innumerable gastric glands to provide a slimy protective layer (Boland 2016).

Lipid digestion in the stomach is a critical step for newborn to offset the lack of pancreatic lipases and bile acid production (Hamosh et al. 1981). At birth, gastric lipase activity already reaches adult level (Lindquist and Hernell 2010) and fat gastric digestion leads in newborn, similarly to adults, to the hydrolysis of 10 to 30% of total esterified fatty acids (Mu and Høy 2004). On the contrary, protein digestion is impaired in newborns due to the combined effect of low pepsin secretion (at 3 months pepsin concentration represents half of the adult one) and activity (only 13% of adult level at birth) and higher post-prandial gastric pH compared to adults (Agunod et al. 1969; Bourlieu et al. 2014) (**Fig. 1.2**). Despite the absence of studies related to mucus secretion and characteristics in child, newborns seem more vulnerable to gastric acidity, which could be linked to a lower gastric mucosa thickness during the 3 first weeks of life (Bourlieu et al. 2014). To favor protein and lipid digestion by gastric pepsin and lipase, the stomach secretes HCl to maintain a low pH.

1.2.3 Gastric cell lining

Different cell types participate in gastric secretions (Johnson et al. 2012; Sherwood and Ectors 2015). The faveolar cells found in the superficial phase of the gastric mucosa and the mucous neck cells in the crypt are the responsible for mucus secretion. Parietal cells secrete HCl (Boland 2016). The chief cells are involved in the enzymatic secretion of pepsinogen and gastric lipase (Gallier and Singh 2012). Finally, deeper in the crypts, G cells secrete hormones such as gastrin, ghrelin, somatostatin or cholecystokinin mediating the overall digestion process (Vasavid et al. 2014).

1.3 Small intestine and colon

1.3.1 Small intestine

1.3.1.1 Anatomy and physiological parameters

The human small intestine, separated into three sections (e.g. duodenum, jejunum and ileum), extends from the pyloric sphincter to the ileo-caecal junction, representing 6-7 m long, the longest part of the digestive tract. The duodenum, jejunum and ileum segments are respectively around 1 m, 2.5 m and 3.5 m in length. The total transit time in the small intestine is around 3 to 4 h (Yuen 2010; Boland 2016). Compared to the stomach, which is considered as an aerobic environment, small intestine is depleted in oxygen (30-50 mmHg O₂) (Zheng et al. 2015). The redox potential becomes negative favoring anaerobic bacterial metabolism.

Values are in ranges between -65 and -200 mV (Boland 2016). pH is comprised between 6 and 8 depending on the segment considered, with huge inter-individual variations (Johnson et al. 2012; Boland 2016). The proximal small intestine has been shown to be more acidic, with pH values between 5.9 and 6.3, while the distal small intestine appears a bit more alkaline with pH values ranging from 7.4 and 7.8 in fasted individuals (Koziolek et al. 2015).

The length of the small intestine is increasing from birth (275 cm) to reach 400 cm at 4 years old (Weaver et al. 1991). All intestinal cell types are fully developed at birth (Moxey and Trier 1978). There is no available data on how absorption surface area and villus length evolve in the first years of age in child. No data regarding the redox potential as well as the pH of the small intestine of infants can be find.

1.3.1.2 Digestive functions

In the small intestine, food bolus is propelled forward by contraction and relaxation movement, termed peristalsis and occurs thanks to longitudinal smooth muscle contractions. Meanwhile, segmentation movements by circular smooth muscle contraction/relaxation enable chyme mixing and increase intra-luminal contact time which aids in digestion, secretion, and absorption (Kumral and Zfass 2018).

The duodenum secretes bicarbonate to neutralize gastric acid and provides an appropriate pH for further enzymatic digestion to occur. It receives secretions of enzymes and bile from the pancreas and liver, respectively (Vasavid et al. 2014). Briefly, in response to food ingestion, the exocrine pancreas secretes a pancreatic juice containing fluids, bicarbonate and diverse enzymes as lipases, proteases and amylases. Some of these enzymes, as trypsin, are secreted in an inactive form and are activated once delivered into the duodenal lumen (Chandra and Liddle 2014). Bile is produced in the liver, stored in the gallbladder and released into the duodenum. It is a complex mixture of bile acids, cholesterol, pigments, lecithin and mineral salts. In the small intestine, bile acids assist in the emulsification and absorption of fatty acids. They also stimulate lipolysis by facilitating binding of pancreatic lipase with its co-lipase (Chandra and Liddle 2014), which results in the formation of small particles of emulsified fats called micelles. Following action of digestive enzymes and bile acids, hydrolyzed nutrients (mono- and disaccharides, amino acids, small oligopeptides and micelles) are ready for uptake. Altogether, the digestion in the luminal phase of the small-bowel is complete for the lipids only, while saccharides and peptides are not all hydrolyzed to single residues. Intestinal epithelial cells will achieve carbohydrates and proteins digestion. Among intestinal cells, enterocytes express on

their apical surface hydrolytic enzymes to perform terminal digestion of polysaccharides and peptides (e.g. enterokinase, aminopeptidase), in concertation with enzymes present in the luminal phase. The majority of nutrient absorption occurs in the jejunum but is completed in the ileum. The distal ileum is the only site where bile salts are re-absorbed (Johnson et al. 2012).

Pancreatic amylase is not detectable before the first month of life and reaches adult levels by 8 months old (Otsuki et al. 1977). On the contrary, intestinal saccharase-isomaltase is already present at birth (Triadou and Zweibaum 1985). In response to milk feeding, intestinal lactase reaches its maximal activity at birth and thereafter decreases to 5-10% of its initial activity during childhood and adolescence (Drozdowski et al. 2010). The expression of pancreatic proteolytic enzymes reaches adult levels few weeks after birth. Duodenal enterokinase, the enzyme activating trypsinogen into trypsin, which in turn activates pancreatic proteases, shows at birth 20% of the activity found in infants and toddlers (Antonowicz and Lebenthal 1977). Pancreatic lipase displays an activity that remains low at birth to increase from 10 weeks of life and reach the adult one by 3 years of age (Cleghorn et al. 1988). At birth, bile salts concentrations in duodenal juice remain very low, representing in average 1.8 mmol/L under 2 days of age (**Fig. 1.2**). Bile composition switches from mainly taurine-conjugated bile salts to glycine-conjugated bile salts after 10 days (Challacombe et al. 1975). This low bile acids pool could explain fat malabsorption in young infants leading to a greater fat delivery into the colon (Murphy and Signer 1974; Watkins et al. 1974). During the first week of life, cholic acid is the major primary bile acid with a 2 to 5:1 ratio with chenodesoxycholic acid, the other main primary bile acid. However, during the first month of life, this proportion falls to 1-2:1, which is also the ratio found in adults (Murphy and Signer 1974).

In the small intestine, glucose and galactose released from lactose digestion are absorbed by enterocyte transporters that are already expressed in newborns. All amino-acid transporters are functional at birth (Malo 1991; Drozdowski et al. 2010) (**Fig. 1.2**). Similarly, the predominant apolipoprotein 48, forming chylomicrons with phospholipids and triglycerides and involved in lipid absorption, is early expressed during gestation (Glickman et al. 1986). Moreover, due to the weak bile salt pool during the first 2 months, absorption of fatty acids and liposoluble vitamins can be impaired (Hamosh et al. 1981).

1.3.2 Colon

1.3.2.1 Anatomy and physiological parameters

The colon, with a significant microbial mass, extends from the ileocecal junction to the anal canal, with a larger diameter, but shorter length (1.5 m) compared to the small intestine and is divided into four sections (ascending, transverse, descending and sigmoid colon). The colonic transit time showed a median of 21.6 h (Rao et al. 2009), with variations according to dietary intakes and individuals. Hence, it fluctuates between 3 to 5 h in the ascending colon, 0.2 to 4 h in the transverse colon and 5 to 72 h in the descending and sigmoid colon (Wilson 2010). The colon is even more anaerobic environment than the small intestine (<10 mmHg O_2), (Zheng et al. 2015) and the redox potential becomes further negative (-415 mV in the ascending, -400 mV in the transversal and -380 mV in the descending colon) (Wilson 2010; Boland 2016). These two parameters favor more microbial growth and metabolism than in the small intestine (Obrenovich et al. 2020). Due to the huge microbial activity, pH should decrease in the colon, but a bicarbonate secretion in the ascending colon counterbalances it (Boland 2016). pH electrodes implanted on the colon wall during colonoscopy in areas free of debris indicate that patients with a normal bowel have a more acidic right colon (pH 7.05 ± 0.32), followed by a more alkaline transverse colon (pH 7.42 ± 0.51), becoming more acid moving towards the rectum (pH 7.15 ± 0.44). The lumen pH mirrors the changes of the wall, but remains more acidic (average value of 6.5) and with more inter-individual variations going from 5 to 8 in the ascending colon and 6 to 8 in the transversal colon (McDougall et al. 1993; Wilson 2010; Koziolok et al. 2015).

After birth, 6 months old children exhibit a colon of $568 \text{ mm} \pm 27 \text{ mm}$ in average, reaching $1224 \text{ mm} \pm 57 \text{ mm}$ in average at 4 years old. The elongation process stops around 5 years old with a length similar to adults (Wozniak et al. 2019). After 2 years old, the mean colonic transit time is not significantly different from adults (mean 31.78 h from various studies) as measured with radio-opaque plastic markers according to a meta-analysis (Southwell et al. 2009). Between birth and 2 years old, colonic transit time and stool frequency decrease, within a mean of 4 stools per day at 1 week old, 2.2 after 1 month and 1.7 stools after 1 year (Lemoh and Brooke 1979). In addition, stool frequency differs with diet, breast-fed infant producing daily fewer dry feces weight than formula-fed infant (Sievers et al. 1993). No data reported the colonic transit time nor the redox potential of each specific segment of the colon in infants. Regarding infant colonic pH, no data is available and only fecal pH value was

reported. Fecal pH varies a lot with age after birth and between individuals according to the evolution of both diet and gut microbiota composition and activity (Bullen et al., 1977; Henrick et al., 2018). It gradually increases with age, with an average fecal pH at 3 days around 5.3 ± 0.6 , reaching 5.7 ± 0.7 for 6-month infant (George et al. 1996), then 5.8 ± 1.1 between 12 to 18 months (Edwards et al. 1994), to be ultimately similar to that of healthy adults with a value around 6.6 ± 0.3 (Osuka et al. 2012) (**Fig. 1.2**).

1.3.2.2 Digestive functions

Peristaltic movements occur in the colon to propel forward the excreta to reach the rectum and exit via defecation. Mixing of watery waste also occurs by segmentation (every 30 minutes) and mass movements (1 to 3 times per day) (Rao et al. 2009). The colon processes indigestible watery-food waste to produce fecal bulk, which in turn is an important determinant of bowel health (Johnson et al. 2012). In the colon, the colonic anaerobic microbes will enable fermentation of food particles partially digested or previously undigested. As only lipid digestion is completed in the luminal phase of the small intestine, these particles contain carbohydrates, but also proteins, particularly when such macronutrients are ingested in high quantities (Yao et al. 2016). The fermentation process of these compounds produces gaseous products including dihydrogen (H_2), Carbon dioxide (CO_2), for some individual's methane (CH_4), and short-chain fatty acids (SCFAs). The three most abundant SCFA, acetate, propionate, and butyrate are playing a primary role in host energy metabolism. The fermentation of proteins also results in the production of hydrogen sulfide (H_2S), ammonia, N-nitroso, amines, phenolic and indolic compounds (Yao et al. 2016). One of the main functions of the colon is to absorb water, electrolytes and short chain fatty acids (SCFA) resulting from microbial fermentation, but also to maintain normal fluid homeostasis.

In particular, newborns display immature small intestinal absorption functions (Pácha 2000). In particular, neonates have a greater nutrient absorptive capacity in the colon than adults, due to the presence of apical brush border hydrolases in the colonic mucosa during the first week of life (Lacroix et al. 1984; Pácha 2000).

1.3.3 Small intestine and colonic cell lining

1.3.3.1 Intestinal epithelium structure and functions

The intestinal epithelium is a complex layer representing the largest epithelium of the body's mucosal surfaces, with an average surface of 32 m², with approximately 2m² referring to the large intestine (Helander and Fändriks 2014). The intestinal epithelium is formed by a single layer of polarized cells organized into crypts and villi in the small intestine but not in the colon. Different cell types compose this layer, with two main functions, digestion/absorption and protection against invaders. The major cell types include enterocytes (termed colonocytes in the colon), goblet cells, Paneth cells and enteroendocrine cells (**Fig. 1.4**). Enterocytes are the only cells with digestive and absorptive functions. The three others cell types are specialized for maintaining the digestive or barrier functions of the epithelium. Intestinal epithelial cells are continuously replaced every 4-5 days, and new cells are produced by stem cells located in crypts. In addition, M cells are found in the ileum and are associated with the immune system (Peterson and Artis 2014).

The functions of each cell types are succinctly described:

- (i) Enterocytes (colonocytes in the colon) – They are the most numerous cell type, covering 80% of the villi in the small intestine, where they express on their apical surface hydrolytic enzymes to perform terminal digestion of polysaccharides and peptides (e.g. enterokinase, aminopeptidase), in concertation with enzymes present in the luminal phase. These cells play an important role in nutrient absorption (e.g. ions, water, mono- and disaccharides, amino acids, oligopeptides, lipids, SCFA), and in secreting immunoglobulins.
- (ii) Goblet cells – Cover around 10% of all intestinal epithelial cells (25% in the colon). They are specialized in the synthesis and continuous secretion of mucus (Birchenough et al. 2015). The mucus layer on itself has a very particular dual function in intestinal physiology. In accordance to the classification of goblet cells into protective cells, the mucus layer controls microbiota interactions with its host by acting as a barrier. However, the mucus layer also represents a nutrient niche favoring microbe maintenance and growth. Details about the structure and functions of the mucus layer in health and disease will be thus provided (see below *paragraph 1.3.4*).

(iii) Paneth cells – Only found in the small intestine, especially in the ileum, and preferentially at the crypt. They synthesize and secrete antimicrobial peptides (AMP) and proteins such as defensins and lysozymes.

(iv) Enteroendocrine cells – Release intestinal hormones or peptides (e.g. glucagon, cholecystokinin) into the bloodstream. They are also known to act as chemoreceptors, detecting harmful substances and initiating protective responses (Van der Flier and Clevers 2009; Kong et al. 2018).

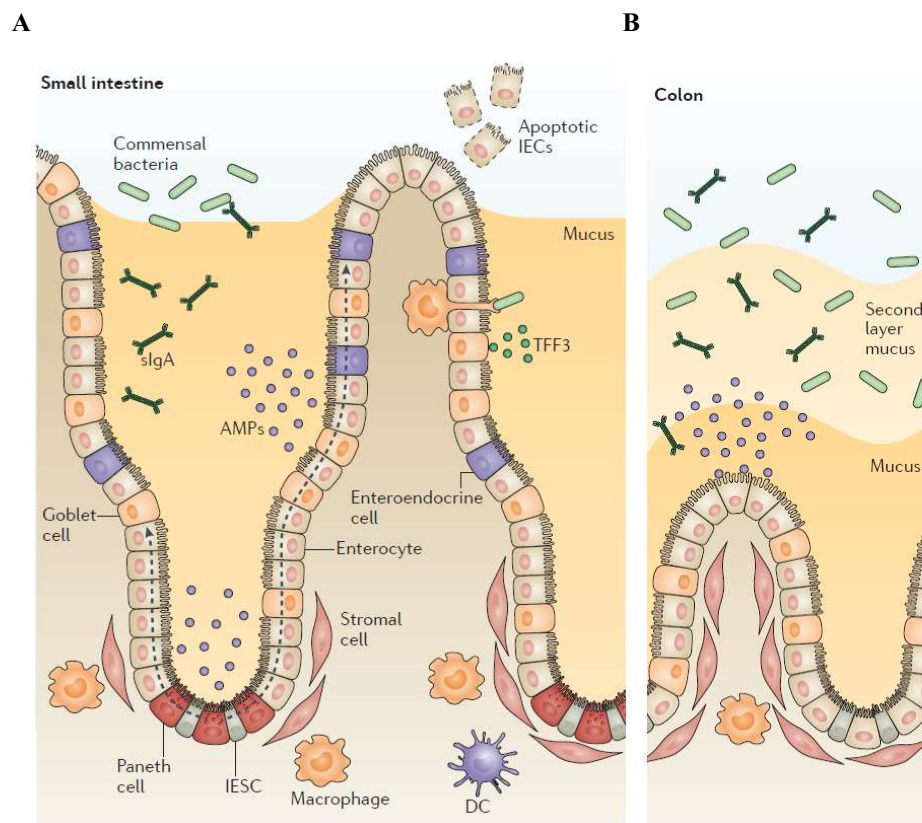


Figure 1.4 Cell lining of the small intestine (A) and colon (B)

Intestinal epithelial cells form a biochemical and physical barrier between the lumen and underlying mucosa. The intestinal epithelial stem cell (IESC) niche controls the continuous renewal of the epithelial cell layer by crypt-resident stem cells. Secretory goblet cells and Paneth cells secrete mucus and antimicrobial peptides (AMP). The transcytosis and luminal release of secretory Immunoglobulin A (SIgA) further contribute to this barrier function. The Trefoil factor 3 (TFF3) is a protein involved in the maintenance and repair of the intestinal mucosa.

AMPs: antimicrobial peptides, DC: dendritic cells, IEC: intestinal epithelial cells, IESC: intestinal epithelial stem cells, IgA: immunoglobulin A, TFF3: Trefoil factor 3.

Modified from Peterson and Artis 2014.

1.3.3.2 The tight control of intestinal permeability

The intestinal epithelium has two functions, which at first sight could seem contradictory. If the epithelium must act as an efficient barrier to protect the host, it must also allow exchanges of compounds like nutrients with the luminal environment. There are several pathways, tightly controlled, for luminal compounds to cross the intestinal epithelium. Such pathways should avoid exposing the host to harmful component like antigens, endotoxins, pathogens, microplastics and other pro-inflammatory substances. Depending on the size, hydrophobicity, and other chemical characteristics of the compound different pathways can be considered. Small hydrophilic and lipophilic compounds can use the transcellular route to cross the plasma membrane of the enterocytes/colonocytes. Transport of amino acids, vitamins, and sugars through enterocytes/colonocytes requires energy consuming epithelial transporters (active transport) (Said 2013). In the colon, SCFAs can use either passive diffusion and active transport mechanisms which are related to various ion exchange transporters (Velázquez et al. 1997). Finally, ions, water, and larger hydrophilic compounds (from 400 Da to 10–20 kDa) use the paracellular route to cross enterocytes/colonocytes (Farré et al. 2020).

There, key players are the different kinds of intercellular junctions including tight junction, *adherens* junction, gap junction, desmosome, and hemidesmosome. Particularly, the tight junction proteins play leading roles in paracellular permeability and finely regulate this route. Tight junctions are dynamic cell-to-cell adhesion complexes that polarizes the intestinal epithelium. They are comprised of four unique groups of transmembrane proteins: claudins, occludins, *zonula occludens* protein and junctional adhesion molecules (Laukoetter et al. 2006). These networks of transmembrane proteins interact between each other and link laterally adjacent cells near the apical surface of the epithelium (Zihni et al. 2016). Thus, the expression levels of these proteins are related to the intestinal barrier integrity (Zeissig et al. 2007; Ahmad et al. 2014; Chelakkot et al. 2018). At least, tight junctions regulate two types of pores. The first one is a high capacity and charge selective pore, which is permeable to small ions and small-uncharged molecules (also known as “pore” pathway). The second one is a much larger pore with low capacity (also known as the “leak” pathway), which is permeable to large ions and molecules regardless of their charge. Mainly claudin proteins regulate the “pore pathway” and in contrast, occludin and *zonula occludens* proteins regulate the “leak pathway”. The permeability of both pathways can be measured using different methods. The transepithelial electrical resistance (TEER) measures the net movement of all ions (cations and anions) through the epithelium. TEER reflects not only the contribution of the paracellular resistance regulated

by tight junctions, but also the transcellular and the sub-epithelial resistance. The permeability of the paracellular leak pathway can be specifically assessed by measuring the flux of large molecules from 4 up to 20 kDa across the epithelium. Alterations in the paracellular pathway are supposed to be relevant in the pathogenesis of several GI diseases as inflammatory bowel diseases (IBD) and metabolic diseases (Zeisel et al. 2019; Farré et al. 2020).

1.3.4 Intestinal mucus

1.3.4.1 Structure

The intestinal mucus is continuously produced and secreted by goblet cells. The goblet cell-to-enterocyte ratio changes along the intestinal tract, with an estimated percentage of goblet cells in the intestinal epithelium of approximately 4% in the duodenum, 6% in the jejunum, 12% in the ileum and 16% in the distal colon. The intestinal mucus also varies in terms of structure and composition according to the considered species (Hugenholtz and de Vos 2018; Etienne-Mesmin et al. 2019b). In human, mucus is found throughout the entire GIT of human from stomach to large intestine, with its thickness and structure varying depending on the segment of the digestive tract considered, but also with cross-sectional differences (**Fig. 1.5**). In the colon, the mucus layer shows a double-layer structure, with an inner layer firmly, for a long time considered attached to the epithelium, and an outer layer superimposed to the first one, constantly shed into the lumen and showing an expanded volume due to proteolytic activities provided by the host but also by commensal bacteria (Atuma et al. 2001; Ijssennagger et al. 2016). Of note, a study revealed differences in mucus organization between the proximal and distal colon of rodents (Kamphuis et al. 2017): in the latter, the mucus layer is attached to the fecal pellet and absent from the surface of the epithelium (Kamphuis et al. 2017).

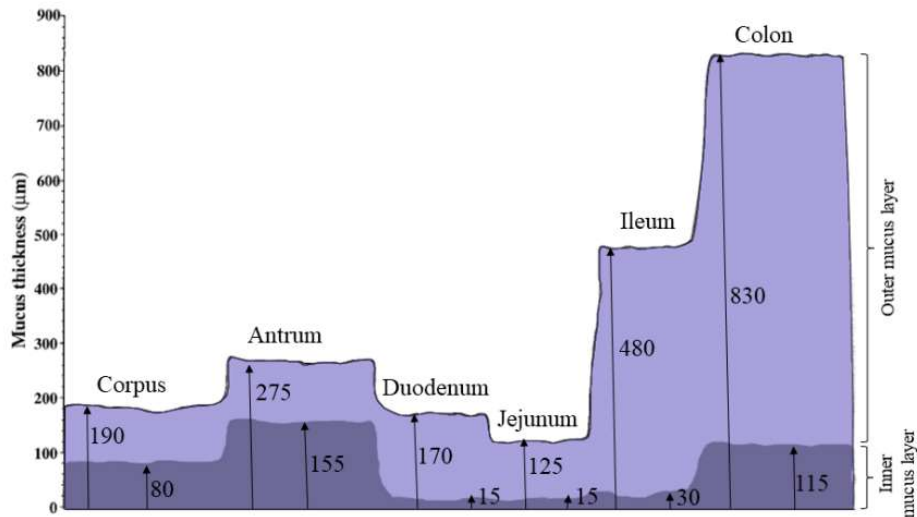


Figure 1.5 Mucus thickness along the human gastro-intestinal tract

Both outer and inner mucus layer thickness are represented all along the digestive tract. Modified from Atuma et al. 2001.

Mucus is a complex viscoelastic adherent secretion composed of water, electrolytes, lipids and the main structural components (around 5%) glycoproteins, which are called mucins. Mucin as polymer consists of a polypeptide backbone with glycan side chains. The core region of the glycans is formed by a combination of three sugars, galactose, N-acetylgalactosamine and N-acetylglucosamine, to which different chains of glycans can be attached. The terminal monosaccharide is usually fucose or sialic acid (Holmén Larsson et al. 2009; Juge 2012). Oligosaccharide chains can also be sulfated, especially in colonic regions (Rho et al. 2005). The glycan moieties are conjugated to proteins, mostly by O-link to serine and threonine but also by N-link to asparagine (Porter and Martens 2017). To date, several MUC genes have been described in human and named based on their order of discovery. Some of them belong to the secreted gel-forming mucin family, while others are classified in the membrane-associated family (**Table 1.1**). Host-secreted mucin 2 (MUC2) glycoprotein is a major constituent of human small intestinal and colonic mucus, while MUC1, MUC5AC, and MUC6 are predominant in the stomach (Sicard et al. 2017).

Table 1.1. Mucins in the human GIT.

Modified from Johansson et al. 2013.

Mucin glycoproteins	Types	Number of amino acids	Cell type expression	Functions
MUC1	Transmembrane	~1,250	Epithelial cells	Signaling, protection
MUC2	Gel-forming	~5,200	Goblet and paneth cells	Protection, lubrication
MUC3	Transmembrane	>2,550	Enterocytes	Apical surface protection
MUC4	Transmembrane	~5,300	Epithelial cells Goblet cells	Signaling, protection
MUC5AC	Gel-forming	>5,050	Mucous cells	Protection, lubrication
MUC5B	Gel-forming	~5,700	Mucous cells Goblet cells	Protection, lubrication
MUC6	Gel-forming	~2,400	Mucous cells	Protection, lubrication
MUC7	Gel-forming	377	Mucous cells	Protection
MUC12	Transmembrane	~5,500	Enterocytes	Apical surface protection
MUC13	Transmembrane	512	Enterocytes	Apical surface protection
MUC16	Transmembrane	~22,000	Epithelial cells	Apical surface protection
MUC17	Transmembrane	~4,500	Enterocytes	Apical surface protection

1.3.4.2 Main functions

The mucus barrier has several functions, a primary one being the lubrication of the epithelium helping the passage of food material along the GIT. Mucin proteins are glycosylated polymers that constitute a carbon and energy source for the growth of resident gut microbiota (Tailford et al. 2015). Accumulating evidence demonstrates a crucial role of the mucus layer in maintaining gut homeostasis (Martens et al. 2018). Notably, it contains a large variety of host antimicrobial molecules (e.g. α and β defensins, IgA and IgM) that are retained within the net-like polymer structure of mucin glycoproteins. In close collaboration with the immune system and the gut microbiota, the mucus is the first line of defense against encroaching bacteria that can breach and persist on the epithelial surface (Johansson and Hansson 2016). In particular, bacteriophages are able to interact with mucus and studies in mice demonstrated that phage

particles are 4-fold more concentrated in mucus layer compared to the lumen content (Barr et al. 2013). Recent studies showed that phages represent key players in limiting bacterial persistence close to the epithelium and may play an important role in the homeostasis of the gut microbiota (Almeida et al. 2019; Sausset et al. 2020; Rasmussen et al. 2020). The mucus layer therefore has a dual role. On the one hand, it lubricates the intestine and acts as a defensive barrier against harmful aggressors. On the other hand, it provides an ecological niche for bacteria by providing adhesion sites and nutrients.

2. Gastro-intestinal gut microbiota

Gut microbes live in close symbiosis with the human host and play a vital role in health. In terms of composition and abundance, the gut microbiota is characterized by pronounced regional differences (described in the section below), influenced notably by physicochemical, nutritional and immunological gradients (Donaldson et al. 2016). In addition to these dissimilarities, intra- and inter-individual variabilities are important (at least at family, genus and species levels), making the awareness of the gut microbiota even more complex, while the phylum level is generally more conserved. A non-exhaustive list of contributing factors can be drawn up with a variation of diet, lifestyle, geographic and ethnical origin, health status, gender, menstrual cycle and age-range. Interestingly, within one individual, the microbial community (at phylum level) is fairly stable over time within adulthood (Donaldson et al. 2016).

This microbial ecosystem contributes to various essential functions for host physiology, including energy extraction from food through fermentation of dietary fibers (Morrison and Preston 2016), vitamin synthesis (Belzer et al. 2017) and development and maturation of the immune system (Kamada et al. 2013). Disruption in gut microbiota composition and activity (termed dysbiosis) can occur and have been associated with negative health outcomes, such as digestive or extra-intestinal diseases like for IBD or metabolic syndrome (Lavelle and Sokol 2020). It is important to mention that in addition to bacteria, other key microorganisms are present in the gut including yeast, fungi, Archaea, viruses and phages, thus constituting the gut microbiota. The following sub-sections will describe only the human gut microbiota (adult and infant) and will focus on the bacterial component.

Microorganisms rapidly colonize infants over the first months or years of life. Currently, no consensus exists concerning the time for microbiota maturity but most studies converged on the time period of 1000 days, i.e. three first years of life (Odamaki et al. 2016; Derrien et al. 2019). Of note, the only available data on resident microbiota in the first years of life are related

to oral and fecal microorganisms and high individual variations in microbiota composition and functions have been reported (Könönen 2000; Pham et al. 2016).

2.1 Biogeography of the gut microbiota

With the advent of next generation sequencing technology, research studies exploring gut microbial community profiles have flourished. Nonetheless, for now, relatively little is known regarding the longitudinal and axial variation of the human gut microbiota. Consequently, most research on host-microbe's interactions are based on studies of the fecal microbiota, which are obviously most easily collected. Moreover, rare studies have started to investigate the mucosal microbiota.

2.1.1 The adult gut microbiota

The following sub-sections will describe only the human adult gut microbiota, a specific focus on the infant microbiota will be given in the *paragraph 2.1.2*.

2.1.1.1 Longitudinal axis

The adult human GIT is estimated to harbor 10^{13} bacteria, which is equivalent to the number of human cells (Sender et al. 2016). The stomach, which was long be considered as a sterile organ, has been found to harbor quite abundant microbial communities dominated at the phylum level by Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria (Bik et al. 2006; Klymiuk et al. 2017; Zhang et al. 2019). The main genera found in stomach are *Streptococcus*, *Prevotella*, *Enterococcus*, *Lactobacillus*, *Veillonella* and *Rothia* (Bik et al. 2006; Engstrand and Lindberg 2013; Jandhyala 2015), the species *Helicobacter pylori* is also prevalent (Bik et al. 2006). The stomach still shelters the lowest number of microbes in the GIT ranging between 10 and 10^3 colony forming units (CFU) per gram of content (Hillman et al. 2017).

In the duodenum and jejunum, the high levels of bile acids, antimicrobials and a short transit time limit bacterial growth to 10^3 to 10^6 CFU.mL⁻¹ with huge interindividual variations. Due to the mild oxygen levels, the microbial communities of these proximal parts of the small intestine are dominated by facultative anaerobic bacteria (Sundin et al. 2017). Few studies have investigated their composition. In a study conducted on five healthy volunteers that have ingested a meal, the duodenum was aspirated 90 min post-intake. Results showed the presence of two dominant phyla Firmicutes and Actinobacteria and four dominant genera with *Streptococcus*, *Actinomyces*, *Propionibacterium* and *Granulicatella* (Angelakis et al. 2015). In

contrast, a study conducted on 20 adults has shown that the jejunum is dominated by Firmicutes, Proteobacteria and Bacteroidetes phyla. It also revealed a recurring core of abundant species in the jejunum belonging to *Streptococcus*, *Prevotella*, *Veillonella*, *Haemophilus* and *Escherichia* genera (Sundin et al. 2017).

In the ileum, due to lower bile salts concentrations, lower level of oxygen, slower transit times and possible retrograde flow by the ileocecal valve from the colonic microbes, bacterial concentrations increase from 10^7 to 10^8 CFU.mL⁻¹ of luminal content (Booijink et al. 2010; Quigley 2013). The ileal microbiota is dominated by the Firmicutes phylum and the following genera *Clostridium*, *Streptococcus*, *Escherichia* and *Veillonella* are the most commonly found. Contrary to the duodenum and jejunum, Bacteroidetes phylum is found in half of the volunteers, but remains still below the levels found in the colon (Booijink et al. 2010; Zoetendal et al. 2012; van den Bogert et al. 2013).

In contrast, colonic conditions exhibit lower cell turnover rate, lower redox potential and a longer transit time. The colon supports a dense and diverse community of bacteria with 10^{10} to 10^{12} CFU.mL⁻¹ of colonic content according to the segment location, mainly anaerobes with the ability to utilize complex carbohydrates undigested from the small intestine. The colonic microbiota is dominated by members of Firmicutes and Bacteroidetes, followed by Proteobacteria and Actinobacteria. The three main dominant genus reported are *Bacteroides*, *Clostridium* and *Prevotella* (Miller et al. 2021). Due to sample accessibility, the numbers of studies focusing on the human fecal bacterial communities is immeasurable compared to studies conducted directly in segments of the GIT. The microbial load of fecal samples is estimated to be around 10^{10} - 10^{11} CFU per gram of feces (Galazzo et al. 2020). Because of their immediate proximity, the fecal microbiota is thought to exhibit similarities with the colonic one. Analysis of fecal materials from healthy adults have shown that bacteria were predominantly members of the phyla Firmicutes and Bacteroidetes, followed by Proteobacteria and Actinobacteria. Moreover, about twenty genera belong to the aforementioned phyla such as *Bacteroides*, *Prevotella*, *Alistipes*, *Eubacterium*, *Ruminococcus*, *Roseburia*, *Faecalibacterium*, *Lactobacillus*, *Enterococcus*, *Blautia*, *Enterobacteriaceae* (family), *Fusobacteria* and hundreds to thousands of species belongs from these genera (Eckburg et al. 2005; Ley et al. 2006; Turnbaugh et al. 2007; Ruan et al. 2020). Among the most represent species, are *Bacteroides* spp. *Escherichia* spp and *Lactobacillus* spp. The figure summarizes the bioregional composition of the gut microbiota along the longitudinal axis (**Fig. 1.6**).

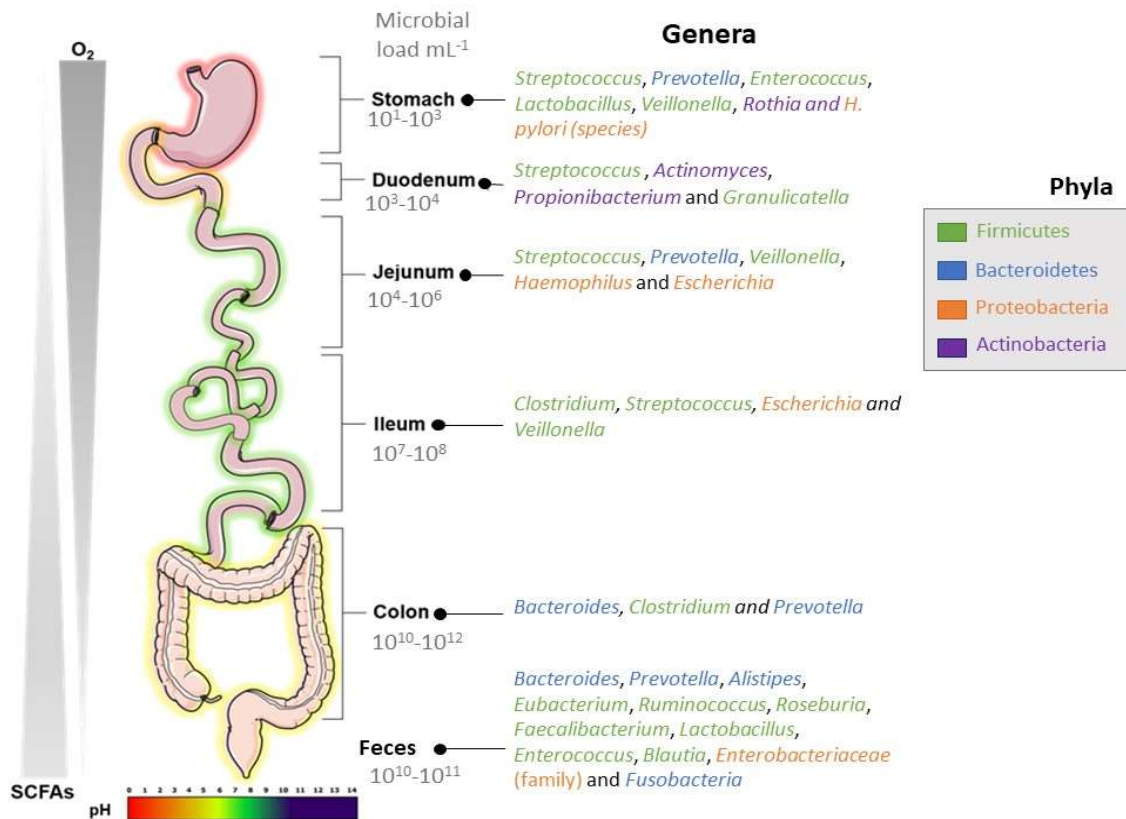


Figure 1.6 Biogeography of the luminal microbial composition of GIT in a healthy adult

The most common genera in each GIT location are represented, belonging to a phylum represented in color (legend on the right). The GIT scheme is also colored according to the pH scale shown at the bottom.

SCFAs: Short Chain Fatty Acids.

Modified from Charlene Roussel's PhD manuscript.

2.1.1.2 Transversal axis

The gut microbiota composition is known to differ along the longitudinal axis of the GIT but it also varies transversally from the lumen to the mucosa due differences in key physiological parameters such as nutrient availability or oxygen gradient.

The mucus layer is considered a well-known microbial niche in the GIT where its colonization is necessary for resident microorganisms to maintain their presence (Ouwkerk et al. 2013). Bacterial mucinases are one of the main actors of this colonization. Described both in commensal bacteria and in pathogenic strains, these enzymes allow access to the mucus layer by proteolysis of the core of mucin proteins then enabling bacterial colonization (Etienne-Mesmin et al. 2019b). To counterbalance mucinase action and maintain its net-like structure that retains the gut microbiota, the mucus contains structural proteins including protease

inhibitors that protect the mucus from extensive degradation (Bansil and Turner 2018). Not all microbial species are adapted to mucosal colonization though and studies have demonstrated that microbial communities from the digestive lumen differ in terms of composition and abundance from the mucus-associated ones, driven by differences in nutrient availability and physicochemical gradients like oxygen availability (Chassaing and Gewirtz 2019). Gastric mucosal samples are more oriented towards Firmicutes and Proteobacteria (Sung et al. 2016). For its part, the mucosal jejunal microbiota displays great similarity with the luminal one (Dlugosz et al. 2015; Sundin et al. 2017). Only one work has studied the ileal mucosa, with an unhealthy volunteer, making difficult any conclusion (Patrascu et al. 2017). Then, most of the studies about differences in microbiota composition between the lumen and the mucus have been conducted in the colonic compartment. Compared to the luminal one, the human colonic mucus layer displays a markedly higher level of Firmicutes, Actinobacteria and Proteobacteria and a lower level of Bacteroidetes (Donaldson et al. 2016; Richard et al. 2018; Chassaing and Gewirtz 2019; Vuik et al. 2019). Especially, mucosal communities are highly enriched in *Bacteroides acidifaciens*, *Bacteroides fragilis*, the mucin-degrader *Akkermansia muciniphila* and in species belonging to the Lachnospiraceae taxa (Donaldson et al. 2016; Pereira and Berry 2017) (**Fig. 1.7**). **Figure 1.7** summarizes the changes in microbiota composition between luminal and mucosal phases of the human gut.

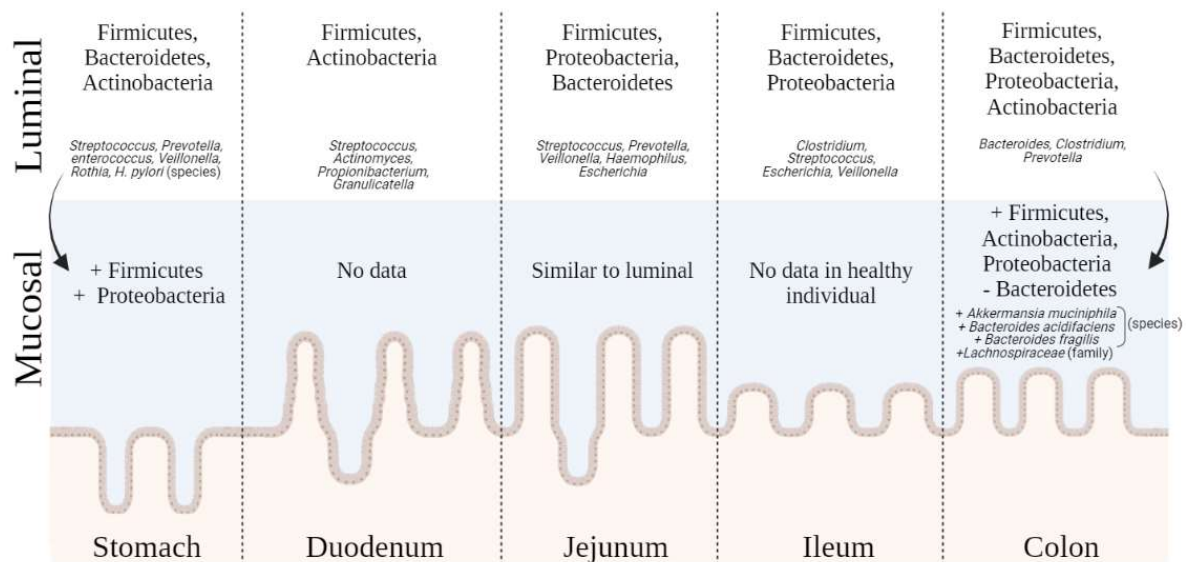


Figure 1.7 Changes in microbiota composition between luminal and mucosal compartments throughout the human gut

The composition of the luminal microbiota (phyla and genera) is represented alongside with changes observed in the mucosal phase of the human gut.

Extracted from Thomas Sauvatre's PhD manuscript.

Differences in microbiota communities are also observed on the transversal axis inside the mucus layer. A gradual density of microbial colonization is found from the outer mucus layer to the intestinal epithelium, mostly due to niche accessibility. The outer colonic layer is more densely colonized thanks to proteolytic activities loosening the net-like structure. The inner colonic mucus layer has for long been believed to be devoid of bacteria in accordance with its more constraining physical properties (Johansson et al. 2013). At tissue scale, single-cell imaging conducted in mouse model revealed the presence of bacteria in close proximity of the epithelium (Earle et al. 2015). Among them, Segmented Filamentous Bacteria have been identified in many vertebrate intestines (humans, rodents, chickens) as commensal strains able to invade this mucus layer without invading the host (Chen et al. 2018a; Hedblom et al. 2018; Ladinsky et al. 2019).

2.1.2 The infant gut microbiota

2.1.2.1 Colonization and composition of infant microbiota

Development of resident communities and their metabolic outcomes are crucial not only for early life maturation, but also for long-term health in children, and to a later extent in adults. Gut microbiota is also mandatory for immunologic programming of infants (Wiertsema et al. 2021) and exerts a key barrier effect against pathogens (Vazquez-Gutierrez et al. 2016).

The colonization process occurring in infants is characterized by a continuous increase in load and diversity of bacteria during the first year of life, and even later on, up to the weaning time (Sulyanto et al. 2019) (**Fig. 1.8, A**). Aerobic and facultative anaerobes are the first colonizers in the neonatal gut due to the presence of oxygen, which limits the colonization of strict anaerobes. Fecal microbiota mainly consists of *Streptococcus*, *Staphylococcus*, *Enterococcus*, *Lactobacillus* and *Enterobacteriaceae* (mainly *Escherichia coli* and related species). Those facultative anaerobe bacteria contribute to the establishment of a reduced environment that is mandatory for strict anaerobes colonization. This important shift seems to occur at 4 months old (Bäckhed et al. 2015) and colonization process of anaerobic microbes belonging to the predominant phyla Bacteroidetes, Firmicutes and Actinobacteria occurs then very quickly. The presence of *Bacteroides* and/or *Bifidobacterium* species that outnumber facultative anaerobes and reach adult-like population densities within the first week of life is even reported from fecal sample analysis (Jost et al. 2012). Both *Bacteroides* and *Bifidobacterium* species are well-known for using most of the substrates available in the neonatal gut from infant milk-based diet (proteins, lactose but also human milk

oligosaccharides) and host-produced components such as mucin glycoproteins (Jost et al. 2015), which could explain this rapid establishment. Another stage of intestinal colonization occurs after several weeks or months, together with an increase in microbial diversity. Some species belonging to Firmicutes (mainly *Lachnospiraceae* and *Ruminococcaceae*) significantly increase over the first year of life in stool samples. Increase in microbial diversity is further associated to diet changes after weaning (Yassour et al. 2016) and for several years. Available reports on fecal samples suggest an early colonization of bacteriophages, but a delayed colonization of fungi, and especially *Archaea* even beyond the weaning time (Vanderhaeghen et al. 2015; Korpela and de Vos 2018).

2.1.2.2 Impact of external factors on oral and intestinal microbiota

Gestational age can strongly affect gut microbiota development (**Fig. 1.8, B**). A decrease in gestational age leads to a delayed colonization of strict anaerobes and a lower diversity, together with an increase in potential pathogenic and facultative anaerobic bacteria (Henderickx et al. 2019). Direct comparison of gut microbiota composition of preterm and term infants showed that *Enterobacter*, *Enterococcus*, *Escherichia* and *Klebsiella* populations, containing well-known opportunistic pathogens, were predominantly present in preterm infants (Arboleya et al. 2012; Henderickx et al. 2019). Environmental and host conditions can partially explain these microbial differences in colonization, together with specific feeding regimen and the frequent use of antibiotics associated with prematurity (Henderickx et al. 2019). Consistent evidence also indicates that the delivery mode has a significant impact on gut microbiota colonization profiles. The primary gut colonizers of babies born vaginally show a high microbial similarity with their mother's vaginal microbiota dominated by *Lactobacillus* and *Prevotella* species. By contrast, this natural exposure is disrupted in caesarean born babies and the primary colonizer species are mostly *Staphylococcus* and *Propionibacterium*. This suggests an initial colonization with microbes found in the hospital environment and later from skin contact with mother as origin (Dominguez-Bello et al. 2010). The feeding mode also strongly affects infant gut microbiota colonization as *Lactobacillus* and *Bifidobacterium* species are more prevalent in breast-fed infants than in formula-fed infants, associated with a decrease in their fecal pH (5.9 in breast-fed compared 6.8 in formula-fed infants) 2 weeks after birth (Simhon et al. 1982). By contrast, a more diverse gut microbiota was observed in formula-fed infants with higher level of *Bacteroides*, *Clostridium* and *Enterobacteriaceae* compared to breast-fed infants (Zhuang et al. 2019). Besides, drugs and especially antibiotics can also profoundly influence infant gut microbiome. Impact of antibiotics on infant gut microbiota

obviously depends on the spectrum, dose, duration and route of treatment (Wang et al. 2016). Maternal intrapartum antibiotic treatments lead to a shift in infant oral and gut microbiota, with a loss of beneficial commensal populations (e.g. *Streptococcaceae* and *Bifidobacteriaceae*) for the benefit of *Proteobacteria* (Gomez-Arango et al. 2017; Zimmermann and Curtis 2020). Similarly, oral use of antibiotics in infants resulted in decreased numbers of gut anaerobic communities, particularly *Bifidobacterium* and *Bacteroides* species (Penders et al. 2006) and/or increased numbers in *Proteobacteria* such as *Enterobacteriaceae* (Arboleya et al. 2015). Intestinal communities in antibiotic-exposed infants are also less mature compared to those of unexposed ones (Bokulich et al. 2016). Lastly, excessive hygiene conditions during early life reduce the continuous microbial exposure which is however essential for the development of a rich gut microbiota in infants (Neu and Rushing 2011; Schmidt et al. 2011).

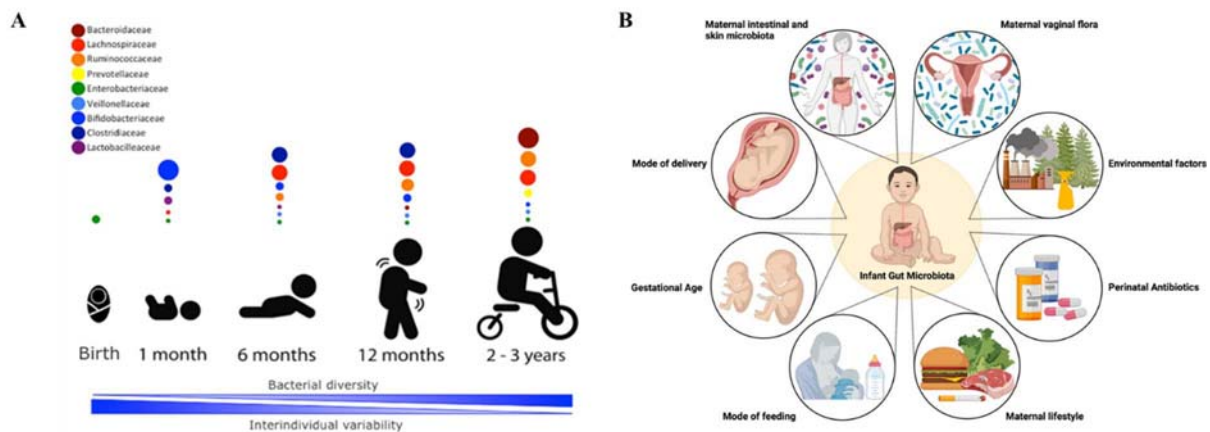


Figure 1.8 Factors shaping the neonatal microbiome and its evolution from birth to adulthood

(A) As diet diversifies with age, the microbiome gradually shifts toward an adult-like configuration, which is usually reached by age 3. Main bacteria associated with the different age-step are indicated. (B) Factors shaping the neonatal microbiome are presented.

Extracted from Arrieta et al. 2014 (A) and Hill et al. 2021 (B)

2.2 Key functions of the gut microbiota

The gut microbiota plays important role in human health and metabolism. This complex microbial ecosystem contributes to various essential functions for host physiology, including energy extraction from food through fermentation of dietary fibers (Morrison and Preston 2016), vitamin synthesis (Belzer et al. 2017) and development and maturation of the immune system (Kamada et al. 2013). The microbiota has also important trophic effects on intestinal epithelia, by favoring the development of intestinal microvilli, epithelial cell differentiation and proliferation (Li et al. 2012). Moreover, gut microbes can promote resistance to colonization

by pathogenic species. This barrier effect can be exerted at several levels by nutrient competition and antimicrobial molecules production by both gut microbes but also intestinal epithelial cells such as Paneth cells (Bäumler and Sperandio 2016) (**Fig 1.9**). The metabolic activities of the gut microbiota of adults and infants will be described in the following subsections.

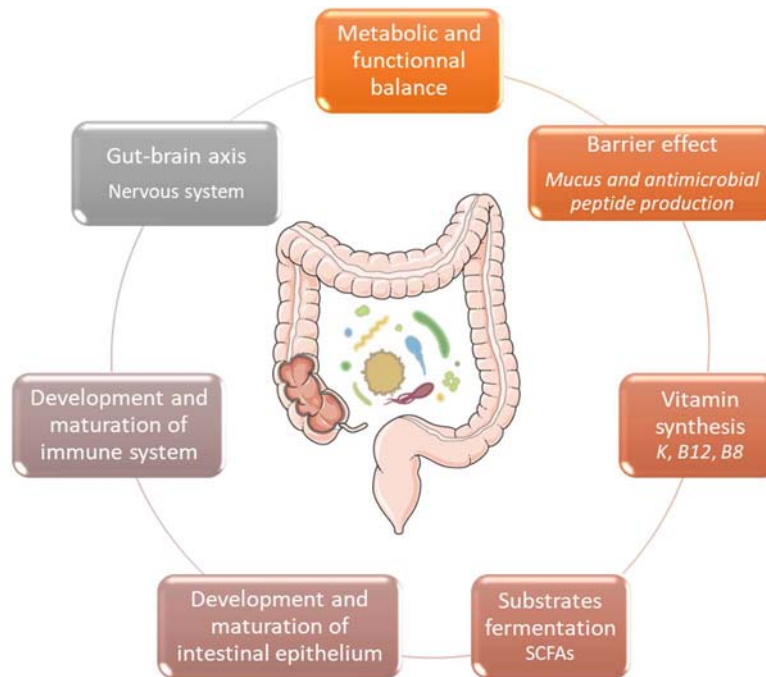


Figure 1.9 Key functions of the human gut microbiota

The gut microbiota achieves numerous functions.

SCFAs: short chain fatty acids

Personal creation

2.2.1 Metabolic activities

2.2.1.1 Gut microbiota activity in adults

Bacterial cells essentially consist of a collection of macromolecules, synthesized by the catabolic breakdown of substrates and the subsequent anabolic assembly of monomers derived from the substrates, collectively termed bacterial metabolism (Madigan 2012). Bacterial metabolism is very vast and diverse, as gut bacteria living in regionalized and changing environments with different nutrient sources, they will adopt different strategies to utilize nutrients and salvage of energy. Thanks to contributing enzymes, gut microbes are involved in polysaccharides (carbohydrate-active enzymes) and polyphenols breakdown (O’Hara and Shanahan 2006).

Under anaerobic conditions, fermentation is the preferred metabolic strategy (Macfarlane and Macfarlane 2003; Madigan 2012). Prior to fermentation, complex polysaccharidic (fibers) and proteic substrates (dietary proteins partially digested or non-dietary proteins such as mucins) have to be degraded to their component monosaccharides (sugars) and amino acids, which are then further oxidized (Topping and Clifton 2001; Tremaroli and Bäckhed 2012). Bacteria from the genera *Bacteroides*, *Bifidobacterium*, *Ruminococcus* and *Roseburia* and some species from *Clostridium*, *Eubacterium* and *Enterococcus* are provided with hydrolytic activity for the breakdown of polysaccharides, resulting in the production of SCFAs (**Fig 1.10**). In adults, total SCFA concentration represents an average ranging from 64 to 77 $\mu\text{mol/g}$ of feces depending on the study (Høverstad et al. 1984; Baxter et al. 2019), and are typically found in a proportion of 3:1:1 with percentages of around 60%, 20% and 20% for acetate, butyrate and propionate, respectively. Propionate, butyrate and acetate account for approximately 95% of colonic SCFA contents (McNabney and Henagan 2017). While the remaining 5% of SCFAs are attributed to valerate, caproate, heptanoate, octanoate and branched SCFAs (e.g. isobutyrate, isovalerate and isocaproate). Acetate is predominantly produced by genera *Bacteroides*, *Clostridium*, *Ruminococcus* and *Eubacterium* and it can then be partially used for butyrate formation. Propionate is mainly produced by genera *Bacteroides*, *Propionibacterium* and *Veillonella* (Bernalier-Donadille 2010). Finally, butyrate-producing species are mainly encountered among Firmicutes in the families *Ruminococcaceae* (e.g. *Faecalibacterium prausnitzii*) and *Lachnospiraceae* (e.g. *Eubacterium rectale*) as well as in *Erysipelotrichaceae* and *Clostridiaceae* (Louis and Flint 2017).

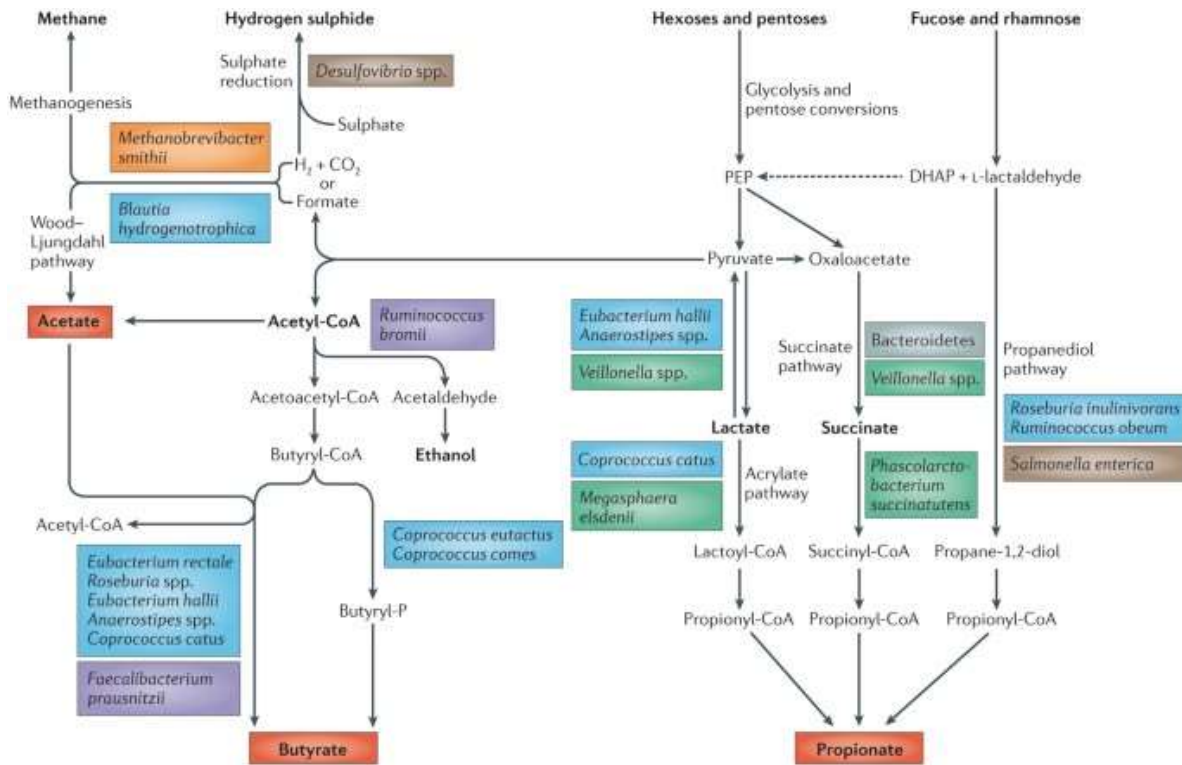


Fig. 1.10 Pathways that are responsible for the biosynthesis of the major microbial metabolites that result from carbohydrate fermentation and bacterial cross-feeding

Extracted from Louis et al. 2014.

Overall, 99% of SCFAs are absorbed by the colonic epithelium. Butyrate alone has been estimated to provide 60-70% of the colonocyte's energy needs, while acetate and propionate are energetic substrates for the liver (McNabney and Henagan 2017). Lastly, SCFA production gradually decreases from the proximal to distal part of the colon (Bernalier-Donadille 2010).

Gut microbiota metabolism also releases about 300 different volatile organic compounds (VOCs) called the volatolome (Garner et al. 2007). Similarly to the gut microbiota composition, healthy fecal samples harbor both common and ubiquitous VOCs (e.g acetone, ethanol, heptanone, butanoic and pentanoic acids) among all human donors tested but also donor-dependent VOCs (Raman et al. 2013). While little is known about potential health risk of these compounds in human health, they are currently used as biomarkers (e.g indol, phenol and ester) of various human diseases including cancer, gastrointestinal and metabolic disorders (Garner et al. 2007; Walton et al. 2013; Arasaradnam et al. 2014). Studies examining microbial VOCs are still scarce and only few databases are available (Lemfack et al. 2014; Abdullah et al. 2015; Agarwal et al. 2016).

Bacterial metabolisms in the gut also lead to the production of various aryl hydrocarbon receptor (AhR) ligands as the indole and its derivatives resulting from tryptophan transformation (Schiering et al. 2017; Shinde and McGaha 2018). AhR is a ligand-activated nuclear transcription factor widely expressed in the body and distributed along the GIT (Lee, Wood and Lee, 2015). This nuclear receptor is retained in an inactive complex in the cytoplasm that, after ligand binding, translocates to the nucleus regulating the expression of target genes like *Cyp1a1*, *Il22* and *Il17* (Lamas et al. 2018; Lamas et al. 2020). AhR recognized xenobiotics as well as natural compounds such as tryptophan metabolites, dietary components and microbiota-derived factors. Finally, AhR signaling orchestrates immune responses which may impact intestinal homeostasis.

2.2.1.2 Gut microbiota activity in infants

The infant gut microbiome metabolizes the complex substrates of milk-based diet, such as proteins, lipids and carbohydrates (especially human milk oligosaccharides) into key metabolites such as SCFAs. The only available data on SCFA production have been obtained from child fecal samples. Whatever the feeding status, total SCFA concentrations found in infant feces between 3 to 5 months were 142 $\mu\text{mol/g}$ in average with a majority of acetate (80%) and less propionate (10%), butyrate (5%) and valerate (2%) (Bridgman et al. 2017). Another more recent study measuring only the three main SCFAs reports a median total concentration of 84.6 $\mu\text{mol/g}$ of feces in 1-year old children, allocated in 72% acetate, 16% propionate and 12% butyrate (Roduit et al. 2019). These authors underline that fecal SCFA concentrations deeply depend on the infant diet. Relative percentages of fecal acetate/propionate/butyrate in child are therefore far from the ideal adult ration of 3:1:1 (den Besten et al. 2013). Breast-fed infants have a lower concentration of fecal SCFAs (99.4 vs 177.8 $\mu\text{mol/g}$) and a higher relative proportion of acetate (86.7 vs 77.3 %) and lactate (7.2 vs 2.3 $\mu\text{mol/g}$) compared to formula-fed infants at 1 month. This is probably due to the more efficient capacity of breast-fed infant microbiota to metabolize human milk substrate (Bridgman et al. 2017) and the greater concentration of oligosaccharides in breastmilk compared to formula (Bode 2015).

Growing infant gut microbiota produces also more VOCs with time from a median of 13 VOCs at birth to 24 at 10 days of life (Frau et al. 2021). Some of these compounds are already produced at birth as aldehydes and acetic acid while others (acids, esters, ketones and alcohol) appear later. Of note, as observed for gut microbiota composition and probably linked, VOC profiles are also different according to the mode of delivery, gestational age (Frau et al. 2021)

and have been used as diagnostic tools for GI diseases in pediatrics (Buijck et al. 2016; Course et al. 2021).

Regarding AhR, data in infants are still at their infancy. Several gut microbiota species including *Bifidobacterium longum*, *B. breve* and *B. bifidum*, particularly present from birth to 6 months, are able to produce AhR ligands as indolelactic acid derivated from aromatic amino acids of breast-milk (Ehrlich et al. 2020; Laursen et al. 2021).

2.2.2 Intestinal immune response surveillance

In addition, gut microbes play a fundamental role in the regulation and maturation of the immune responses. Located at the interface between the lumen and the underlying host, the epithelial barrier acts as the first line of defense and monitor unfavorable activation of the host immune system. Disruption of this barrier leads to immune system activation. The immune system is composed of two parts. First, the innate immunity is acting as the first line of immunological defense present in the gut lumen and interacts with the microbiota *via* nonspecific innate immune receptors expressed on the different cell types in the mucosa (enterocytes, polynuclear cells, mast cells, macrophages and dendritic cells). Second, the adaptive immune system allows a highly targeted immune response of the host against the aggressors. The adaptive immune system interacts with gut microbiota *via* highly specific receptors expressed on lymphoid cells.

The activation of the innate immunity by microbial proximity occurs *via* the recognition of conserve microbe-associated molecular patterns (MAMPs). Of note, when these patterns originate from pathogens, they are termed pathogen-associated molecular patterns (PAMPs). MAMPs (and PAMPs) include diverse microbial components such as lipopolysaccharide (LPS), lipid A, flagelin, nucleic acids (Takeuchi and Akira 2010). Those MAMPs are recognized by pattern recognition receptors (PRR) such as toll like receptors (TLR), the C-type lectin receptors and NLRs (NOD-like receptors) of eukaryotic cells, triggering the host innate immunity and downstream signaling pathways (Ausubel 2005; Bailey et al. 2005).

Part 2 – *In vitro* models of the human gut

Research on the fate, transformation and effects of food pollutants, including MPs, upon human intake remains limited, and hampered by obvious ethical and regulatory reasons. *In vivo* studies in mammals cannot address to all the questions raised, due to major gut characteristics differences depending on species (Hugenholtz and de Vos 2018). Furthermore, animal experiments are restricted by the 3R European rules which widely encourage the development of alternative *in vitro* approaches to reduce the number of animals used in research. Then, *in vitro* models of the human digestive tract are of uppermost importance for human gut research. Even though *in vitro* gut models cannot obviously integrate nervous or endocrinal systems, they can reproduce all or part of the following physicochemical and microbial parameters of the human digestive tract: temperature, pH, transit time, mechanical constraints, supply of digestive enzymes and bile salts, passive absorption of nutrients and water, condition of oxygenation (especially anaerobiosis in colonic compartments) and complex, metabolically active and compartmentalized resident microbiota from human origin. The incoming part will review the available *in vitro* gut and cellular models that could be used to evaluate the impact of MP exposure throughout human digestive tract. The main infant *in vitro* gut models currently available (from birth to 3 years old) are also described. Noteworthy, some parts of this state of the art on infant *in vitro* gut models have been published in the same review article mentioned in the part 1, in the journal *Biotechnology Advances*, and redrafted / updated for the present section.

REVIEW: FOURNIER E, ROUSSEL C, DOMINICIS A, LEY D, PEYRON M-A, COLLADO V, MERCIER-BONIN M, LACROIX C, ALRIC M, VAN DE WIELE T, CHASSARD C, ETIENNE-MESMIN L, BLANQUET-DIOT S. *In vitro* models of gut digestion across childhood: current developments, challenges and future trends. *Biotechnol Adv.* 2022 Jan-Feb; 54:107796. doi: 10.1016/j.biotechadv.2021.107796.

1. *In vitro* gut models

1.1 Gastric and small intestinal models

1.1.1 Mono-compartmental simulators

1.1.1.1 Static mono-compartmental models

Static mono-compartmental models (also termed batch) are the simplest option to reproduce gastric or small intestinal conditions (**Table 2.1**). In such models, only one or two digestive conditions are fixed at start in a unique vessel, underestimating thereby the complexity of the human GI physiology. Due to their simplicity, these models have been extensively used to simulate gastric or small intestinal digestion and do not necessary own a name (Bengoa et al. 2018). We can still quote the Simulated Gastric Fluid (SGF), which simulates peptide hydrolysis by stirring the food sample with pepsin and pH maintenance between 2 and 3 for 30 to 120 min (Egger et al. 2016). Nonetheless, in this case the complexity of the gastric physiology is undervalued since the gastric pH is not progressively dropped, and the enzymatic cocktail, classically found in the stomach is not included. Other batch models can include a gastric phase followed in the same vessel by the small intestinal one (Hur et al. 2011; Brodkorb et al. 2019). To cite only one of them, the Oomen system was developed to study pollutant bioaccessibility (Oomen et al. 2003). This model mimics in the same compartment at 37°C, the successive oral, gastric and duodenal phases and adapted the specific secretions, pH and digestive time according to the phase considered. These simple-to-use and cheap models offer the flexibility to test unlimited conditions and are often used for screening assays.

Table 2.1 Main *in vitro* static mono-compartmental digestive models of the upper adult GIT

Batch systems are included, but they do not necessarily have a name due to their wide availability.

Digestive simulators of the upper GI tract		
Mono-compartmental models		
<i>Static models</i>		
Simulated Gastric Fluid (SGF)	Simple stirring of food with pepsin	Egger et al. 2016
COST INFOGEST static model	Successive (oral), gastric and intestinal digestion in the same vessel (pH, T°)	Brodkorb et al. 2019
Oomen system	Successive oral, gastric and duodenum digestion in the same vessel (pH, T°) Used to study pollutant bioaccessibility	Oomen et al. 2003

Existing adult static models were adapted to infant specific digestive conditions, as described in **Table 2.2**. The first static child model was developed in 2010 by Dupont and colleagues from INRAE in France (Dupont et al. 2010). The model was adapted to 2 weeks - 3 months old infants. During the gastric phase, pH was adjusted at 3 instead of 2.5 for adult protocol and pepsin concentration was decreased by 8-fold. In the intestinal phase, bile salts amount was reduced 4-fold and phosphatidylcholin, trypsin and chymotrypsin concentrations were decreased by 10-fold compared to adult conditions. Then, the adult international consensus protocol of *in vitro* static digestion (Minekus et al. 2014) was adapted several times to specific child conditions (Cattaneo et al., 2017 ; Ménard et al., 2018). In particular, Dupont's team set up a new static *in vitro* model for full-term newborns -28 days of life- (Ménard et al. 2018). In this model, both gastric and intestinal phases lasted 60 min, as for adult conditions. However, pH values were adapted, with a gastric pH increased to 5.3 (vs 3 in adult) while intestinal pH was reduced to 6.6 (vs 7 in adult). Gastric pepsin was largely reduced (268 U/mL vs 2000 U/mL) while gastric lipase remained equivalent to adult concentration and only slightly adapted to infant body weight (19 U/mL vs 21 U/mL). Intestinal juice concentrations reproduced by porcine pancreatin and bovine bile were largely reduced, with a 22-fold and 3-fold reduction compared to adult levels, respectively. Very recently, another digestion model

was implemented by Hunan Agricultural University in China, for infants ranging from birth to 6 months old (Luo et al. 2019a), based on the publication from Bourlieu and colleagues (Bourlieu et al. 2014). Of note, this model integrates kinetics of gastric pH (with four steps: pH 6.5 from 0–30 min, 6.0 from 30–60 min, 5.5 from 60–90 min and 5.0 from 90–120 min), allowing to mimic the slower pH decrease in infant compared to adult. Intestinal digestion was set up at pH 7 as in other models. The composition of simulated gastric and intestinal fluids was also adapted to infant conditions. The gastric phase was mimicked with 0.4 mg/mL pepsin and 0.87 mg/mL lipase, both added at the beginning of each pH steps. During the intestinal phase, 5 mg/mL of porcine bile extract and 1.6 mg/mL of porcine pancreatin and porcine pancreatic lipase were added. Kamstrup's team, from University of Copenhagen in Denmark, also provided advices to design an improved digestion model for neonates under 2 months old (Kamstrup et al. 2017). This model aims to integrate various parameters, such as volume, pH, osmolality, gastric pepsin and lipase, intestinal lipase and bile salts, and distinguishes fasted state from fed state. During fasted state, gastric phase pH was set at 2.9, while a pH kinetic was considered during the fed state increasing to 6.4, 30 min after feeding and decreasing to 3.9, after 50-min digestion. For the intestinal phase (2h), the pH was maintained at 6.5 during fed state compared to 7.0 for fasted state. For the fasted state, an osmolality of 298 and 308 mosmol/kg of intestinal content was recommended for the gastric and intestinal phases respectively, compared to 291 and 296 mosmol/kg for the fed state. In gastric fluids, lipase activity was set up at 100 tributyrin units (TBU)/mL while pepsin activity was 63 U/mL/kg of body weight. Lastly, in intestinal fluids, lipase activity was set at 50 TBU/mL and bile salts concentration at 1 mM and 4.7 mM for fed and fasted states, respectively. From Kamstrup's work, Klitgaard and collaborators developed the immediate transfer digestion model (ITDM) reproducing the stomach and small intestine of 0 to 2 months newborns (Klitgaard et al. 2017). They used the same parameters, as previously described (Kamstrup et al. 2017), except for the gastric pH (adjusted at 6.4), and the use of pancreatic extract instead of intestinal lipase.

Table 2.2 Main characteristics of the current infant *in vitro* static models mimicking the upper GIT.

* when compared to adult condition

Abs : absorption; Asc : ascending colon; Duo : duodenum; Des : descending colon; Jej : jejunum; Ile : ileum; SSF : Simulated Salivary Fluid; SGF : Simulated Gastric Fluid; SI : small intestine; SIF : Simulated Intestinal Fluid; Stom : stomach; RT : residence time; T°C : temperature; Trans : transverse colon; N/A : not applicable; (-) : information not available; X : not simulated in the model






MODELS	Age	pH / time (min)							Food	Digestive secretions Nutritive medium	Abs	Microbiota	Mucosal phase	References
		Transit time TT or Retention time RT (min or h)		Small intestine			Large intestine							
		Oral phase	Stomach	Duo	Jej	Ile	Asc	Trans						
Static mono-compartmental models														
<i>In vitro</i> infant digestion model (INRAE France)	2 weeks - 3 months	X	pH=3 / 30 min	pH=6.5 / 30 min	X	Milk	Pepsin concentration reduced 8-fold* Bile salts concentration reduced 4-fold*	X	Dupont et al., 2010					
			TT = N/A	TT = N/A			Phosphatidylcholin, trypsin and chymotrypsin concentrations reduced 10-fold*	X						
	28 days	X	pH=5.3 / 60 min	pH=6.6 / 60 min	X	Liquid infant formula + SGF + SIF	Pepsin concentration reduced 7-fold* Pepsin activity : 268 U/mL Bile salts concentration reduced 3-fold* Porcine pancreatin reduced 22-fold* Bovine bile reduced 3-fold*	X	Menard et al., 2018					
<i>In vitro</i> infant gastric digestion (Hunan Agriculit Univ, China)	-	X	pH=6.5 / 0-30 min pH=6 / 30-60 min pH=5.5 / 60-90 min pH=5 / 90-120 min	pH=7 min / 120 min	X	X	Pepsin : 0.4 mg/mL Lipase : 0.8667 mg/mL Pancreatin : 1.6 mg/mL Bile salts : 5 mg/mL	X	Luo et al., 2019					
			TT = N/A	TT = N/A										
			Fasted pH=2.9 / 50 min Fed pH=6.4 / 50 min											
<i>In Vitro</i> Model Simulating Gastro-Intestinal Digestion (Univ Copenhagen, Denmark)	Neonates <2 months old	X	TT = N/A	TT = N/A	X	X	Gastric lipase activity : 100 TBU/mL Pepsin activity : 63 U/mL/kg of BW Intestinal lipase activity : 50 TBU/mL Bile salts : 50 mM	X	Kamstrup et al., 2017					
			Fasted pH=2.9 / 50 min Fed pH=6.4 / 50 min											
ITDM - Immediate Transfer Digestion Model (Univ Copenhagen, Denmark)	0 - 2 months	X	Fasted pH=2.8 / 50 min Fed pH=6.4 / 50 min	Fasted pH=7 / 120 min Fed pH=6.5 / 120min	X	Infant formula	<u>Fasted state</u> Gastric lipase activity : 100 TBU/mL Pepsin activity : 741.2 U/mL Pancreatic lipase : 250 TBU/mL Pancreatic extract Bile salts : 50 mM (sodium taurocholate) <u>Fed state</u> Gastric lipase activity : 17 TBU/mL Pepsin activity : 126 U/mL Pancreatic lipase : 50 TBU/mL	X	Klitgaard et al., 2017					
			TT = N/A	TT = N/A										



1.1.1.2 Dynamic mono-compartmental models

To cleverly appreciate the physical and/or chemical changes affecting ingested compounds in the stomach, dynamic mono-compartmental models have also been developed (Table 2.3). Depending on the *in vitro* systems considered, they offer a large spectrum of parameters to follow such as the continuous changes in pH (pH drop) and secretion flow rates (e.g. pepsin, lipase), the peristalsis, and physical breakdown or the gastric emptying as well. Different models are available and we can quote the human gastric simulator –HGS- or Riddet model (Kong and Singh 2010), the dynamic gastric model -DGM- (Mercuri et al. 2011), the TIM- advanced gastric compartment (TIM-agc) (Minekus 2015) and the Gastric Digestion Simulator –GDS- (Kobayashi et al. 2017). Interestingly, the HGS model is the one that faithfully and precisely reproduces the contractive motility of the stomach by a series of rollers that continuously impinge and compress the compartment wall with increasing amplitude (Kong and Singh 2010; Ferrua and Singh 2015). On its side, the TIM-agc consists of a body part that gradually contracts to simulate gastric tone and consequent reduction of gastric volume during emptying. Two antral parts (proximal and distal) are distinguished and contracted separately to reproduce the antral contraction waves physiologically observed (Minekus 2015). In addition to gastric mono-compartmental models, a system mimicking the small intestine has been recently developed and set-up to work with very small reactive models : the Smallest Intestine (TSI) model (Cieplak et al. 2018). The three intestinal digestion phases (duodenum, jejunum and ileum) are mimicked successively in a sole vessel with a working volume of 12 mL by adjusting pH, bile salt and pancreatic enzyme concentrations. Remarkably, TSI reproduces also small intestine absorption through a dialysis system and incorporates the ileal microbiota to study the microbial behavior during small intestinal passage.

Table 2.3 Main *in vitro* dynamic mono-compartmental digestive models of the upper adult GIT

Digestive simulators of the upper GI tract		
Mono-compartmental models		
<i>Dynamic models</i>		
Human Gastric Simulator (HGS) 	Mixing by peristaltic motion and mechanical breakdown by a roller rotation system, emptying time and dynamic digestive secretions	Kong and Singh 2010
Dynamic Gastric Model (DGM) 	Mixing by water pressor piston, emptying time and dynamic gastric secretions	Mercuri et al. 2011
TIM-advanced gastric compartment (TIM-agc) 	Mixing peristaltic motion between proximal and distal antrum and no mechanical breakdown	Minekus 2015
Gastric Digestion Simulator (GDS) 	Mixing by peristaltic motion and mechanical breakdown by a roller rotation system, dynamic digestive secretions	Kobayashi et al. 2017
The Smallest Intestine (TSI) 	Three intestinal digestion phase mimicked successively in the same vessel with a working volume of 12 mL	Cieplak et al. 2018

As for static mono-compartmental models, dynamic ones were adapted to specific infant conditions (**Table 2.5**). The Israel Institute of Technology developed a gastric model for infant under 2 years of age (Shani Levi et al. 2013). A unique vessel is water-jacketed, temperature-controlled, continuously stirred and filled with a gastric medium containing sodium chloride, hydrochloric acid and pepsin. Time of gastric digestion was increased in infant compared to adult (4 vs 2h). pH kinetics during the gastric digestion was monitored and adapted to infant digestive conditions: 6.5 (0-30 min), 6.5 (30-150 min), 4.5 (150-240 min) and 3.5 at 240 min. Lastly, pepsin concentration was reduced compared to adult level (210 vs 240 U/mg protein).

1.1.2 Dynamic bi-compartmental models

To study the fate of ingested pollutants along the digestive tract, it is required to simulate further steps of the human digestion, the intestinal digestive secretion deliveries and the associated transit time. In response, bi-regionalized or multi-compartmental simulators have

been developed (**Table 2.4**). Most of the time, bi-compartmental models mimic only the stomach and the proximal small intestine by controlling the pH, temperature, transit time and bile concentration (Mainville et al. 2005; Tompkins et al. 2011; Levi and Lesmes 2014; Ménard et al. 2014). The DIDGI[®], for Dynamic Gastrointestinal Digester, was developed by INRAE in France for adult and is composed with two consecutive temperature-controlled compartments reproducing the stomach and small intestine (Ménard et al. 2015). Interestingly, a Teflon membrane (2-mm holes) reproduces the pylorus sieving effect. Several sensors allow the control of temperature, pH and redox potential and variable-speed pumps ensure meal flow monitoring, compartment emptying, and supply of hydrochloric acid and sodium carbonate, bile and digestive enzymes. Adapted from the Mainville system, the In Vitro Digestive System (IViDiS) was developed by the Institut Rosell Inc and is composed with two vessels reproducing the stomach and the duodenum (Tompkins et al. 2011). Noteworthy, food could be sequentially added to the system in order to reproduce a realistic and dynamic ingestion while the duodenum is represented using a tube (immersed in a water bath) with constrictions which, with a specific pumping profile, simulate peristaltic movement. Lastly, the “near real” *in vitro* human stomach and small intestine system (DHSI-II) was recently developed by Soochow University (Wang et al. 2019) to simulate the real human morphology, dimensions and inner wrinkles of the stomach presented with a “J” shape. Peristaltic contractions were also reproduced by electromechanical instrument composed of a series of motors, rollers and eccentric wheels. Collectively, all existing models, including the multi-compartmented TNO gastrointestinal system 1 (TIM-1) model (described below *paragraph 1.1.3*), operate with relatively large volumes, and thus require large amounts of sample material for testing, which can increase considerably the experimental costs. Therefore, an additional GI *in vitro* model have been build-up to collect smaller volumes and increase throughput, the Tiny-TIM (TNO), which is an adaptation from the initial TIM-1 where together with the stomach compartment, a single compartment is used to reproduce the adult small intestine (Schaafsma 2005).

Table 2.4 Main *in vitro* dynamic bi-compartmental digestive models of the upper adult GIT

Digestive simulators of the upper GI tract		
Bi-compartmental models		
Dynamic Gastrointestinal Digester (DIDGI)	Mimic the stomach and small intestine (pH, T°, redox potential, transit time, enzyme and bile progressive supply), pylorus sieving effect reproduced with a Teflon membrane	Ménard et al. 2015
In Vitro Digestive System (IViDiS)	Mimic the stomach and duodenum (pH, T°, transit time and bile concentration)	Tompkins et al. 2011
The “near real” <i>in vitro</i> human stomach and small intestine system (DHSI-II)	Similar gastric morphology (“J” shape), dimensions and inner wrinkles to the real human stomach, peristaltic contractions produced by electromechanical instrument composed of a series of motors, rollers and eccentric wheels	Wang et al. 2019
Tiny-TIM	Simplified and downscaled TIM (one compartment for the whole intestine)	Schaafsma 2005

The first bi-compartmental model set-up for infant conditions (birth to 6 months neonate) was adapted from the DIDGI[®] (Ménard et al. 2014) (**Table 2.5**). The volume and flow rates of secretions were adapted from another multi-compartmental infant *in vitro* model – the TNO gastrointestinal model or TIM described later in this section - (Blanquet et al. 2004) or from piglet *in vivo* data, with 1,250 U/mL pepsin (0.25 ml/min) and 60 U/mL lipase (0.25 mL/min) in the stomach compartment, and 1% porcine bile (0.5 mL/min) and 10% porcine pancreatin (0.25 mL/min) in the small intestinal compartment. In the gastric compartment, half time gastric emptying was set at 70 min based on human *in vivo* studies (Bourlieu et al. 2014), while the pH followed an acidification profile based on piglet data. The intestinal half time emptying was set at 200 min according to human data (Bourlieu et al. 2014) and pH was maintained at 6.5. It should be emphasized that the validation of the infant model based on protein digestibility was performed based on *in vivo* piglet studies and not humans’ ones. Klitgaard and collaborators also improved their immediate transfer digestion model into a dynamic system simulating infant stomach and small intestine (0 to 2 months) called the Continuous Transfer Digestion Model (CTDM) (Klitgaard et al. 2017). The same parameters as previously described were

implemented but a continuous transfer of GI fluids was set up between two distinct vessels reproducing the gastric and small intestinal compartments. Gastric and intestinal digestions started together and the transfer rate between the two vessels was adjusted to mimic gastric emptying of neonates. The GI transfer ends after the complete emptying of the gastric vessel (around 150 min) and intestinal digestion is stopped later at 180 min. Interestingly, the CTDM was adapted to incorporate the gastric pH drop following meal ingestion based on an old report from Mason (Mason 1962): gastric pH is maintained at 6.4 during the first 30 min and then undergoes a gradual drop to 5.1 (30-120 min). The Tiny-TIM (Schaafsma 2005), described above, was adapted to specific infant conditions in the TIMpediatric (Havenaar et al. 2013a; Havenaar et al. 2013b). In two publications, Havenaar's team from TNO adapted the Tiny-TIM to 6 to 18 months child ingesting a light meal (Havenaar et al. 2013b), and term-neonates (0-1 month), infants (1-6 months) or toddlers (6-24 months) ingesting formula milk, formula milk or mixed fruit sauce and milk, juice and cereals, respectively (Havenaar et al. 2013a). In these studies, the main distinct parameter compared to adult was a less pronounced gastric pH drop, while maintaining the same intestinal pH (6.5). For 6-18 months child, the gastric pH drops from 6.5 to 4.5 within 150 min digestion vs 5.5 to 1.7 in adult (Havenaar et al. 2013b). This was further adjusted from 6.7 to either 4, 3.2 or 2.4 for neonate, infant and toddlers, respectively (Havenaar et al. 2013a). The gastric emptying time was similar for neonate and infant (half time emptying of 60 min) but longer for toddler (70 min), with a total digestion time of 6h for the three kid categories. Digestive secretions were adapted to the amount of protein intake from food matrix and not the age (Havenaar et al. 2013b). Lastly, a dissolution model was developed and adapted from the existing dissolution paddle apparatus, the United States Pharmacopeia (USP) apparatus III of adults to simulate pH conditions and fluid volumes of the stomach, small intestine and proximal colon of infant (Karkossa et al. 2017). Intriguingly, the simulated age was not mentioned in this study. Two heated-controlled vessels were filled with the infant adapted dissolution media. The gastric phase was reproduced in one vessel during 30 min. At the end of this phase, all the gastric vessel content was transferred into a second vessel for the intestinal and proximal colonic phase by adding the specific intestinal media (lasting 4h for the small intestine and 8h for the proximal colon). Gastric pH was either set at 1.8 or 4 in two different experiments to test the gastric pH range reported *in vivo*, while the small intestinal and proximal colonic pH were maintained both at 6.8. For the gastric medium, a simulated gastric fluid without pepsin (SGFsp), at pH 1.8 was considered. The intestinal medium is reproduced by a pH 6.8 bicarbonate based simulated intestinal fluid (Carbonate-SIF, CarbSIF) containing 120 mM sodium chloride and 5 mM potassium.

Table 2.5 Main characteristics of the current infant *in vitro* dynamic mono- and bi-compartmental models mimicking the upper GIT

* when compared to adult condition

Abs : absorption; Asc : ascending colon; Duo : duodenum; Des : descending colon; Jej : jejunum; Ile : ileum; SI : small intestine; Stom : stomach; RT : residence time; T°C : temperature; Trans : transverse colon; N/A : not applicable; (-) : information not available; X : not simulated in the model

MODELS	Age	Transit time TT or Retention time RT (min or h)					Food	Digestive secretions Nutritive medium	Abs	Microbiota	Mucosal phase	References	
		Oral phase	Stomach		Small intestine								
			Large intestine		Duo	Jej							Ile
Dynamic mono-compartmental models													
<i>In vitro</i> infant gastric digestion (Israel Inst Technol, Israel)	<2 years	X	pH=6.5 to 3.5 / 0 to 240 min		X		Sodium chloride Hydrochloric acid Pepsin	X	X	X	Shami-Levi et al., 2013		
			TT = N/A										
Dynamic bi-compartmental models													
DIDGI® Dynamic Digester (INRAE, France)	-	X	pH (acidification curve) T _{1/2} = 70 min β=1.23	pH=6.5 T _{1/2} = 200 min β=2.2	X	Milk protein, dairy meat, vegetables, fruits, emulsionsate	Pepsin : 1250 U/mL ; 0.25 mL/min Lipase 60 U/mL ; 0.25 mL/min Bile: bile solution (1 %) ; 0.5 mL/min Porcine pancreatin : pancreation solution (10%) ; 0.5 mL/min	X	X	X	Ménaud et al., 2014		
												0-2 months	X
6-18 months	Yes	pH 6.5 to 4.5 / 0-150 min TT = N/A	pH 6.5	X	Light meal	α-Amylase Gastric lipase Bile Pepsin Pancreatin	X	Havenaar et al. 2013b					
Tiny TIM TNO gastrointestinal system 1 (TNO, Netherlands)	0-1 month	Yes	pH=6.7 to 4 / 0-120 min	pH 6.5	X	Formula milk		Passive (Jej + Ile)	X	X	Havenaar et al. 2013a		
TIM pediatric TNO gastrointestinal system 1 (TNO, Netherlands)	1-6 months	Yes	pH=6.7 to 3.2 / 0-120 min	pH 6.5	X	Formula milk or mixed fruit sauce	Amylase Gastric lipase Pepsin Pancreatin	Passive (Jej + Ile)	X	X	Havenaar et al. 2013a		
	6-24 months		pH=6.7 to 2.4 0-140 min			Milk, juice and cereals							
New pediatric two-stage dissolution model (Univ Greifswald, Germany)	-	X	pH=1.8-4 / 30 min	pH=6.8 / 240 min	pH=6.8 / 480 min	Water Apple juice / sauce Yoghurt, Pudding SGF - SIF	SGF <i>sive</i> pepsin CarbSIF with 120 mM sodium chloride, 5 mM potassium chloride, and 15 mM sodium bicarbonate	X	X	X	Karkossa et al., 2017		

Upper GI tract models



1.1.3 Multi-compartmental simulators of the upper gut

Then, to increase the complexity and relevance of *in vitro* gut models, only few systems became innovative by combining both bio-regionalization and dynamisms of the upper GI digestion (**Table 2.6**). So far, the TIM-1 is the main system answering to these criteria. This model has been well-validated *in vitro* / *in vivo* correlation studies (Blanquet-Diot et al. 2012). Noteworthy, the TIM-1 faithfully reproduces the physicochemical parameters of the human upper GI tract (e.g. stomach, duodenum, jejunum and ileum) and integrates the passive absorption of small molecules thanks to a dialysis system connected to the jejunal and ileal compartments (Cordonnier et al. 2015; Uriot et al. 2021). This system also allows the mimicking of the body temperature, the temporal and longitudinal changes in gastric and intestinal pH kinetics, the dynamism of the chyme transit and mixing, and the sequential delivery of the stomach, pancreatic and biliary digestive secretions (**Fig 2.1**). Nonetheless, this device does not integrate the anaerobiosis nor the digestive microbiota found in the human small intestine.

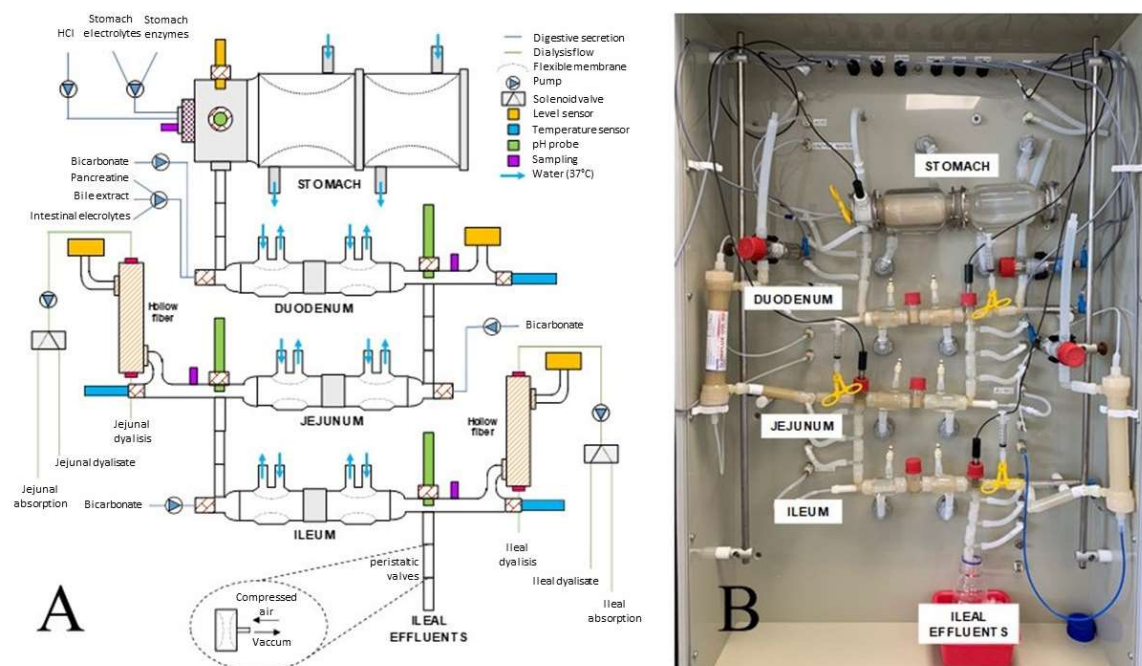


Figure 2.1 Schematic representation (A) and picture (B) of the TIM-1 system

Built from personal source.

One additional device, the Engineered Stomach and small Intestinal (ESIN), was developed to overcome some limitations encountered in the TIM-1 (Guerra et al. 2016). ESIN allows a close imitation of real food size particles entering the stomach and reproduces a differential gastric emptying between liquid and solid particles, and was also designed to allow a progressive anaerobiosis in the small intestinal compartments as well as the introduction of an ileal microbiota. However, ESIN has only been developed recently and is still under validation.

Table 2.6 Main *in vitro* dynamic multi-compartmental digestive models of the upper adult GIT

Digestive simulators of the upper GI tract		
Multi-compartmental models		
TNO gastrointestinal Model 1 (TIM-1)	Mimic from the stomach to the ileum (pH, T°, enzymes, peristaltic movements, enzyme secretions, passive absorption)	Uriot et al. 2021
Engineered Stomach and Small Intestine (ESIN)	Mimic from the mouth to the ileum. Progressive meal intake with conservation of particles and differential stomach emptying between liquid and solid particles, possibility to reproduce the ileal microbiota	Guerra et al. 2016

As described for the previous models, dynamic multi-compartmental systems has been also adapted to the digestive physiology of infants (**Table 2.7**). The Model of an Infant Digestive Apparatus (MIDA) developed by the University of Naples in Italy (Passannanti et al. 2017) is composed of four compartments reproducing the esophagus, stomach, pyloric sphincter and small intestine of 6-month infants. Each of them, except for the pyloric sphincter, is represented by two concentric tubes, the inner containing the digestive medium while the outer holds a temperature-controlled water flow to maintain a constant body temperature. As an elegant development, relevant stomach and small intestinal dimensions are used and adapted to 6-month-old infant conditions. The small intestinal compartment is made of five tubular sections continuously squeezed during digestion in order to reproduce peristaltic and segmentation motions. The overall digestion lasts 248 min with the following sequence: oral phase (8 min), gastric phase (2h), intestinal phase (2h). The oral (oesophagus), gastric and intestinal compartments remain static and maintained at pH 7, 3 and 6, respectively. The oral


pH is adjusted according to *in vivo* data, the static gastric pH is chosen to represent mean data observed between the first months to second year of life as no data specific for 6 months infant was found. The intestinal pH used corresponds to adult value. The α -amylase used in the oral phase is added based on *in vivo* data in adults at a concentration of 75 U/mL. Every 10 min, 10 mL of the incubated food bolus are injected into the esophageal compartment, together with gastric fluids composed of electrolytes and pepsin (if tested foods contain proteins). Surprisingly, a high pepsin concentration was used in this study (1000 U/mL), compared to other child models and was based on data from adult models whereas *in vivo* data in child are available (Tavares et al. 2014). During the gastric phase, the stomach is hand squeezed every 15 min after sampling, which is not relevant compared to *in vivo* data. At the end of gastric phase, the pyloric sphincter, opening and closing in a physiologically relevant manner (4 times per min in infant), allows the release in the intestinal compartment of a relevant chyme volume, depending on digested food. Gastric emptying is derived from a power exponential mathematical equation with parameters adapted to infant conditions and half-time emptying calculated according to food caloric density. During the intestinal phase, α -amylase, glucoamylase and porcine pancreatic lipase are added depending on the digested food. The TIM-1 model described above was also adapted to child digestive conditions, as related in several publications from TNO (Havenaar et al. 2013b; Havenaar et al. 2013a) and from our laboratory (Blanquet et al. 2004; Roussel et al. 2016a). This is the only model adapted to child digestion that reproduces passive absorption of small molecules and water. TIM-1 was adapted to simulate different age ranges and meal conditions: 6 months to 3 years old child ingesting a milk formula (Blanquet et al. 2004) and 6 months to 2 years child drinking a glass of water (Roussel et al. 2016a). In the seminal study from Blanquet et al. (2004), half-time chyme delivery in both gastric (80 min vs 30 min -fast GI passage- or 70 min -slow GI passage-) and ileal (200 vs 160 min) compartments was increased in child compared to adult. While intestinal pH values of duodenal, jejunal and ileal compartments remained similar, gastric pH slope was less pronounced in child compared to adult (from 6.5 to 3.5 vs 4.5 to 1.5 -fast GI passage- or 5.5 to 1.7 -slow GI passage-). In this study, all digestive secretions were reduced in child compared to adult conditions: gastric pepsin (525 vs 600 U/mL) and lipase (30 vs 37.5 U/mL) activities, as well as porcine bile salts (1% vs 4% from 0-30 min and 2% from 30-360 min) and pancreatin (2 vs 7%) concentrations. Since absorption mechanisms seem already mature at birth, the same dialysis conditions were applied in the jejunal and ileal compartments in child conditions as in adult conditions. Recently, Roussel and collaborators (2016) adapted the TIM-1 to similar child age for the simulation of drinking a glass of water (instead of a milk formula).

Half-time of chyme delivery was also increased both in gastric (20 min vs 15 min) and ileal compartments (190 min vs 150 min) compared to adult conditions. Gastric pH remained higher in child than in adult model, even if the pH set-up value was lower with water due to the absence of milk buffering effect. In this study, pepsin and lipase activities were not modified compared to adult condition, while pancreatic juice and porcine bile salts concentrations were reduced as previously described in Blanquet et al. (2004).

Table 2.7 Main characteristics of the current infant *in vitro* dynamic multi-compartmental models mimicking the upper GIT

* when compared to adult condition

Abs : absorption; Asc : ascending colon; Duo : duodenum; Des : descending colon; Jej : jejunum; Ile : ileum; SI : small intestine; Stom : stomach; RT : residence time; T°C : temperature; Trans : transverse colon; N/A : not applicable; (-) : information not available; X : not simulated in the model

MODELS	Age	pH / time (min)							Food	Digestive secretions Nutritive medium	Abs	Microbiota	Mucosal phase	References	
		Oral phase	Stomach	Small intestine			Large intestine								
				Duo	Jej	Ile	Asc	Trans							Des
Upper GI tract models 	MIDA - Model of an Infant Digestive Apparatus (Univ Naples, Italy) 6 months 6 months – 3 years TIM1 - TNO gastrointestinal system 1 (TNO, Netherlands)	pH=7/2 min	pH=3 / 120 min	pH=6 / 120 min			X	Rice starch-based food, rice-cream based food SSF - SGF - SIF	α-amylase in SSF and SIF Pepsin in SGF Glucoamylase in SIF Pancreatic lipase in SIF	X	X	X	Passamanti et al., 2017		
		TT = N/A	T _{1/2} = 20-28 min	TT = N/A	pH=6.5	pH=6.8								pH=6.8	Milk formula
		X	pH=6.5 / 0-30 min pH=6.5 / 30-150 min pH=4.5 / 150-240 min pH=3.5 at 240 min T _{1/2} = 70 min β=1.23	pH=6.4 pH=6.9 pH=7.2 T _{1/2} = 120 min β=1.7	pH=6.4 pH=6.9 pH=7.2 T _{1/2} = 200 min β=2.2	Water	Pepsin 130 IU/min Lipase 5 IU/min Pancreatic juice 3.5% Bile salts 2% Trypsin	Passive (Jej + Ile)	X	Roussel et al., 2016					

1.1.4 Conclusion and current limits of digestive simulators of the upper gut

Taken together, available data indicate that scientists reached a consensus when developing *in vitro* models by setting-up a less pronounced gastric pH drop in child compared to adults, while maintaining the same intestinal pH. Developments that are more controversial are observed regarding GI secretions where broad range of concentrations and enzyme activities are used between *in vitro* models. Nevertheless, a decrease in pepsin and intestinal lipase activities and bile salts concentrations are the marked parameters changed in infant models compared to adult conditions. Likewise, many discordances persist for gastric and intestinal transit times depending on studies. This discrepancy can be explained by the paucity of *in vivo* data in healthy neonates, infants and toddlers due to their invasive character. Also, the presence of the food matrix is poorly considered in most studies. The age simulated is varying widely between studies, with models set-up on age range rather than a precise age.

As seen with the previous examples, the *in vitro* simulators of upper human GI tract are more and more complex and accurate to simulate human digestion. However, they still present some limits. The chemical products used (e.g. enzymes, bile salts) are never from human origin and thus, potentially far from human physiology. More importantly, the host part is missing. In consequence, the absorption phenomenon, when included, is limited to passive absorption. Lastly, it is important to underline that none of these models reproduces the microbial component of the child small intestinal environment, focusing mainly on digestive mechanical and physicochemical parameters and almost no model respects the anaerobic status of the GIT compartments. Then, the cross talk between the human microbiota and the immunity cannot obviously be reproduced in those *in vitro* systems. The *in vitro* gut models integrating human microbiota are developed in the next section.

1.2 *In vitro* lower gut models

The development of bioreactors able to reproduce the fermentation process of the human gut originated from the awareness that fecal microbiota significantly differ from the *in vivo* colon microbiota, both in terms of community composition and metabolic activity (Van de Wiele et al. 2015). Thus, the common purpose of fermentation systems is to cultivate a complex fecal microbiota under controlled colonic environmental conditions for carrying out microbial modulation and metabolism studies. There is no consensus in the preparation of fecal samples. Aguirre et al. (2015) demonstrated comparable microbial diversity and metabolic activity

between pooled and not pooled human feces (Aguirre et al. 2015), while Van de Wiele et al. (2015) revealed that “the artificially high microbial diversity in pooled inocula creates disturbances in the cross-feeding processes between microorganisms that are adapted to one another in each of the separate microbiomes”. Therefore, authors advised to study inter-individual variability through separate fecal experiments (Van de Wiele et al. 2015). Finally, the use of either fresh stool or fecal samples processed with conservative treatments (e.g. frozen or lyophilized) for bioreactor inoculation could also modify the kinetic of implantation in the *in vitro* models (Chen et al. 2019b; Pérez-Burillo et al. 2021).

1.2.1 Static mono-compartmental models

Again, static systems or batch represent the simplest option. In batch systems, fecal microbiota is diluted in a nutritive medium constantly stirred (average fecal concentration of 10% w/v) and cultured during 24 to 48h in a temperature-controlled vessel maintained under anaerobiosis (by flushing with nitrogen and/or carbon dioxide). Upon inoculation, the batch remains static with no further medium input or output. Lastly, a static mini colon model has been recently developed, named the Copenhagen MiniGut (CoMiniGut), developed by University of Copenhagen in Denmark (Wiese et al. 2018). It is composed of small replicate bioreactor unites with a working volume of 5 mL allowing multiple tested conditions or a higher number of biological replicates (**Table 2.8**).

Available batch models of the infant colon were set-up by using fecal samples from infants of 5 to 11 months in the model of Gamage’ team from Macquarie University in Australia (Gamage et al. 2017), and 1 to 5 months by Ahlborn and colleagues from Grassland Research Center in Australia (Ahlborn et al. 2020) (**Table 2.8**). Ahlborn’s team uses pre-digested raw sheep and cow milk as nutritive medium according to the simple and static method proposed by Minekus (Minekus et al. 2014). The suspension is mixed with a cysteine solution to reduce the medium and baby feces diluted in phosphate buffer. The mixture is incubated at 37 °C with constant flushing with carbon dioxide and fermentation lasts up to 24h. Gamage and colleagues use predigested cereal products according to an *in vitro* digestion protocol adapted to infant conditions, but after perform fermentation under anaerobic conditions for a 48-h period without any adaptation of the basal medium simulating adult large intestinal conditions (Gamage et al. 2017). The rapidity and high throughput of batch models render them very useful for large screening studies but this approach is limited by short-time fermentation but also accumulation of metabolites and pH decrease that could impede microbial activities (Payne et al. 2012). The

CoMiniGut, initially designed to reproduce the adult colon, was also further used to mimic 6-month infant conditions (Wiese et al. 2018). However, except the use of infant stool, none of the operating parameters was adapted to a 6-month infant physiology. Besides, the microbiota profiles reported in this work suggest that the system is not well- controlled (maybe due to very small reactor volumes) and microbiota composition is abnormal for infant with dominance of Proteobacteria, low Firmicutes and almost no Actinobacteria.

Table 2.8 Main *in vitro* static mono-compartmental models of the lower adult GIT

Fermentation systems		
Static mono-compartmental models		
<i>Adult</i>		
Copenhagen MiniGut (CoMiniGut)	Five low volume-bioreactors (5 mL) for high throughput	Wiese et al. 2018
<i>Infant</i>		
Gamage's model	5 to 11 months, successive oral, gastric, small intestine and colonic digestions in the same vessel Upper digestive part digestion: redigested cereal products according to an <i>in vitro</i> digestion protocol adapted to infant condition Fermentation medium not adapted to infant	Gamage et al. 2017
Ahlborn's model	1 to 5 months Nutritive medium adapted: pre-digested raw sheep and cow milk	Ahlborn et al. 2020
Copenhagen MiniGut (CoMiniGut)	Used only infant stools to inoculate bioreactors. No adaptation of the operating parameters	Wiese et al. 2018

1.2.2 Dynamic mono- or multi-compartmental models

Dynamic colon models rely on continuous or semi-continuous (and not batch) fermentation systems, with regular input of nutritive medium and regular output of fermentation medium, this allows to define for each bioreactor (for multi-compartmental models) a specific residence time that can be extrapolated to *in vivo* transit time. Most of the culture media used to simulate the composition of ileal effluents are derived from the initial one described by Macfarlane and colleagues from University of Reading in UK for adult colon models (Macfarlane et al. 1989). As for batch systems, bioreactors are temperature-controlled and

maintained under anaerobiosis. Colonic pH is continuously monitored and controlled, and redox potential can be measured in real time in some of the models. Human colonic conditions can be reproduced by a single vessel or by multiple bioreactors in series representing different anatomical parts of the colon (ascending, transverse and descending colon). Prior to start an experiment and for a stable metabolic activity, the initial fecal inoculum needs a suitable adaptation period to the environmental conditions found in the respective colon compartments. The length of stabilization will relate to the residence time that is imposed in the bioreactor and can vary greatly between 8 to 20 days (Van de Wiele et al. 2015; Verdier et al. 2021). As for batch models, intermediate or end-products of microbial fermentation such as gas and SCFA can be measured in the fermentation media or atmospheric phase of bioreactors and microbiota composition is determined.

1.2.2.1 Dynamic mono-compartmental models

Various dynamic mono-compartmental models have been developed to mimic average human colonic conditions in a single vessel, such as the ARCOL from our laboratory or the Polyfermentor Intestinal Model (PolyFermS) from Lacroix's team in ETH Zurich in Switzerland (**Table 2.9**). ARCOL is a single-stage device whose have the particularity that the anaerobiosis is maintained by the sole activity of the gut microbes whereas most fermentor systems are maintained anaerobically by regular flushing with CO₂ and/or N₂ gases (Thévenot et al. 2013) (**Fig. 2.2**). This model has been recently adapted in the M-ARCOL configuration to maintain the mucus-associated gut microbiota in a separated compartment based on the Simulation of the Mucosal Human Intestinal Microbial Ecosystem -M-SHIME- (see below in the *paragraph 1.3*). Briefly, a glass vessel filled with mucin-alginate beads is connected to the main bioreactor allowing the continuous flow of luminal medium on mucin beads and the replacement of beads without flushing with gases.

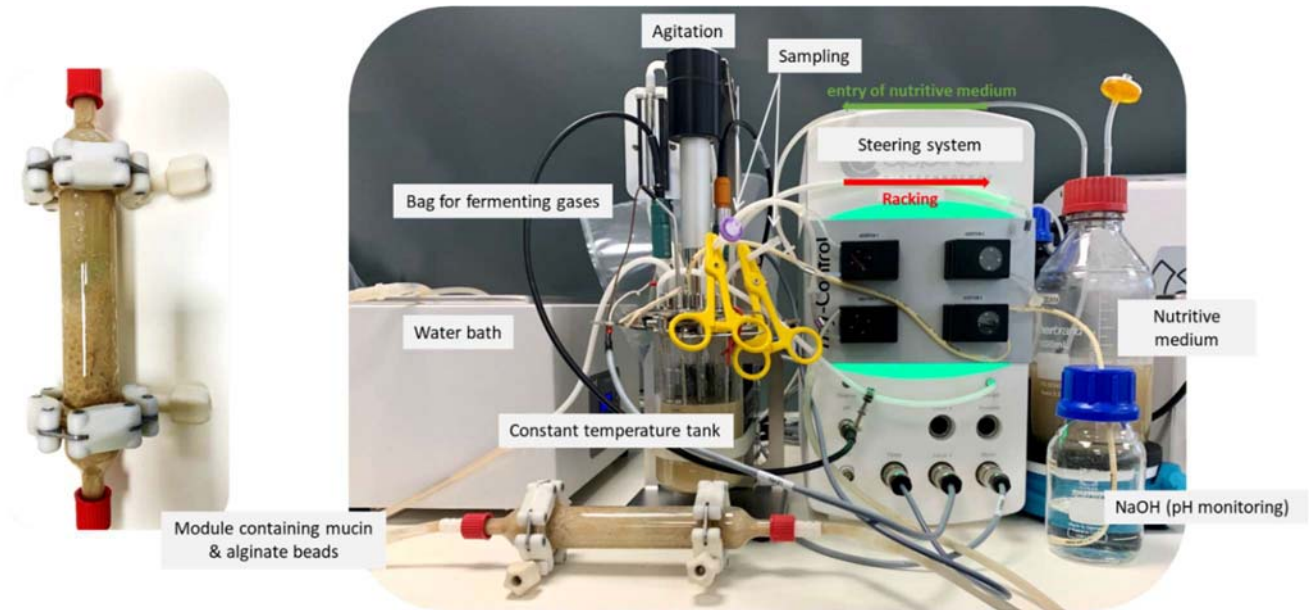



Figure 2.2 Picture of the M-ARCOL model with its mucin-alginate bead module

With originality, PolyFermS bioreactors are seeded with immobilized fecal microbiota and used to continuously inoculate with the same microbiota different second-stage reactors mounted in parallel, avoiding the washout of the most sensitive populations (Poeker et al. 2018). Lastly, several mini dynamic colon models have been recently developed as the minibioreactor arrays (MBRAs) (Auchtung et al. 2015) developed by the Alkek Center for Metagenomics and Microbiome Research and even more freshly, the mini colon model (MiCoMo) set up by McGill University (Jin et al. 2022). They are composed of small replicate bioreactor units with a working volume of only 15 and 30 mL, respectively. Contrary to the CoMiniGut described above, these systems reproduce the feed cycle allowing longer fermentations (several weeks), and thus, the development of a more complex microbiota.

Table 2.9 Main *in vitro* dynamic mono-compartmental digestive models of the lower adult GIT

Fermentation systems		
Dynamic mono-compartmental models 		
ARTificial COLon (ARCOL)	Anaerobiosis guaranteed by the sole activity of the gut microbiota (no flushing) (M-ARCOL includes a mucosal phase (mucin-alginate beads))	Thévenot et al. 2013
Polyfermentor Intestinal Model (PolyFermS)	Mimic the adult proximal colon (pH, T°, transit time.), 1 to 5 replicate bioreactors	Poeker et al. 2018
Minibioreactor arrays (MBRAs)	Composed of small replicate bioreactor units with a working volume of only 15 mL	Auchtung et al. 2015
Mini colon model (MiCoMo)	Composed of small replicate bioreactor units with a working volume of only 30 mL	Jin et al. 2022


The first dynamic mono-compartmental colon model adapted to child conditions (**Table 2.10**) was the PolyFermS (Cinquin et al. 2004). In this study, the continuous *in vitro* model reproduces the infant colon from 4 to 6 months old. The nutritive medium was made according to Macfarlane's adult medium and adapted to infant digestion of a standard formula, considering infant digestibility indices. In particular, protein fraction was reduced, and whey proteins, starch, maltodextrin and lactose were added to replace complex fibers. Bile salts, which are less concentrated in the GI tract of infant, were also reduced compared to adult (0.05 g/L vs 0.4 g/L, respectively). Three different successive residence time and pH values were applied in order to reproduce the three colonic parts in a single bioreactor: 4h/5.7, 8h/6.2 and 12.5 h/6.8 (residence time/pH). Residence time and colonic pH were not modified compared to the adult model. Of note, the innovative microbiota immobilization technique on mixed gellan-xanthan gel beads allows modeling infant gut microbiota even with a very low amount of fecal sample is available and ensures recovery and viability of dominant cultivable bacteria from infant feces such as *Bifidobacteria* (Cinquin et al. 2004; Pham et al. 2019). Other publications from ETH Zurich in Switzerland reported further adaptations of the PolyFermS model. First, Dostal and colleagues simplified the model to simulate only the proximal colon of 2.5 years old child with the increase of retention time to 8h at pH 5.7 (Dostal et al. 2015). In many studies, the selected retention time relies on a general rule stating that proximal colon transit time is

estimated to be about one-third of the total colonic transit time. The infant adapted medium used was developed previously (Le Blay et al. 2009) and mainly relies on the reduction of biliary salts concentrations from 0.4 to 0.05 g/L (Macfarlane et al. 1998). Then, Pham and collaborators further adapted PolyFermS for 2-month-old formula-fed infant (Pham et al. 2019). Different pH values were tested in order to represent the proximal colonic pH of breast-fed (pH 5) or formula-fed infant (pH 7), as well as different retention times (5h or 10h). The nutritive medium was adapted to younger infant according to Cinquin's study, by removing maltodextrin, increasing lactose (6.4 vs 5 g/L) and whey proteins (8.1 vs 7.2 g/L) and decreasing casein concentrations (0.5 vs 1.2 g/L) (Cinquin et al. 2004). Lastly, Doo and collaborators again extended potentialities of the proximal colon PolyFermS for 6-month-old formula-fed infant (pH 6, retention time 7.4 h) and 8-month-old African infant living in poor environmental conditions and fed with a mixed diet (pH 5.8, retention time 6.1h) (Doo et al. 2017). A very close 6-month colonic model was developed by University of Agronomic Science and Veterinary Medicine in Romania, the mono-compartmented system of *in vitro* colonic simulation (GIS1), using the same operating parameters and medium composition as described by Cinquin (Vamanu et al. 2013). The only notable change is the addition of twenty 10-mm diameter glass balls to better simulate the peristalsis motion. These mono-compartmental colonic models are interesting, but do not consider the spatial regionalization of the colon observed *in vivo*.

Table 2.10 Main characteristics of the current infant *in vitro* dynamic mono-compartmental models mimicking the lower GIT

* when compared to adult condition


Abs : absorption; Asc : ascending colon; Duo : duodenum; Des : descending colon; Jej : jejunum; Ile : ileum; SI : small intestine; Stom : stomach; RT : residence time; T°C : temperature; Trans : transverse colon; N/A : not applicable; (-) : information not available; X : not simulated in the model

MODELS	Age	Transit time TT or Retention time RT (min or h)				Food	Digestive secretions Nutritive medium	Abs	Microbiota	Mucosal phase	References		
		Oral phase	Stomach	Small intestine								Large intestine	
				Duo	Jej							Ile	Asc
Dynamic mono-compartmental models													
Lower GI tract models 	4 - 6 months	X	X	X	pH=5.7 RT = 4h	pH=6.2 RT = 8h	pH=6.8 RT = 12.5h	X	Derived from Macfarlane adult medium and adapted to infant digestion	X	Child fecal sample (fresh) n=4 + gellan-xanthan gel beads	Mucin in medium (porcine gastric type II)	Cinquin et al. 2004
		X	X	X	pH=5.7 RT = 8h	X	X	X	Derived from Le Blay et al. 2009	X	Child fecal sample (fresh) n=1 + gellan-xanthan gel beads	Mucin in medium (porcine gastric type III)	Dostal et al., 2015
	2 months	X	X	X	Various pH RT = 5h	X	X	X	From Cinquin infant medium (6 months old)	X	Child fecal sample (fresh) n=2 + gellan-xanthan gel beads	Mucin in medium	Pham et al., 2019
		6 months 8 months	X	X	X	pH = 6 RT = 7.4 pH = 5.8 RT = 6.1	X	X	Derived from formula-fed infants Le Blay et al. 2010	X	Child fecal sample (fresh) n=2 + gellan-xanthan gel beads	Mucin in medium	Doo et al. 2017
	6 months	X	X	X	pH=5.6-5.9	pH=6.2-6.5	pH=6.6-6.9	Standard infant formula	Gastric juice (3 g/L pepsin + 3 g/L mucin) Bile salts (1, 2, 3, 4%) Pancreatin Derived from Macfarlane adult medium and adapted to infant digestion	X	Pool of child fecal samples (fresh) n=1 infant (several days of fecal collection)	Mucin in medium	Vamanu et al., 2013

1.2.2.2 Dynamic Multi-compartmental models

Different multi-stage models have been developed to answer this limitation (**Table 2.11**), among which the continuous three-stage colon system developed by Gibson, Cummings and MacFarlane (Gibson et al. 1988) or the PolyFermS which can be set up with the three colonic compartments depending on the level of regionalization needed (Fehlbaum et al. 2015). The Gibson model was adapted by Danisco in Finland to a four-stage colonic fermentation system reproducing the ascending, transverse and descending colon and the sigmoid/rectum area named the EnteroMix (Mäkivuokko et al. 2005). Furthermore, TIM-2, developed by the TNO and that can simulate the three parts of the human colon, is very different from the traditional bioreactors (Minekus et al. 1999). It consists of an assembly of compartments, which reproduce the peristalsis and passive absorption of water and fermentation products thanks to dialysis fibers, as initially created in the TIM-1. As a drawback, this system monitors the microbiome only on a short timeframe (1 week maximum) and give therefore poor chance to the gut microbes to be well stabilized (a 48h stabilization period has been reported). Besides, the anaerobiosis remains difficult to maintain since the system is not a bioreactor. Based on Macfarlane's three-stage colonic model and SHIME model, the Human colonic model (HCM) is a recent model developed by Miami University reproducing also the three part of the colon ; ascending, transverse, and descending colons (Xu et al. 2020). Noteworthy, the nutritive medium was digested according to a simple batch protocol prior to its addition in the fermenters and pooled human fecal samples were used to inoculate the system.

Table 2.11 Main *in vitro* dynamic multi-compartmental models of the lower adult GIT


Fermentation systems		
Dynamic multi-compartmental models 		
Three-stage Polyfermentor Intestinal Model (PolyFermS)	Can be set up with three link-compartments corresponding to the three parts of the colon or with only the proximal and distal colon	Fehlbaum et al. 2015
EnteroMix	4 compartments (ascending, transverse and descending colon and the sigmoid/rectum area)	Mäkivuokko et al. 2005
TNO gastrointestinal Model 2 (TIM-2)	1-3 compartments Integration of peristalsis and passive absorption	Minekus et al. 1999
The Human Colonic Model (HCM)	3 colonic compartments Nutritive medium pre-digested	Xu et al. 2020

Regarding infant conditions, ETH Zurich in Switzerland provided an update of the PolyFermS system by developing a three stages model of the child colon using three serial reactors (Cinquin et al. 2006) (**Table 2.12**). All the parameters were adapted to 6-months infant based on *in vivo* data. The total mean retention time is set at 13h and fixed in each compartment at 4, 5 and 4h, respectively. pH is only controlled in the two first reactors and set at 5.9 (proximal colon) and 6.2 (transverse colon), and naturally stabilized between 6.6-6.7 in the last reactor (distal colon). A similar system was developed by University of Agronomic Science and Veterinary Medicine, the multi-compartmental GIS2, an adaptation of the GIS1 composed by three reactors in series (Vamanu et al. 2015). The EnteroMix, initially developed by Danisco in Finland for adult studies (Mäkivuokko et al. 2005) was also used for infant gut research by inoculating the model with infant stools under 1 year old (Salli et al. 2019). To generate enough fecal inoculum, infant stools produced within a week were frozen, pooled, diluted in the nutritive medium, and amplified prior to inoculation. Anymore, none of the other parameters has been adapted to the specific infant colon conditions. This complex model reproduces from the ascending colon to the sigmoid/rectum area, with four glass vessels and ensures for each of them a control of pH (5.5, 6.0, 6.5 and 7.0, in the ascending, transverse, descending and sigmoid /rectum parts, respectively). Each vessel is inoculated with the amplified fecal inoculum and after 3h, the fermentation begins with the addition of 3 mL of nutritive medium to the first vessel. Every 3-hours, 3 mL are transferred from one vessel to the following one. Finally, 12h later, 3 mL are discarded from the last compartment. The entire experiment lasts 48 h.

Table 2.12 Main characteristics of the current infant *in vitro* dynamic multi-compartmental models mimicking the lower GIT

* when compared to adult condition

Abs : absorption; Asc : ascending colon; Duo : duodenum; Des : descending colon; Jej : jejunum; Ile : ileum; SI : small intestine; Stom : stomach; RT : residence time; T°C : temperature; Trans : transverse colon; N/A : not applicable; (-) : information not available; X : not simulated in the model

MODELS	Age	Transit time TT or Retention time RT (min or h)			pH / time (min)			Food	Digestive secretions Nutritive medium	Abs	Microbiota	Mucosal phase	References	
		Oral phase	Stomach	Small intestine		Large intestine								
				Duo	Jej	Ile	Asc							Trans
Dynamic multi-compartmental models														
Lower GI tract models 	Three-stage PolyFermS - Polyfermentor Intestinal Model (ETH Zurich, Switzerland)	6 months	X	X	X	X	X	X	X	X	X	X	Cinquin et al., 2006	
		6 months	X	X	X	pH=5.9	pH=6.2	pH=6-6.7	-	From Cinquin medium	X	Child fecal sample (fresh) n=2 + gellan-xanthan gel beads	Mucin in medium (porcine gastric type II)	Vamanu et al., 2015
						RT = 4 h	RT = 5 h	RT = 4 h						
GIS2 - <i>In Vitro</i> Colonic Fermentation Model (Univ Agronom Sci Vet Med, Romania)	6 months	X	X	X	pH=5.5	pH=6.2	pH=6.3	-	From Vamanu medium	X	Child fecal sample (fresh) n=1	Mucin in medium (porcine gastric type II)	Salli et al. 2019	
					RT = not mentioned									
EnteromIX® - Danisco (Finland)	< 1 year	X	X	X	pH=5.5	pH=6	pH=6.5 (des) pH=7 (distal)	-	From Macfarlane adult medium	X	Child fecal sample (fresh) n=2	Mucin in medium (porcine gastric type III)		

1.2.3 Conclusion and limits of fermentation models for the lower human gut

Adaptation of critical parameters, such as pH and retention time, that widely influence gut microbiota composition and activity, is a key feature of mono and multi-compartmental colonic models (Duncan et al. 2009; Tottey et al. 2017). Unfortunately, in some of the current developments mentioned above, these parameters have not been adapted to specific child colonic conditions, with adult parameters remaining the standard. This probably results from paucity of *in vivo* data or high individual variability, especially when all differentiated colon compartments are considered. Despite their diversity and flexibility, those fermentation models undoubtedly present some limits. Under the adult or child conditions, they always exhibit lower gut microbiota diversity compared to human original fecal samples, suggesting that they are not yet capable of supporting the full range of species that are living in the human gut (Van de Wiele et al. 2015; Pham and Mohajeri 2018). Similarly, SCFAs that play a major role in gut homeostasis are not absorbed by most of these models, or only by passive mechanisms, which may have an impact on gut microbiota (Pham and Mohajeri 2018).

1.3 Whole gastro-intestinal models

Multi-stage continuous bioreactor systems have been also extended to include the anterior segments of the GIT. They allow to perform microbiota studies not only in the colonic compartment but also in the upstream's ones, notably the ileum. The SHIME[®] developed in the 90's by Ghent University in Belgium (Molly et al. 1993) and the Multicompartmental Dynamic Model of the Gastrointestinal System (SIMGI), which imitates the original SHIME[®] (Barroso et al. 2015), are the two well-known multi-stage bioreactors including the human microbiota (**Table 2.13**). They simulate nearly entirely the GI transit from the stomach to the descending colon (Ménard et al. 2015; Van de Wiele et al. 2015). Importantly, by integrating upper compartments of the gut and their physiological impact on the whole GIT, these models gathered digestive simulators and fermentation models, even if the simulation of the upper GI part in these models remain less complex than in gastric and small intestinal model such as the TIM-1. The main difference between SHIME[®] and SIMGI[®] is that the latter includes the peristaltic movements of the stomach. Nevertheless, the SHIME[®] remains the originally and internationally known system with special features that are constantly developed in this system. As explained earlier, the mucosal microbiome was already known to fundamentally differ from the luminal microbiome in composition with specific surface-attached mucosal microbes. This niche remains difficult to access in human, and to answer that, the SHIME system was

optimized for mimicking the mucosal microbial colonization, called M-SHIME. It consists of the incorporation of type 2 mucin-agar covered microcosms (polyethylene netting) within the bioreactors. To mimic mucosal desquamation and allow a physiological wash-out of mucin-adhered microbes, the microcosms are regularly replaced (Van den Abbeele et al. 2012).

Table 2.13 Main whole gastrointestinal models of the adult GIT

Digestive simulators / fermentation systems		
Simulation of the Human Intestinal Microbial Ecosystem (SHIME)	Mimic mouth, stomach, duodenum, ileum and the three stages of the large intestine.	Molly et al. 1993
Multicompartmental Dynamic Model of the Gastrointestinal System (SIMGI)	Stomach, small intestine and three stages of the large intestine Peristaltic movements of the stomach	Barroso et al. 2015
Mucosal SHIME (M-SHIME)	M-SHIME includes a mucosal phase (mucin-agar microcosms)	Van den Abbeele et al. 2012


The SHIME model was adapted to reproduced infant conditions -from 3 to 18-months- in the Baby SHIME (De Boever et al. 2001) (**Table 2.14**). Baby SHIME is a semi-continuous model, composed of six temperature-controlled vessels reproducing the stomach, duodenum, jejunum/ileum and ascending, transverse and descending parts of the colon. The colon compartments are inoculated with a pooled of three feces collected from 3 to 12 months infants. pH values are not adapted to child conditions but only maintained between fixed limits ranging for stomach from 3.8 to 4.1 and for small intestine from 5.5 and 6, as previously set for adult conditions (Molly et al. 1993). The pH values of ascending (between 5.5 and 6.0) and transversal colon (between 6.0 and 6.3) compartments were also set up based on adult data, while the last vessel corresponding to the descending colon was maintained from 6.3 to 6.5 based on 10-18 months infant stool pH. For each GI compartment, the transit time is set at 3, 3, 2, 8, 12 and 8h, respectively, as inferred from infant whole gut transit time reported in *in vivo* studies on 6-week old infants (McClure and Newell 1999) and 1 to 4 years toddlers (Weaver and Steiner 1984; Walker and Walker 1985). The nutritive medium used, based on a milk formula intended for babies aged 4 to 18 months, is added to the first gastric vessel, three times per day. Another baby-SHIME was adapted from the M-SHIME using fresh pooled fecal samples from 6 month-old infants allowing the development of the specific mucosal microbiota

(Van den Abbeele et al. 2019). Lastly, the SHIME system was also very recently adapted to mimic toddler digestion (Bondue et al. 2020). Colonic compartments were inoculated with fecal samples collected from healthy formula-fed children aged between 1 and 2 years. Apart from using feces from toddlers, the model was further adapted by changing the composition of the nutritive medium even if there is no information about it in the publication. pH and retention time parameters were based on adult data.

Table 2.14 Main characteristics of the current infant *in vitro* complete gastro-intestinal models

* when compared to adult condition

Abs : absorption; Asc : ascending colon; Duo : duodenum; Des : descending colon; Jej : jejunum; Ile : ileum; SI : small intestine; Stom : stomach; RT : residence time; T°C : temperature; Trans : transverse colon; N/A : not applicable; (-) : information not available; X : not simulated in the model

MODELS	Age	pH / time (min)						Food	Digestive secretions Nutritive medium	Abs	Microbiota	Mucosal phase	References
		Transit time TT or Retention time RT (min or h)		Large intestine									
		Oral phase	Stomach	Small intestine	Asc	Trans	Des						
Complete gastro-intestinal models													
Upper+ lower GI tract models 	3 - 12 months	X	pH=3.8-4.1 / 180 min	pH=5.5-6 / 180 min	pH=5.5-6 / 120 min	pH=5.5-6	pH=6-6.3	pH=6.3-6.5	-	X	Pool of child fecal samples (fresh) n=19	Mucin in medium	De Boever et al., 2001
			RT = N/A	RT = N/A	RT = 8 h	RT = 12 h	RT = 8 h						
	6 months	N/A	pH=3.8-4.1 / 90 min	pH=6 / 180 min	RT = 32 h	pH=5.5-6	pH=6-6.3	X			X	Pool of child fecal samples (fresh) n=3	Mucin-agar beads (porcine gastric type III)
Toddler SHIME® - Simulator of the Human Intestinal Microbial Ecosystem (Univ Liege, Belgium)	1 - 2 years old	N/A	pH=3.8-4	pH=5.9-6.1	pH=5.4-5.6	pH=6-6.3	pH=6-6.5			X	Pool of child fecal samples (fresh) n=3	-	Bondué et al., 2020

2. *In vitro* models of the intestinal barrier

All the gut models mentioned above are relevant models to reproduce digestive luminal conditions (rarely mucosal ones), but none of them integrates intestinal cells. Therefore, complementarily to *in vitro* systems modelling the upper or lower human GI tract, *in vitro* models reproducing epithelial, goblet and/or immune intestinal host cells, with various levels of complexity from monoculture models to *ex vivo* organs, can be useful to investigate the uptake, fate and effects of various ingested pollutants (pre-digested or not) on the human gut barrier.

2.1 *In vitro* cell models

2.1.1 Mono-culture models

Hundreds of intestinal epithelial cell lines have been established from tumors, several of which have been extensively used and characterized. The most widely used include Caco-2, HT-29, T84, LoVo, LS174T, DLD-1, SW480, and SW620 (Bjerknes and Cheng 2006) (**Table 2.15**). Caco-2 cell line, the most frequently used, was originally isolated from a human colorectal adenocarcinoma, and spontaneously differentiates upon confluence to a monolayer of polarized enterocytes within 14 to 21 days, expressing microvilli, transporters and brush border enzymes (Hidalgo et al. 1989). Also isolated from a human colorectal adenocarcinoma, HT-29 cells are commonly used and especially the clone HT29-MTX which can be differentiated into a mucus-secreting phenotype by growth under metabolic stress conditions (Lesuffleur et al. 1990). When grown on Transwell filter supports, some HT29-MTX clones (e.g. MTX-D1 and MTX-E12) form polarized monolayers mostly constituted of mature goblet cells secreting an adherent mucus layer of 150 μm thickness composed with MUC2 and MUC5AC (Hews et al. 2017) as revealed by Alcian Blue staining (Behrens et al. 2001). Among HT-29 cell derivatives, the LS174T colon carcinoma cell line also secretes mucus, and notably secretes mature MUC2 and MUC5AC (van Klinken et al. 1996) but does not produce an adherent mucus layer (Navabi et al. 2013). T84 colonic adenocarcinoma cell line is also extensively used as intestinal epithelial model. They also differentiate in goblet-like cells and produce mucin (McCool et al. 1990). While Caco-2 cells are often described as more reminiscent to small intestinal enterocytes than colonocytes, T84 cells harbor distinct morphological, biochemical and functional characteristics closer to original colonic ones (Devriese et al. 2017). Several intestinal epithelial cell lines have been also produced from

normal tissue from various species but they are relatively undifferentiated and the cells are usually described as “crypt-like.” Some of them have been derived from human: the human fetal small intestine (HIEC) (Perreault and Beaulieu 1996), and the human adult colon as NCM460 (Moyer et al. 1996) and NCOL-1 (Deveney et al. 1996). However, to date, no cell culture model was adapted to specific infant conditions.

2.1.2 Co-culture models

To better modelling human intestinal epithelia, mixed cultures of enterocyte-like Caco-2 cells and mucus-producing HT29-MTX cells have been widely used in permeability studies (Hilgendorf et al. 2000; Lozoya-Agullo et al. 2017) (**Table 2.15**). Various seeding ratios were proposed in the literature but the one with 90 % Caco-2 and 10% of HT29-MTX was the closest to physiological conditions in term of cellular repartition and epithelial permeability (Chen et al. 2010; Pan et al. 2015). However, some studies reported that this co-culture model secretes mucus patches upon itself rather than a proper continuous mucus layer (Dorier et al. 2017; García-Rodríguez et al. 2018; Gillois et al. 2021), while some others state that co-cultures seeded out on Transwell inserts form a continuous mucus layer similar to cultures of HT29-MTX cells grown alone (Poquet et al. 2008; Béduneau et al. 2014). More complex co-cultures were also developed to incorporate inflammatory cell lines to better simulate the intestinal epithelium. A triple co-culture system combining Caco-2 cells, HT29-MTX and differentiated M cells, which are Caco-2 able to differentiate into M-cells following addition of Raji-B lymphocytes, have been mostly used (Antunes et al. 2013; Vernay et al. 2020). Another co-culture of Caco-2 cells with macrophages and dendritic cells on Transwell filter inserts is also used especially in xenobiotic and nanoparticle interactions studies (Leonard et al. 2010). Following the same purpose, association of Caco-2, HT-29-MTX and human monocyte cells (THP-1) have been also used (Li et al. 2013). Co-culture models have been extensively used in pollutant studies to evaluate pesticide residues (Guibourdenche et al. 2021) or for various silver or (Gillois et al. 2021)- and copper oxide nanoparticles (Büttner et al. 2022).

2.1.3 Gut-on-a-chip

Another cellular approach to simulate a human intestinal epithelium is the ‘Gut-on-a-Chip’ system, where Caco-2 cells are grown on a porous membrane support in a microfluidic device (**Table 2.15**). While the cell membrane support is maintained under cyclic strain mimicking peristaltic motion, the chambers above and below the cell membrane are constantly

perfused with medium, thereby generating low shear stress. These stream disturbances upon the cells are particularly relevant of the *in vivo* conditions, where the mucosal surface is under turbulent flow (De Weirdt and Van de Wiele 2015). Furthermore, this turbulent environment stimulates the formation of 3D intestinal villi similar to those found in the small intestine (Kim et al. 2012), and the differentiation of Caco-2 cells into four types of differentiated epithelial cells including enterocytes, mucus-secreting, enteroendocrine and Paneth cells (Kim and Ingber 2013). In addition, Caco-2 epithelium grown in the Gut-on-a-Chip model displays enhanced barrier function and mucus production as compared to static Caco-2 cell cultures (Kim and Ingber 2013). The gut-on-a-chip devices is a suitable tool to follow intestinal inflammation (Shin and Kim 2018). The Human Microbial Cross-talk (HuMix) model is another microfluidic device enabling the culture of human cell lines like Caco-2 cell with commensal gut bacteria under anaerobic conditions. In contrast to the Gut-on-a-Chip system, the epithelial cells, which do not produce mucus, are separated from the bacteria by a membrane coated with porcine gastric mucin (Shah et al. 2016). Gut-on-a-chips represent valuable tools to assess toxicity of environmental pollutants providing a complex microenvironment comparable to the *in vivo* situation (Yang et al. 2021). A gut-on-a-chip model was developed by Kulthong and his team and used to investigate the potential cell association and transport of a mixture of 17 dioxin congeners (Kulthong et al. 2018). No gut-on-a-chip model was adapted yet to the infant-specific conditions but Jalili-Firoozinezhad and his team inoculated adult intestine chips with complex microbiome isolated from infant stool samples (Jalili-Firoozinezhad et al. 2019). However, these models were not applied yet to toxicological studies.

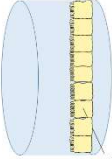
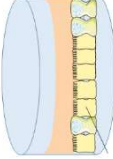
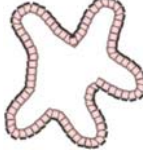
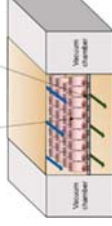
2.2 Human enteroids/colonoids and *ex vivo* organ cultures

As illustrated above, cell lines models and their diverse complexity levels provide valuable tools in pollutant studies. However, all these models are still limited by the absence of the tissue microenvironment. New technologies have been developed which enable the generation of self-propagating spheres of primary intestinal epithelial cells (“mini-guts”). Enteroids or colonoids are derived from adult stem cells isolated from the crypts of human small intestinal or colonic tissues, respectively (Jung et al. 2011; Sato et al. 2011) (**Table 2.15**). As these enteroids are not derived from cancer cells, their differentiated cells are supposedly closer to human physiology. The apical side of the epithelium can be on the inside or on the outside side of the enteroids (Co et al. 2019). Culture approaches using human intestinal biopsy samples therefore represent an upscale platform. The advantages of *in vitro* organ culture of intestinal biopsies vs cell line culture models include the presence of healthy non-transformed cells with

all major intestinal epithelial cell types (enterocytes, goblet cells, Paneth cells and neuroendocrine cells), underlying basement membrane and mucosal tissue, and the production of mucus. Gut *ex vivo* systems are used in various research topics such as in the evaluation of the metabolism and toxicity of various xenobiotics (de Graaf et al. 2010). Human intestinal organoids were used to assess polystyrene nanoparticle accumulation and impact on intestinal cells (Hou et al. 2022), but also to investigate the effect of prolonged ethanol exposure on colonoids (Devall et al. 2020) or the toxicity of cisplatin or 5-fluorouracil on intestinal organoids (Grabinger et al. 2014). Human intestinal samples from infants under 1 year of age (Matsumoto et al. 2021) and from fetal intestine (Chusilp et al. 2020) were also used to form intestinal organoids but no toxicological study was conducted yet with such models.

Table 2.15 Summary of the different *in vitro* models used to simulate the human intestinal barrier

Modified from Etienne-Mesmin *et al.* 2019.

Types of models	Description	References
<i>In vitro</i> cell models		
<i>Monoculture models</i>		
Intestinal epithelial cell lines	Tumor-derived intestinal epithelial cell lines: Caco-2, HT-29, T84, LoVo, LS174T, DLD-1, SW480, and SW620	Bjerknes and Cheng 2006 McCool <i>et al.</i> 1990
	Normal tissue-derived intestinal epithelial cell lines: HIEC, NCM460, NCOL-1	Perreault and Beaulieu 1996 Moyer <i>et al.</i> 1996 Deveney <i>et al.</i> 1996
<i>Co-culture models</i>		
	* Mixed culture of enterocytes, mucin secreting cells, inflammatory cells etc. e.g. Caco-2, HT29-MTX, M-cells, THP-1	Lozoya-Agullo <i>et al.</i> 2017 ; Vernay <i>et al.</i> 2020 ; Li <i>et al.</i> 2013
<i>Ex-vivo</i> organ cultures		
<i>Intestinal organoids</i>		
	* Generation of self-propagating spheres of primary intestinal epithelial cells * Enteroids = derived from adult stem cells isolated from the crypts of human small intestine * Colonoids = derived from adult stem cells isolated from the crypts of human colonic tissue	Jung <i>et al.</i> 2011 Sato <i>et al.</i> 2011
<i>Gut-on-a chip</i>		
	* Reproduction of the multicellular structures, cell-cell and tissue-tissue interactions, and the native microenvironment * Closely reproduction of the <i>in vivo</i> situation	Kim <i>et al.</i> 2012 Kim and Ingber 2013 Shah <i>et al.</i> 2016

2.3 Coupling *in vitro* gut models with cell cultures

Recent advances have been undertaken to combine *in vitro* digestion or fermentation models with cellular systems (Déat et al. 2009; Marzorati et al. 2014; Cai et al. 2017; Van Rymenant et al. 2018; Defois et al. 2018; Lu et al. 2019b; Calatayud et al. 2021). The easiest way is to directly apply filtered or diluted samples from the *in vitro* systems onto cell cultures, such as co-cultures of enterocytes and immune cells in Transwell systems. Combining *in vitro* digestion model with cell culture model was already done between batch oral, gastric and small intestine digestion models and Caco-2 culture to investigate the impact of cooking on speciation and cellular uptake of arsenic in rice (Lee et al. 2018) and to evaluate the effect of sodium copper chlorophyllin on mercury absorption from fish (Hwang and Shim 2008). Simple batch fermentation systems were also used coupled with intestinal epithelial TC7 cells to investigate the impact of food contaminants on the human gut microbiota and in fine the inflammatory host cell response caused by microbial metabolites following the pollutants-gut microbiota interactions (Defois et al. 2018). Such coupling between the SHIME and a co-culture of Caco-2 and HT-29 cells or Caco-2 alone was also used to investigate the bioaccessibility and metabolism of arsenic from various food matrices (Calatayud et al. 2018), but also the effect of human gut microbiota on the bioaccessibility of trace elements from vegetables (Cai et al. 2017). The SHIME was also coupled with a co-culture of Caco-2 and TC7 cells to evaluate both the impact of the organophosphorothionate pesticide chlorpyrifos alone on the intestinal barrier and the potentiality of inulin, when co-exposed with the pesticide, to prevent such effects (Réquilé et al. 2018). *In vitro* gut models can also be coupled with complex cell-containing units, such as the Host Microbiota Interaction (HMI) module (Marzorati et al. 2014). The HMI module has been specifically designed to be connected to continuous fermentation models such as the SHIME model to study indirect host-microbe interactions in the GIT (**Fig 2.3**). It consists of two separated compartments, the upper one simulates the luminal side of the GIT and contained mixed microbiota while the lower compartment is composed of enterocytes simulating the host. Furthermore, the double layer is composed of an upper mucus layer and a lower semi-permeable polyamide membrane providing mucosal area for gut bacteria colonization, protecting enterocytes from direct exposure to the microbiota, allowing bilateral transport of low molecular weight metabolites and of oxygen creating microaerophilic conditions (Marzorati et al. 2014). In addition, HMI module reproduces shear forces, as intestinal flow and peristalsis known to impact the gut microbiota as well. Such device has been never used in toxicological studies nor under infant conditions.

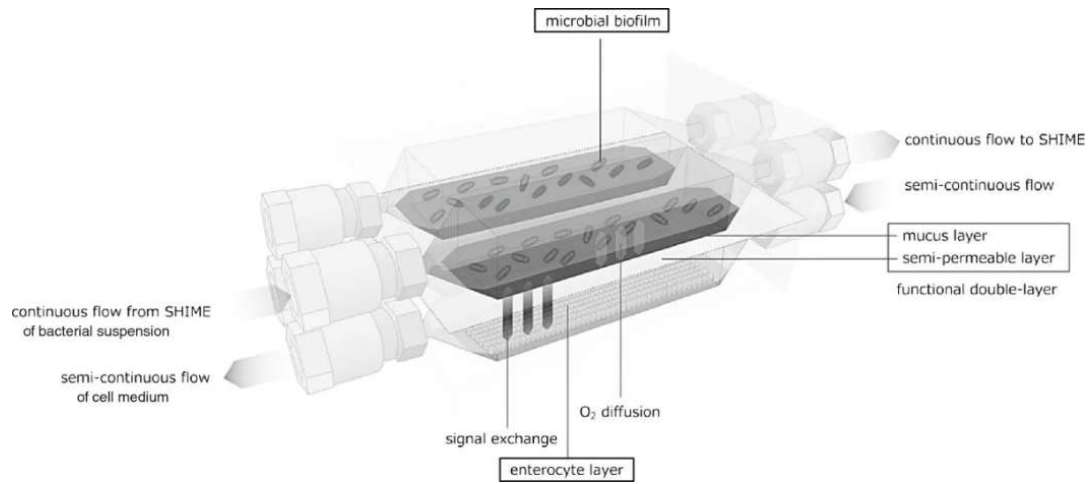


Figure 2.3 Scheme of the HMI module for host-microbiota interaction studies in the GIT

A polyamide semipermeable membrane and a mucus layer form a double functional layer that separates the luminal compartment (upper one) from the lower compartment containing enterocyte cell lines. The HMI module allows to study the bacterial adhesion under relevant shear forces and microaerophilic conditions. It allows the reciprocal exchange of signals and metabolites between compartments and it allows the exposure of cell lines to a complex microbial community, representative for the human colon, for up to 48 h.

Extracted from Marzorati et al. 2014.

Gut-on-a-chip devices should be also coupled to fermentation models to study host-microbiota interactions (Shin and Kim 2018) but they were not used until now to study pollutants. However, a recent study has proposed a new compartmentalized gut-on-a-chip system for pharmacological and toxicological analyses combining three different compartments (de Haan et al. 2021). The first one consists in a digestion-on-a-chip system corresponding to three micromixers representing the mouth, stomach and intestinal phase of digestion with the associated digestive secretions connected to one another using tubing. The second compartment is composed by another micromixer, to mix the chyme and diluted it with cell culture matrix before its introduction in the apical side of a flow-through Transwell system containing a co-culture of Caco-2 and HT29-MTX-E12 cells. Finally, a last compartment assures the continuous sampling of apical and basal chambers of the Transwell system and retains compounds of interest by a complex system of valves and tubing. These compounds are subsequently analyzed by quadrupole time-of-flight mass spectrometry. This model was tested with two drug models, the omeprazole and the verapamil.

Noteworthy, this part summed up existing *in vitro* models of the upper and lower gut and of the intestinal barrier but my PhD work was mainly focused on colonic and cell culture models with some preliminary insights into the application of upper gut models to address MP-related issues

Part 3 - Microplastics: From environmental pollution to threat for human health

Plastic production has significantly increased worldwide over the last decades, from 1.7 million tons in the 1950s to over 367 million tons in 2020 (PlasticsEurope, 2021), because of its success as a multifunctional, resistant, easy-to-process and affordable material. Due to these unique properties, plastics are central to modern living and constitute a vital source for innovation-driven growth. In 1957, a French intellectual, Roland Barthes, celebrated them in his book *Mythologies*. “The hierarchy of substances is abolished, and a single one replaces them all – the whole world can be plasticized and even life itself since, we are told, they are beginning to make plastic aortas”. However, the extensive use of plastics has become tainted by the continuous rise of plastic pollution worldwide. The negative impacts related to their end-of-life cannot be set aside and the deleterious consequences on the environment and organisms resulting from plastic pollution are now well-established (Bucci et al. 2019). Each organism is exposed, from fish or worms to mammals (Toussaint et al. 2019). Inevitably, after fragmentation, plastics enter the food chain with probable effects on human gut homeostasis (Lu et al. 2019a; Prata et al. 2019a). Barely mentioned five years ago, the emerging question of MPs as a threat to human health is today one of the most active research topics in the field. After a definition of plastics and MPs, this part focuses on the human digestive tract as the first site of MP exposure in the body. Of note, some parts of this state of the art have been published in a review article in the journal *Journal of Hazardous Materials* and in a book chapter in Springer Nature and have been redrafted / updated for the present section.

REVIEW: **FOURNIER E**, ETIENNE-MESMIN L, GROOTAERT C, JELSBK L, SYBERG K, BLANQUET-DIOT S, MERCIER-BONIN M. Microplastics in the human digestive environment: A focus on the potential and challenges facing *in vitro* gut model development. *J. Hazard. Mater.* 2021 Aug; 415:125632. doi: 10.1016/j.jhazmat.2021.125632.

BOOK CHAPTER: **FOURNIER E**, ETIENNE-MESMIN L, BLANQUET-DIOT S, MERCIER-BONIN M. Impact of microplastics in human health. In: ROCHA-SANTOS T, COSTA M, MOUNEYRAC C (eds) *Handbook of microplastics in the environment*. Springer International Publishing, Cham, pp 1-25. doi: 10.1007/978-3-030-10618-8_48-1.

1. From plastics to microplastics

1.1 Plastic definition and regulations

1.1.1 Characteristics of plastics

Plastics are carbon polymers, which are water-insoluble, easily molded and shaped under heat and pressure. They are mainly derived from fossil fuel as oil, coal or natural gas but can also be bio-based (mainly from cellulose, lignin and polysaccharides) (**Fig. 3.1**). Today, the five major plastics produced are high- or low-density polyethylene (HDPE or LDPE), polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC) and polyethylene terephthalate (PET) while 50% of global demands come from PE and PP and around 40.5% of all plastic production is used for packaging (PlasticsEurope, 2021). However, the extensive production of plastics, coupled with their stability, has led to their persistence in the environment. Plastic wastes have different fates: they can be reused, recycled, energy recovered (burned), deposited at dumping sites or lost in the environment (European Parliament, 2008) (**Fig. 3.1**). Plastic wastes are transported by rivers, runoff, tides, winds and catastrophic events but also come directly from fishing, aquaculture gears and ships. Great Pacific Garbage Patch, or the seventh continent, in the Pacific Ocean is evidence of the magnitude of the problem. It is a 1.6 million square kilometers of 79 thousand tons of floating plastic garbage (more than three times the size of Spain). Borrelle and colleagues recently underlined that plastic pollution is a planetary threat and extraordinary efforts to transform the global plastics economy are needed (Borrelle et al. 2020).

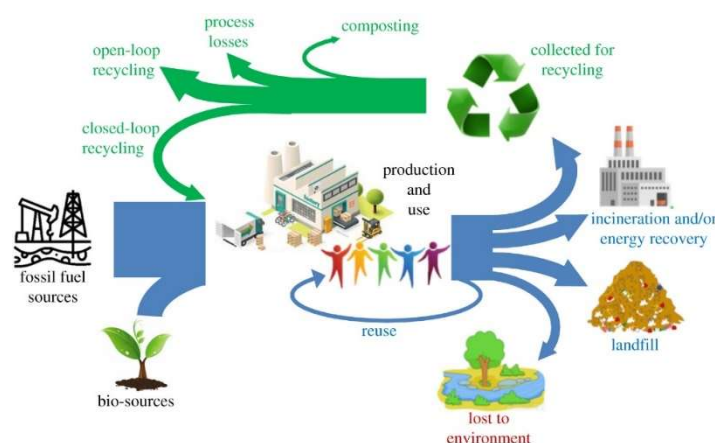


Fig 3.1 The “Life cycle” of plastics

Plastics are made from fossil-fuel or bio- sources. After use, they are either reuse, incinerated, deposited on dumping sites, recycled or lost in the environment.

Extracted from Bucknall, 2020

1.1.2 Regulations around plastics

Facing these critical issues, there is an increasing effort from governments to implement policy measures, legislative instruments and regulatory laws related to plastic pollution. In the European Union (EU), the European Strategy for Plastics in a Circular Economy (European Commission, 2018) stressed the need to tackle the challenges related to plastics. It aims at moving from a linear “take-make-consume-discard” pattern to a closed loop in which materials are continually reused, recycled and do not leak into the environment. Moreover, the Commission banned ten single-use plastic products in 2021 targeting 70% of the marine plastic wastes (Commission Implementing Decision, 2021/1752). Not only EU but numerous actions have been developed all around the world to address plastic pollution, like a complete ban of using, producing or selling plastic bags in Kenya since 2017, followed by many other countries. However, the COVID-19 sanitary crisis, which has confirmed the essential role of plastics in our daily life, is forcing to rethink plastic problem worldwide. Klemeš and colleagues showed that demand for medical products and packaging was sharply increasing during the COVID-19 pandemic (Klemeš et al. 2020). The city of Wuhan, the COVID-19 epicenter in China, experienced a massive increase of medical waste from between 40 and 50 tons per day before the outbreak to about 247 tons per day in March 2020 (You et al. 2020). A similar situation might arise in the food industry and packaging, textiles and other services that had decided to temporarily limit reusable, delay restriction reforms on plastic products, thereby contributing to the increasing plastic pollution (Patrício Silva et al. 2021). In addition, the massive production and use of disposal face masks to curb the infection and prevent virus transmission led to additional annual plastic waste and MPs, for the thirty-six nations considered worldwide (including USA, UK, EU and Asian countries as China), of 4.2 million tons and 9774 thousand tons, respectively (Shukla et al. 2022). In such context, the use of disposable face masks has been added to the list of potential MP sources (Aragaw 2020).

1.2 MP formation and characteristics

While observable plastics are only the tip of the iceberg, abundant smaller plastic particles accumulate in the environment. Plastic debris can be classified according to their size. MPs are defined as “any synthetic solid particles or polymeric matrices, with regular or irregular shape and with size ranging from 1 μm to 5 mm, of either primary -voluntarily manufactured- or secondary -released from plastic debris weathering- manufacturing origin, which are insoluble in water” (Frias and Nash 2019).

The majority of MPs derives from weathering and subsequent breakdown of plastic debris (Fig. 3.2). In the marine environment, it is primarily the UV-B radiation in sunlight that leads to photo-oxidative degradation of plastics and once initiated, the degradation can proceed through thermo-oxidation. Other types of degradation processes, like biodegradation, are orders of magnitude slower compared to light-induced oxidative degradation and hydrolysis is usually not a significant mechanism in seawater (Andrady 2011; Mattsson et al. 2015). MPs are found in all environmental media from marine to ground water, lakes, sediments, soil and even in the atmosphere (Miranda et al. 2019; Wu et al. 2019b). They represent 94% of floating plastic garbage in terms of pieces number. Moreover, MPs may be directly released from domestic and industrial sources (Weinstein et al. 2016), as well as from plastic products used for food packaging, fast-food delivery and water consumption (Hernandez et al. 2019; Fadare et al. 2020).

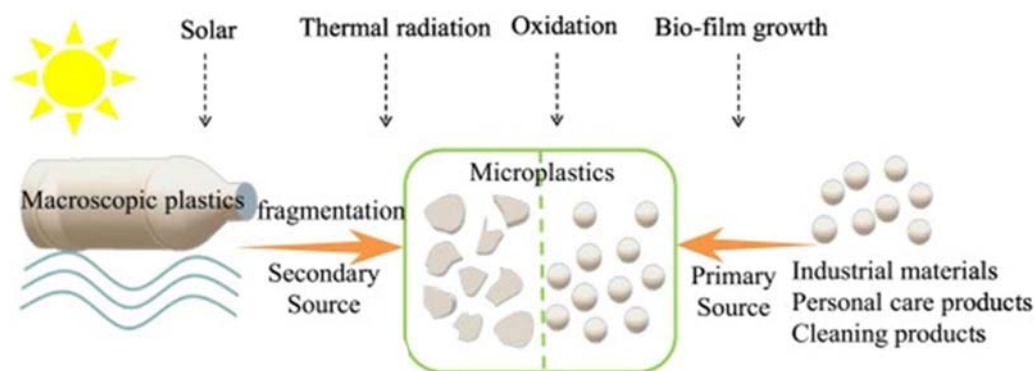


Fig 3.2 MP sources and formation

MPs derive from weathering and breakdown of plastic debris in the environment (e.g. photo- and thermo- oxidations, biodegradation), named secondary MPs, or are directly made by industrials (primary MPs).

Modified from Guo and Wang 2019.

The increasing use of outdoor PE goods may also generate an important release of MPs by fragmentation due to constant exposure to an open-air environment of these products, which are generally of poor structure and recycled material (Mehmood et al. 2022). Other unexpected sources, as toothpaste containing voluntarily added MPs (Chidhambaram et al. 2022), degradation of kitchen sponge, mainly composed with PET and Nylon PA6 (Luo et al. 2022b), dog poop bags (Mai et al. 2021) or cigarette butts (Belzagui et al. 2021), represent additional MP amounts in the environment, for instance representing in India 1.4 billion g of MPs per year only for toothpaste. Efforts for plastic product recovery and recycling have increased drastically in the recent years to minimize plastic pollution. However, plastic recycling processes, mostly

operable only once with PET, may generate important amounts of MPs in wastewater, discharge effluent and sludge. This was recently shown for PET flakes processing using post-consumer PET bottles as raw material (Guo et al. 2022).

2. MPs: vector of contaminants and microorganisms

2.1 Sorption of chemical pollutants and heavy metals onto MPs

MPs may be associated with different chemicals (**Fig. 3.3**), and notably pollutants adsorbed from the surrounding environment and thereby having their environmental fate altered (Wang et al. 2018; Hahladakis et al. 2018). This “vector effect” is a potential concern facing the extent of MP pollution (Syberg et al. 2015) even though its environmental importance is still debated. A study of the interaction between biota and silver-contaminated MPs in zebrafish showed that MPs altered the pollutant fate but not necessarily by increasing its uptake, illustrating that plastics can bind contaminants, which are further less available for uptake (Khan et al. 2015). Similarly, Kleinteich and colleagues found that contaminant bioavailability in soil decreased as a function of binding to MPs (Kleinteich et al. 2018). MPs can thus both serve as a source and a sink for other contaminants, in a case-by-case specific manner. The process is complex and depends on the pollutant, the composition and state of the MPs, as well as the conditions surrounding the particles.

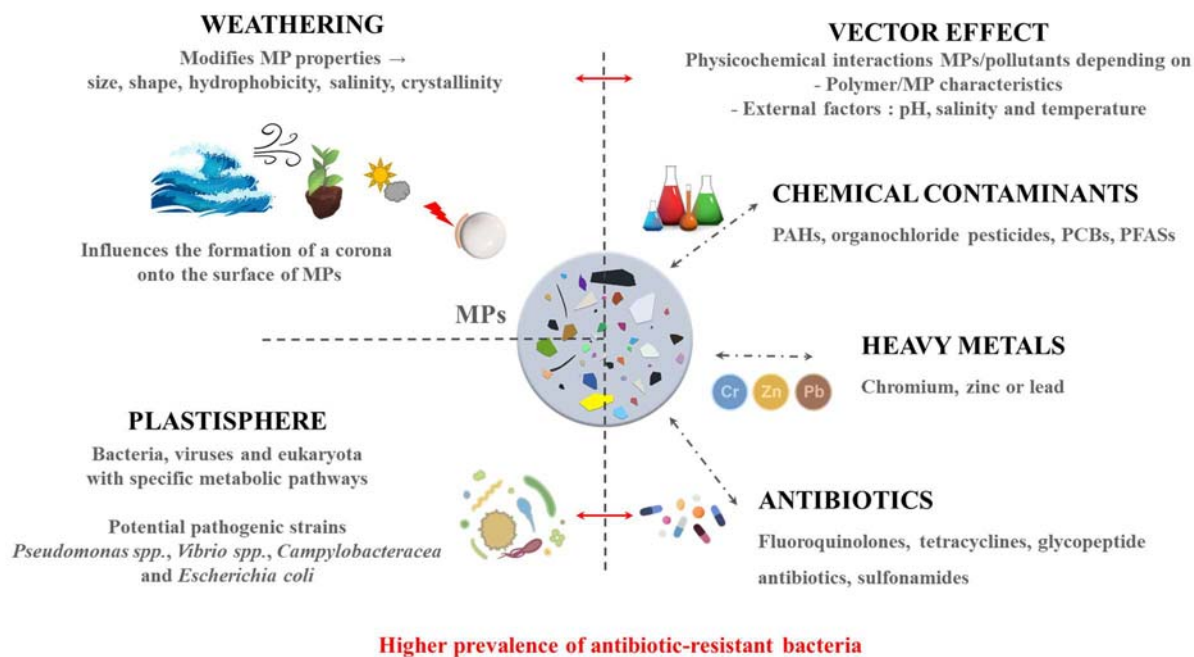


Fig 3.3 MPs: vectors for contaminants and microorganisms

MPs could be vectors of various contaminants (e.g; chemicals, heavy metals, antibiotics) but also microorganisms (e.g. bacteria, viruses, eukaryota) potentially pathogens. These physico-chemical interactions are dependent on MP properties (e.g. size, shape, hydrophobicity) and formed a biomolecular corona on the MP surface.

MPs: microplastics; spp: species; PAHs: polycyclic aromatic hydrocarbons; PCBs: polychlorinated biphenyls; PFASs: perfluoroalkylated substances
Extracted from Fournier et al. 2021.

Sorption of chemical pollutants (e.g. organochloride pesticides and polychlorinated biphenyls -PCBs-, polycyclic aromatic hydrocarbons -PAHs- and other benzene-ring derivatives, perfluoroalkylated substances -PFASs-) onto MPs has been widely investigated, as reviewed (Yu et al. 2019). Sorption is highly governed by physico-chemical characteristics (e.g. polymer matrix structure combining highly ordered crystalline regions and less structured amorphous regions, particle size, surface roughness) as well as environmental conditions (Velez et al. 2018). Lowering pH protonates the surface of MPs, leading to enhanced sorption of anionic pollutants. Likewise, when salinity increases, cation competition increases, inducing sorption or desorption of contaminants (Yu et al. 2019). The presence of ionic surfactant may also influence adsorption of hydrophilic pollutants. Indeed, MP adsorption capacity could increase from three to twenty-six times according to the co-existing surfactants (Xia et al. 2020). Adsorption typically occurs in the crystalline regions whereas internal partition is more likely to happen in the amorphous regions (Velez et al. 2018).

Furthermore, weathering of MPs is important for the association of pollutants. Weathering affects the physico-chemical properties of MPs as size, crystallinity or oxygen-containing groups (**Fig. 3.3**) and, thus, changes contaminant sorption profile onto MPs (Liu et al. 2019b). For example, weathered MPs contain more surface oxygen-containing groups than pristine ones, leading to greater contaminant sorption, especially for the most hydrophilic ones (Budhiraja et al. 2022). Moreover, after etching and/or UV radiation, specific surface area and pore volume of MPs are increased, by formation of nanovoids and larger cracks in the surface, which also favors contaminant adsorption process (Li et al., 2018; Velez et al., 2018). During fragmentation, chemicals are also susceptible to migrate to the plastic surface and then to be released (Schrank et al. 2019).

MPs also adsorb and concentrate heavy metals (**Fig. 3.3**) as chromium (Cr), zinc or lead (Godoy et al. 2019; Nguyen et al. 2022). Sorption of heavy metals onto MPs is generally due to electrostatic interactions between metal ions and polar sites on the plastic surface. As for other contaminants, weathered MPs contain more heavy metals than their pristine counterparts (Holmes et al. 2012b; Vedolin et al. 2018; Wang et al. 2019b). Adsorption is enhanced in waters with high chemical and biological oxygen demands as urban wastewater and irrigation water. However, after maximum adsorption, metals tend to slowly desorb from MPs (Godoy et al. 2019). Acidic conditions, like those encountered in gastric juice during digestion (see Part 1), may promote the leaching of metals bound to MPs (Teuten et al. 2009).

2.2 Pathogenic bacteria and antibiotics on MPs, potential role in spread of antimicrobial resistance in the gut

Microorganisms, including bacteria, are able to aggregate and adhere to biological or non-biological surfaces, in complex communities commonly referred to as biofilms (Lopez et al. 2010). Common environmental surfaces for biofilm formation include rocks, weeds and woods, but also plastic particles as they offer a suitable support for the formation of biofilm communities. In contrast to other floatable materials, like wood and weeds, plastic particles provide a long-lasting surface preserving their inhabitants from influence of environmental variations (Lobelle and Cunliffe 2011; De Tender et al. 2017). Accordingly, in recent years, marine plastic particles have been reported to harbor diverse microbial species (**Fig. 3.3**), including viruses, associated in dense multi-species biofilm structures (Zettler et al. 2013; Kirstein et al. 2016; Li et al. 2022b). Of note, MPs may also serve as various assimilable organic carbon sources, varying according to the MP types. These plastic-derived nutrients then

contribute to bacterial growth and ‘plastisphere’ shaping (Chen et al. 2021a). In addition, it was shown that biofilms on MPs have a higher sorptive capacity for various pollutants compared to pristine MPs, which may increase the toxicological characteristics of plastic particles. For instance, biofilm formed on PE MPs was shown to enhance adsorption and stabilization of Cu(II) and tetracycline on MPs (Wang et al. 2020b). On contrary, a more recent study showed that most contaminants tested (e.g. Atrazine, estrone, diclofenac and fluconazole) exhibited a lower concentration on biofilm-attached MPs than on virgin ones and that only few compounds exhibited enhanced adsorption, thus reminding to consider multifactorial effects when evaluating the potential of MPs as carriers (Zhang et al. 2022b).

The microbial inhabitants associated with MPs include potentially pathogenic bacterial species, like *Pseudomonas* spp., *Vibrio* spp., *Campylobacter* spp. and *Escherichia coli* (Zettler et al. 2013; McCormick et al. 2014; Bryant et al. 2016; Kirstein et al. 2016; Curren and Leong 2019; Wu et al. 2019b). A more recent study highlighted biofilm formation at the PE MP surface after incubation in raw sewage from municipal wastewater treatment works. Metagenomic analysis showed the presence of potentially pathogenic bacteria as *Arcobacter cryaerophilus*, *Aeromonas salmonicida*, *Vibrio areninigræ* and *Vibrio navarrensis*, demonstrating the potential carrier properties of MPs for wastewater-borne pathogenic bacteria in municipal sewage (Lai et al. 2022). In addition, fungal pathogens, such as cryptococcal and Phoma-like species, were accumulated at the MP surface (Gkoutselis et al. 2021). Viruses were also detected on MPs (Li et al. 2022b) and, within MP-associated biofilms, were protected from surrounding environmental factors (Moresco et al. 2022).

Altogether, these observations have led to the hypothesis that MPs colonized by enteric pathogens may constitute a novel vector for transmission of infectious diseases, possibly indirectly through contamination of food and drinks, although the dose transmitted by this route would most likely not reach infectious levels. A more concerning aspect is the association of antibiotics on MPs (**Fig. 3.3**). For some antibiotics, concentrations selecting for resistance have been reported. Specifically, levofloxacin (fluoroquinolone) can be adsorbed in concentrations up to 1.2 mg/g (Yu et al. 2020) onto PVC, whereas ciprofloxacin (fluoroquinolone), amoxicillin (penicillin), and tetracycline can be adsorbed to polyamide (PA) in concentrations ranging from 1 to 3 mg/g (Li et al. 2018). Finally, sulfamethoxazole was found to be strongly adsorbed onto PA and PE MPs in concentrations of 0.4 and 0.1 mg/g, respectively (Razanajatovo et al. 2018; Guo et al. 2019). These results point out that some of the most common MP particles are not only vectors for potential pathogenic microbes, but also favour hitch-hiking of microbes that

are resistant to antimicrobials. In support of this, recent studies have shown a significantly higher prevalence of antimicrobial resistance genes in microbiomes isolated from MPs than from seawater (Wang et al. 2020a; Xue et al. 2020; Yu et al. 2020) whereas other studies have highlighted the ability of MPs to act as habitats for increased gene exchange. Thus, high concentrations antibiotics and bacteria on MP surfaces could drive the spread and sharing of antimicrobial resistance genes (Arias-Andres et al. 2018; Yang et al. 2019; Xiang et al. 2019; Wang et al. 2020a). Accordingly, apart from enhancing the development of antibiotic resistant bacteria, ingestion of MPs may facilitate transfer of antimicrobial resistance genes to the intestinal microbiota.

3. Human oral exposure to MPs

While more and more studies evaluate the toxicological issue of MP exposure on various organisms, besides their exposure to MPs through personal care products (Qi et al. 2022), humans daily ingest MPs through beverages, food, food packaging but also from air (**Fig. 3.4**).

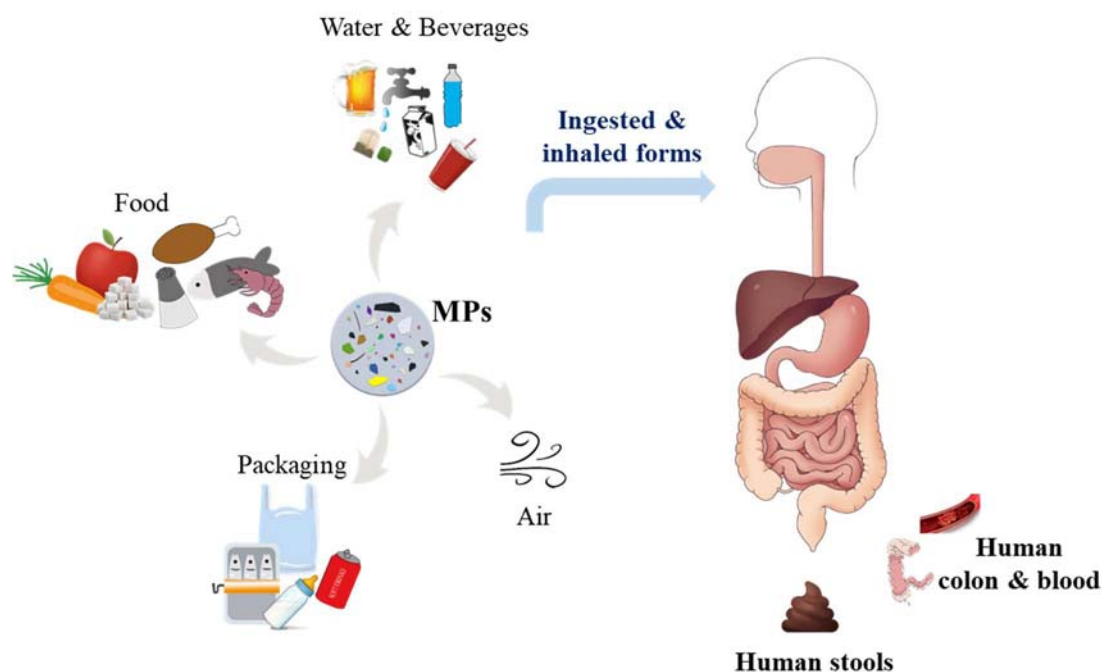


Fig 3.4 Human GIT exposure to MPs

Human GIT is exposed to MPs through air inhaled but also water, beverage and food ingested. MP contamination may originate from packaging. MPs were detected in human stool, colon and blood.

MPs: microplastics

3.1 Drinking water and beverages

MP contamination has been reported in various beverages, including bottled and tap water. In the study reported by Mason and colleagues (Mason et al. 2018), samples from bottled water were processed by Nile red staining, 1.5- μm filtration and analyzed using an optical microscope; they were then classified in two size ranges: larger than 100 μm (polymer identity further confirmed with Fourier transform infrared (FTIR) spectroscopy) and between 6.5-100 μm . In the study of Kosuth and collaborators (Kosuth et al. 2018), samples from tap water were processed by a 2.5- μm filtration, analyzed using a dissection microscope and only particles not stained by the Rose Bengal were counted; no confirmation by FTIR was performed. Under these conditions of sample analysis, bottled water seemed to have the highest particle concentration (ranging from 0 to 10,000 particles/L), compared to tap water (ranging from 0 to 61 particles/L) (Kosuth et al. 2018; Mason et al. 2018). These differences in contamination level have also been found by Danopoulos and colleagues, with concentrations of 628 MPs/L and 4,889 MPs/L for tap and bottled water, respectively (Danopoulos et al. 2020). After tap water analysis from fourteen different countries, the most abundant shapes reported were for 98.3% fibers being between 0.1 to 5 mm in size (Kosuth et al. 2018). For bottled water from various brands in nine different countries, fragments (66%) and fibers (13%) were predominant, with 95% of particles being between 6.5 and 100 μm in size. PP, PET and PE were the most abundant polymers (Koelmans et al. 2019; Danopoulos et al. 2020). MP contamination in tap water can derive from environmental water pollution but also from the degradation of plastic water pipes, amplified by water disinfectants commonly used as chlorine dioxide and ozone exposure (Vertova et al. 2019; Zhang et al. 2022c). For bottled water, the presence of MPs results from bottle degradation, mainly through ultraviolet radiations or during bottling process (Mason et al. 2018).

Other studies have shown the contamination of beer (from 2 to 79 fibers/L, from 12 to 109 fragments/L and from 2 to 66 granules/L), milk but also various refreshments (ranging from 10 to 100 MPs/L) and white wines (2,563-5,857 particles/L) (Liebezeit and Liebezeit 2014; Diaz-Basantés et al. 2020; Prata et al. 2020). In addition, common soft drinks, energy drinks and cold tea contain MPs, in 48 samples out of 57 tested, ranging from none to 28.00 ± 5.29 particles/L with various polymer types (e.g. PA, PET), shapes (fibers and fragments), colors and sizes (0.1-3 mm) (Shruti et al. 2020).

3.2 Air

Atmospheric contamination with MPs was shown in the planetary boundary layer but also in the free troposphere (0.09-0.66 MPs/m³) at 2877 meters above sea level (Allen et al. 2021). Numerous studies have reported air contamination by MPs and PE, PP, PS and PET were generally the major polymers found, with fibers as the predominant shape (more than 90%). Synthetic textiles are the main source of airborne MPs even though other sources may occur, such as from degradation of larger plastics in landfills or from incineration, from traffic or re-suspended dust. Meteorological conditions like wind, rain and snow events, as well as human activities, affect the distribution and deposition of airborne MPs (Chen et al. 2019a). Recently, it was shown that air conditioner filters could act as a source of MP fibers, leading to heavy accumulation after long time running (Chen et al. 2021b). Indoor air of different apartments was investigated and all were contaminated with MPs (1.7-16.2 particles/m³) (Vianello et al. 2019). In addition, dust samples from offices, university classrooms and dormitories, business hotels but also residential apartments were contaminated by MPs and especially of polyester, PE and PP types (Zhu et al. 2022). MP fibers can be ingested by eating air-dust exposed food but also by dust ingestion, particularly for young children (Dris et al. 2017; Fang et al. 2022). This route of exposure is important for infants with a median daily intake of inhaled PET-based MPs estimated between 4,000 and 150,000 ng/kg of body weight (bw) per day (from 360 to 150,000 ng/kg bw/day in adults, depending on their living area) (Zhang et al. 2020b). Through fiber deposition from dust fallout in a house during a meal, adults may consume 13,731 to 68,415 MPs/year/capita, which represents a higher exposure than after mussel consumption (from 123 to 4,620 MPs/year/capita) (Catarino et al. 2018). As a confirmation of the inhalatory route of exposure for MPs, analysis of human sputum from 22 patients suffering from respiratory diseases showed the presence of 21 types of MPs, mainly in polyurethane and polyester and smaller than 500 µm in size (Huang et al. 2022c). In addition, a recent study using FTIR spectroscopy detected a total of 0.69 ± 0.84 MPs/g of tissue in 13 different human lung tissues, with MPs mainly composed of PP, PET and resin (Jenner et al. 2022).

3.3 Food

In 2016, the European Food Safety Authority (EFSA) published an overview on the presence of MPs (and also smaller-sized nanoplastics) in food, with a focus on seafood (EFSA 2016). MPs have been detected in all the major livestock production systems as chicken, poultry and ruminants but also in more than 200 edible marine species (Toussaint et al. 2019;

Ramachandraiah et al. 2022). MPs in bivalves, shrimps and fish reach average concentrations of 0.2-4 particles/g, 0.75 particles/g and 1–7 particles/individual, respectively. In addition, contamination in honey (40 to 660 fibers; 0-38 fragments/kg of honey), sugar (217 ± 123 fibers and 32 ± 7 fragments/kg of sugar), salt (from 0 to 19,800 particles/kg of salt), canned sardines and sprats was reported (Bouwmeester et al. 2015; Karami et al. 2018; Peixoto et al. 2019). A study reported MP pollution ranging from 52,050 to 233,000 particles/g depending on fruit and vegetable types, with fruits being more contaminated than vegetables. Among each group, apples were the most polluted while lettuces represented the least contaminated. This MP contamination seems to originate from soil pollution, leading to root absorption and translocation in other tissues (Oliveri Conti et al. 2020).

3.4 Potential MP contamination from food packaging and processing

This contamination, which acts as a direct source of human exposure, probably originates from processing and/or food packaging steps but could also be already present in raw materials (**Fig 3.4**). Opening a plastic packaging (e.g. containers, bags, tapes, caps) by scissoring, tearing with hands, cutting with knives or twisting manually may generate MPs (0.46–250 particles/cm in average), depending on the plastic properties like size, density, stiffness or thickness (Sobhani et al. 2020). Simple actions as cutting meat on a plastic cutting board could be a source of MP contamination (Habib et al. 2022). Even the type of cooking method matters. Interestingly, according to the shellfish type and cooking method, MP ingestion differs, suggesting frying for cooking mussels, boiling for clams and winkles, and steaming for scallops (Li et al. 2022a).

As reported in the previous section, common soft drinks, energy drinks, cold tea and beers contain MPs, which seems to originate from synthetic textiles and packaging (Shruti et al. 2020). For instance, Winkler and colleagues demonstrated in plastic bottles release of MPs (90% of particles < 5 μm) from HDPE caps and from PET bottlenecks to a lesser extent on their surface after a series of bottle openings/closings, with great differences in cap abrasion between brands (Winkler et al. 2019). Bottles themselves were not a source of MP release after exposure to mechanical stress mimicking squeezing/crushing effects. In another study (Hernandez et al. 2019), plastic teabags were shown to release MPs (as well as smaller-sized nanoplastics) into steeped tea. The composition of released particles matched of the ones from original teabags (nylon and PET). Furthermore, disposable paper cups, currently used for coffee and tea, are coated by a hydrophobic plastic film onto their internal surface to make it waterproof. When

filled with hot water (85-90°C), the film was deteriorated and then released MPs. Within 15 min, 25,000 micron-sized plastic particles were found to be released for a total liquid volume of 100 mL (Ranjan et al. 2021).

As for beverage packaging, MPs from food packaging are able to migrate into food, especially in high-fat products due to the hydrophobic properties of plastics and during heating (Ajaj et al. 2021). A recent study showed the presence of MPs (ranging from 3 to 29 particles per container, fibers as the dominant shape) in take-out food containers made of PP, PS, PE or PET. The highest abundance occurred in PS containers with a rough surface. This contamination mainly originated from flaking from container inner surfaces and/or atmospheric fallout, at an extent depending on the polymer type. This could represent an additional intake of up to 203 MPs per person and per week for people who order take-out food from 4 to 7 times per week (Du et al. 2020; He et al. 2021). Store-bought rice was also contaminated with MPs and especially of PE, as well as instant (pre-cooked) rice containing fourfold higher MP levels than standard rice, suggesting potential contamination from industrial processing (Dessi et al. 2021). Finally, canned fish contains MPs (PET polymer and fiber shape), probably originating from the cleaning and canning processes (Akhbarizadeh et al. 2020).

Infants may also be exposed through PP feeding bottles that could release 16,200,000 particles/L in average, mainly during sterilization process and filling with high-temperature liquids (Li et al. 2020b). In addition, polyethersulfone and polysulfone, commonly used as membrane materials in dairy processes, were identified as the main polymer types of MPs contaminating milk samples from various brands (Kutralam-Muniasamy et al. 2020). In another study, a slight trend of increase in MP amount with the degree of milk processing and packaging conditions was depicted, with the main MP types found in farm milk, processed liquid milk and reconstituted powdered milk being PE, polyester, PP, polytetrafluoroethylene (PTFE) and PS (Da Costa Filho et al. 2021).

3.5 Human consumption

As described above, humans are exposed to numerous MP sources (Rist et al. 2018) but to date, few studies have clearly quantified human exposure to MPs integrating dietary and inhalation sources. Importantly, a large part of inhaled particles may enter the digestive tract through the mucociliary clearance mechanisms of the lung after inhalation (Toussaint et al. 2019; Prata et al. 2019a). In the first study in the field (Cox et al. 2019), it was shown that a child with a typical American diet consumes per day 106 (female) and 113 (male) MPs and

inhales 97 (female) to 110 (male) of these particles. Regarding adults with a typical American diet, they consume 126 (female) to 142 (male) MPs and inhale 132 (female) to 170 (male) particles per day. However, Cox and colleagues pointed out that, according to the geographical localization and dietary habits, MP exposure could vary (Cox et al. 2019). For instance, when daily water intake recommendations are achieved through ingestion of bottled water, MP intake is increased by 22-fold compared to tap water consumption (Cox et al. 2019). In addition, seafood products are among the major food contributors for MP intake known to date. As illustrated in Japanese, known to be one of the largest consumers of fish and seafood products, they could ingest up to 154 MPs per day only through seafood products. However, data from other food groups consumed daily are now required for identifying additional sources of MP exposure in human. Recently, Senathirajah and colleagues used the current knowledge to estimate the global average rate of MP ingestion in human, which was in the range 0.1-5 g of MPs per week (Senathirajah et al. 2021). Beyond differences in experimental conditions and analytical methods used across studies, their analysis was limited by a scarcity of data for certain food categories (honey, sugar and fish: limited available data; pasta, oil, milk, bread, rice, meat and wheat: absence of data at the time of analysis), which led to only investigate four types of consumables with the most robust data (e.g. drinking water, salt, beer and shellfish). Similarly, the probable contribution of MPs from plastic utensils/food packaging was excluded due to data limitations (Senathirajah et al. 2021). Nevertheless, these pioneering studies constitute a first reliable step towards health risk assessment in human.

In such context, Schwabl's team was the first to report the presence of MPs in stools collected from healthy volunteers worldwide, with an average of 20 particles per 10 g of stool, composed of various polymer types (PP and PET being the most abundant) and sizes (between 50 and 500 μm) (Schwabl et al. 2019). Although this study only investigated MPs larger than 50 μm for a small number of subjects ($n=8$, 3 males, 5 females, aged 33-65 years) with only 1 stool sample provided per participant, it is still striking that MPs are found in all analyzed samples. Later studies, like the one conducted by Zhang and colleagues on 26 young male students from China, confirmed stool contamination by MPs, with 1 to 36 particles per g of stool, between 20 to 800 μm in size and mainly composed with PP (Zhang et al. 2021c). A contamination with even more MPs (i.e. from 20.4 to 138.9 particles per g of wet weight feces) was more recently reported in 8 Chinese and these particles were principally composed with PS, PP and PE and smaller than 300 μm in size (Ho et al. 2022). Further studies on larger cohorts should now be performed to correlate types and amounts of fecal MPs to geographic

area, dietary habits, other potential sources of MP ingestion and health impacts. Recently, Ibrahim and colleagues detected MPs in all the 11 human colectomy samples analyzed, with in average 331 particles per individual, mainly composed with filaments and fibers of polycarbonate, PA and PP (Ibrahim et al. 2021). Moreover, the presence of MPs, composed with PET, PE and styrene polymers, was reported in human blood (1.6 µg/mL in average) of 22 healthy volunteers, suggesting particle absorption (Leslie et al. 2022).

Data in early life are scarce. However, already in the mother's womb, fetus seems to be exposed to MPs as they were detected in the placenta and meconium from newborns (Ragusa et al. 2021; Braun et al. 2021). After birth, infants are highly exposed through dust breathing, dust ingestion during roaming (Zhu et al. 2022), but also during bottle feeding, as both milk (Kutralam-Muniasamy et al. 2020; Da Costa Filho et al. 2021) and feeding bottles (Li et al. 2020b; Li et al. 2021) contain MPs. Breastfed infants are not saved from MP exposure as plastic particles were detected in human breastmilk samples (Ragusa et al. 2022). In addition, silicone-rubber baby teats suckling represents an exposure source as their steam sterilization conducts to MP release (Su et al. 2021).

Altogether, these data converge towards a ubiquitous presence of MPs in air, food items, drinking water and beverages, with probable consequences on human health for adults but also in at-risk populations, like infants. However, a debate still remains on whether the associated risk is real or overrated (Backhaus and Wagner 2020). As underlined by Toussaint and colleagues (2019), it is still difficult to assess human oral exposure to MPs, due to the lack of a standardized methodological approach, including sampling/analytical procedures, as well as reference materials (for different polymers in different matrices). To illustrate this urgent need, a recent international laboratory intercomparison between 34 laboratories, using seven different identification and quantification methods, highlighted the discrepancies in particle numbers reported in dissolvable soda tablets (van Mourik et al. 2021).

4. Consequences of oral exposure to MPs on intestinal homeostasis impairment: a focus on virgin particles

4.1 Physico-chemical transformations of MPs during digestion

As a first site for MPs after ingestion, the GIT is of utmost importance. The potential impact of MPs on intestinal homeostasis impairment depends on their initial physico-chemical properties (e.g. particle size and distribution, shape, surface coating, weathering), as well as any changes in these properties as they pass through the GIT, which has changing pH, transit time, redox potential and other chemical characteristics. Therefore, it is important to unravel the physico-chemical transformations of MPs (size, shape, surface properties, formation of a biomolecular corona etc.) during human digestion.

To date, studies are scarce and mainly focused on *in vitro* models exposing MPs to simulated digestive fluids. Using a three-stage *in vitro* system mimicking the saliva, gastric and intestinal phases of human digestion, Stock and colleagues demonstrated that digestive fluids were not able to degrade five types of MPs (PE, PP, PVC, PET and PS) into smaller fragments and to modify their shape, size or surface texture (Stock et al. 2019c). Likewise, in a dynamic three-stage *in vitro* digestion system mimicking the mouth, stomach and small intestinal human conditions, Liu and colleagues showed no alteration of PS MPs in terms of chemical composition (Liu et al. 2020a). In contrast, Tong and his team observed fragmentation of PE MPs after 4 h in artificial gastric juice (Tong et al. 2021). Moreover, Tamargo and collaborators reported a progressive amorphization of PET MP surface during digestion following the INFOGEST protocol, which was associated with a higher organic matter deposition on the particle surface. These physico-chemical transformations were reinforced during colonic fermentation using a complex *in vitro* system, SIMGI, described in Part 2, that reproduces the different regions of the lower gut (ascending, transverse and descending colon) (Tamargo et al. 2022). While no consensus exists yet concerning MP degradation during digestion, all authors mentioned above described the formation of a biomolecular corona onto the particle surface, due to the adsorption of organic compounds such as proteins, mucins and lipids during digestion process. Owing to its biological impact (Monopoli et al. 2012) and its ability to modify MP physico-chemical properties, such as hydrophobicity or surface charge as shown for PS particles (Liu et al. 2020a), the characteristics of the biomolecular corona formed around the MPs during their transit in the digestive tract should be more deeply studied to better understand the behavior of the MPs in the gut, and particularly their uptake rate.

As seen above, digestive fluids may influence the physico-chemical characteristics of MPs during their transit in the GIT. Reciprocally, MPs are able to interfere in the digestive processes, as illustrated *in vitro* by Tan and colleagues for lipid digestion (Tan et al. 2020). These authors showed that five types of MPs (PS, PET, PE, PVC, and poly(lactic-co-glycolic acid -PLGA-) reduced lipid digestion in an *in vitro* system reproducing the main processes of human digestion occurring in the mouth, stomach and small intestine. PS particles exhibited the highest inhibition rate by decreasing the bioavailability of lipid droplets via the formation of large lipid-MP heteroaggregates, and by adsorbing lipase with subsequent alterations in the enzyme secondary structure and activity (Tan et al. 2020).

4.2 Impact of MPs on the intestinal barrier: coupling *in vivo* and *in vitro* models

The intestinal barrier on its luminal side involves epithelium, mucus and microbiota, and their crosstalk provides, in an orchestrated manner, a physical, chemical, and biological line of defense for the host towards MPs, which has been investigated by *in vivo* (rodents) and *in vitro* models.

4.2.1 Uptake and tissue accumulation in rodents

There are different routes of MP uptake in the GIT due to their size range, as reviewed by EFSA (EFSA, 2016). Cells involved in microparticle (and also nanoparticle) transport are notably specialized M-cells overlying Peyer's patches, which belong to the mucosa-associated lymphoid tissues, and transport large structures, amongst which antigens, bacteria and viruses, to the immune system. MP particles of less than 10 μm could then be trapped into the intestine by M-cells (**Fig 3.5**), according to the adherence on mucus, high adherence to the loosely adherent mucus layer leading to a quick clearance and fewer absorption time (Ensign et al. 2012). A marked decrease in uptake efficiency by M-cells of particles between 200 and 500 nm diameter was reported compared to smaller sizes (Powell et al. 2010). Generally, small-sized particles seem to better translocate across the gut than larger ones (Wright and Kelly 2017). For MPs between 5 and 110 μm diameter, persorption, a paracellular transport, especially in desquamation zones and between the villi, appears to be the most important uptake mechanism (Wright and Kelly 2017). Not only the size, but also the hydrophobicity, surface functionalization and charge and biomolecular corona, influence MP uptake ability.

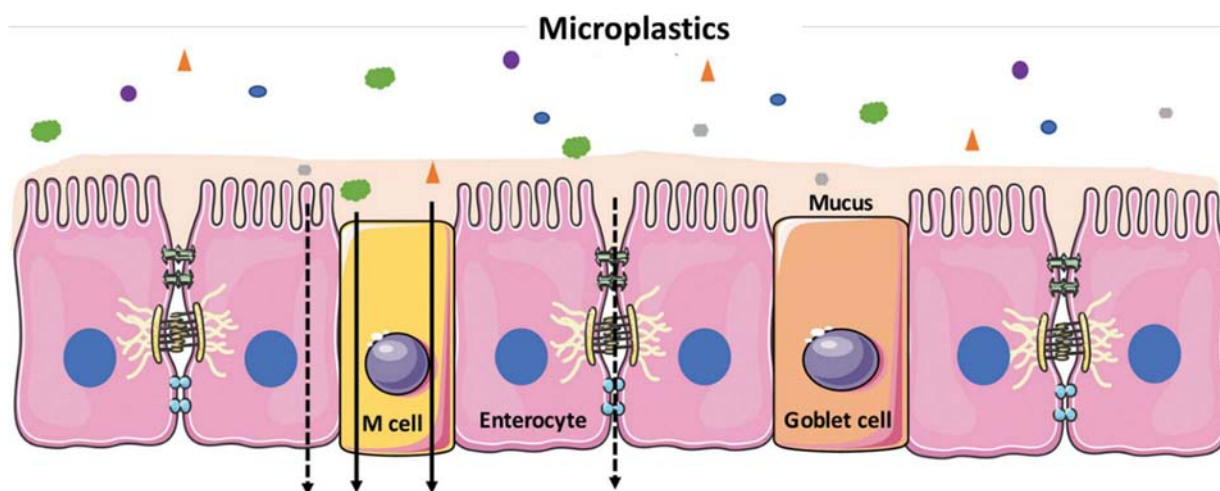


Fig 3.5 Transport of MPs through intestinal cells

Solid arrows indicate the predominant route of transport for MPs (M or microfold cell-based transcytosis) while dotted arrows indicate their secondary routes of transport (paracellular or transcellular transport).

Extracted from Banerjee et al. 2021

The first studies investigating the fate of MPs in the GIT upon ingestion in mammals (rats and mice) have used (fluorescent) spherical PS MPs, usually 2 μm diameter size, as they are convenient for detection through diverse microscopy techniques, such as scanning electron microscopy, transmission electron microscopy or fluorescence/confocal microscopy (Carr et al. 2012). After administration to rats, it was demonstrated that MP uptake is a fast process, as particle absorption in intestinal tissue already took place after 5 min of ingestion, and significantly higher amounts of MPs were found in the epithelia of the proximal part of the small intestine compared to the distal one (Carr et al. 2012). Accumulation of these MPs was visible at the level of the microvilli of enterocytes, in the intercellular spaces, in the epithelial-stromal boundary and in the blood vessels. Interestingly, accumulation in Peyer's patches was much lower. Nevertheless, 5 weeks after a single-dose ingestion, the few MPs that were monitored in this region persisted while at the level of enterocytes, MPs had disappeared. In mice, Stock and co-workers reported that only few MPs were detected by fluorescence microscopy in the intestinal walls of animals after oral exposure three times per week for 28 days by oral gavage with a mixture of 1 μm , 4 μm and 10 μm PS MPs, corresponding to an administered dose of 1.25 mg/kg bw, 25 mg/kg bw, and 34 mg/kg bw, respectively (Stock et al. 2019b). No histological lesion nor inflammatory response was observed. In contrast, in mice fed 600 $\mu\text{g}/\text{day}$ (corresponding to 30 $\mu\text{g}/\text{kg bw}/\text{day}$ for a mouse weight of approximately 20 g) of PE MPs (10–150 μm) for 5 consecutive weeks, the histological scores of colon and

duodenum were significantly higher than the ones determined for non-treated animals (Li et al. 2020a).

In mammals, it is thus not clear whether MP exposure leads to gut inflammation. Results depicted yet are highly dependent on treatment conditions (duration, doses) but also on the polymer type and size of MPs and potentially on their shape and biomolecular corona. In addition, as also underlined by Stock and co-workers, doses administered *in vivo* in animal models have to be consistent with levels realistically expected for human exposure (Stock et al. 2019b). With this in mind, most studies highlight MP presence in the gut and other tissues of many species, including rodents, in a MP size-dependent manner (Lu et al. 2019a). In rats, 6% of MPs (PS, 870 nm) were found in the circulation 15 min after oral administration (Eyles et al. 1995) while nano-sized particles (PS, 50 nm) led to 34 % of absorption, with a main accumulation in the liver (Jani et al. 1990). Following a 4-week exposure period, 20 µm and 5 µm PS MPs accumulated in the gut, liver and kidney of mice in a MP size-dependent localization. The biggest particles were consistently distributed in the three tissues while the smallest ones accumulated more in the gut of exposed mice (Deng et al. 2017). However, the relevance of this work has been discussed by others (Braeuning 2019), notably due to the fact that the quantity of particles detected in the organs massively exceeded the quantity of particles administered during the study.

4.2.2 Impact of MPs on gut epithelium *in vivo* and *in vitro*

In their study on mice orally exposed for 6 weeks to two different sizes of PE MPs, 36 and 116 µm, administered either individually or as a mixture at a dose of 100 µg/g of food, Djouina and co-workers showed an increase in crypt depth throughout the intestinal tissues (which could contribute to intestinal tumorigenesis), defective recruitments of some intestinal immune cells, from the proximal portion of the small intestine to the colon, and modifications of the gut microbiota (Djouina et al. 2022), which will be described in the following section. Qiao and his team evaluated in mice the effects of differentially charged MPs: negatively (carboxylated) and positively (aminated) charged PS MPs (5 µm). Gavage of these particles for 28 days led to a decrease in tight junction gene expression and protein level, only significant for aminated PS MPs. The effect of positively-charged PS particles was then more important than that achieved with negatively charged ones while uncharged MPs had no significant impact (Qiao et al. 2021). Interestingly, targeting a sensitive population, i.e. patients suffering from inflammatory bowel disease, a recent study highlighted major intestinal aggravations in mice

with dextran sodium sulfate (DSS)-induced colitis (Luo et al. 2022a). While gavage with PS MPs (5 μm , either 2.5 or 25 mg/L) alone only caused minimal effects on the intestinal barrier function, for animals with colitis, additional PS MP exposure led to an increased colonic permeability, accompanied by a shorter colon length and reduced mucus secretion. This study suggests the critical need for more research on the health risks of MPs for sensitive individuals.

In vitro, studies investigating uptake, fate and effects of MPs on the gut epithelial barrier have been performed using human epithelial Caco-2 cell monolayers. This cell line, originally isolated from a human adenocarcinoma, spontaneously differentiates upon confluence to a monolayer of polarized enterocytes, expressing microvilli, transporters and brush border enzymes (see Part 2). MPs were shown to disrupt the intestinal epithelial barrier, as measured by a decreased TEER, with a particle transport regulated by tight and adherent junctions (Carr et al. 2012). In addition, on the same cell model, increasing the MP exposure concentration (from 10^{-8} to 10^{-1} mg/mL) and duration (24 and 48 h) led to a higher cytotoxicity albeit at a slight extent (maximal loss of viability of 10%) (Wu et al. 2019c). Stock and collaborators studied particle uptake in Caco-2 monoculture acutely-exposed (24 h) to PS MPs of three different sizes (1, 4 and 10 μm) (Stock et al. 2019b). The highest uptake into Caco-2 cells (of about 3%) was observed for 4 μm particles. Of note, ethanol, which acts as a “loosener” of tight junctions (TJs), facilitated an increased particle passage across the epithelium (Carr et al. 2012). Interestingly, Liu and colleagues showed that the formation of a biomolecular corona onto the PS MP surface after *in vitro* digestion was probably a player in the reduction of cytotoxicity and impairment of the transport function observed in Caco-2 cells, particularly for small-sized digested particles (0.1 μm) compared to their larger counterparts (5 μm) (Liu et al. 2020).

Questions concerning the pivotal role of simulated digestive fluids (e.g. physico-chemical transformations of MPs, biomolecular corona formation) are then increasingly addressed, especially the consequences on uptake and toxicity (Liu et al. 2020). Of note, further insights on healthy vs inflamed gut conditions have been proposed *in vitro* to study the effect of inflammatory processes on plastic particle toxicity (Busch et al. 2021a). Other interesting developments are currently under investigation. For instance, Yu and his team investigated the potential impact of aged-PS MPs on Caco-2 cell toxicity (Yu et al. 2021). The authors showed that photo-transformation of PS MPs was a process in which carbon-containing functional groups were gradually and partially transformed into oxygen-containing ones. Toxicity results pointed out that, with an increase in PS MP concentration and an extension of light irradiation time, the survival rate of Caco-2 cells gradually decreased and the integrity of cell membrane

was disrupted. The increased cytotoxicity could be explained, at least in part, by the fact that toxicity of oxygen-containing functional groups is greater than that of carbon-containing ones. This work reveals the importance to investigate, beyond virgin particles, more representative forms in MP toxicology studies. Recent literature reports have enlarged assays on gut toxicity, and related organs, to other cell lines than Caco-2 cells. For instance, Gautam and colleagues reported the effects of two different sizes (30.5 ± 10.5 and 6.2 ± 2.0 μm) and different concentrations of PE MPs on six human cell lines, representing various tissues or cells (Gautam et al. 2022). Interestingly, PE MPs did not have the same effects on all kinds of cells. In intestinal epithelial Caco-2 cells, while PE MP exposure did not substantially lower cell viability, the highest concentration (1000 $\mu\text{g}/\text{mL}$) for both sizes of MPs slightly reduced cell viability and increased oxidative stress, as illustrated by a higher level of nitric oxide (NO). Moreover, using human colonic cells, CCD-18 cells, Bonanomi and colleagues revealed potential human health cancer risk of PS MP chronic exposure (Bonanomi et al. 2022). Besides these developments, there is a growing trend to use more advanced cellular models that combine multiple intestinal cell types (see Part 2), including immune cells (e.g. M-cells (des Rieux et al. 2007; Carr et al. 2012; Stock et al. 2019b)) and/or mucus-secreting HT29-MTX cells, as detailed in the following paragraph.

4.2.3 Impact of MPs on intestinal mucus *in vivo* and *in vitro*

Besides gut epithelium, intestinal mucus as a first barrier, with size- and charge-exclusion properties, plays a role of uppermost importance, as reviewed by Gillois and colleagues (Gillois et al. 2018). Mucus may trap MPs, thereby decreasing their toxicity to the host. However, it is possible that MPs more easily cross the mucus layer because of their physico-chemical transformations after contact with the intestinal content, as reported for dietary microparticles (Powell et al., 2007).

To date, a few studies have investigated *in vitro* the interactions between MPs and intestinal mucus. To overcome the absence of a mucus protective barrier in Caco-2 cells, a Caco-2/HT29-MTX co-culture has been developed as a more physiologically relevant intestinal model to mimic a mucus-producing epithelium (Lozoya-Agullo et al. 2017). Considering Caco-2/HT29-MTX (mucus model) co-culture vs Caco-2 monoculture exposed to PS MPs of three different sizes (1, 4 and 10 μm), Stock and collaborators surprisingly found a significantly higher uptake rate for both the 1 μm and 4 μm particles in the co-culture compared to the monoculture (no significant difference with the 10 μm particles) (Stock et al. 2019a).

However, it should be noted that numerous studies have highlighted that mucus in this Caco-2/HT29-MTX co-culture model does not form a uniform layer over the cell surface, but rather a patchy structure (Gillois et al. 2021) while others depicted a mucus layer completely covering the cell surface (Mahler et al. 2009). These discrepancies stress the necessity of examining mucus characteristics, and thus barrier properties, when HT29-MTX cells are used for the toxicological assessment of MPs *in vitro*. A three-dimensional *in vitro* intestinal cell model, composed with a mixture of Caco-2 and HT29-MTX cells in combination with immune cell types (i.e. human blood monocyte-derived macrophages and dendritic cells), was developed for testing environmentally relevant MPs (PP, PA and polyurethane (PU), 50-500 μm) after 6, 24 and 48 h of an aerosolized exposure, chosen to avoid particle floating in the cell culture medium (Lehner et al. 2020). Whatever MP type and exposure conditions, no significant deleterious effect was reported in terms of cytotoxicity, barrier integrity disruption and pro-inflammatory response.

In vivo, recent studies in mice highlighted the deleterious effects of MPs on mucus homeostasis pathways. After exposure to 0.5, 5 and 50 μm PS MPs at 100 $\mu\text{g/L}$ and 1000 $\mu\text{g/L}$ for 6 weeks, mucus secretion in the colon was significantly decreased as shown by Alcian blue-periodic acid Schiff staining, which was supported by the down regulation of genes involved in mucus synthesis/secretion pathways (*Muc2*, *Muc1*, *Retnlb* and *Klf4*) in the colon (Lu et al. 2018; Jin et al. 2019). Two more recent studies with PE MPs reported contrasted findings. In their study on mice orally exposed for 6 weeks to two different sizes of PE MPs (36 and 116 μm) administered either individually or as a mixture at a dose of 100 $\mu\text{g/g}$ of food, Djouina and colleagues highlighted an increase in mucosal and mucin areas and an upregulation of *Muc2* transcripts in the colon, reflecting dysregulation of colonic mucosa differentiation (Djouina et al. 2022). Sun and co-workers showed that oral exposure to PE MPs (1–10 μm) at a dose of 0.2 $\mu\text{g/g}$ bw/day in mice decreased mucin production but not *Muc2* mRNA level (Sun et al. 2021). Furthermore, as introduced above, exposure to PS MPs (5 μm , either 2.5 or 25 mg/L) in mice with dextran sodium sulfate (DSS)-induced colitis led to reduced mucus secretion, in addition to histopathological damage and inflammation aggravation (Luo et al. 2022a).

4.2.4 Impact of MPs on gut microbiota *in vivo* and *in vitro*

The third member of the gut barrier is the intestinal microbiota. This huge community of microorganisms present in the GIT raised an increasing interest the past few years, with a recent focus on the potential impact of MPs on this ecosystem (Fackelmann and Sommer 2019). To

date, only one study has been carried out on the human gut microbiota. A single dose of PET MPs (0.166 g), previously digested using a standardized static *in vitro* digestion protocol (INFOGEST method), was administered for 72 h in the SIMGI gut model inoculated in parallel with fecal samples from two human adult donors (Tamargo et al. 2022). The authors reported compartment- and donor-dependent effects. Following PET MP exposure, a decrease in *Bacteroidota* (previously named *Bacteroidetes*) was observed in the three colonic compartments for the two donors. An increase in *Desulfobacterota* was also reported in the three compartments for one of the donors but not in the transverse colon for the other one. The *Synergistetes* population was increased in the transverse and descending colon for a donor but only in the descending colon for the other one. Surprisingly, while *Proteobacteria* were increased in the transverse colon of one donor, they were decreased in the ascending and transverse colon of the second one, following PET MP exposure.

In animal models, the more and more studies conducted on mice orally exposed to MPs have revealed microbiota composition modifications in the cecal or fecal contents (Lu et al. 2018; Jin et al. 2019). A lot of work has been reported to date on PS MPs. For instance, mice exposed to PS MPs (0.5 μm and 50 μm) in drinking water at two different concentrations (100 $\mu\text{g/L}$ or 1000 $\mu\text{g/L}$) for 5 weeks exhibited microbiota alterations. Impaired α and β -diversities and a significant reduction in the abundance of *Firmicutes* and α , β and γ -*Proteobacteria* were reported in the cecal content of treated mice in all conditions using a PCR-based quantification assay. The *Actinobacteria* abundance was reduced only after exposure to 1000 $\mu\text{g/L}$ of PS MPs (Lu et al. 2018; Jin et al. 2019). Analysis of 16S rDNA sequencing of cecal content demonstrated a decrease in the relative abundance of *Firmicutes*, *Bacteroidetes* and *Verrucomicrobia* at the highest dose for both MP sizes (Lu et al. 2018) and of *Actinobacteria* after exposure to 5 μm PS MPs at a concentration of 1000 $\mu\text{g/L}$ (Jin et al. 2019) while *Proteobacteria* proportion increased (Lu et al. 2018; Jin et al. 2019). In addition, at the genus level, 15 types of bacteria were significantly affected; in particular, *Bifidobacterium*, *Prevotella* and *Veillonella* decreased while *Coprococcus* and *Anaeroplasm*a increased in the cecal content following exposure with 1000 $\mu\text{g/L}$ of PS MPs (Jin et al. 2019). A more recent study of Wen and colleagues showed microbiota composition disturbance, increasing its pathogenicity, in mice chronically exposed to PS MPs at a dose of 18 or 180 $\mu\text{g/kg}$ bw/day for 90 days (Wen et al. 2022a). According to 16S rDNA sequencing results, oral exposure to MPs decreased the abundance of *Bacteroidetes* (B) and slightly changed the abundance of *Firmicutes* (F), resulting in an elevated F/B ratio. Moreover, a significantly elevated proportion of *Proteobacteria* and

Enterobacteriaceae, the main lipopolysaccharide sources, was reported. In addition, exposure to MPs elicited a dramatical increase in abundance of potentially pro-inflammatory bacteria and pathogens, such as *Desulfovibrio*, *Alistipes*, *Oscillospiraceae* and *Allobaculum*. Meanwhile, MP exposure also remarkably decreased the proportion of *Dubosiella*, *Blautia* as well as *Butyricocccaceae*. The deficiency in these gut bacteria led to reduced anti-inflammatory ability and SCFA production, which might account for gut inflammation and elevated pathogenic bacteria. Interestingly, Spearman correlation analysis between the changed gut bacteria and functional profiles showed that PS-MPs enriched *Desulfovibrio* and *Alistipes* had a negative correlation with the mRNA expression level of colonic TJ proteins (Occludin, zonula occludens-1 -ZO-1- and Claudin-1) as well as the contents of superoxide dismutase and glutathione. The potential synergic effect of diet and PS MP exposure (20 mg/kg bw/day with 5, 50, 100 and 200 μm particle sizes for 10 weeks) was recently evaluated in mice. For mice fed a normal or a high-fat diet, MP exposure led to a decrease in the richness and diversity of the gut microbiota (Huang et al. 2022a). Relative abundance of *Bacteroidetes* was increased while *Firmicutes* phylum was decreased in PS-MP treated mice. At the family level and for mice fed a normal or a high-fat diet, PS MP exposure reduced the relative abundance of *Muribaculaceae* and *Helicobacteraceae*, while as previously observed in other studies, it increased the relative abundance of *Prevotellaceae*, *Enterobacteriaceae*, *Desulfovibrionaceae* and *Rikenellaceae*.

In comparison with PS MPs, less studies have been conducted to assess the impact of other MP polymer types on rodent gut microbiota. After exposure to 6, 60, and 600 $\mu\text{g}/\text{day}$ of PE MPs incorporated in the diet during 5 weeks in mice, an increase in gut microbial abundance and diversity from fecal samples was reported by 16S rDNA metagenomics data. Treated-mice showed a higher abundance of *Staphylococcus*, as well as a reduction in *Parabacteroides* abundance compared to untreated animals (Li et al. 2020a). Another recent study, conducted on mice orally exposed for 6 weeks with two different sizes of PE MPs (36 and 116 μm), administered either individually or as a mixture at a dose of 100 $\mu\text{g}/\text{g}$ of food, showed modifications of the gut microbiota, characterized at the order level by less abundant *Verrucomicrobiales* and more abundant *Gastranaerophilales* in exposed mice (Djouina et al. 2022). The gut microbiota of mice was also modified after exposure to 2 μm PVC MPs (0.5 mg/day) for 60 days (Chen et al. 2022). While α -diversity was not modified, β -diversity analysis indicated a clear distinction in the microbial composition between the control and exposed groups. In particular, lower *Bacteroidetes* and higher *Verrucomicrobia*, *Firmicutes*, *Proteobacteria*, and *Patescibacteria* levels were observed in the PVC-MPs treated group

compared with the control group. In addition, exposure to PVC MPs augmented at the genus level the relative abundances of *Parasutterella*, *Roseburia*, *Dubosiella* and *Akkermansia* but reduced those of *Aerosphaera*, *Aquamicrobium*, *Brevibacterium*, and *Ruminiclostridium* 5. Specifically, PVC MP exposure decreased the relative abundances of several beneficial bacteria, including *Muribaculaceae*, *Prevotellaceae* and *Enterorhabdus*, which are associated with SCFA production (Liang et al. 2021b; Wang et al. 2021a). Furthermore, PVC MP exposure increased the abundance of opportunistic pathogenic bacteria, such as *Parasutterella*, *Blautia*, *Staphylococcus* and *Mucispirillum* (Chen et al. 2018b; Herp et al. 2021).

In the above studies, the effects of MP exposure on gut microbiota have been evaluated in healthy mice. Recently, the comparative impact of 500 µg/L PS MPs on healthy mice or mice with intestinal immune imbalance (via DSS-induced colitis) showed a deeply disturbed colonic microbial community for the latter (Liu et al. 2022). In particular, the relative abundance of *Proteobacteria* and *Bacteroidetes* was important in PS-MP exposed colitis group while lactobacilli were decreased in both colitis and PS-MP exposed colitis groups.

As presented above, the existing studies focused on the bacterial fraction but none investigated the impact of MP exposure on the composition and activity of the fungal population (“mycobiome”) in the gut (Sánchez 2019), which is increasingly recognised as a key player in intestinal health and disease (Richard and Sokol 2019). Furthermore, a limitation for many of studies reported for gut bacteria is that they mostly rely on 16S rDNA gene sequence analysis, which does not allow species level resolution. To fully resolve microbial issues, there is a need to go beyond community profiling by employing high-resolution molecular analysis of species genotypes in combination with cultivation dependent phenotypic characterizations, as well as functional metabolic activities as SCFA, AhR ligand and VOC production (see Part 1).

To understand more deeply the interactions between MPs and gut microbiota, some studies have been performed on specific bacteria. For instance, Tong and his team showed the adherence of *Helicobacter pylori*, a resident of the human stomach, to PE MP surface, in both aqueous solution and artificial gastric juice, forming a biofilm that was conducive to the rapid colonization of gastric epithelial cells by the bacteria (Tong et al. 2021). This cooperation between *H. pylori* and PE MPs exacerbated the release of inflammatory factors, causing in mice the worsening of gastric mucosal tissue damage. Interestingly, Tang and colleagues recently confirmed adhesion of *E. coli*, a member of the human gut microbiota, to PE MP surface within 30 s forming subsequent protection toward sodium hypochlorite disinfection (Tang et al. 2022).

Lastly, little is known about the microbial degradation ability of MPs in mammals. However, some microorganisms from the gut microbiota of soil waxworms are able to degrade MPs. The gut of the larvae *Plodia interpunctella* contains two bacterial strains, *Enterobacter asburiae* YT1 and *Bacillus* sp. YP1, able to degrade PE. Indeed, it was observed the formation of pits and cavities on the PE surface and addition of carbonyl groups (Yang et al. 2014). Bacteria extracted from the gut of *Lumbricus terrestris*, members of the phyla *Actinobacteria* (*Microbacterium awajiense*, *Rhodococcus jostii*, *Mycobacterium vanbaalenii* and *Streptomyces fulvissimus*) and *Firmicutes* (*Bacillus simplex* and *Bacillus* spp), were able to reduce the size of low density PE MPs (from 150 μm to an average of 53.1–41.3 μm) in a gamma sterilized soil culture after 21 days (Huerta Lwanga et al. 2018).

According to the huge and diverse microbial communities in the human gut, it seems plausible that some members could harbor MP degradation ability. Indeed, after comparison between bacteria identified as able to degrade MPs (Jacquin et al. 2019) and bacteria found in the human gut microbiota, according to the database of Plaza Oñate and his team (Plaza Oñate et al. 2019), bacteria belonging to *Proteobacteria*, *Actinobacteria*, *Bacteroidetes* and *Firmicutes* phyla were present in both; however, to date, except the first attempt of Tamargo and colleagues on PET MPs during colonic fermentation in the SIMGI gut model (Tamargo et al. 2022), no rigorous study has been carried out on the possible plastic degradation metabolism by the human gut microbiota.

A summary of the major findings *in vivo* and *in vitro* on the gut epithelium, mucus and microbiota is displayed in Fig 3.6.

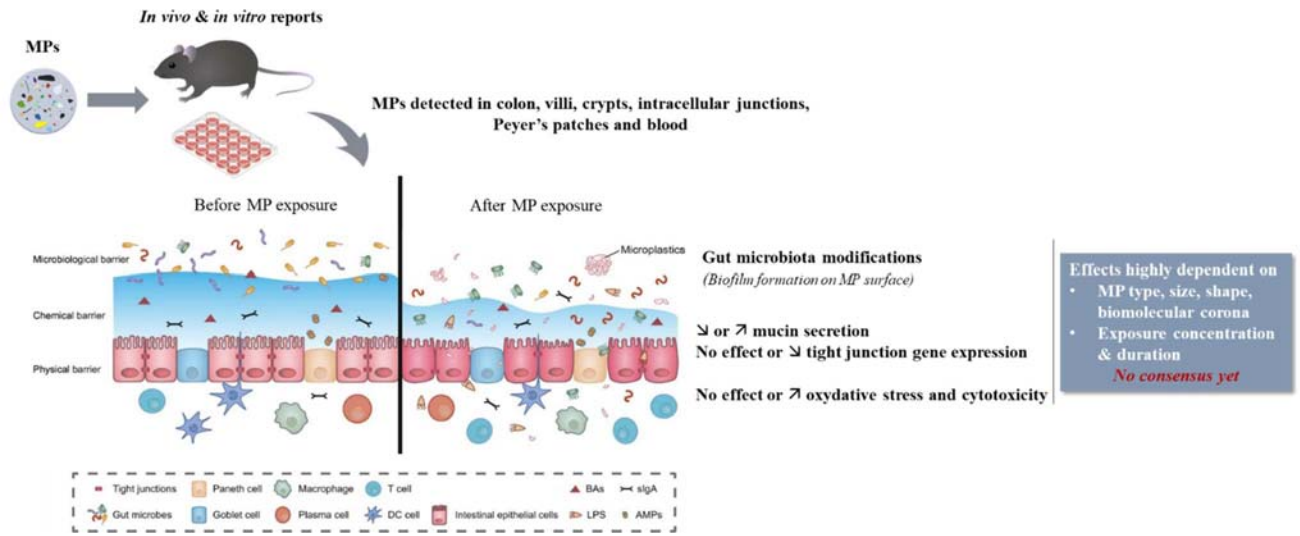


Fig 3.6 Possible influence of MPs on the intestinal barrier as reported by *in vivo* and *in vitro* studies

Exposure to MPs may lead to an increase or decrease in mucus secretion, changes in the composition and abundance of intestinal microorganisms and, potentially, an increase in oxidative stress and cytotoxicity and a decrease in tight junction protein expression. Noteworthy, the immune response after MP exposure was not included in this state-of-the-art part.

MPs: microplastics

Adapted from Huang et al. 2021

Section 2: Results

Chapter I - Comparative methods for fecal sample storage to preserve gut microbial structure and function in an *in vitro* model of the human colon

In *vitro* gut models, such as the Mucosal Artificial Colon (M-ARCOL), provide timely and cost-efficient alternatives to *in vivo* assays allowing mechanistic studies to better understand the role of human microbiome in health and disease. Using such models implies to work with human fecal samples. Processing fresh stool samples within 6 h after collection is not always easily manageable and storage methods, as freezing or lyophilization, may be valuable options. Then, it raises the question on the most suitable methods for preserving initial gut microbiota specificities. However, the effects of preservation methods on microbial structure and function in *in vitro* gut models have been poorly investigated. This study thus aimed to assess the impact of short-term storage and cryopreservation on the kinetics of lumen and mucus-associated microbiota colonization in the M-ARCOL model to finally select the most suitable option for our following experiments on the impact of oral exposure to MPs. Fecal samples from two healthy volunteers were subjected to different preservation protocols: freezing 48 h at -80°C (raw freezing stool, RFS) or freezing 48 h at -80°C with glycerol as a preservative agent (frozen stool, GFS), or lyophilization with maltodextrin/trehalose (lyophilized stool, LS) compared to fresh fecal samples. This work has been published in *Applied Microbiology and Biotechnology* (IF = 5.56) in 2020.

Comparative methods for fecal sample storage to preserve gut microbial structure and function in an *in vitro* model of the human colon

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Comparative methods for fecal sample storage to preserve gut microbial structure and function in an in vitro model of the human colon

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Abstract

In vitro gut models, such as the mucosal artificial colon (M-ARCOL), provide timely and cost-efficient alternatives to in vivo assays allowing mechanistic studies to better understand the role of human microbiome in health and disease. Using such models inoculated with human fecal samples may require a critical step of stool storage. The effects of preservation methods on microbial structure and function in in vitro gut models have been poorly investigated. This study aimed to assess the impact of three commonly used preserving methods, compared with fresh fecal samples used as a control, on the kinetics of lumen and mucus-associated microbiota colonization in the M-ARCOL model. Feces from two healthy donors were frozen 48 h at -80°C with or without cryoprotectant (10% glycerol) or lyophilized with maltodextrin and trehalose prior to inoculation of four parallel bioreactors (e.g., fresh stool, raw stool stored at -80°C , stool stored at -80°C with glycerol and lyophilized stool). Microbiota composition and diversity (qPCR and 16S metabarcoding) as well as metabolic activity (gases and short chain fatty acids) were monitored throughout the fermentation process (9 days). All the preservative treatments allowed the maintaining inside the M-ARCOL of a complex and functional microbiota, but considering stabilization time of microbial profiles and activities (and not technical constraints associated with the supply of frozen material), our results highlighted 48 h freezing at -80°C without cryoprotectant as the most efficient method. These results will help scientists to determine the most accurate method for fecal storage prior to inoculation of in vitro gut microbiome models.

Key points

- *In vitro* ARCOL model reproduces luminal and mucosal human microbiome.
- Short-term storage of fecal sample influences microbial stabilization and activity.
- 48 h freezing at -80°C : most efficient method to preserve microbial ecosystem.
- Scientific and technical requirements: influencers of preservation method.

Keywords Human gut microbiota · Fecal sample · Preservation method · In vitro M-ARCOL model

Charlotte Deschamps and Elora Fournier contributed equally to this work.

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Introduction

The human gastrointestinal tract (GIT) harbors a complex community including 100 trillion microorganisms including Bacteria, Fungi, Archaea and virus-like particles, collectively referred as gut microbiota. Bacteria mainly represent this tremendous number of microbes with the highest proportion found in the colon (Sender et al. 2016). It is now well established that each individual has a unique gut microbiota composition with bacteria belonging to the predominant phyla *Bacteroidetes*, *Firmicutes*, *Actinobacteria* and to a lesser extent to *Proteobacteria* and *Verrucomicrobia* (Cheng et al. 2013; Ndeh and Gilbert 2018). This diverse microbial ecosystem provides benefits to the host through its role in digestion and assimilation of dietary nutrients, immunity, and protection against invasion by pathogenic microorganisms (Rooks and Garrett 2016; Andoh 2016). Short chain fatty acids (SCFAs) and gas are the main fermentation products derived from microbiota-accessible carbohydrates and dietary proteins, which exert crucial physiological effects as energy supply and trophic factors (Lavelle and Sokol 2020). Perturbations in gut microbiota structure or function (termed dysbiosis) have been associated with many gastrointestinal and extra-digestive disorders (Cheng et al. 2020).

Many insights into the complex interactions between the host and the members of the gut microbiota have been performed in well-validated in vivo rodent models or using in vitro cell-based approaches (Pearce et al. 2018). However, these approaches are hampered by differences between animal and human digestive physiology (including resident microbiota) and the ignorance of other niches encountered during gastrointestinal passage prior to host cell interactions, respectively. Alternative solution is to use in vitro models enabling the maintenance of human gut ecosystem while reproducing the main physicochemical parameters of the human gut, i.e., pH, temperature, retention time, nutrient availability, and anaerobiosis. The TNO gastrointestinal model TIM-2 (Minekus et al. 1999), the PolyFermS (Zihler Berner et al. 2013), the Simulator of Human Intestinal Microbial Ecosystem (SHIME) (Molly et al. 1993), and the Artificial Colon (ARCOL) (Cordonnier et al. 2015) are among the most relevant and well-validated in vitro models reproducing the human colonic environment. ARCOL has been recently upgraded to distinguish luminal and mucosal microenvironments of the colon, leading to the M-ARCOL configuration. These models are suitable platforms allowing mechanistic studies on the role of gut microbiota in health and disease (Fleury et al. 2017). One of their main advantages is the possibility to set them up for an extended period of time, under controlled laboratory settings, without any ethical constraints and independently from the host.

To reproduce the microbial ecosystem of the human colon, these in vitro models are inoculated with a liquid suspension

of feces collected from human volunteers (Aguirre et al. 2014). Overall, fresh stools are preferably used for bioreactor inoculation to be as close as possible from in vivo situation, but scientific or technical constraints may lead to require fecal sample storage (due to geographic restriction or long-term experiments with the same fecal inoculum). Several studies have already investigated the impact of various storage protocols for downstream gut microbiota nucleic acid analysis. These studies have focused on the impact of preserving methods, mainly freezing at different temperatures (4 °C, –20 °C, or –80 °C) with the addition of cryoprotectants (ethanol, RNA later, DNA shield) on the microbial composition of stool (Al et al. 2018; Bassis et al. 2017; Choo et al. 2015; Flores et al. 2015; Gorzelak et al. 2015; Song et al. 2016). To date, only one study has evaluated the impact of freezing preservative techniques on the onset of luminal (but not mucus-associated) gut microbiota in in vitro gut models (Aguirre et al. 2015).

Therefore, in the present study, we investigated the impact of short-term storage and cryopreservation on the establishment of luminal and mucus-associated gut microbiota in M-ARCOL. To this endeavor, before inoculation in the in vitro gut model, fecal samples from two healthy volunteers were subjected to different preservation protocols: freezing 48 h at –80 °C (raw freezing stool, RFS), freezing 48 h at –80 °C with glycerol as a preservative agent (glycerol frozen stool, GFS), or lyophilization with maltodextrin/trehalose (lyophilized stool, LS). Impact of preservation methods was assessed by monitoring microbiota composition and microbial metabolic activity (gas and SCFA production) throughout the fermentation process after inoculation with RFS, GFS, or LS and compared at each time point to fresh stool (FS) condition.

Materials and methods

Fecal sample collection and treatments

Fecal samples used to inoculate the M-ARCOL model were collected from two healthy volunteers, a female aged 22 years old (donor A) and a male aged 52 years old (donor B). Both donors have no history of antibiotic treatment or probiotic consumption for 3 months prior to sample collection. Immediately after defecation, fecal samples were transferred into a sterile recipient, placed in an airtight anaerobic box (GENbag anaer gas pack systems, Biomerieux, France), transported, and processed at the laboratory within 6 h. In an anaerobic chamber (COY laboratories, Grass Lake, USA), stool samples were manually homogenized in a hermetic freezer bag and then divided into 4 pieces of 16 g each: (i) one fresh aliquot was used to generate the fresh stool (FS) condition; (ii) the second aliquot of raw stool was directly frozen at –80 °C for 48 h without any preservative (raw

frozen stool, RFS condition); (iii) the third aliquot of stool was resuspended in 45 mL sterile 10% glycerol and frozen at -80°C for 48 h (glycerol frozen stool, GFS condition); (iv) the last aliquot of stool was resuspended in 45 mL of a maltodextrin/trehalose solution, then frozen at -80°C for 12 h before lyophilization at -15°C during 36 h, as described in patent n°WO 2017/103225 (Reygner et al. 2020) (lyophilized stool, LS condition). Prior to inoculation into M-ARCOL, fresh and stored fecal samples were resuspended with 30 mM sterile sodium phosphate buffer and filtered (500 μm inox sieve).

Description and set-up of M-ARCOL model

Artificial COLon is a one-stage fermentation system (MiniBio, Applikon, Delft, The Netherlands), used under semi-continuous conditions, which simulates both microbial and physicochemical conditions encountered in the human colon (Blanquet-Diot et al. 2012; Thévenot et al. 2015). The model has been recently adapted to distinguish specific microenvironments found in the digestive lumen and in the mucosa (Van den Abbeele et al. 2009), resulting in the Mucosal Artificial Colon configuration (M-ARCOL). It consists of a pH and temperature-controlled main bioreactor reproducing the luminal colonic medium connected to a glass vessel containing mucin beads and reproducing the mucus-associated microbiota. A sterile nutritive medium (Thévenot et al. 2015) containing various sources of carbohydrates, proteins, lipids, minerals, and vitamins was sequentially introduced into the main bioreactor to simulate sequential input of ileal effluents. To ensure anaerobic condition at the beginning of fermentation, the bioreactor was operated with an initial sparging with O_2 -free N_2 gas. Afterwards, during the fermentation course, anaerobic condition inside the bioreactor was maintained by the sole activity of resident microbiota and by ensuring the airtightness of the system. Redox potential was constantly measured using a redox sensor (Mettler Toledo, Columbus, USA) and corrected into an Eh value by adding 200 mV to the recorded values, according to the manufacturer's instructions. In the present study, M-ARCOL was set up to reproduce the mean conditions found in a healthy human adult colon with a fixed temperature of 37°C , a constant pH of 6.3 maintained by the addition of 2 M NaOH, a stirring speed at 400 rpm, and a mean retention time of 24 h.

Mucin beads and mucin compartment

Mucin from porcine stomach type II (Sigma-Aldrich, Saint-Louis, USA) and sodium alginate (Sigma-Aldrich, Saint-Louis, USA) were diluted in sterile distilled water, at a concentration of 5% and 2%, respectively. To produce mucin beads, the mucin/alginate solution was dropped using a peristaltic pump into a 0.2 M solution of sterile CaCl_2 under agitation. Mucin beads were introduced into the airtight glass

compartment (total area of beads 556 cm^2) connected to the bioreactor and allowing a continuous flow of the luminal medium on mucin beads. Mucin bead compartment was kept at 37°C during the experiment using a hot water bath. Every 2 days, mucin/alginate beads were renewed by fresh sterile ones under a constant flow of CO_2 to avoid oxygen entrance.

Experimental design and sampling

Experimental design and sampling in the M-ARCOL are presented in Figs. 1 and 2, respectively. For each experiment, four bioreactors inoculated with the fecal sample from the same donor (fresh or stored) were run in parallel, corresponding to the four tested conditions (FS, RFS, GFS, and LS). Two series of experiments were performed with fecal samples from two different donors (corresponding to two biological replicates). All the fermentations were run under batch conditions for 24 h and then under semi-continuous conditions for 8 additional days. Samples from the main bioreactor, referred to as luminal microbiota, were collected daily for microbiota characterization and SCFA analysis. Additional samples from the mucin bead compartment, referred to as mucus-associated microbiota, were collected every 2 days for microbiota characterization. Mucin/alginate beads were washed thrice in sterile phosphate buffer saline (PBS) and stored at -80°C for downstream analyses. Samples were also harvested daily from the atmospheric phase of the bioreactor to ascertain anaerobic conditions and evaluate gas composition. The daily total volume of gas produced was also measured.

DNA extraction

Genomic DNA was extracted from bioreactor samples and mucin/alginate beads using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) following manufacturer's instructions with the following modifications. Prior to DNA extraction, bioreactor samples were centrifuged (2000g, 10 min), and the pellet was subjected to mechanical disruption using a bead beater (3 min, 20 beat/s) with 300 mg sterile glass beads (diameter ranging in size from 0.1 to 0.6 mm), incubated (70°C , 5 min), and centrifuged again (12,000g, 1 min). Mucin/alginate beads were subjected to the following modifications prior to DNA extraction: 10 min of incubation with citrate buffer (37°C), as previously described by Capone et al. (2013), before vortexing (maximal speed, 3 min) and centrifugation (8000g, 1 min). DNA integrity was checked by agarose gel electrophoresis. DNA quantity was evaluated using the Qubit dsDNA Broad Range Assay Kit (Invitrogen, Carlsbad, USA) with a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, USA). Samples were stored at -20°C prior to microbiota analysis (qPCR and 16S metabarcoding).

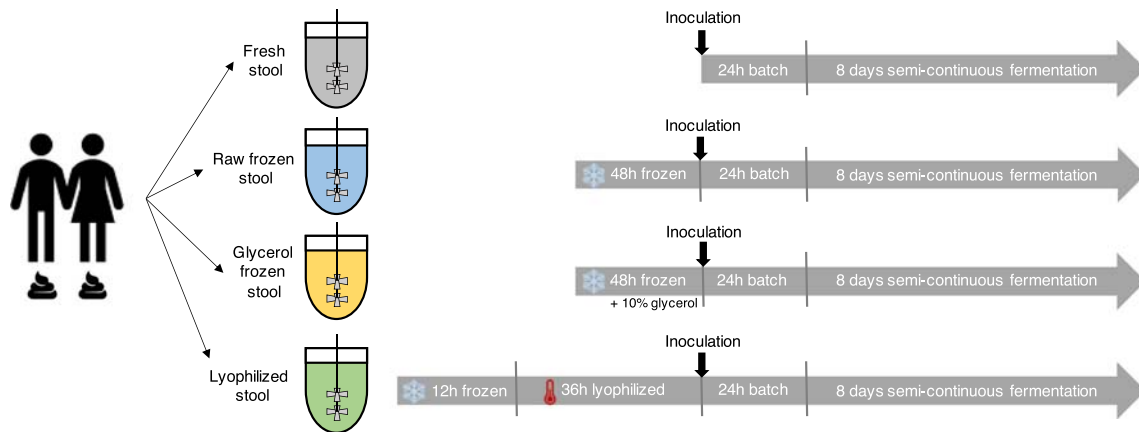


Fig. 1 Experimental design of the in vitro fermentation experiments in the M-ARCOL. Stools from two donors (one male, one female) were used to inoculate two independent experiments (two biological replicates) of four bioreactors, allowing testing four conditions in

parallel: fresh stool (control), raw frozen stool (48 h, -80°C), glycerol-frozen stool (48 h, -80°C), and lyophilized stool (36 h, -15°C). Each fermentation was carried for a total period of 9 days including 24 h of batch and 8 days of semi-continuous fermentation

Quantitative PCR

Total bacteria, *Bacteroidetes*, and *Firmicutes* were quantified by qPCR. Primers and qPCR conditions are listed in Table 1. Real-time PCR assays were performed in a Mx3005P apparatus (Stratagene, San Diego, USA) based on SYBR Green technology using Takyon™ Low Rox SYBR® kit (Eurogentec, Seraing, Belgium). Each reaction was run in duplicate in a final volume of 10 μL with 5 μL of Master Mix, 0.45 μL of each primer (10 μM), 1 μL of DNA sample (10 ng/ μL), and 3.1 μL of ultra-pure water. Amplifications were carried out using the following ramping profile: 1 cycle at 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 60°C for 1 min. A melting step was added to ensure primer specificity. Standard curves were generated from 10-fold dilutions of

bacterial DNA (isolated from reference strain) and allowed the calculation of DNA concentrations from extracted samples.

16S metabarcoding and data analysis

The bacterial V3-V4 region of 16S ribosomal DNA (rDNA) and the archaeal 16S rDNA were amplified with primers described in Table 1. Amplicons were generated using a Fluidigm Access Array followed by high-throughput sequencing on an Illumina MiSeq system (Illumina, USA) performed at the Carver Biotechnology Center of the University of Illinois (Urbana, USA). Raw data are available at NCBI under the Sequence Read Archive database with accession no. PRJNA638256. Bioinformatic analysis was performed

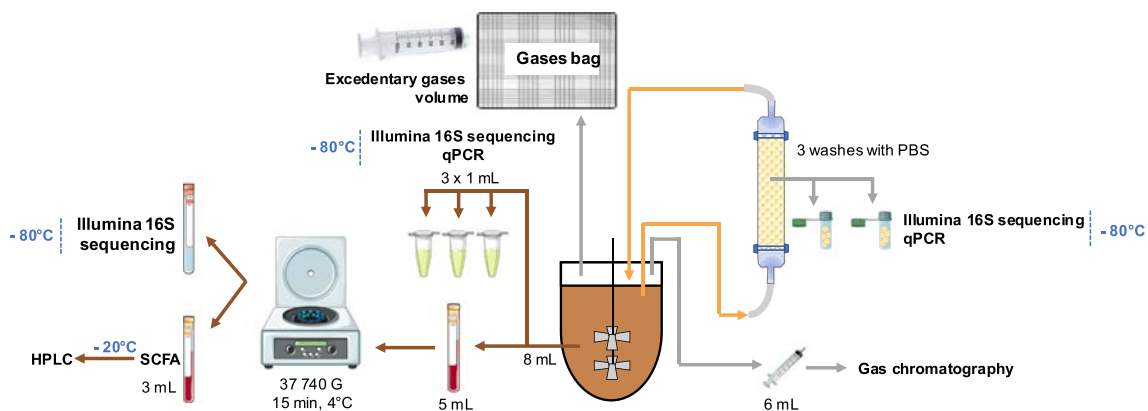


Fig. 2 Experimental set-up and sampling procedure during M-ARCOL experiments. Every day, samples were collected from the fermentation medium and frozen at -80°C for downstream analysis. After centrifugation, the supernatant was used for short chain fatty acid measurement while DNA was extracted from the pellet for further 16S metabarcoding and qPCR analyses. Daily overflow gas was measured, and gas

composition was determined by gas chromatography. Every 2 days, mucin beads were collected to determine the composition of mucin-associated microbiota by qPCR and 16S metabarcoding. HPLC: high-performance liquid chromatography; PBS: phosphate buffer saline; SCFA: short chain fatty acids

Table 1 Primers used for qPCR and 16S metabarcoding

Primer name	Sequence 5'-3'	Target	Annealing temperature (°C)	References
Primer qPCR				
BAC338R	ACTCCTACGGGAGGCAG	Total bacteria	58	Yu et al. (2005)
BAC516F	GTATTACCGCGGCTGCTG			
789cfbF	CRAACAGGATTAGATACCCT	<i>Bacteroidetes</i>	61	Bacchetti De Gregoris et al. (2011)
cfb976R	GGTAAGGTTCTCGCGTAT			
928F-Firm	TGAAACTYAAAGGAATTGACG	<i>Firmicutes</i>	61	Bacchetti De Gregoris et al. (2011)
1040FirmR	ACCATGCACCACCTGTC			
Primer 16S				
V3_F357_N	CCTACGGGNGGCWGCAG	Bacteria	-	Klindworth et al. (2013)
V4_R805	GACTACHVGGGTATCTAATCC			
Arch349F	GYGCASCAGKCGMGAAW	Archaea	-	Takai and Horikoshi (2000)
Arch806R	5GGACTACVSGGGTATCTAAT			

with QIIME 2 (version 2020.2) (Bolyen et al. 2019). Raw sequence data were demultiplexed and quality filtered using the q2-demux plugin followed by denoising with DADA2. Diversity analysis was estimated using q2-diversity after samples were rarefied. All amplicon sequence variants (ASVs) were aligned with mafft and used to construct a phylogeny with fasttree2. Taxonomy was assigned for Bacteria and Archaea to ASVs using the q2-feature-classifier coupled to SILVA release 132 database (Quast et al. 2012).

Gas and SCFA analysis

Analysis of O₂, N₂, CO₂, CH₄, and H₂ produced during the fermentation process was performed using HP 6890 gas chromatograph (Agilent Technologies, Santa Clara, USA) coupled with a micro-TCD detector (Agilent Technologies, Santa Clara, USA). Two series columns, Molecular Sieve 5A and Porapack Q (Agilent Technologies, Santa Clara, USA), were used. Gas composition was determined using calibration curves made from ambient air (78.09% N₂, 20.95% O₂, 0.04% CO₂) and three gas mixtures A (5% CO₂, 5% H₂, 90% N₂), B (19.98% CO₂, 80.02% H₂), and C (19.89% CO₂, 19.88% CH₄, 20% H₂, 40.23% N₂).

For SCFA analysis, 2 mL of luminal samples were centrifuged (5000g, 15 min, 4 °C) and 900 µL of supernatant was diluted at 1/10 in the H₂SO₄ 0.04 M mobile phase, vortexed, and filtered (0.22 µm). The three major SCFAs (acetate, propionate, and butyrate) were quantified by high-performance liquid chromatography (HPLC) (Elite LaChrom, Merck HITACHI, USA) coupled with a DAD diode. The HPLC column (150 × 7.8 mm) contains a stationary phase of polystyrene divinylbenzene grafted with sulfonic groups, negatively charged, and carries an eluent containing acidified water. Data were obtained and analyzed by the EZChrom Elite software at 205 nm. SCFA concentrations were calculated from calibration curves established from known concentration

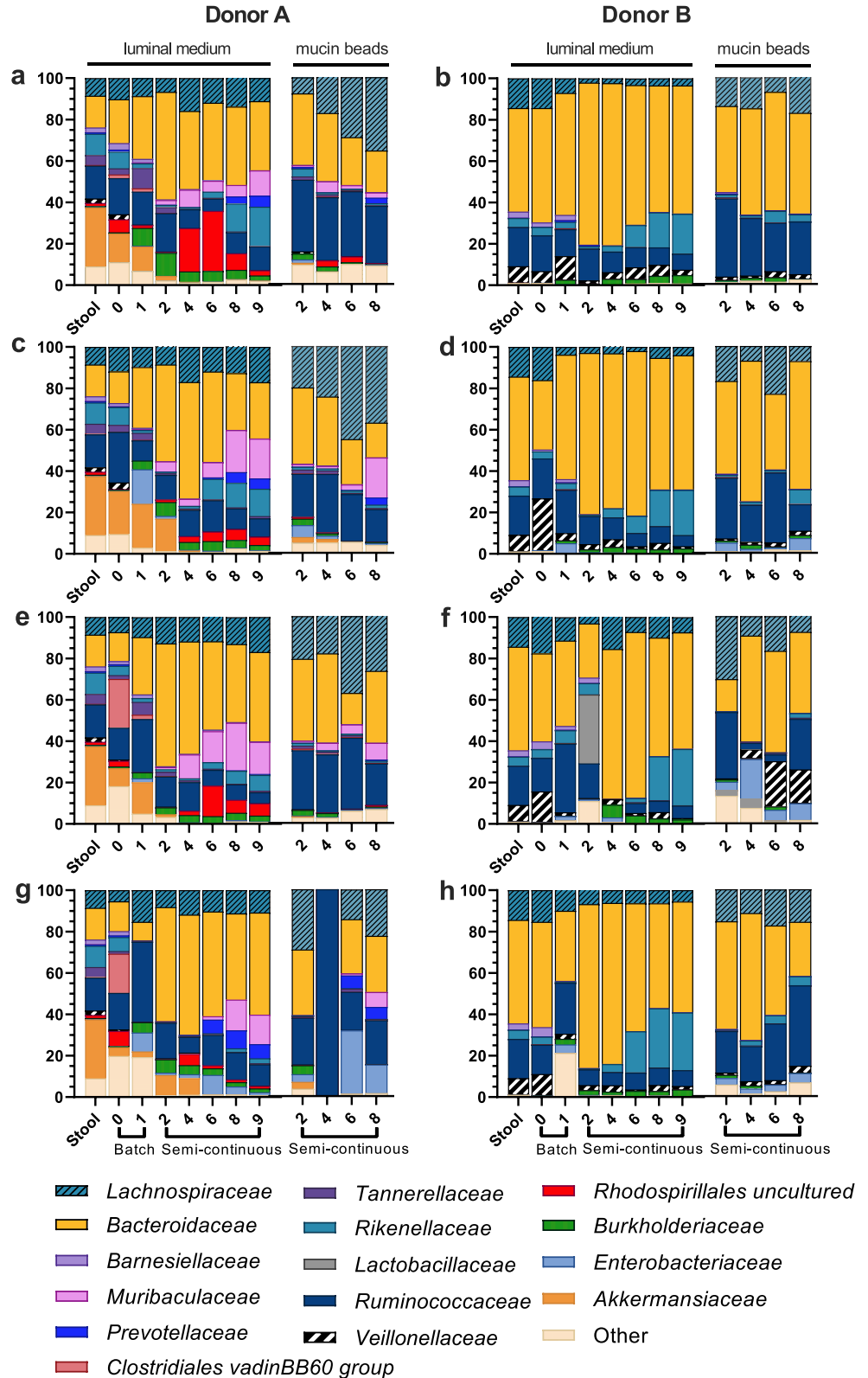
solutions of acetate, propionate, and butyrate (0, 10, 25, and 40 mM).

Results

Microbiota composition of initial fecal samples

Microbiota from starting stools (without any treatment) appeared highly different between the two donors (289 OTUs for donor A, 201 OTUs for donor B). As revealed by 16S metagenomics data, stool microbiota from donor A was mainly composed of *Firmicutes* (34%), *Bacteroidetes* (33%), and *Verrucomicrobia* (28%) while donor B was almost exclusively constituted by *Firmicutes* (42%) and *Bacteroidetes* (57%) (Figure S1). Predominant family taxa (stool samples; Fig. 3) emphasize divergences in the structure of fecal microbiota. The most abundant families in donor A were *Akkermansiaceae* (29%), *Ruminococcaceae* (16%), *Bacteroidaceae* (15%), *Rikenellaceae* (10%), and *Lachnospiraceae* (9%). Lower number of different families was identified for donor B with mainly *Bacteroidaceae* (50%), *Ruminococcaceae* (19%), *Lachnospiraceae* (14%), and *Veillonellaceae* (8%). In the same way, when considering species diversity on initial fecal samples, Shannon's index indicates that donor A exhibited a higher microbial diversity than donor B (5.5 versus 4.2) (stool samples; Fig. 4). This important discrepancy was also confirmed by data from whole metagenome shotgun analysis performed on initial stools in the frame of another study (data not shown) with microbial richness higher in stool from donor A (326 species) compared with donor B (239 species). Methanogenic *Archaea* were detected only in the donor A's stool and identified as *Euryarchaeota*, *Methanobacteriaceae*, and *Methanobrevibacter* at the phylum, family, and genus levels, respectively (data not shown).

Fig. 3 Relative abundance of bacterial families retrieved in the initial stools and in the luminal medium and mucin beads from M-ARCOL. Fecal samples from two donors (donor A: left panel; donor B: right panel) were submitted to different preservative protocols: control non-treated fresh stool (FS, **a, b**), raw frozen stool (RFS, **c, d**), glycerol-frozen stool (GFS, **e, f**), and lyophilized stool (LS, **g, h**). Composition of luminal medium and mucin bead samples from M-ARCOL inoculated with fresh or treated stools was determined at the family level by analysis of 16S amplicon sequencing data



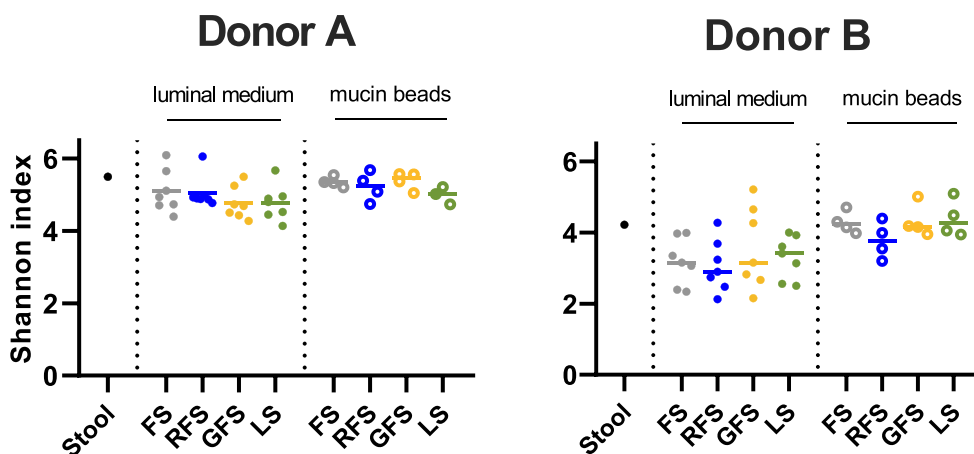


Fig. 4 Effect of different stool storage conditions on Shannon diversity index values. Fecal samples from two donors (donor A: left graph; donor B: right graph) were submitted to different preservative protocols: control non-treated fresh stool (FS, in gray), raw frozen stool (RFS, in blue),

glycerol-frozen stool (GFS, in yellow), and lyophilized stool (LS, in green). Shannon diversity index was determined in the initial stool and in the luminal medium and mucin bead samples collected from M-ARCOL inoculated with fresh or treated stools

Effect of stool short-time storage on microbial composition and stabilization in M-ARCOL

In the present study, we assessed the effect of different preservative methods on luminal and mucus-associated gut microbiota composition and compared at each time point during fermentation to fresh stool used as a positive control. Two parameters were followed to determine the most efficient method: (i) time of stabilization of microbial profiles in M-ARCOL and (ii) microbiota profiles obtained after stabilization compared with the fresh stool.

Luminal medium For the FS condition, total bacteria level reached and stabilized at 10^{10} copies of 16S rDNA/mL in the luminal phase at day 2 (qPCR data; Fig. 5a). Similar results were obtained for RFS and GFS conditions, while for LS condition, this stabilization level at 10^{10} copies of 16S rDNA/mL was only reached at day 4. Population levels of *Firmicutes* and *Bacteroidetes* stabilized at 10^9 and 10^{10} copies of 16S rDNA/mL, respectively, with a 2-days delay for LS condition compared with the FS control and the two other experimental conditions (Fig. 5 b and c). Regarding bacterial composition at the family level, stabilization time of major luminal populations is reached after 6–8 days (depending on the donor A or B) for all preservative methods, as observed for control FS condition (Fig. 3). At day 9 (end of the experiment), bacterial microbiota kept consistent differences between the two donors with families of *Bacteroidaceae* (33% in donor A versus 62% in donor B), *Rikenellaceae* (19% versus 19%), *Muribaculaceae* (12% versus 0%), *Ruminococcaceae* (12% versus 8%), *Prevotellaceae* (5% versus 0%), and *Lachnospiraceae* (11% versus 3%). Microbial profiles of control and preservative treatments were comparable at the phylum level at day 9 (Figure S1), but differences may be highlighted for donor A at the family level. Specifically,

Prevotellaceae decreased from 5 to 0.5% in GFS, while *Rikenellaceae* decreased from 19 to 2% in LS. Also, microbial profiles obtained for RFS were very similar to those from FS for both donors. Interestingly, *Akkermansiaceae* (from the *Verrucomicrobia* phylum), only present in donor A, disappeared after 2 days of semi-continuous fermentation from luminal medium in all conditions except for LS where these microbes persist until day 4 (Fig. 3). Species diversity of all luminal-associated microbiota defined by the Shannon's index was not affected during fermentation with values close to 5 in donor A and 3 in donor B (Fig. 4). β -diversity analysis revealed no impact of preservative treatment on microbial structure while samples highly clustered according to the donor (data not shown). Regarding methanogenic Archaea, whatever the treatment *Methanobrevibacter* population is present in the luminal phase of M-ARCOL and remains stable from day 2. In addition, Archaea were not impacted by any of the preservative techniques (data not shown).

Mucin beads For mucin beads, qPCR analysis revealed that total bacteria, *Bacteroidetes* and *Firmicutes* stabilized around 10^8 copies of 16S rDNA/mL for both FS and RFS conditions (Fig. 5). These populations stabilized at a slightly lower level for GFS, especially for *Bacteroidetes* (10^7 copies of 16S rDNA/mL), and the level of *Bacteroidetes* regularly decreased through the experiment for LS. At the phylum level, stabilization of the mucus-associated microbiota was reached between days 4 and 6 depending on the donor, for both FS and treated stools (Figure S1). At day 8, for donor A, no difference was observed between FS, RFS, and GFS, whereas an increase in *Proteobacteria* was noticed in LS condition (14% versus 2% in FS) (Figure S1). For donor B, none of the profiles obtained at day 8 were similar from those of FS. In particular, GFS and LS conditions were characterized by an increase in *Actinobacteria* compared with control fresh stool

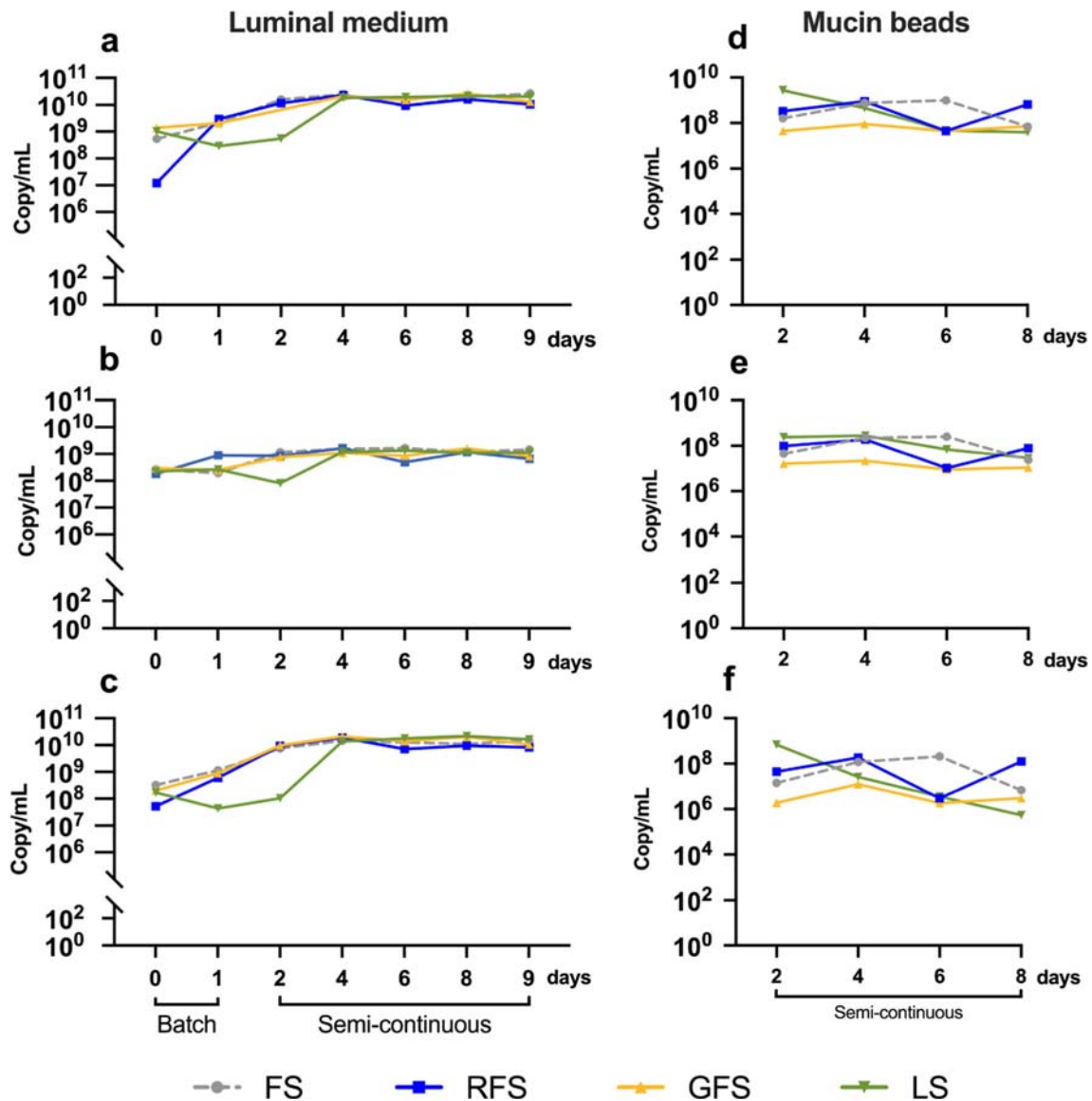


Fig. 5 Effect of different stool storage conditions on qPCR enumeration of main bacterial groups. Fecal samples from two donors were submitted to different preservative protocols: control non-treated fresh stool (FS, gray circles), raw frozen stool (RFS, blue squares), glycerol-frozen stool (GFS, yellow triangles), and lyophilized stool (LS, green triangles). Total

bacteria (a, d), *Firmicutes* (b, e), and *Bacteroidetes* (c, f) were evaluated by qPCR in luminal medium (top panel) and mucin bead samples (bottom panel) collected from M-ARCOL inoculated with fresh or treated stools. Results are expressed as mean copy of 16S rDNA per mL ($n = 2$)

(at day 8, 4% and 1%, respectively, versus 0.2% for FS). At the family level, at the end of the experiments, all microbial profiles from processed stools originated from both donors differed from that of FS control (Fig. 3). For donor A, RFS condition seemed to benefit *Muribaculaceae* (19% versus 2.5% in FS) while LS condition was associated with an increase in *Enterobacteriaceae* (14% versus 0.1% in FS). For donor B, *Enterobacteriaceae* also increased in all treatment conditions compared with FS (6% for RFS, 8% for GFS, and 5% for LS, versus 0.07% for FS). In addition, *Veillonellaceae*'s proportion raised in GFS compared with FS (16% versus 2%), as well as *Ruminococcaceae* in LS compared with FS (39% versus

25%), at the expense of *Bacteroidaceae* (26% versus 49%). Interestingly, *Akkermansiaceae* was able to maintain in mucin beads up to day 4, with higher levels at day 2 in LS (3.4%) and RFS (2.8%) conditions compared with FS (1%) and GFS (0.6%). As for luminal medium, no difference in Shannon's index values was observed between treated and fresh stools for mucin beads, with values close to 6 in donor A and 4 in donor B (Fig. 4). Similarly, β -diversity analysis revealed no impact of preservative treatments on mucus-associated microbiota (data not shown). Archaea were also detected in mucin beads and *Methanobrevibacter* population was not impacted by any treatment (data not shown).

Effect of stool short-time storage on microbial activity in M-ARCOL

As an efficient fermentation yields to production of gut microbiota-derived metabolites among which SCFA and gas are important functional end-products, both were monitored in M-ARCOL. As previously described for microbiota composition, the efficiency of preservative methods was determined by analyzing stabilization time and comparing final levels with the one obtained for control FS.

Gas Gas composition (CH₄, CO₂, N₂, and H₂) was determined in the atmospheric phase of M-ARCOL throughout fermentations and expressed as mean relative percentages (Fig. 6). O₂ levels remained during all the fermentations below 1%,

indicating that the anaerobiosis was efficiently maintained by the sole microbiota activity. In addition, CH₄ was detected only during experiments inoculated with fecal samples from donor A, in accordance with 16S metabarcoding data on methanogenic Archaea. For FS control condition, gas composition reached stable values at day 4 and day 3 in donors A and B, respectively. Stabilization was reached at a similar period for all other tested conditions except for GFS in donor B where stable state was reached only after day 7. Stable gas pattern of donor A was composed of around 20% N₂, 65% CO₂, and 15% CH₄, while that of donor B (non-methane producer) was characterized by about 20% N₂ and 80% CO₂. For donor A, gas profiles observed after stabilization were all similar to that of FS, even if H₂ production was observed until day 4 in GFS and LS conditions only.

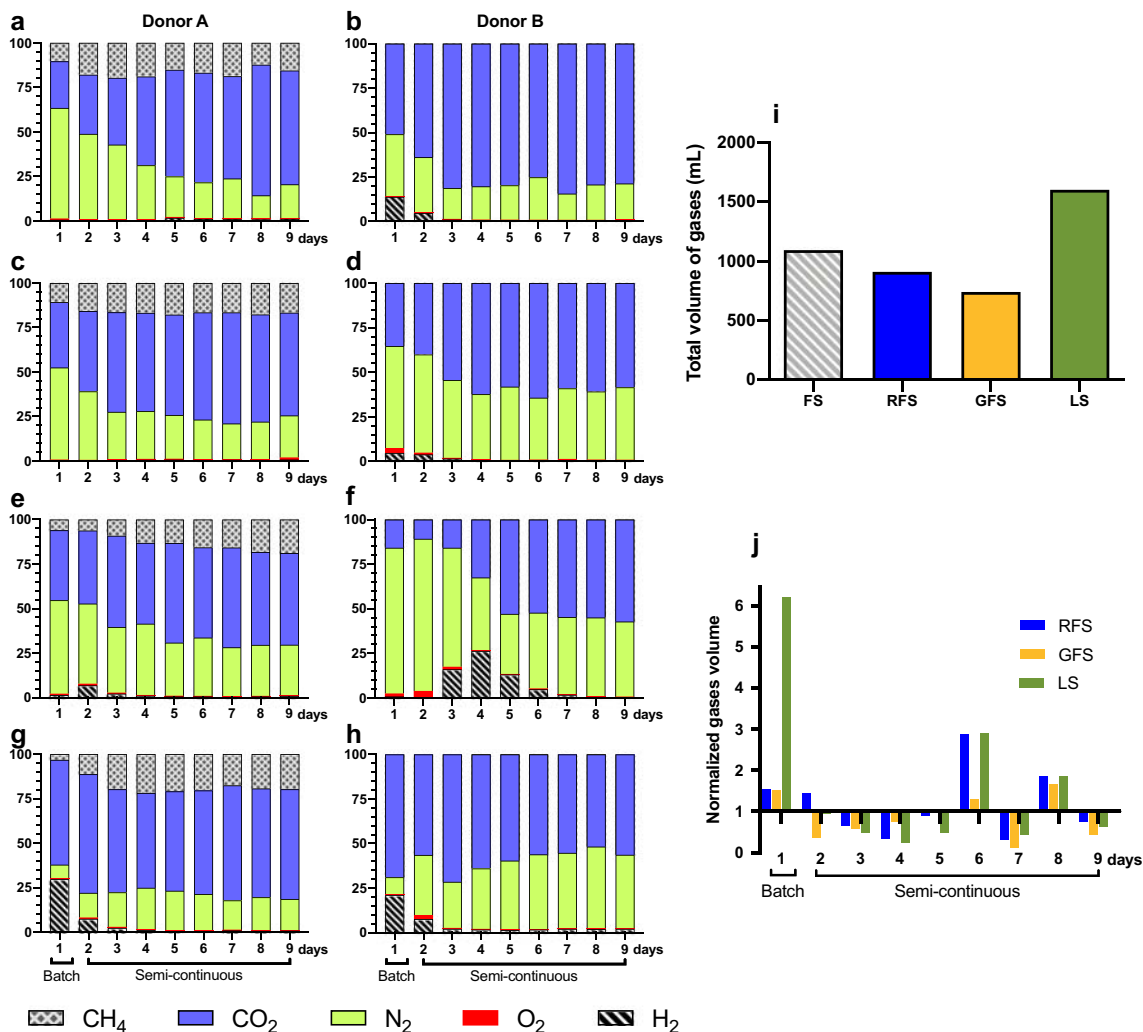


Fig. 6 Gas composition and production in M-ARCOL according to stool storage conditions. Fecal samples from two donors (donor A: left panel; donor B: right panel) were submitted to different preservative protocols: control non-treated fresh stool (FS, a, b), raw frozen stool (RFS, c, d), glycerol-frozen stool (GFS, e, f), and lyophilized stool (LS, g, h). Gas composition was analyzed by gas chromatography and results were

expressed as relative percentages (graphs a to h) or as total mean volumes (in mL) produced during the fermentations (graph i). Daily gas volume productions were also averaged ($n = 2$) and normalized to FS condition (graph j). CH₄: methane, CO₂: carbon dioxide, N₂: nitrogen, O₂: dioxygen, H₂: dihydrogen

Conversely for donor B, all gas profiles after fecal storage differed from FS with a higher percentage of N₂ (40 *versus* 20%) and consequently a lower percentage of CO₂ (60 *versus* 80%). Uncommonly, an increase in H₂ production was noticed from day 3 to day 7 with GFS for donor B. On average, a higher total gas volume (1600 mL *versus* 1100 mL) was measured in LS condition when compared with FS (Fig. 6i), mainly due to a sharp increase in gas production (multiply by 6 compared with FS) at day 1 (i.e., during batch experiment) (Fig. 6j). Lastly, during semi-continuous fermentations, average daily gas production seemed to be lower for all the conservative methods compared with FS, and particularly for GFS condition.

SCFA The three main SCFAs, acetate, butyrate, and propionate were quantified in the luminal medium of M-ARCOL and

expressed as mean relative percentages (Fig. 7a–h). During experiments with FS, SCFA profiles were stabilized at day 3 for the two donors. Similar stabilization time was observed for RFS while an additional 1 or 2 days was necessary to reach stable profiles under GFS and LS conditions. At day 9 for FS control, SCFA composition was stabilized with 48% acetate, 30% propionate, and 22% butyrate for donor A, compared with 58% acetate, 22% propionate, and 20% butyrate for donor B. Profiles obtained after stabilization for both donors are stackable with those obtained with FS regardless of tested conditions (RFS, GFS, or LS). As previously observed for microbial structure, stabilization profiles exhibited a more pronounced donor-dependent effect than a storage-dependent effect. However, for donor B, a distinct increase in acetate percentages was noticed from day 1 to day 4 with GFS (Fig. 7f). When total SCFA concentration was calculated

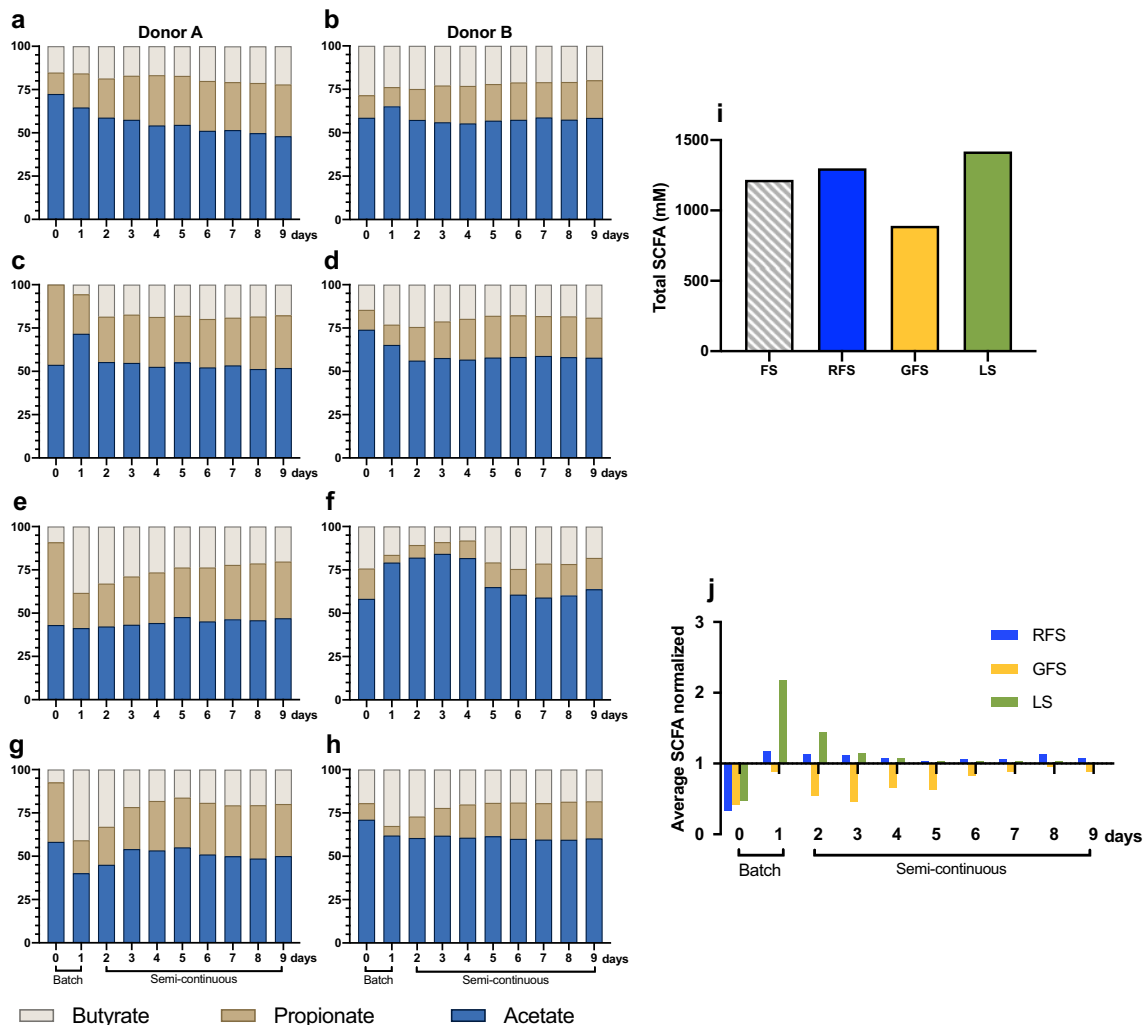


Fig. 7 SCFA composition and production in M-ARCOL according to stool storage conditions. Fecal samples from two donors (donor A: left panel; donor B: right panel) were submitted to different preservative protocols: control non-treated fresh stool (FS, a, b), raw frozen stool (RFS, c, d), glycerol-frozen stool (GFS, e, f), and lyophilized stool (LS, g, h). Main SCFA (acetate, propionate, and butyrate) were analyzed by

liquid chromatography and results were expressed as relative percentages (graphs a–h) or as total mean concentrations (in mM) produced during fermentations (graph i). Daily SCFA concentrations were also averaged ($n = 2$) and normalized to FS condition (graph j). SCFA: short chain fatty acids

throughout the total duration of the fermentation process (Fig. 7i), similar levels were obtained for FS and RFS, while a slight increase was observed for LS (1420 mM *versus* 1220 mM) together with a greater decline for GFS (890 mM *versus* 1220 mM). These data are confirmed by daily normalization of total SCFA concentrations with FS values (Fig. 7i). A sharp increase in SCFA production was evidenced at day 1 for LS (multiply by 2 *versus* FS) while reduced levels were maintained from day 2 to day 9 with GFS.

Redox potential Redox potential was recorded during the overall fermentation process in M-ARCOL as an indicator of microbial fermentative activity (Figure S2). For FS control, stable levels were obtained from day 3 and low values confirmed an important reduced colonic environment (-225 mV). Similar or lower time was required to reach stabilization time for LS (3 days) and RFS (1 day) whereas increased time period was needed to obtain a stable redox potential for GFS (4 days). In addition, for donor B, abnormal elevated values were noticed until day 3 with GFS (up to $+200$ mV). However, once stabilized, all conditions showed similar levels compared with FS.

Discussion

Inoculation of *in vitro* gut models with fecal samples from patients or healthy donors aims to reproduce the complexity and diversity of the microbial ecosystem encountered within human colon, with obvious differences in microbial structure and activity compared with initial stools due to fecal-colon and *in vivo-in vitro* transitions. Use of fresh stools is not always feasible; defining an appropriate preservation method of fecal samples is crucial to prevent loss of important microbial populations and maintain efficient fermentation activities in the bioreactor. To date, a unique study had investigated the effects of preservation methods on gut microbial structure and functions using the TIM-2 *in vitro* human colon model (Aguirre et al. 2015). For the first time, in the current study, we assessed the effect of three preservation methods on the luminal and mucus-associated microbiota using M-ARCOL *in vitro* gut model and, in a unique way, we investigated both bacterial and archaeal populations. As fecal microbiota exhibits important inter-individual variability, this study was conducted on fecal samples collected from two healthy donors, chosen to represent “extreme” conditions, i.e., donor A is a methane producer female while donor B is a non-methanogen male (Kim et al. 2020; Chaudhary et al. 2018). Likewise, donor A harbors a much higher microbial diversity than donor B, very likely associated due to differences in dietary habits (De Filippis et al. 2016; De Angelis et al. 2019).

Conservative treatments used previously by others for stool conservation were tested before inoculation of M-ARCOL and

compared with the fresh stool condition. The simplest alternative to fresh feces is stool freezing at -80 °C without any preservative treatment nor fecal samples mixing (“raw” samples), leading to the RFS condition in our study. To improve the stability of fecal microbiota during freezing and subsequent storage, different cryoprotectants have been previously described in the literature, such as dimethylsulfoxide, polyethylene glycol, RNA later, ethanol, or glycerol (Flores et al. 2015; Gaci et al. 2017; Gorzelak et al. 2015; Khanna et al. 2016). This later (GFS condition) has been chosen as it is undoubtedly the most frequently applied for bacterial storage and currently recommended and used in clinical practice for fecal microbiota transplantation (Camarota et al. 2017, 2018, 2019). Lastly, lyophilization has been tested as a new alternative to freezing for fecal sample storage (LS condition) since various protocols of this process have been recently developed for fecal microbiota transplantation (Burz et al. 2019; Reygner et al. 2020). Lyophilization with trehalose/maltodextrin solutions appears as a suitable mean for preserving highly sensitive oxygen populations and sub-dominant species (Burz et al. 2019; Reygner et al. 2020). This preservation method also allows the maintenance of major functional activities of gut microbiota and a better revivification after reheating. Maltodextrin and trehalose sugars increase media viscosity, thus limiting crystallization and osmotic disturbance to prevent cell lysis (Al et al. 2018; Burz et al. 2019). Interestingly, Reygner and colleagues demonstrated that freeze-drying in maltodextrin/trehalose or freezing in glycerol better preserved SCFA for 12-months storage compared with samples frozen without any cryoprotectant (Reygner et al. 2020). In the present study, time of storage was restricted to 48 h for all the tested conditions as our primary aim was to study the impact of preservative methods and not that of storage which is recognized to further influence microbial structure and activity (Song et al. 2016; Gaci et al. 2017).

To offer a complete picture of the effects of such preservative methods on gut microbiota in *in vitro* gut models, we investigated in M-ARCOL both luminal and mucus-associated gut microbiota and fermentation activity through gas and SCFA measurements. Mucin beads were recently integrated in M-ARCOL based on a technology similar to that implemented in M-SHIME (Van den Abbeele et al. 2009), with some adaptations allowing the physiological maintenance of anaerobiosis through microbiota activity. *In vitro* colonic models involving a mucosal phase represent a valuable alternative to consider the fine-scale spatial organization present *in vivo* in the human gut microbial ecosystem. In particular, mucin beads allow a better capture of initial fecal microbial diversity and favor the development of specific mucus colonizers (e.g., *Ruminococcaceae* and *Lachnospiraceae*) and thus the restoration of a high *Firmicutes/Bacteroidetes* ratio, as observed *in vivo* (Liu et al. 2018). Our results showed that all the preservative treatments allowed the maintaining inside M-ARCOL of a complex and functional microbiota for the two donors A and B. Interestingly, methanogenic Archaea are not impacted by treatments neither in luminal nor in

Table 2 Scientific and technical comparative data on preservative methods applied to fecal samples. From low € to high €€€€ costs; from low ★ to high ★★☆☆ score. F/B ratio: Firmicutes/Bacteroidetes ratio; ↗ increase or ↘ decrease; NA: not applicable

			Fresh stool	Raw frozen stool	Glycerol frozen stool	Lyophilized stool	
Scientific aspects (compared to FS)	Composition	Stabilization time	Days 6-8	Days 6-8	Days 6-8	Days 6-8	
		Final microbial profiles	NA	No loss of microbial diversity as measured by Shannon index Increase of <i>Proteobacteria</i> and <i>Actinobacteria</i> in the mucin beads			
	Activity	Stabilization time	Gas	(A) day 4 (B) day 3	(A) day 3 (B) day 4	(A) day 5 (B) day 7	(A) day 4 (B) day 5
			SCFA	(A) day 3 (B) day 3	(A) day 2 (B) day 3	(A) day 5 (B) day 7	(A) day 4 (B) day 4
		Final metabolic profiles	NA	↗ N ₂ (donor B)	↗ N ₂ (donor B) ↘ Total gas and total SCFA production	↗ N ₂ (donor B) ↗ Total gas production	
Score (/5)			5/5	4/5	2/5	3/5	
Technical requirements	Preparation steps		No	One step (i) Freezing	Two steps (i) Preparation including sterilization of the glycerol solution (ii) Freezing	Three steps (i) Preparation including sterilization of the maltodextrin/trehalose solution (ii) Freezing (iii) Lyophilization	
	Time to implement the method		Immediate	Immediate	Up to 2 hours	Up to 2 hours	
	Duration of storage		Few hours	Months (Dorsaz et al. 2020)	Months (Costello et al. 2015)	Years (Reygner et al., 2020)	
	Costs		€	€€	€€€	€€€€	
	Storage facility		No storage	★	★	★★★	
	Ease of sending		★★	★★	★★★	★★★★	
	Score (/5)			4/5	3/5	3/5	4/5
TOTAL RANKING			1	2	3	2	

mucosal phase. Maintaining anaerobiosis without flushing with N₂ or CO₂ is certainly a key parameter enabling the persistence of Archaea in vitro (Gaci et al. 2017). Nevertheless, applying preservative methods seemed to limit the expansion of some dominant populations normally present in the mucosal phase, leaving the opportunity for *Proteobacteria* (e.g., *Enterobacteriaceae*) and *Actinobacteria* (mainly with GFS) to colonize this ecological niche. In particular, *Enterobacteriaceae* (opportunistic pathobionts and enteric pathogens) are well-known efficient colonizers of unoccupied ecological niches after transient changes in microbiota composition (Chassaing et al. 2014; Kamada et al. 2013).

To represent all the facets of the human colonic ecosystem, stabilization time was evaluated on both microbial activity (gas and SCFA measurement) and composition (qPCR and 16S rRNA sequencing). In control condition (FS), a stabilization period of 2–3 days was necessary for metabolic activities compared

with 6–8 days for microbiome structure, in accordance to previous studies in in vitro gut models (Zihler Berner et al. 2013; Fehlbaum et al. 2015). Therefore, when considering all these parameters, a stabilization time of 8 days (corresponding to eight residence times) was observed in the M-ARCOL, in accordance to Tottey et al. (2015). Differences in stabilization time and/or in final stabilized levels for each parameter analyzed compared with FS control were observed, indicating that freezing stool without any preservative (RFS condition) was the most efficient method, followed by lyophilization and then freezing with glycerol. These results are in accordance with the studies from Tang and colleagues demonstrating that frozen fecal microbiota is as effective as fresh fecal sample for fecal microbiota transplantation (Tang et al. 2017) and from Choo's study showing the efficiency of freezing at – 80°C without cryoprotectant (Choo et al. 2015). Similarities between fresh and raw frozen stools can be explained by the absence of stool mixing and destructuration that may help

microbiota to withstand temperature decrease. Compared with raw fecal freezing, freezing with 10% glycerol or lyophilization increased *in vitro* stabilization time in the bioreactor with notable changes in SCFA and gas production. This could be explained by the important microbial metabolism of cryoprotectants at the beginning of fermentations that may negatively affect repeatability between experiments. An increased level of acetate was thus observed until day 4 for glycerol-treated stool experiment of donor B, reflecting glycerol metabolism by gut microbiota-acetate producers (De Weirdt et al. 2010). These results contrast with those from Aguirre and colleagues that did not observe any modification of SCFA production in the TIM-2 after 1-week freezing at -80°C with 1.5 g glycerol (Aguirre et al. 2015). This difference may be explained by different settings between the two studies, since in TIM-2 study, storage time was longer while the total duration of the fermentation experiment was shortened, and mucin beads were absent. A sharp increase in gas and SCFA production was also evidenced in both donors with lyophilized samples, which may reflect the consumption by gut microbiota of maltodextrin, an intermediate product of starch hydrolysis, and trehalose, a diholoside (Collins et al. 2018). In general, adding glycerol as a preservative agent led to a reduced metabolic activity compared with all the other conditions, suggesting cellular damages during the preservative step. Interestingly, 16S metabarcoding demonstrated that lyophilization seemed to preserve beneficial microbes such as *Akkermansiaceae* (only identified in donor A) for a longer period than for the other treatments, as previously described (Burz et al. 2019; Reygnier et al. 2020).

Our results showed that raw freezing of stools is the most efficient short-term preservative technique. Other parameters, such as technical constraints, need to be considered to determine the most appropriate method to inoculate *in vitro* gut bioreactors (Table 2). Shipping frozen stools is challenging since the samples require specific preserving containers (usually shipping proceeds with dry ice). Samples can face temperature fluctuations during storage (freezing failure due to power outage) and transport (exposure to high temperatures that increase the risk of thawing-freezing cycles) that may influence further microbial colonization and activity in *in vitro* gut models. Lyophilized stool samples encompass the above concerns and represent a good alternative since they are easier to deliver (no requirement of specific freezing containers), they can be stored at room temperature and require less storage capacity. However, this technique requires time and expensive specific equipment (lyophilizer price is about 8 to 55 k\$).

To conclude, the present study gives useful insight into the effects of short-term stool preservative methods, namely freezing and lyophilization, on further gut microbiota structure and fermentation activity in an *in vitro* model of the human colon. All the preservative treatments allowed the maintaining inside M-ARCOL of a complex and functional microbiota.

Considering both stabilization time of microbial profiles/metabolic activities, we showed in the current study that 48 h freezing at -80°C without cryoprotectant was the most efficient method. This result has to be balanced by technical constraints associated with the use of frozen fecal materials.

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Authors' contributions EML and BDS conceived and designed research. FE, LF, DC, and UO performed the experiments, analyzed, and interpreted data. VC provided technical assistance during fermentations and SCFA analysis. CC, KN, and TM helped to design lyophilization protocol. CMS and AM provided help for bioinformatics analysis. EML, BDS, FE, and DC wrote the manuscript with input from co-authors. All authors critically revised and approved the manuscript.

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Data availability Data transparency.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval No ethical approval was obtained because this study did not involve a prospective evaluation, did not involve laboratory animals, and only involved non-invasive procedures (fecal sample collection).

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Chapter II - Microplastics: what happens in the human digestive tract? First evidences in adults using *in vitro* gut models

As presented in Section 1 part 3, the last half-century has sustained a bloom in plastic production leading to massive environmental pollution. Accumulated plastics can be degraded into smaller pieces known as MPs found in the air, drinking water but also in the food chain. MPs have been detected in human feces and colon biopsies attesting of their transit along the gut after oral exposure. In addition, previous *in vivo* studies conducted in murine models have highlighted gut microbiota modulation following MP exposure. While the human gastro-intestinal tract is one of the first exposed surfaces, the fate and potential effects of MPs in the human digestive sphere are, up to now, largely unknown. The aim of this study was then to investigate the interactions of virgin PE MPs with human gut microbiota under adult colonic conditions simulated by the M-ARCOL model. This *in vitro* gut model was inoculated with fecal samples collected from four different adult donors and processed freshly as it appeared the best conservative method reported in Chapter I. Bi-directional interactions between the gut microbiota and PE MPs were evaluated for both luminal and mucosal compartments. Finally, luminal M-ARCOL supernatants before and after PE MP exposure were applied on co-culture of intestinal epithelial and mucus-secreting goblet cells (Caco-2 and HT29-MTX, respectively) to investigate the potential indirect impact of gut microbe metabolites after PE MP exposure on cell-related inflammatory response, mucin synthesis and barrier. function.

Microplastics: what happens in the human digestive tract? First evidences in adults using *in vitro* gut models

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Microplastics: What happens in the human digestive tract? First evidences in adults using in vitro gut models

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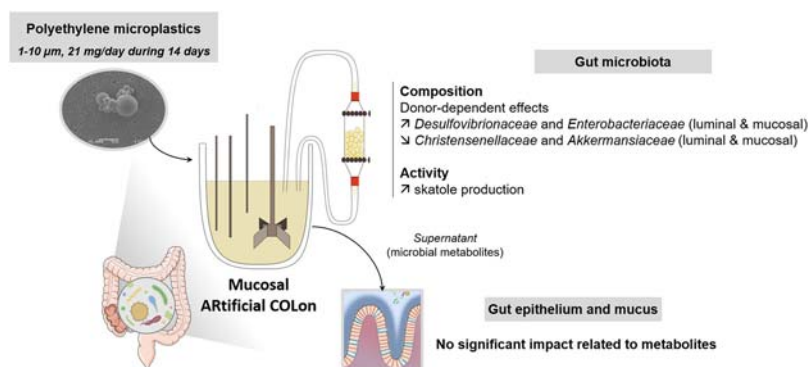
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HIGHLIGHTS

- Repeated exposure to PE MPs induced adult donor-dependent effects in M-ARCOL.
- The composition of the human gut microbiota was affected by repeated exposure to PE MPs.
- Common or distinct effects were observed between luminal and mucosal gut microbiota.
- Production of indole, 3-methyl-skatole increased after repeated exposure to PE MPs.

GRAPHICAL ABSTRACT



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ABSTRACT

Microplastics (MPs) are ubiquitous in the environment and humans are inevitably exposed to them. However, the effects of MPs in the human digestive environment are largely unknown. The aim of our study was to investigate the impact of repeated exposure to polyethylene (PE) MPs on the human gut microbiota and intestinal barrier

Abbreviation: AhR, aryl hydrocarbon receptor; ASV, amplicon sequence variant; DSC, differential scanning calorimetry; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GC-MS, gas chromatography-mass spectrometry; HRP, horseradish peroxidase; IL-8, interleukin-8; LDPE, low density polyethylene; LY, lucifer yellow; MP, microplastic; MUC2, mucin 2; MUC5AC, mucin 5AC; M-ARCOL, mucosal artificial colon; OCLN, occludin; Papp, apparent permeability coefficient; PE, polyethylene; qPCR, quantitative polymerase chain reaction; SCFA, short chain fatty acid; SPME, solid-phase microextraction; TEER, trans-epithelial electrical resistance; TGA, thermogravimetric analysis; VOC, volatile organic compound; ZO-1, zonula occludens-1.

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using, under adult conditions, the Mucosal Artificial Colon (M-ARCOL) model, coupled with a co-culture of intestinal epithelial and mucus-secreting cells. The composition of the luminal and mucosal gut microbiota was determined by 16S metabarcoding and microbial activities were characterized by gas, short chain fatty acid, volatilomic and AhR activity analyses. Gut barrier integrity was assessed via intestinal permeability, inflammation and mucin synthesis. First, exposure to PE MPs induced donor-dependent effects. Second, an increase in abundances of potentially harmful pathobionts, *Desulfovibrionaceae* and *Enterobacteriaceae*, and a decrease in beneficial bacteria such as *Christensenellaceae* and *Akkermansiaceae* were observed. These bacterial shifts were associated with changes in volatile organic compounds profiles, notably characterized by increased indole 3-methyl- production. Finally, no significant impact of PE MPs mediated by changes in gut microbial metabolites was reported on the intestinal barrier. Given these adverse effects of repeated ingestion of PE MPs on the human gut microbiota, studying at-risk populations like infants would be a valuable advance.

1. Introduction

While plastics were described as a revolutionary material in the 1950s, they have increasingly become a global environmental threat affecting all ecosystems (Wang et al., 2021; Su et al., 2022). In the environment, plastic debris undergo a progressive weathering, releasing smaller plastic particles (Zhang et al., 2021a). In their attempt to reach a consensus on the definition of microplastics (MPs) that incorporates all the most important characteristics, Frias and Nash (2019) defined them as any synthetic solid particles or polymeric matrices, with regular or irregular shape and with size ranging from 1 μm to 5 mm, of either primary (i.e. voluntarily manufactured) or secondary (i.e. released from plastic debris weathering and fragmentation) manufacturing origin, which are insoluble in water (Frias and Nash, 2019). Many types of plastic types are produced worldwide, but the most prevalent polymer is polyethylene (PE) (Anon, 2021).

The deleterious consequences on the environment and organisms resulting from plastic pollution are now well established (Bucci et al., 2019). Inevitably, MPs enter the food chain, with converging studies reporting their presence in drinking water and several foods (Fournier et al., 2020). This has raised concern about the potential health effects of MPs after ingestion (Lehel and Murphy, 2021; Provencher et al., 2020), since their presence has been assessed in human blood (Leslie et al., 2022), colonic tissues (Ibrahim et al., 2021) and stools (Zhang et al., 2021a, 2021a; Schwabl et al., 2019). However, assessing the health risk of MPs in humans remains a global challenge to date. In particular, little is known about the fate of these particles in the human digestive tract. During their transit, MPs encounter the gut barrier composed, in its luminal side, of intestinal epithelium and mucus and gut microbiota, which prevents the host from translocation of exogenous aggressors in the form of foreign particles and pathogens (Pelaseyed et al., 2014; Birchenough and Johansson, 2020). The mucus layer, a viscoelastic gel that lines and protects the intestinal epithelium, constitutes the first line of physical, chemical and biological defense of the host. Secreted by goblet cells, mucus is notably composed with complex glycoproteins, called mucins (MUC2 in the human intestine) (Etienne-Mesmin et al., 2019). The gut microbiota refers to this complex and diverse community of microbes from almost all kingdoms of life, composed of bacteria, viruses, fungi, archaea and protozoa harbored along the human gut and especially in the colon (Rooks and Garrett, 2016; Thursby and Juge, 2017). These microorganisms, coexisting and interacting in the gut, contribute to various essential functions for host physiology. For instance, they play a role in the regulation of host immunity but also in the participation in the metabolism of drugs, toxins and xenobiotics (Claus et al., 2016; Andoh, 2016; Lindell et al., 2022). In addition, they enable the breakdown of undigested food (e.g. dietary fibers), resulting in significant production of secondary metabolites, such as gas, short chain fatty acids (SCFAs) (Kalantar-Zadeh et al., 2019; Morrison and Preston, 2016), volatile organic compounds (VOCs) (Raman et al., 2013) and aryl hydrocarbon receptor (AhR) ligands mainly derived from tryptophan metabolisms (Lamas et al., 2018). In particular, SCFAs and AhR ligands are involved in the preservation of the integrity of intercellular tight junctions in the intestinal epithelium, thereby establishing

the link between the gut microbial activity and the maintenance of effective intestinal barrier function. Thus, deciphering the potential interactions between MPs and the gut microbiota, as well as the intestinal mucus and epithelium, is a critical issue that has been little explored to date. Studies in rodents have shown a variety of effects of MPs, depending on particle size, polymer type, shape, surface charge, as well as duration of exposure, mode of administration and dose tested. These effects included altered mucus synthesis and secretion and dysbiosis of the microbiota, which could exacerbate diseases (Jin et al., 2019; Lu et al., 2018; Li et al., 2020; Djouina et al., 2022; Sun et al., 2021), accompanied by local inflammation, oxidative stress and metabolic disruption. Murine model investigations are still widely used in toxicological studies because they have physiological relevance to a certain extent. However, due to differences in rodent/human physiology and gut microbiota (Hugenholtz and de Vos, 2018) but also due to the incentive for in vitro approaches by the application of European 3R (Replacement, Refinement and Reduction) principle rules (Prescott and Lidster, 2017), dynamic in vitro colonic models mimicking the human physiological conditions, including gut microbiota, represent powerful alternative tools, which have been little explored for MPs (Fournier et al., 2021a). The main studies in the in vitro literature to date have focused on cell culture experiments simulating the epithelium/mucus barrier. As for in vivo studies, various impacts on cell cytotoxicity, permeability and inflammation have been described, depending on polymer type, particle size, concentration and surface properties (Gautam et al., 2022; Liu et al., 2020a; Stock et al., 2022; Zhang et al., 2021b). In this work, we focused on polyethylene (PE), one of the major manufactured polymers (Anon, 2021), and widely found in flexible and rigid packaging for food, but also cosmetics, medical or industrial applications. It is also the most abundant type of plastic polluting marine environments (Erni-Cassola et al., 2019). We hypothesized that exposure to PE MPs could affect the human adult digestive environment in vitro under realistic conditions (i.e. repeated exposure to a human-relevant dose with potential inter-individual variability). To address these questions, we investigated the impact of a 2-week daily exposure to PE MPs on the human gut microbiota composition and metabolic activity (production of gas, SCFAs and VOCs, AhR activation) in four healthy adult volunteers using the in vitro Mucosal Artificial Colon model (M-ARCOL). This model is a one-stage fermentation system, which simulates the mean physico-chemical and microbial parameters of the human colon, including not only the luminal but also mucosal microbiota through an external mucin-alginate beads compartment (Deschamps et al., 2020). Co-cultures of human Caco-2 and mucus-secreting HT29-MTX intestinal cells were then incubated with M-ARCOL luminal supernatants collected after the 2-week exposure period in order to decipher the potential effects on intestinal mucus and epithelium (cytotoxicity, permeability, inflammation).

2. Materials and methods

2.1. Characterization of MPs

PE microspheres (CPMS-0.96 1–10 μm - 0.2 g; non-fluorescent

particles) were purchased from Cospheric (USA). The initial powder was suspended (2.625 mg/mL) in a sterile deionized water solution with 0.01 % (w/v) Tween 80. Particle size was determined using the smile-view software from scanning electron microscopy images captured with the JSM 6060LV microscope (Jeol, Japan). To determine the polymeric composition, the powder was analyzed using differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA). The DSC

measurements were performed on a Mettler DSC3⁺ device (N.V. Mettler-Toledo S.A., Belgium). The equipment is calibrated with Indium. A few mg of material were placed into an aluminum pan and then subjected to a temperature cycle ranging from 25 °C to 200 °C under a nitrogen gas flow. A rate of 10 °C/min was applied to heat and cool the material during the experiment. TGA measurements were performed on a Mettler TGA2 device (N.V. Mettler-Toledo S.A., Belgium). The analysis was

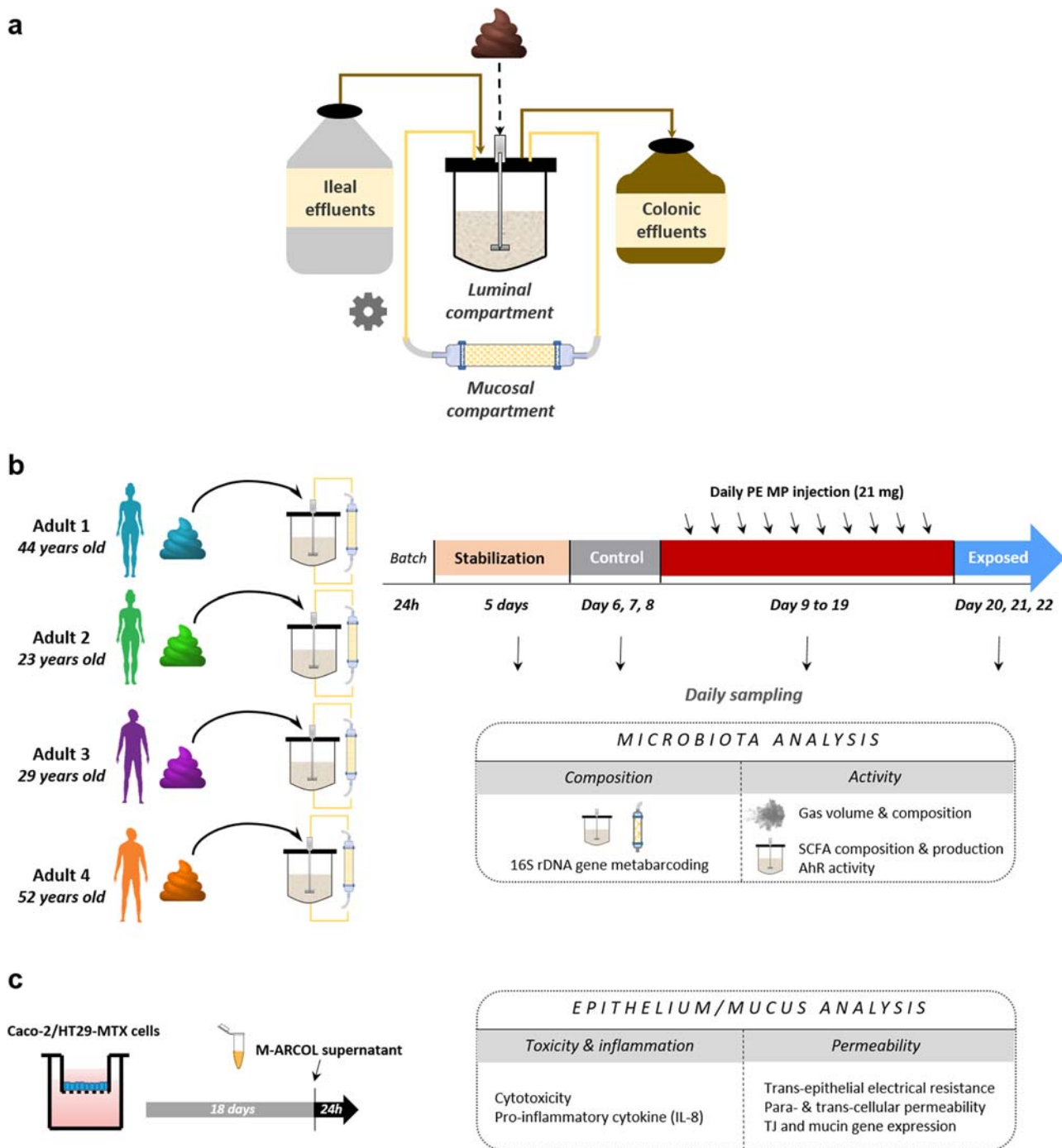


Fig. 1. Outline of in vitro fermentation in M-ARCOL and Caco-2/HT29-MTX cell culture experiments. a) Schematic representation of the M-ARCOL colonic model. b) M-ARCOL experimental design and analysis. Four bioreactors inoculated with fecal samples from four different adults were run in parallel. After an 8-day stabilization phase, PE MPs were added daily into the bioreactor tank at a concentration of 21 mg for 14 days. The last three days of the stabilization period were chosen as control days ('control', day 6, 7, 8) while the last three days of the exposure period were selected as exposure days ('exposed', day 20, 21, 22). Gas and luminal fermentation medium were sampled daily while mucin-alginate beads were collected every other day for further analysis of gut microbiota structure and activity. c) Cell culture experimental design and analysis. Caco-2 and HT29-MTX intestinal cells in co-culture (90/10) were seeded using inserts. After an 18-day cell growth period, cells were exposed during 24 h to day 8 or day 22 luminal supernatants of M-ARCOL and cytotoxicity, inflammation, intestinal permeability and mucin synthesis were evaluated. AhR: aryl hydrocarbon receptor; IL-8: interleukin-8; MP: microplastic; PE: polyethylene; SCFA: short chain fatty acid; TJ: tight junction.

performed at a heating rate of 10 °C/min under a nitrogen gas flow up to 800 °C.

2.2. Fecal sample collection and treatment

Donors were selected according to their sex (two males, two females), their age (age range between 23 and 52 years old) and their dietary habits (flexitarian and western like diet). All selected donors had no history of antibiotic treatment, drug or probiotic consumption for 3 months prior to sample collection.

Immediately after defecation, the fecal samples were transferred to a sterile container placed in an airtight anaerobic box (GENbag anaer gas pack systems, Biomerieux, France) and transported at ambient temperature. They were then processed at the laboratory within 6 h after defecation. In an anaerobic chamber (COY laboratories, USA), 11 g of each fresh stool sample were suspended in 110 mL of 30 mM sterile sodium phosphate buffer and filtered (500 µm stainless steel sieve).

2.3. Description and set-up of the M-ARCOL model

The Mucosal Artificial Colon (M-ARCOL) is a one-stage fermentation system (MiniBio, Applikon, The Netherlands), operated under continuous conditions, which simulates the physico-chemical and microbial conditions (both luminal and mucosal environments) encountered in the adult colon (Deschamps et al., 2020; Thévenot et al., 2015). The operating parameters (temperature, pH, retention time, volume, stirring speed) as well as the composition of the nutritive medium are given in Table S1. The M-ARCOL model is composed of a main bioreactor (luminal compartment) connected to an external glass compartment (mucosal compartment) containing mucin-alginate beads (Fig. 1a). These beads were prepared with mucin from porcine stomach type II (Sigma-Aldrich, USA) and sodium alginate (Sigma-Aldrich, USA), as previously described (Deschamps et al., 2020). The beads were stored at 4 °C before introduction into the hermetically sealed glass compartment (total beads area 556 cm²) connected to the bioreactor. M-ARCOL was set-up, based on in vivo data, to reproduce the average conditions found in the colon of a healthy human adult and the nutritive medium was adapted to mimic the composition of ileal effluents, based on previous studies (Deschamps et al., 2020; Thévenot et al., 2015) (Fig. 1a). The temperature was maintained at 37 °C, the average retention time was set at 24 h and the pH was maintained at a constant value of 6.3 by addition of 2 M NaOH. After an initial N₂ flush upon introduction of the fecal inoculum, anaerobiosis was maintained inside the bioreactor by the activity of the resident microbiota alone, to mimic as closely as possible the in vivo situation. Mucin-alginate beads were renewed every 2 days while maintaining the external compartment under anaerobiosis through a constant flow of CO₂.

2.4. Experimental design and sampling in M-ARCOL

The experimental design and sampling are shown in Fig. 1b. Four M-ARCOL bioreactors were inoculated with fecal samples from four adult donors and operated in parallel. Fermentations were performed under continuous conditions for 22 days, after a 24-h batch microbial amplification. An 8-day microbiota stabilization phase was applied and the three last days of this period (i.e. days 6, 7 and 8) were chosen as 'control days'. Subsequently, to mimic human chronic exposure, 14 days of daily exposure to PE MPs (21 mg of MPs in 8 mL of a deionized water solution containing 0.01 % (w/v) Tween 80) were performed and the three last days (i.e. days 20, 21 and 22) were selected as 'exposed days'. Samples from the main bioreactors, termed luminal microbiota, were collected daily for microbiome characterization (storage at -80 °C) and SCFA analysis (storage at -20 °C). Additional samples from the mucin bead compartment, termed mucosal microbiota, were collected every 2 days (when the mucin-alginate beads were replaced) for microbiota characterization. The beads were washed twice in sterile phosphate buffer

saline -PBS- (pH 7.1) and stored at -80 °C before downstream analyses. The medium surrounding the mucin-alginate beads was also collected at the same time. Additional luminal samples were collected at day 8 (control) and day 22 (exposure) for cell culture experiments, scanning electron microscopy, and volatilomic analysis. Samples were also collected daily from the atmospheric phase of the bioreactors to verify anaerobic conditions and determine gas composition. The daily extra volume of gas produced by microbial fermentation was also measured using a syringe connected to the gas bag.

2.5. Extraction of DNA

Genomic DNA was extracted from luminal and mucosal samples using the QIAamp Fast DNA Stool Mini Kit (12830-50, Qiagen, Germany) following the manufacturer's instructions with the following adjustments. Prior to DNA extraction, luminal samples were centrifuged (2000 g, 10 min, 4 °C) and the pellet was mechanically disrupted using a bead beater (5 min, 20 beat/sec) with 300 mg of sterile glass beads (diameter ranging from 0.1 to 0.6 mm), incubated (70 °C, 5 min) and centrifuged (12,000 g, 1 min, 4 °C). Mucosal samples were subjected to the following modifications prior to DNA extraction: 10 min of incubation with citrate buffer (37 °C), as previously described (Capone et al., 2013), before vortexing (maximal speed, 3 min) and centrifugation (8000 g, 1 min). DNA integrity was verified by agarose gel electrophoresis and by Nanodrop 2000 analysis (Thermo Fisher Scientific, USA). The DNA quantity was assessed using the Qubit dsDNA Broad Range Assay Kit (Q32851, Invitrogen, USA) with a Qubit 2.0 Fluorometer (Invitrogen, USA). Samples were stored at -20 °C before microbiota analysis (qPCR and 16S metabarcoding).

2.6. Total bacteria quantification by qPCR

Total bacteria were quantified by qPCR using primers BAC338R and BAC516F with hybridization temperature set at 58 °C (Yu et al., 2005) (Table S2). Real-time PCR assays were performed on a Biorad CFX96TM Real-Time System (Bio-Rad Laboratories, USA) using the Takyon™ Low Rox SYBR® 2X MasterMix blue dTTP kit (B0701, Eurogentec, Belgium). Each reaction was run in duplicate in a final volume of 10 µL with 5 µL of Master Mix, 0.45 µL of each primer (10 µM), 1 µL of DNA sample (10 ng/µL) and 3.1 µL of ultrapure water. Amplifications were performed as follows: 1 cycle at 95 °C for 5 min, followed by 40 cycles at 95 °C for 30 s and 60 °C for 1 min. A melting step was added to ensure primer specificity. The standard curve was generated from 10-fold dilutions of bacterial DNA (extracted from a fermentation sample), allowing the calculation of DNA concentrations as described in (Deschamps et al., 2020).

2.7. 16S metabarcoding and data analysis

The bacterial V3-V4 region of the 16S ribosomal DNA (rDNA) was amplified with primers V3_F357_N and V4_R805 for the bacterial fraction and Arch349F and Arch806R for the methanogenic Archaea. Amplicons were generated using a Fluidigm Access Array followed by high-throughput sequencing on an Illumina MiSeq system performed at the Carver Biotechnology Center of the University of Illinois (Urbana, USA).

Bioinformatics analysis was performed by GeT-Biopuces platform (INSA/Toulouse Biotechnology Institute, Toulouse, France) with R software version 4.1.1 (2021-08-10) and rANOMALY package (Theil and Rifa, 2021). The demultiplexed raw sequence data were filtered by quality and the denoising process was performed using DADA2 version 1.20.0 (Callahan et al., 2016). Reads with N bases or low phred quality score (under 2) were eliminated and reads under 100 pb length were removed. Decontamination steps were performed to filter out sequences corresponding to PhiX DNA used as a spike-in control for MiSeq runs and chimeric sequences were filtered out. Taxonomic affiliation of all

amplicon sequence variants (ASVs) was performed with *idtaxa* function from DECIPHER package version 2.20.0 (Murali et al., 2018) using SILVA release 138 (Quast et al., 2013) and GTDB bac120_arc122 (Parks et al., 2022) databases (60 % bootstrap cut-off). To improve the completeness of taxonomic affiliation, alignments were carried out using BLAST (Altschul et al., 1990) (98 % identity and coverage) on representative sequences of unassigned ASVs or assigned with incomplete taxonomy. A phylogenetic tree was constructed based on the representative ASVs sequences using the functions of the *phangorn* package version 2.7.1 (Schliep et al., 2017). α - and β -diversity indexes were calculated using the *diversity_alpha_fun* function from *rANOMALY* package (Theil and Rifa, 2021). The diversity of the microbiota across the samples was described using a Bray-Curtis dissimilarity-based redundancy analysis (RDA) using *vegan* R-package version 2.5–7 (Oksanen et al., 2011). The impact of the different parameters studied (i. e. total gas and SCFA production, gas and SCFA composition, type of sample (luminal or mucosal), time and sex of donors) on the dissimilarities between the groups was evaluated using PERMANOVA permutation tests (999 permutations).

2.8. Gas analysis

The analysis of O₂, N₂, CO₂, CH₄ and H₂ produced during the fermentation process in the atmospheric phase of the main bioreactors was performed using a HP 6890 gas chromatograph (Agilent Technologies, USA) coupled with a micro-TCD detector (Agilent Technologies, USA) (Deschamps et al., 2020). Two series columns, Molecular Sieve 5A and Porapack Q (Agilent Technologies, USA), were used. Gas composition was determined using calibration curves made from ambient air (78.09 % N₂, 20.95 % O₂, 0.04 % CO₂) and 3 gas mixtures A (5 % CO₂, 5 % H₂, 90 % N₂), B (19.98 % CO₂, 80.02 % H₂) and C (19.89 % CO₂, 19.88 % CH₄, 20 % H₂, 40.23 % N₂).

2.9. Analysis of SCFAs

2 mL of each luminal sample was centrifuged (5000 g, 15 min, 4 °C) and 900 μ L of supernatant was diluted at 1/10 in 0.04 M H₂SO₄ mobile phase, vortexed and filtered (pore size 0.22 μ m). The three major SCFAs (acetate, propionate and butyrate) were quantified by high-performance liquid chromatography (HPLC) (Elite LaChrom, Merck HITACHI, USA) coupled to a DAD diode (Deschamps et al., 2020). The HPLC column (150 \times 7.8 mm) contained a negatively charged sulfonic-grafted polystyrene divinylbenzene stationary phase and carried an eluent containing acidified water. Data were obtained and analyzed by the EZChrom Elite software at 204 and 205 nm. SCFA concentrations were calculated from calibration curves established from solutions of known increasing concentration of acetate, propionate and butyrate (0, 10, 25 and 40 mM).

2.10. Measurement of AhR activity

The aryl hydrocarbon receptor (AhR) activity of luminal M-ARCOL luminal supernatants (see sample preparation in Section 2.14) after exposure to PE MPs (day 22) compared to control conditions (day 8) was measured using a luciferase reporter assay method, as previously described (Lamas et al., 2016). Briefly, mouse hepatocellular carcinoma H1L1.1c2 cells, containing a stably integrated dioxin response element-driven firefly luciferase reporter plasmid pGudLuc1.1, were seeded into 96-well plates at 10⁵ cells/well in minimum essential medium Eagle alpha modification (MEM- α) medium (with 10 % (v/v) heat-inactivated fetal calf serum -FCS-, 1 % (v/v) penicillin/streptomycin and 1 % (v/v) Geneticin G418) and cultured (37 °C, 5 % CO₂) 24 h before stimulation with M-ARCOL luminal supernatants. After incubation, the wells were washed with 100 μ L PBS (pH 7.0), and 50 μ L of Promega lysis buffer (pH 7.8) was added to each well. The plates were shaken for 1 h for cell lysis. After addition of 100 μ L of

luciferase reagent (Promega, France), luciferase activity was measured using a luminometer (Tecan, Switzerland). Experiments were performed in triplicate. All values were normalized, based on the cytotoxicity of the samples using the lactate dehydrogenase activity assay (Promega, USA).

2.11. Volatolomic analysis

Volatile organic compounds (VOCs) from control (day 8) and exposed (day 22) luminal samples were analyzed by solid-phase microextraction (SPME) coupled with gas chromatography-mass spectrometry (GC-MS), as previously described (Defois et al., 2017, 2018). Briefly, the day before analysis, 2.2 mL of a saturated NaCl solution (360 g/L) was added to each sample (0.8 mL). The mixture vials were closed under a flow of N₂, vortexed for homogenization, and thawed for 24 h at 4 °C. The following steps were then performed with an automated sampler (AOC-5000 Shimadzu, Japan): (i) preheating of the sample to 40 °C for 10 min in the shaker (500 rpm), (ii) SPME trapping (75 μ m carboxen/polydimethylsiloxane, 23-gauge needle, Supelco, USA) of the VOCs for 30 min at 40 °C, and (iii) thermal desorption at 250 °C for 2 min in splitless mode in the GC inlet. Further VOC analysis was performed by GC/MS-full scan (GC2010, QP2010 +, Shimadzu, Japan). VOCs were injected in a DB-5MS capillary column (60 m \times 0.32 mm \times 1 μ m, Agilent, USA) according to the GC-MS settings used by Defois and collaborators (Defois et al., 2017, 2018). Provisional identification of VOCs was performed on the basis of mass spectra, by comparison with mass spectral libraries (Wiley Registry 12th Edition / NIST 2020), and retention indices (RI), by comparison with published RI values and with those of our in-house database. The peak area of the tentatively identified compounds was determined for each of the targeted molecules using a mass fragment selected for its specificity and freedom from co-elution.

2.12. Scanning electron microscopy (SEM) analysis

Luminal and mucosal samples of adult 3, selected as having the most diverse bacterial community in the initial stool inoculum (see Section 3.2), were deposited on SEMPore filters (Jeol, Japan) and fixed for 12 h at 4 °C in 0.2 M sodium cacodylate buffer at pH 7.4 containing 4 % (w/v) paraformaldehyde and 2.5 % (v/v) glutaraldehyde. Filters were washed 10 min in sodium cacodylate buffer (0.2 M, pH 7.4) and post-fixed 1 h with 1 % (w/v) osmium tetroxide in the same buffer. The filters were then washed 20 min in distilled water. Dehydration by graded ethanol was performed from 25° to 100° (10 min each) and completed in hexamethyldisilazane (HMDS) for 10 min. Samples were mounted on stubs using adhesive carbon tabs and sputter-coated with gold-palladium (JFC-1300, JEOL, Japan). Analysis was carried out using a scanning electron microscope JSM-6060LV (Jeol, Japan) at 5 kV in high-vacuum mode.

2.13. Raman spectroscopy mapping

Raman spectroscopy mapping was performed using a Raman spectrometer (Alpha300R Apyron, Witec, Germany) equipped with a confocal microscope. Exposed luminal samples (day 22, adult 3) were vacuum filtered using Anodisc filter (pore size 0.2 μ m, diameter 25 mm). The filters were excited with a Coherent sapphire laser (532 nm, laser power 10 mW) and the Raman spectra were recorded in backscattering configuration using a 50X objective (Zeiss, Germany) with a numerical aperture of 0.55 to achieve a theoretical spatial resolution of 590 nm in the focus plane. Single Raman spectra were recorded 10 times with an integration time of 1 s. For each filter, maps between 900 and 2500 spectra were recorded with a step of 1.33 μ m and 2 μ m, respectively. For each mapping, the recorded signal (Raman signal plus background) was integrated over a 20 cm⁻¹ spectral range centered at 1294 cm⁻¹ corresponding to the CH₂ twisting vibration band of PE. The signal value was then automatically transformed by the software in a

color scale (from black to bright yellow).

2.14. Intestinal cell lines and cell culture conditions

The human colon adenocarcinoma Caco-2 cell line was obtained from the European Collection of Cell Cultures (ECACC, UK) and used at passage 58–62. The mucus-secreting colon adenocarcinoma HT29-MTX cell line was kindly provided by Dr. Thécla Lesuffleur (INSERM Lille, France) and used at passage 10–16. Both cell lines were maintained with culture medium (Dulbecco/Vogt modified Eagle's minimal essential medium -DMEM- without phenol red and 4.5 g/L glucose), supplemented with 1 % (v/v) penicillin/streptomycin, 1 % (v/v) non-essential amino acids (NEAA), 1 % (v/v) Glutamax and 10 % (v/v) heat-inactivated fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5 % CO₂/95 % air. The medium was changed every 2–3 days and cells were split upon 80 % of confluence using Tryple express. Intestinal cells (90 % Caco-2/10 % HT29-MTX) (Gillois et al., 2021) were seeded in 24-well inserts of 1- μ m pore size (Millipore, Merck Millipore SAS, France) and maintained in the culture medium. Starting on day 17, the FBS in the culture medium was replaced with 1 % (v/v) Insulin Transferrin Selenium (ITS) to limit interference with the samples to be tested. After 18 days, Caco-2/HT29-MTX co-cultures were apically incubated for 24 h with luminal samples of M-ARCOL, collected on day 8 (control) and day 22 (exposed), centrifuged (5000 g, 15 min, 4 °C) and diluted 20-fold (Fig. 1c). Experiments were performed with 3 biological replicates, 5 technical replicates for the day 8 (control) and 6 technical replicates for the day 22 (exposed).

2.15. Cell viability

To determine the potential cytotoxic effect of M-ARCOL supernatant samples on Caco-2/HT29-MTX cells and to establish sub-toxic conditions for testing on inserts, different dilutions (1/2, 1/5, 1/10, 1/20, 1/30 and 1/50 in cell culture medium) were evaluated. Cell viability was tested in triplicate with CytoTox 96® non-radioactive cytotoxicity assay (Promega, France) after 24 h of exposure. In 96-well plates, 25 μ L of apical supernatants of the cell culture was diluted in 25 μ L of ITS, 1 % culture medium and mixed with 50 μ L of substrate. The plates were then incubated for 15 min at room temperature in the dark. The reaction was stopped with 50 μ L of stop solution (1 M CH₃COOH) and absorbance at 490 nm was read with a Spark spectrophotometer (Tecan, Switzerland).

2.16. Intestinal permeability

Starting on day 12, the transepithelial electrical resistance (TEER) was continuously monitored in real time using the CellZScope2 device (NanoAnalytics, Germany). In addition, after the 24-h contact time with the 20-fold diluted supernatants of M-ARCOL, the inserts were transferred into a new 24-well plate containing 1 mL of Hank's balanced salt solution -HBSS- (pH 7.7). Lucifer yellow -LY- (0.4 mg/mL, molecular weight 457 g/mol) and horseradish peroxidase -HRP- (0.4 mg/mL, molecular weight 44,000 g/mol) were dissolved in HBSS and added to the apical compartment. After 2 h at 37 °C under 5 % CO₂/95 % air atmosphere, samples were taken from the apical and basolateral compartments. For LY measurement, samples were analyzed in duplicate using a Spark multimode plate reader (Tecan, Switzerland). Excitation and emission wavelengths were 405 nm and 535 nm, respectively. Total HRP was determined by an ELISA assay. Briefly, 96-well flat-bottomed black plates (Greiner, Dutcher, France) were coated overnight at 4 °C with 50 μ L of 10 μ g/mL mouse polyclonal to HRP (Abcam, France) in PBS. Plates were blocked with PBS-1 % bovine serum albumin (BSA) prior to incubation with apical and basolateral samples collected from inserts. Rabbit polyclonal anti-HRP biotin (Abcam, France) was added at a concentration of 10 μ g/mL before the addition of fluorescein-5-isothiocyanate (FITC) conjugated streptavidin (Becton Dickinson, France) for 20 min and the fluorescence intensity measured at 485 nm/

525 nm using a Spark microplate reader (Tecan, Switzerland). The apparent permeability coefficient (Papp in cm/s) was determined by the following equation: $Papp = Cb / (S \times C0)$, where Cb represents the level of LY or HRP accumulated in the basolateral compartment after 2 h, S represents the membrane area (0.33 cm²) and C0 is the initial concentration of the marker (0.4 mg/mL -LY- or - HRP) in the apical compartment.

2.17. Interleukin-8 (IL-8) level measurement

The apical and basolateral media from the co-cultures were collected after the contact time of 24-h with 20-fold diluted supernatants of M-ARCOL and stored at -20 °C until analysis. Interleukin-8 (IL-8) levels were measured using the Quantikine Human IL-8 ELISA kit (DY208-05, R&D Systems, USA). Briefly, in Maxisorp 96-well plates (Nunc, France), biotinylated Goat polyclonal anti IL-8 was added at a concentration of 10 ng/mL before adding HRP-conjugated streptavidin (Becton Dickinson, France) for 20 min. Absorbance intensity was measured at 450 nm and 540 nm using a Spark microplate reader (Tecan, Switzerland).

2.18. Intestinal gene expression

Total RNAs were extracted from cells for each insert using the All-Prep RNA Mini kit (Qiagen, France) according to the manufacturer's instructions. Quantity and quality of extracted RNA samples were monitored using Nanodrop (Nanophotometer Implen, Dutscher, France) and Bioanalyzer (Agilent Technologies, USA), respectively. RNAs were reverse transcribed using enzyme iScript reverse transcription supermix (Biorad, France). Sample concentration was adjusted to 50 ng/ μ L. 384-well plates were filled by an Agilent Bravo Automated Liquid Handling Platform (Agilent Technologies, France). All wells contained 5 μ L of the following mix: 2.5 μ L of IQ SYBR green Supermix (Biorad, France), 1.5 μ L of each primer set and 1 μ L of cDNA sample. Amplification was performed using a ViiA7 Real-Time PCR System (Applied Biosystems, ThermoFisher Scientific, France). Thermal cycling conditions were as follows: 3 min denaturation at 95 °C followed by 40 cycles at 95 °C for 15 s and 45 s at 60 °C, and a melting curve step. The primer sequences of the targeted genes (genes encoding tight junction (TJ) proteins (zonula occludens-1 -ZO-1-, occludin -OCLN-) and genes involved in mucin synthesis (mucin 2 -MUC2-, mucin 5AC -MUC5AC-) are listed in Table S2. Raw data that passed quality control were analyzed with LinRegPCR (version 2021.2) and then normalized against the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a reference housekeeping gene.

2.19. Statistical analysis

Statistical analyses on gut microbiota activity (gas, SCFAs and AhR activity), α -diversity indexes (number of observed ASVs and Shannon index) from metabarcoding data, as well as results from cell culture experiments obtained in control (day 8) and exposed (day 22) groups were processed using GraphPad Prism software version 9.3.1 for Windows (GraphPad Software, USA). Data normal distribution was verified by combining Anderson-Darling, D'Agostino & Pearson, Shapiro-Wilk and Kolmogorov-Smirnov tests and homoscedasticity was checked using the Fisher test. Then, appropriate statistical analysis was applied (either one-way ANOVA, Kruskal-Wallis or Welch's tests) and significance was considered for $p < 0.05$. For gut microbiota results, differential analyses (DESeq2, metagenomeSeq, metacoder) were performed on ExploreMetabar software using rANOMALY package (Theil and Rifa, 2021; Rifa and Theil, 2021). Principal coordinate analysis (PCoA) of unweighted UniFrac distances was performed and significance between groups was assessed with a permutational multivariate analysis of variance (PERMANOVA) using ADONIS (999 permutations) with non-parametric tests. Volatolomics data were processed using the

Statistica Software (v.13) (StatSoft, France). Student's t-tests ($p < 0.05$) were performed for the VOC abundances determined in control (day 8) and exposed (day 22) groups and principal component analyses (PCA) were performed on the discriminant VOCs selected to visualize the structure of the data.

3. Results

3.1. Characterization of PE MPs

It was observed that MPs were spherical in shape with 71 % of the total number included in the size range defined by the supplier (1–10 μm). Of these, 50 % were in the size range between 1 and 2 μm (Fig. S1a). The DSC scans obtained from the heat of the initial MP powder showed an endothermic peak characteristic of melting of a PE material (Fig. S1b). The temperature at the maximum of the peak was measured to be about 112 °C, which is more relevant for a low-density PE (LDPE). Upon cooling of the material from its melting state, the crystallization peak appeared with a defined onset temperature of about 103 °C. No other effect could be observed in the temperature range studied. The thermal decomposition of the material was then analyzed by TGA (Fig. S1c). Only one weight loss was observed with a maximum of the degradation rate measured at approximately 467 °C. The decomposition was complete, 100 % of the material was lost during decomposition. Overall, these results confirmed that MPs were composed of pure PE polymer.

3.2. Characterization of initial human stool microbiota

A total of 199, 176, 205 and 140 ASVs were observed for adults 1, 2, 3 and 4, respectively, associated with the following Shannon indexes: 4.01, 3.97, 4.04 and 3.66 (data not shown). In addition, qPCR analysis revealed 3.88×10^9 , 9.39×10^8 , 4.38×10^9 and 6.02×10^9 16S rDNA gene copies/g of sample for adults 1, 2, 3 and 4, respectively (Fig. S2a). Regarding archaea, only *Methanobrevibacter* were detected with sequences number equal to 599, 1254, 3006 and 0 for adults 1, 2, 3 and 4, respectively. Only in adult 3, 4 sequences of *Methanomassiliicoccus* were also observed (Fig. S2b). At the phylum level, relative abundance profiles were donor-dependent but harbored mainly *Bacteroidota*, *Firmicutes*, *Verrucomicrobia* and *Proteobacteria* (Fig. S3a). The most significant differences between donors were observed in family abundance (Fig. S3b). While *Bacteroidaceae*, *Lachnospiraceae*, *Ruminococcaceae*, *Veillonellaceae*, *Akkermansiaceae* and *Rikenellaceae* were predominant, some family populations were specific to each donor. For instance, while adult 1 exhibited *Acidaminococcaceae*, adult 2 harbored *Prevotellaceae*, *Sutterellaceae* and *Clostridia UCG-017* family; adult 3, which had the most diverse inoculum, had *Christensenellaceae*, *Acutalibacteraceae*, *Prevotellaceae* and *Clostridia UCG-014* family. Finally, adult 4, which had the least diverse inoculum, was unremarkable despite the presence of *Clostridia UCG-014* family.

3.3. Bidirectional relationships between PE MPs and human gut microbiota during repeated exposure in M-ARCOL

3.3.1. Impact of exposure to PE MPs on the human gut microbiota composition

As shown in Fig. S2a, the total bacteria level was constant throughout the total duration of fermentation for both the lumen ($1.04 \times 10^{10} \pm 1.09 \times 10^9$, $3.40 \times 10^9 \pm 8.73 \times 10^8$, $7.07 \times 10^9 \pm 7.99 \times 10^8$, $9.96 \times 10^9 \pm 1.01 \times 10^9$ 16S rDNA gene copies/g of sample for adults 1, 2, 3 and 4, respectively) and mucus-associated ($4.78 \times 10^8 \pm 2.08 \times 10^8$, $3.83 \times 10^8 \pm 1.19 \times 10^8$, $8.61 \times 10^8 \pm 2.80 \times 10^8$, $8.35 \times 10^8 \pm 3.05 \times 10^8$ 16S rDNA gene copies/g of sample for adults 1, 2, 3 and 4, respectively) microbiota. The number of *Methanobrevibacter* sequences fluctuated during the fermentation and did not stabilize during the control period (Fig. S2b). However, archaeal

populations were maintained over the 22-day period. From day 14, *Methanobrevibacter* sequences were detected in adult 4 in the luminal and mucosal compartments while none were reported in the inoculum.

Regarding the gut bacterial communities, the same major phyla of the initial inoculum were encountered but with different relative abundances, in a donor-dependent manner (Fig. S4a). This donor-dependent effect can be illustrated by the two-dimensional PCoA plot, based on the unweighted UniFrac distance matrix, over the days preceding exposure (stabilization phase including control days) (Fig. S4b). Clustering is highlighted according to the different donors for luminal and mucosal microbiota. For the control period (days 6, 7, 8), the profiles at the phylum level stabilized, with only minor variations in the observed relative abundance. With respect to the impact of exposure to PE MPs, no striking differences in exposed and non-exposed microbiota were notable at this taxonomic level.

At the family level (Fig. 2a), the donor effect remained noticeable and the relative abundance of families differed by donor. Interestingly, the impact of exposure to PE MPs was more striking at this taxonomic level. In particular, the results of the differential analysis at the family level, considering the mean of the data obtained for the four individuals (Fig. 2b), notably showed an increase in *Dethiosulfovibrionaceae* ($p < 0.005$), *Enterobacteriaceae* ($p < 0.0001$), *Desulfovibrionaceae* ($p < 0.005$) after exposure to PE MPs for luminal and mucosal microbiota. Of note, a significant increase in *Desulfovibrionaceae* due to exposure to PE MPs was observed for each of the four donors, whereas an increase in *Enterobacteriaceae* was found for three donors (adults 1, 3 and 4) in the luminal compartment (Fig. S5). In the mucosal compartment, a significant decrease in *Christensenellaceae* and *Akkermansiaceae* was observed for two donors (adults 3 and 4).

Regarding the diversity of the gut microbiota, exposure to PE MPs did not significantly impact the bacterial α -diversity indexes for luminal (Fig. 3a) and mucosal (Fig. 3b) microbiota. However, the number of observed ASVs tended to decrease after exposure in the microbiota of adults 1 and 2 while it slightly increased for adults 3 and 4. Representation of β -diversity by a two-dimensional PCoA plot, based on the unweighted UniFrac distance matrix between control and exposed days for luminal and mucosal microbiota, revealed a clear distinction for adults 3 and 4 (Fig. 3c).

3.3.2. Influence of the human gut microbiota on the surface properties of PE MPs

Conversely, we investigated the influence of the gut microbiota on the surface properties of PE MPs. To this end, luminal and mucosal samples from adult 3 before (day 8) and after exposure (day 22) were first analyzed by SEM (Fig. 4a). For the control conditions, as expected, a huge amount of microorganisms of various morphologies (bacilli, cocci and coccobacilli) were present in the luminal compartment but also some bacillus-like bacteria were observed on the surface of the mucin-alginate beads. For the exposed conditions, considering both luminal and mucosal samples, bacteria were in close contact with PE MPs. Furthermore, in order to decipher potential physico-chemical modifications of the PE MP surface due to these interactions, luminal samples were analyzed by Raman spectroscopy (Fig. 4b). The Raman spectrum of MPs in the luminal medium after exposure was identical to that obtained from the PE particles present in the initial suspension, showing that contact with the gut microbiota during fermentation does not induce major changes in the polymeric surface composition of MPs.

3.4. Impact of repeated exposure to PE MPs on the human gut microbiota activity in M-ARCOL

3.4.1. Gas and SCFA production

The mean total gas and SCFAs daily produced were first studied as indicators of fermentation activities (Fig. 5a, b). Although exposure to PE MPs had no significant impact on these two parameters, the average daily total gas production (Fig. 5a) tended to decrease in adult 2 and

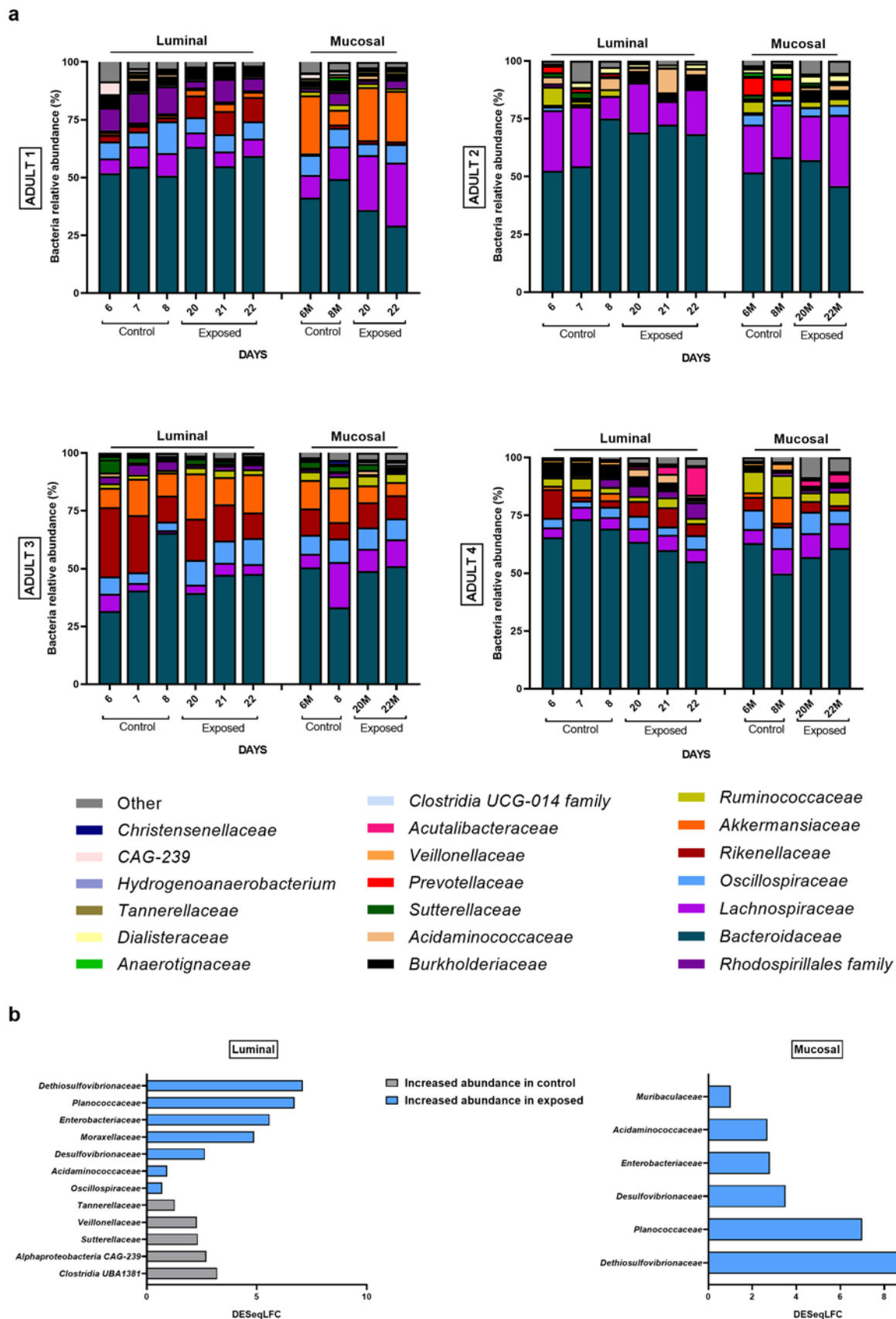


Fig. 2. Effect of repeated exposure to PE MPs in M-ARCOL on luminal and mucosal bacterial microbiota composition at the family level. Bacterial composition was determined by 16S rDNA gene metabarcoding. a) Relative abundance of the 20 main bacterial populations at the family level during the control and exposed periods for each donor. M represents the medium surrounding the mucin-alginate beads. b) Differential Analysis (DESeq2, metagenomeSeq, metacoder, $p < 0.05$) at the family level between control and exposed days ($n = 4$).

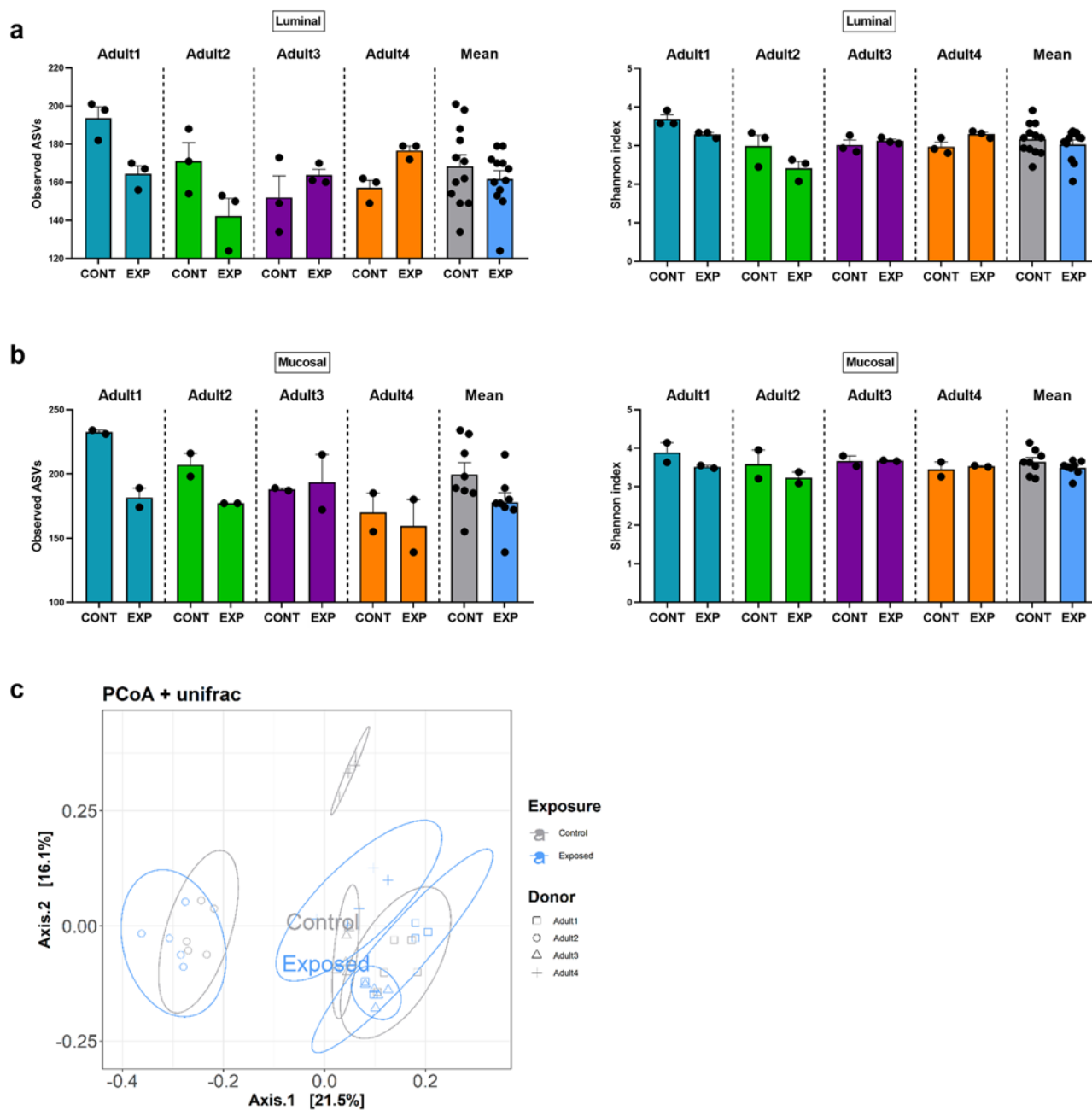


Fig. 3. Effect of repeated exposure to PE MPs in M-ARCOL on α - and β -diversity indexes of bacterial communities. Bacterial composition was determined by 16S rDNA gene metabarcoding. Bacterial α -diversity was calculated and expressed as the number of observed ASVs (a) and as the Shannon index (b) for each donor and as a mean ($n = 4$) (mean \pm SEM). Bacterial β -diversity was also represented (c) using a two-dimensional PCoA plot, based on the unweighted UniFrac distance matrix, on luminal and mucosal samples. ASVs: amplicon sequence variants; CONT: control, EXP: exposed.

increase in adult 4 after exposure while the average daily total SCFA production (Fig. 5b) tended to decrease for adult 4. Only adult 2 produced significantly less acetate after exposure to PE MPs (56.9 ± 0.2 vs 54.2 ± 0.1 mM, Brown-Forsythe ANOVA, $p = 0.04$) while acetate production only tended to decrease in adult 4 and to increase in adults 1 and 3. For propionate, adult 3 tended to produce less propionate daily after exposure to PE MPs, as did adult 4. For adult 2, propionate production tended to increase after exposure. Mean daily butyrate production was not significantly impacted by exposure to PE MPs although it was significantly decreased for adult 4 (26.5 ± 0.8 vs 17.1 ± 0.3 mM, one-way ANOVA, $p = 0.03$). The kinetics of total daily gas and SCFA production were also followed (Fig.S6a, b). The profiles of relative gas and SCFA composition (Fig.S6c) were stable for the control period with the exception of the gas profile of adult 2, beginning to produce H_2 at day 5

and up to 25 % for all the remaining days. Of note, adult 3 produced a peak of H_2 (14 %) on day 9, the day after the first injection of PE MPs. Interestingly, adult 4 began to produce CH_4 on day 13. In an attempt to integrate the different datasets, an RDA of the bacterial β -diversity was implemented, based on 16S rDNA gene metabarcoding and metabolic data from the luminal phase only and excluding the strong donor-effect potentially masking the influence of other factors (Fig. 5d). A clear distinction between control and exposed days was observed and several variables explained the difference between the four gut microbiota: total gas and SCFA production, propionate and acetate production. However, the main factor explaining those differences was the exposure to PE MPs.

3.4.2. Volatolome

Another robust indicator, although little studied to date in the

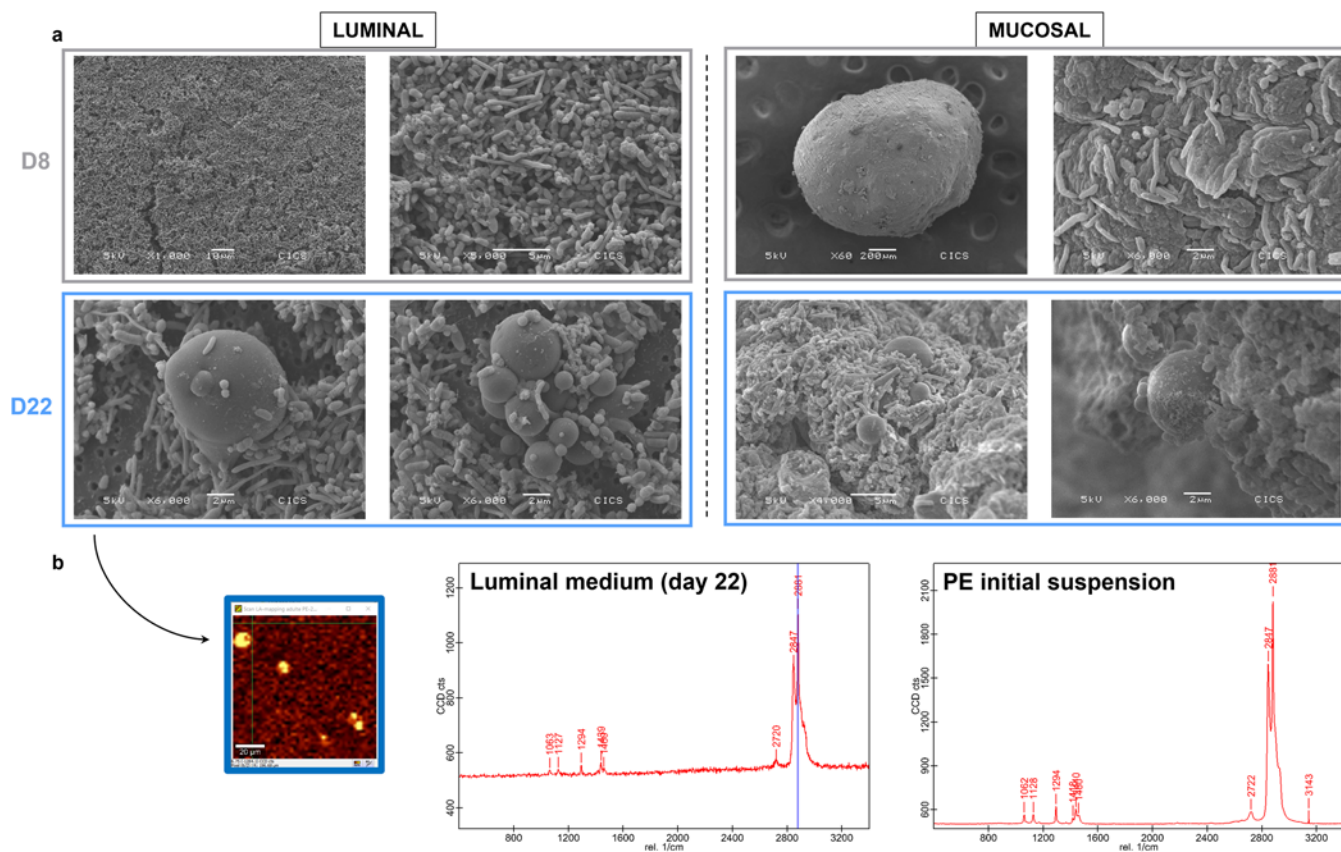


Fig. 4. Scanning electron microscopy observations of PE MPs/gut microbiota interactions in M-ARCOL and Raman spectroscopy analysis of PE MPs at the end of exposed period. (a) Representative scanning electron microscopy images of luminal samples and mucin-alginate beads for adult 3 before (upper panels) and after (lower panels) exposure to MPs. The analysis was performed on mucin-alginate beads after 48 h of colonization in the mucosal external compartment. The right panel at day 8 shows a typical mucin-alginate bead and its colonization by gut microbiota. (b) Representative Raman spectroscopy for adult 3 of PE MPs (map on the left, PE MPs are coloured in yellow; Raman spectra on the right) in the luminal sample at day 22 compared to the initial suspension of PE MPs. PE: polyethylene.

literature, of gut microbiota activity is its volatolome. VOCs were identified and quantified for day 8 as 'control' and day 22 as 'exposed' from the luminal samples of the four donors. While a total of 183 VOCs were detected, five had significantly differential abundance between control and exposed samples ($n = 4$, $p < 0.05$) (Table 1). The PCA using the five discriminant VOCs clearly showed the presence of two clusters, corresponding to the control and exposed groups (Fig. 6). Thus, exposure to PE MPs induced specific changes in the volatolomic profile of the gut microbiota. The abundance of these five discriminant VOCs, including three hydrocarbons (pentane, 3-methyl-, cyclopentane, 1,1,3-trimethyl-, 1-heptene, 2,4-dimethyl-), one alcohol (3-nonyl-2-ol) and N-heterocycle compound (indole, 3-methyl-), is shown in Table 1. While the abundance of the three hydrocarbon and alcohol compounds was significantly reduced after exposure to PE MPs, the abundance of indole, 3-methyl- was significantly increased (35-fold increase).

3.4.3. Activation of AhR

Finally, we investigated the ability of M-ARCOL luminal supernatants to activate AhR after exposure to PE MPs (day 22) compared with control conditions (day 8) using a reporter cell line. AhR activity was not significantly different after exposure from that obtained with controls (Fig. 7). However, AhR activity tended to be lower for all adults, except for adult 2 for which it was significantly higher (one-way ANOVA, $p < 0.001$), suggesting a potential defect in the microbiota to produce metabolites known to activate this receptor involved in regulation of intestinal homeostasis.

3.5. Impact of M-ARCOL luminal supernatants on intestinal barrier integrity using a Caco-2/HT29-MTX cell model

We then investigated the potential impact of M-ARCOL luminal supernatants after exposure to PE MPs (day 22) compared with control conditions (day 8) on the intestinal barrier integrity using co-cultures of Caco-2 and mucus-secreting HT29-MTX cells grown in inserts. The toxicity assay with 1/2, 1/5, 1/10, 1/20 and 1/50 (v/v) dilutions of M-ARCOL luminal supernatants showed no cytotoxicity for 1/20 and 1/50 dilutions (data not shown). Therefore, in the following experiments on Caco-2/HT29-MTX cells, 1/20 (v/v) diluted supernatants were used.

The intestinal barrier function was first assessed by TEER and LY transport measurements. A decrease in TEER values, associated with an increase in LY translocation, is indicative of an increase in intestinal permeability and thus an impairment of intestinal barrier function (Geirnaert et al., 2017). Here, TEER was monitored in situ and in real time with the cellScope2 system. The difference between the mean TEER values before and after the 24-h incubation period was calculated for all conditions and termed Δ TEER. This parameter was not significantly differentially impacted by the supernatants of the exposed microbiota compared with the control, although it tended to be lower for adults 2 and 3 and higher for adult 4 (Fig. 8a). Consistently, the LY P_{app} was not statistically different between the supernatants from exposed and control gut microbiota, but tended to be higher for adults 1 and 2 and lower for adult 3 (Fig. 8a). Transcellular permeability was also assessed by measuring HRP apparent permeability measurement. Similarly, no significant difference was observed between supernatants from exposed and control gut microbiota, although, as with LY P_{app} , HRP P_{app} tended to be higher for adults 1 and 2 and lower for adults 3 and 4

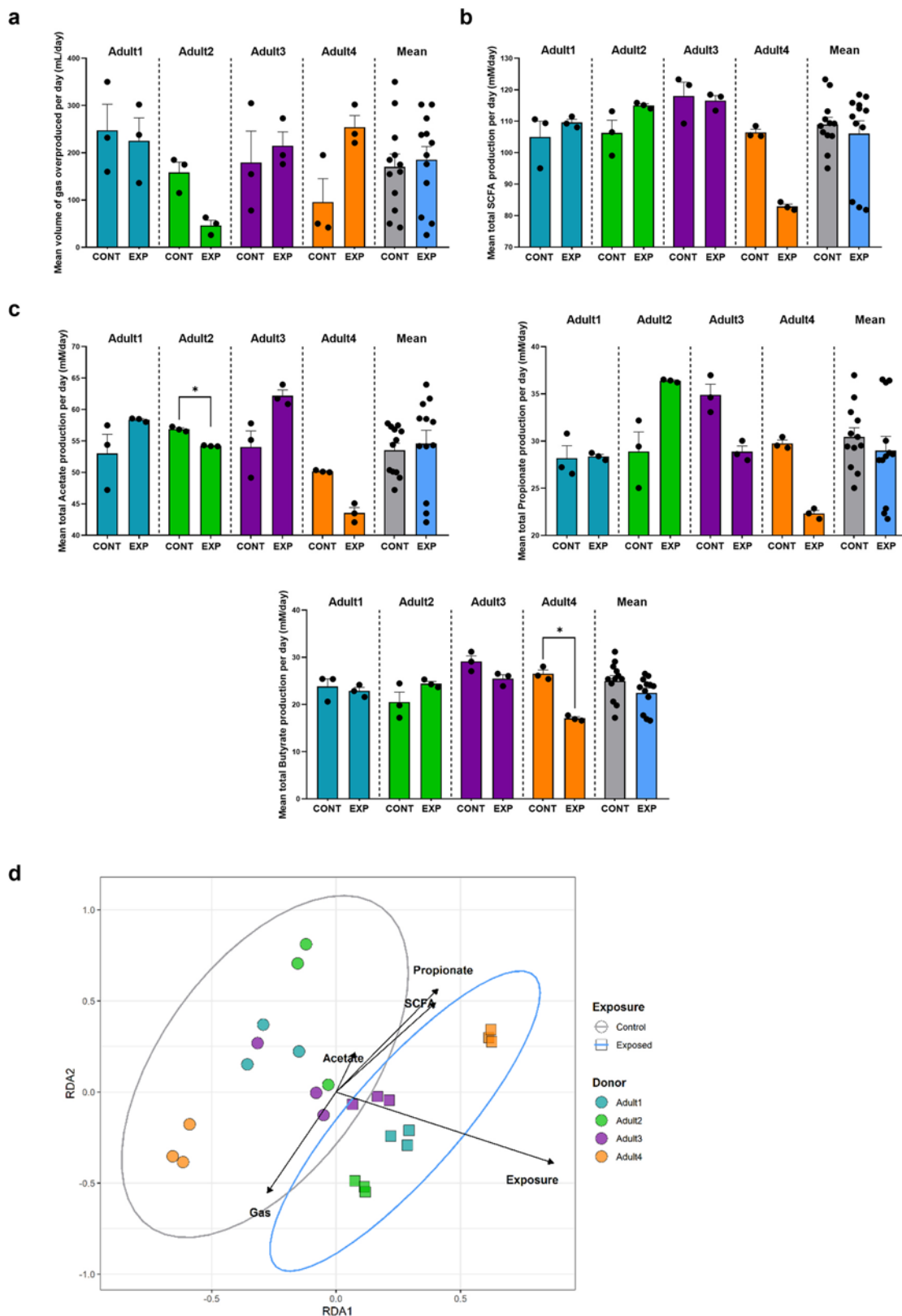


Fig. 5. Impact of repeated exposure to PE MPs on gas and short chain fatty acid production in M-ARCOL. (a) Daily gas production (mean \pm SEM in mL) and (b) Total SCFA production (mean \pm SEM in mM) for each donor and on average (n = 4). (c) Daily production of acetate, propionate and butyrate (mean \pm SEM in mM) for each donor and on average (n = 4). Statistical differences were assessed by one-way ANOVA with $p < 0.05$ for significance. (d) Distance-based redundancy analysis (RDA) based on 16S rDNA gene metabarcoding and metabolic data (gas and SCFAs) applied to luminal samples and excluding the donor effect. *CONT*: control; *EXP*: exposed; *SCFAs*: short chain fatty acids.

Table 1
Volatile organic compounds in the volatolome of the fecal microbiota as significantly altered by the repeated exposure to PE MPs.

Candidate markers	Tentatively identification ^a	m/z ^b	Exp. RI ^c	Ref. RI ^d	CAS number	p value	Control (day 8)	Exposed (day 22)	Under/over production compared to the control
<i>Hydrocarbons</i>									
pentane, 3-methyl-	MS+RI	71	561	578	96-14-0	***	1.6 (18 %)	0.32 (48 %)	↘
cyclopentane, 1,1,3-trimethyl-	MS	97	728		4516-69-2	***	1.5 (13 %)	0.38 (40 %)	↘
1-heptene, 2,4-dimethyl-	MS+RI	70	841	855	19549-87-2	*	16 (19 %)	7 (53 %)	↘
<i>Alcohols</i>									
3-nonyl-2-ol	MS	43	1110	NA	26547-25-1	*	11 (36 %)	4.2 (46 %)	↘
<i>N-heterocycle compounds</i>									
indole, 3-methyl-	MS+RI	58	1197	1192	693-54-9	*	12 (149 %)	418 (73 %)	↗

Values are the mean of abundances ($\times 10^5$) of each candidate marker with its standard deviation (in brackets).
^aMS + RI, mass spectrum and RI agree with literature data; MS, mass spectrum agrees with literature spectrum; ^bMass fragment used for area determination; ^{c,d}Retention indices (RI) on a DB5 capillary column from experimental run (c) or bibliographic data (d); NA: Non-available; ↘: under production compared to the control; ↗: over production compared to the control; *p < 0.05; **p < 0.01; ***p < 0.001 Bonferroni corrected value.

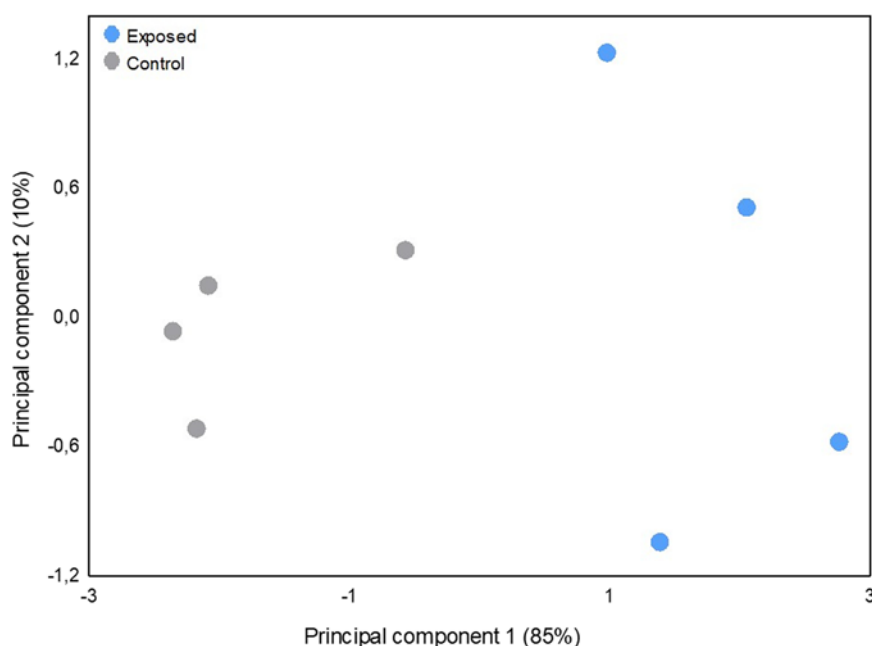


Fig. 6. Impact of repeated exposure to PE MPs in M-ARCOL on volatile organic compounds produced by the gut microbiota in the luminal compartment. Volatile organic compounds (VOCs) were analysed for each adult donor (n = 4) at day 8 and day 22. Principal component analysis (PCA) of the control and exposed days including the five discriminant VOCs described in Table 1.

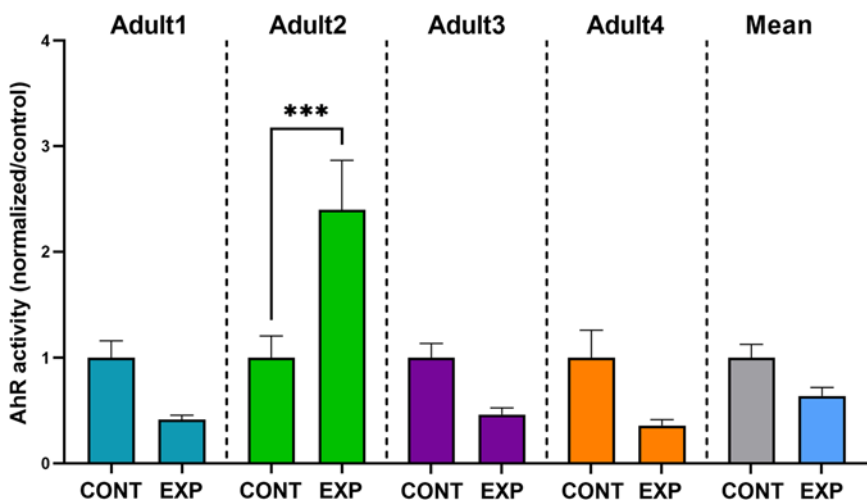


Fig. 7. Ability of luminal M-ARCOL luminal supernatants to activate AhR after exposure to PE MPs. AhR activity is expressed as fold changes relative to the luciferase activity of the control and after normalisation based on sample cytotoxicity using the lactate dehydrogenase activity assay. All results are given for each individual donor and the mean is calculated (mean \pm SEM, n = 4). Statistical differences were assessed by one-way ANOVA with p < 0.05 for significance. AhR: aryl hydrocarbon receptor; CONT: control; EXP: exposed.

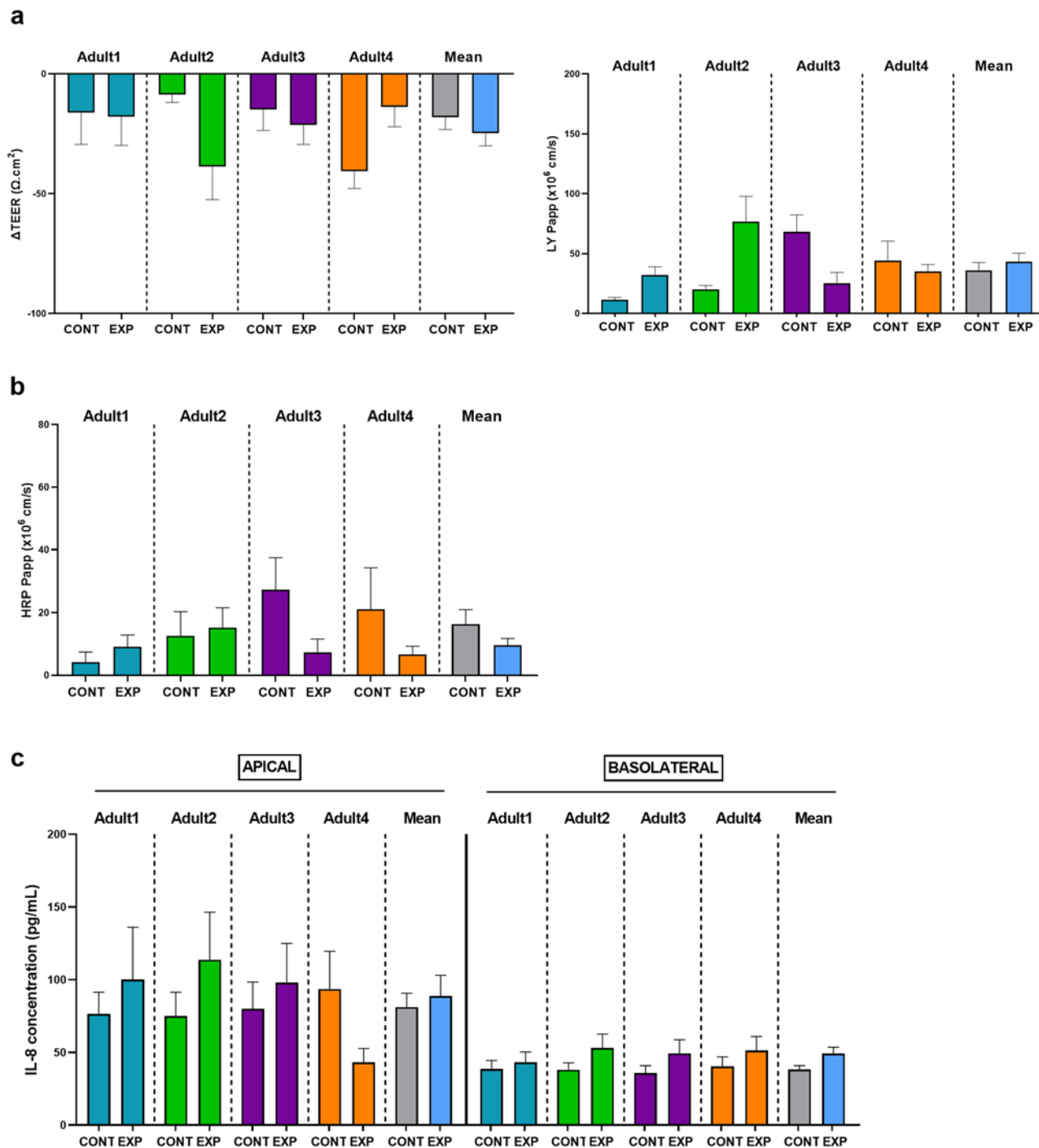


Fig. 8. Impact of luminal supernatants of M-ARCOL before and after repeated exposure to PE MPs on intestinal barrier integrity using a Caco-2/HT29-MTX co-culture. (a) Δ TEER (difference in $\Omega\cdot\text{cm}^2$ between mean TEER after and before the 24-h incubation period) and LY transport (Papp in cm/s) as an indicator of intestinal paracellular permeability. (b) HRP transport (Papp in cm/s) as an indicator of intestinal transcellular permeability. (c) Mean IL-8 concentration (in pg/mL) in the apical and basolateral media of the co-culture. All results are given by donors and on average ($n = 4$) and expressed as means \pm SEM. CONT: control; EXP: exposed; HRP: horseradish peroxidase; IL-8: interleukin-8; LY: lucifer yellow; Papp: apparent permeability coefficient; TEER: trans-epithelial electrical resistance.

(Fig. 8b). Fully consistent with these permeability measurements, gene expression of *ZO-1* and *OCN* was not statistically different between the supernatants from exposed and control gut microbiota (Fig.S7a). Of note, unlike *OCN*, *ZO-1* gene expression tended to decrease in adults 1, 2 and 3 for the exposed gut microbiota supernatant compared with the control. Regarding mucin synthesis, no significant differences in *MUC2* and *MUC5AC* gene expression were observed (Fig.S7b). Interestingly, *MUC5AC* gene expression tended to increase in adults 1 and 3 for the exposed gut microbiota supernatant compared with the control.

Finally, no significant difference in apical production of the pro-inflammatory cytokine IL-8 was reported between the supernatants of exposed and control gut microbiota, but it tended to be higher for all adults except adult 4 (Fig. 8c). In the basolateral compartment, IL-8 production, generally lower than in the apical compartment, tended to be increased for the exposed gut microbiota supernatant compared with the control for all donors.

4. Discussion

Given the converging and critical evidence of human exposure to MPs, our study is the first to assess in adults the impact of PE MPs on the human digestive ecosystem *in vitro* under realistic conditions, i.e. repeated exposure for 14 days at a dose (21 mg/day) in agreement with exposure data reported so far in the literature for humans (from 14 to 714 mg per day) (Senathirajah et al., 2021). To this endeavor, we simulated the human adult digestive environment by coupling the M-ARCOL colonic model with a co-culture of human intestinal cells mimicking the mucus/epithelium axis.

The gut microbiota has been identified as a key player in the host toxicity of xenobiotics (Claus et al., 2016). While the gut microbiota has a high capacity to metabolize various environmental chemicals, xenobiotics also alter the composition and/or metabolic activity of gut microbial communities (Lindell et al., 2022). Our study focused on elucidating the interplay between the gut microbiota and PE MPs. It should be noted that the initial fecal microbiota used for M-ARCOL inoculation showed strong inter-individual variabilities, which were maintained *in vitro* throughout the experiment, as evidenced by adults 3 and 4. However, both were profoundly affected by exposure to PE MPs as clear changes in β -diversity were observed after treatment, accompanied by a trend of increasing α -diversity (observed ASVs) for the luminal microbiota. Despite the inter-individual variability, specific effects were associated with the lumen and mucus-associated microbiota. While an increase in well-known gut pathobionts *Dethiosulfovibrionaceae*, *Enterobacteriaceae*, *Desulfovibrionaceae* (Liu et al., 2020b; Zhang et al., 2021c; Pittayanon et al., 2019) was observed after exposure to PE MPs for both luminal and mucosal microbiota, a decrease in *Christensenellaceae* and *Akkermansiaceae*, two populations associated with a healthy state (Waters and Ley, 2019; Hasani et al., 2021), were observed for two donors in the mucosal compartment. Some of the variations in the structure of the gut microbiota obtained here are close to the microbial specificities described in irritable bowel syndrome and inflammatory diseases of the gastrointestinal tract (Pittayanon et al., 2019; Zhu et al., 2018). To date, only one study has investigated the impact of MPs on the human gut microbiota under physiologically relevant digestive conditions *in vitro*. Tamargo and her team used a complex *in vitro* colonic system, SIMGI®, which recreates the different regions of the lower gut (ascending, transverse and descending colon) (Tamargo et al., 2022). A single dose of polyethylene terephthalate (PET) MPs (0.166 g), previously digested by a standardized *in vitro* static digestion method, was administered for 72 h into the SIMGI® model inoculated in parallel with fecal samples from two donors. The authors observed compartment- and donor-dependent effects. Following exposure to PET MPs, a decrease in *Bacteroidota* (previously named *Bacteroidetes*) was observed in all three colonic compartments for both donors. An increase in *Desulfovibrionota* was also reported in all three compartments for one donor but not in the transverse colon for the other. The *Synergistetes* population was increased in the transverse and descending colon for one donor but only in the descending colon for the other. Surprisingly, while *Proteobacteria* were increased in the transverse colon of one donor, they were decreased in the ascending and transverse colon of the second donor. In our experiments, we also observed an increase in *Synergistetes* and *Proteobacteria* at lower taxonomic levels.

Despite major discrepancies between human and mouse digestive conditions and gut microbiota characteristics (Hugenholtz and de Vos, 2018), it is interesting to report effects on gut microbiota composition after exposure to PE MPs in murine models. An increase in the abundance and diversity of the fecal microbiota has been reported after exposure to PE MPs (10–150 μm) (Li et al., 2020), which was associated with a higher abundance of *Staphylococcus*, as well as a reduction in *Parabacteroides* abundance, compared to untreated animals. More recently, variations in the composition of the gut microbiota have been reported in mice orally exposed to PE MPs of two sizes (36 and 116 μm), characterized in particular by less abundant *Verrucomicrobiales* and

more abundant *Gastranaerophilales* compared to their unexposed counterparts (Djouina et al., 2022). While the affected populations reported in the above studies were different from those we observed, similar results were found *in vivo* with other types of MP polymer. Notably, exposure of mice to 2- μm polyvinylchloride (PVC) MPs (Chen et al., 2022) led to a higher level of *Proteobacteria* in the exposed group compared with the control, whereas exposure to polystyrene (PS) MPs (mixture of 5-, 50-, 100- and 200- μm particles) in mice fed a normal or high-fat diet resulted in an increase in the relative abundance of *Enterobacteriaceae* and *Desulfovibrionaceae* (Huang et al., 2022). In addition, another study with 5- μm PS MPs also showed a significant increase in *Proteobacteria* and *Enterobacteriaceae* as well as potentially pro-inflammatory bacteria and pathogens, such as *Desulfovibrio* and *Oscillospiraceae*, which was also observed in our study (Wen et al., 2022). These observations highlight the importance of identifying, in addition to the exposure scenario, the main properties of the MPs involved, e.g. type or size of plastic, as highlighted previously (Stock et al., 2022).

Our study is the first one investigating the potential effect of exposure to PE MPs on the production of gas, VOCs and SCFAs by the human gut microbiota. Interestingly, the effect of PE MPs on these parameters was not so striking, but the trend, as with gut microbiota composition, appeared to be donor-dependent. To date, studies examining microbial VOCs are scarce and only a few databases indexing these microbial VOCs are currently available (Agarwal et al., 2016; Abdullah et al., 2015; Lemfack et al., 2014). However, these compounds play important roles in biological interactions between organisms and are increasingly used as biomarkers for various human diseases, including cancer, gastrointestinal and metabolic disorders (Arasaradnam et al., 2014; Schenkel et al., 2015; Walton et al., 2013; Garner et al., 2007). Exposure to PE MPs significantly altered the microbial volatile profile, characterized by five major discriminant VOCs. While the abundance of three hydrocarbon and one alcohol compounds was decreased after exposure to PE MPs, the abundance of indole, 3-methyl- was drastically increased. This compound, also known as skatole, is a tryptophan-derived metabolite absorbed by the gut epithelium and synthesized by the gut microbiota in the small intestine and the colon from indole-3-acetic acid via a decarboxylation process (Yokoyama and Carlson, 1979). Because its production requires two steps mediated by at least two different bacterial species (*Bacteroides*, *Clostridia* or *Escherichia coli* and *Lactobacillus*, *Clostridium*, *Desulfovibrio* or *Bacteroides*) (Cook et al., 2007; Smith and Macfarlane, 1997), its level is generally low. Interestingly, the *Desulfovibrio* population, which is increased in our study, might be involved in skatole production (Shi et al., 2020) and might explain, at least in part, the increased skatole production. In healthy individuals, skatole was not detected during fermentation in the large intestine (Smith and Macfarlane, 1997), whereas high skatole concentrations were reported in the gut of patients suffering from saccharo-butyric putrefaction (Herter, 1908) and in the serum of patients with hepatic encephalopathy (Suyama and Hirayama, 1988). This drastic increase in indole, 3-methyl- or skatole abundance may indicate potential microbiota-mediated gastrointestinal dysregulation after exposure to PE MPs and thus warrants further investigations.

Finally, we evaluated the ability of the gut microbiota to activate AhR after exposure to PE MPs. Several environmental chemicals have been characterized as triggering an AhR-mediated pro-inflammatory response in different cell types (Henley et al., 2004; Tamaki et al., 2004; Pei et al., 2002). The gut microbiota has the ability to produce tryptophan-based AhR ligands (e.g. tryptamine, indole-3-acrylic acid, indole-3-acetic acid, indole-3-acetaldehyde or indole-3-aldehyde) influencing the intestinal epithelial integrity but also the development, function, production and maintenance of several key mucosal immune cells and mediators (Lamas et al., 2018; Agus et al., 2018; Roager and Licht, 2018; Rothhammer and Quintana, 2019). Of note, skatole was shown to have a moderate agonist effect on human AhR compared with other microbial intestinal tryptophan catabolites (Vyhřídlová et al., 2020). A deficit in AhR ligand production by the gut microbiota has been

reported in inflammatory bowel disease, celiac disease and obese patients compared to healthy subjects (Lamas et al., 2016, 2020; Natividad et al., 2018). In our study, AhR activity tended to be lower with the supernatant of PE-MP exposed gut microbiota compared to the respective control for all donors except adult 2. These results suggest a lower production of tryptophan-based AhR ligands by the gut microbiota after exposure to PE MPs, which could promote intestinal inflammation, as previously observed in celiac disease (Lamas et al., 2020).

We next investigated how the gut microbiota may interact reciprocally with PE MPs. SEM images revealed the presence of microorganisms attached to the surface of PE MPs. The formation of “biofilm-like” microbial communities on the surface of PET MPs was already reported by Tamargo and colleagues (Tamargo et al., 2022), but not on PE MPs. In the environment, depending on the MP surrounding medium, biofilms of various microorganisms can be deposited on the particle surface. These specific communities are called the ‘plastisphere’ (Zettler et al., 2013). *Alphaproteobacteria*, *Gammaproteobacteria* and *Bacteroidia* were identified as the core microbiome of the PE-associated biofilms in seawater (Tu et al., 2020) and are also phyla encountered in the gut microbiota. In addition, Tang and colleagues recently confirmed the adhesion of *E. coli* (*Enterobacteriaceae* family), a well-known member of the gut microbiota, on the surface of PE MPs (Tang et al., 2022). While Tamargo and co-workers reported a progressive amorphisation of the PET MP surface especially during colonic fermentation in SIMGI®, no major modification of the polymeric composition of PE MPs was demonstrated in our study by Raman spectroscopy, after contact with the gut microbiota. Interestingly, biodegradation of LDPE was reported in enrichment cultures with this polymer as the main carbon source for two environmental species from *Proteobacteria* phylum (*Microbulbifer* sp and *Alcanivorax borkumensis*, the latter being described as an excellent candidate for degradation of petroleum-based plastics such as LDPE), while the *Desulfobionaceae* family would include members of the LDPE plastisphere (Delacuvellerie et al., 2019). These results support the hypothesis of interactions between microorganisms and PE MPs in the gut. Thus, the potential degradation of MPs (PE but also other types of polymer) by the human gut microbiota would deserve further research, although the limited contact time in the human gut during gastrointestinal transit does not favor plastic degradation, compared to longer exposure to soil or water microorganisms in the environment (Akarsu et al., 2022; Tareen et al., 2022; Giangeri et al., 2022).

We next evaluated for the first time the impact of M-ARCOL luminal supernatants before and after exposure to PE MPs exposure on the co-culture of Caco-2 and mucus-secreting HT29-MTX human intestinal cells to simulate the gut microbiota/mucus/epithelium axis. No major effects were observed on intestinal para- and trans- permeabilities, mucin synthesis or IL-8 production, probably due to the few changes observed in the profiles of gut microbiota metabolites (e.g. SCFAs or AhR ligands) after exposure. Although no studies to date have evaluated the indirect effects of PE MPs, mediated by changes in microbial metabolites, on the mucus/epithelium barrier, the direct impact of exposure to PE MPs on these parameters has already been studied in vivo. In particular, in mice exposed to PE MPs of two sizes (36 and 116 μm), the authors reported an increase in mucosal and mucin areas and, in particular, an upregulation of *Muc2* transcripts in the colon, reflecting dysregulation of colonic mucosa differentiation (Djouina et al., 2022). Moreover, Sun and colleagues showed for smaller PE MPs (1–10 μm) a decrease in mucin production but not *Muc2* mRNA levels in mice (Sun et al., 2021). A pro-inflammatory state after exposure to PE MPs was also reported in mice with 10–150 μm particles (Li et al., 2020). *In vitro*, the impact on intestinal cells, in monoculture or co-culture, has been evaluated via direct exposure to MPs. For example, exposure of Caco-2 cells to two sizes of PE MPs (30.5 \pm 10.5 and 6.2 \pm 2.0 μm) did not significantly reduce cell viability except slightly at high concentration (1000 $\mu\text{g}/\text{mL}$) (Gautam et al., 2022) but induced oxidative stress, as illustrated by nitric oxide and reactive oxygen species markers. Interestingly, Hesler and colleagues studied the impact of PS small-sized

particles (500 nm) on a co-culture of Caco-2 cells with HT29-MTX-E12 cells, but found no significant cytotoxicity, unless applied at very high concentrations, nor barrier integrity impairment, thus highlighting a possible protective effect of the mucus layer (Hesler et al., 2019).

Despite the substantial progress regarding the impact of MPs on the human digestive environment, our study has some limitations. First, we only considered virgin PE microbeads even though the spherical shape is not fully representative of ingested forms (e.g. fibers or fragments (Fournier et al., 2021a)). Indeed, MPs are complex particles, characterized by many different parameters, including the type of polymer, size, shape, surface properties, presence of adsorbed contaminants (Yu et al., 2019) and/or the formation of a biomolecular corona, for example after human digestion (Stock et al., 2019). Furthermore, while virgin MPs can simulate a direct source of human exposure (e.g. from food packaging), the use of weathered particles is important to simulate environmental exposure as weathering leads to strong physico-chemical changes in MPs (Liu et al., 2019). This highlights the critical need to disentangle the effects of “real-life” MPs, which are likely to exhibit distinct physico-chemistry, behavior and toxicity towards the human digestive environment.

In addition, we only studied healthy adult volunteers here. However, assessing the toxicological consequences on at-risk populations such as infants, in fit-for-purpose *in vitro* gut models (Fournier et al., 2021b), would be highly relevant since it has been recently shown that MPs are more abundant in the stools of infants than in adults (Zhang et al., 2021a). Similarly, pathophysiological conditions (e.g. patients with inflammatory bowel disease (IBD)) could be simulated in the M-ARCOL gut model. Indeed, in these “sensitized” individuals with defective mucus/epithelial barriers and dysbiotic gut microbiota (Stange and Schroeder, 2019), the fecal concentration of MPs was significantly higher than that in their healthy counterparts (Yan et al., 2022). Interestingly, a positive correlation was found between the fecal concentration of MPs and the IBD severity (Yan et al., 2022), although the underlying mechanisms remain far from understood.

5. Conclusions

Our study investigated for the first time the impact of a 2-week repeated exposure to PE MPs at a relevant dose on the human digestive ecosystem in adults, by coupling the *in vitro* colonic model M-ARCOL with a co-culture of Caco-2/HT29-MTX intestinal cells to simulate the gut microbiota/mucus/epithelium axis. Taken together, our results showed effects of PE MPs on the luminal and mucosal gut microbiota, both in terms of composition and metabolic activity, to a different degree and in an individual-dependent manner. Such exposure may promote the development of human pathobionts (e.g. *Desulfobionaceae* and *Enterobacteriaceae*, as seen both for the luminal and mucosal compartments) at the expense of beneficial bacteria (e.g. *Christensenellaceae* and *Akkermansiaceae* in the mucosal microbiota). The production of gas and metabolites (i.e. SCFAs and tryptophan-based AhR ligands) by the human gut microbiota after exposure to PE MPs was not significantly affected although the microbial volatolome was altered with, in particular, an increased production of indole, 3-methyl- (or skatole). We also focused on the interactions of microorganisms from the gut microbiota with PE MPs without biodegradation at the fermentation time scale used in our experiments, probably due to the chemical stability of this type of polymer. Furthermore, no significant impact of exposure to PE MPs, as mediated by changes in gut microbial metabolites, was reported on the mucus/epithelium barrier in terms of permeability and inflammation. Further studies are now needed to characterize the specific “gut plastisphere” in terms of composition but also metabolism. Other developments can be envisaged for the M-ARCOL/cell culture combination, such as the use of more representative MPs (morphology, surface properties (weathering)), including a digestion step, which can influence physico-chemical properties of MPs (e.g. formation of biomolecular corona). The consequences of MP exposure

on at-risk populations like infants, as well as in pathophysiological conditions (e.g. inflammatory bowel disease patients), could also be determined for a better human health risk assessment.

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CRedit authorship contribution statement

Elora Fournier: Conceptualization; Data curation; Formal analysis; Methodology; Funding acquisition; Investigation; Supervision; Writing - original draft. **Mathilde Leveque:** Data curation; Formal analysis; Investigation; Methodology; Validation. **Philippe Ruiz:** Methodology; Validation. **Jeremy Ratel:** Formal analysis; Investigation; Validation; Writing - review & editing. **Claude Durif:** Investigation. **Sandrine Chalancon:** Investigation. **Frederic Amiard:** Formal analysis; Methodology; Validation; Writing - review & editing. **Mathieu Edely:** Formal analysis; Methodology. **Valerie Bezirard:** Methodology. **Eric Gaultier:** Methodology; Validation. **Bruno Lamas:** Methodology; Validation; Writing - review & editing. **Eric Houdeau:** Resources. **Fabienne Lagarde:** Methodology; Validation; Writing - review & editing. **Erwan Engel:** Formal analysis; Validation; Writing - review & editing. **Lucie Etienne-Mesmin:** Conceptualization; Funding acquisition; Project administration; Supervision; Validation; Writing - review & editing. **Stéphanie Blanquet-Diot:** Conceptualization; Funding acquisition; Project administration; Supervision; Validation; Writing - review & editing. **Muriel Mercier-Bonin:** Conceptualization; Funding acquisition; Project administration; Supervision; Validation; Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Financial interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be considered as a potential conflict of interest.

Data availability

Raw data are available at NCBI under the Sequence Read Archive database in the BioProject n°PRJNA831017.

Ethics approval

This study is a non-interventional study with no additions to usual clinical cares. The protocol does not require approval from an ethics committee according to the French Public Health Law (CSP Art L 1121-1.1).

Environmental implication

The last half-century has sustained a bloom in plastic production leading to environmental pollution, with formation of microplastics (MPs). Their omnipresence along the food chain has raised concern for human health. While the gastro-intestinal tract is the first exposed surface, the impact of MPs in the human digestive sphere is largely unknown. We investigated the effects of a repeated oral exposure to polyethylene MPs using an original *in vitro* approach. This work provides a better understanding of interactions between MPs, gut microbiota and mucus, considering inter-individualities, and increases our knowledge on the health hazards of exposure to environmental MP pollution.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jhazmat.2022.130010](https://doi.org/10.1016/j.jhazmat.2022.130010).

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Chapter III - A child is not an adult: development of a new *in vitro* model of the toddler colon

In previous Chapter II, we clearly demonstrated an effect, which was donor-dependent, of oral exposure to PE MPs on gut microbiota composition and functions in M-ARCOL simulating adult conditions. However, early life is a critical period where gut ecosystem and functions are being established with significant impact on health. In addition, even though MP exposure data in infants are largely unknown, we can assume possible health hazard in this at-risk population, due to the existence of numerous contamination sources (bottle- and breast-feeding, dust breathing and ingestion during roaming and baby teat suckling). We then decided to investigate the impact of such exposure on the immature infant gut microbiota. As a first step, the implementation of an *in vitro* gut model, adapted to toddler digestive physiology, was required. To this end, an exhaustive literature review was conducted to update M-ARCOL with specific physicochemical (pH, transit time and nutritional composition of ileal effluents) and microbial parameters from toddlers in the age range of 6 months to 3 years, resulting in the Toddler M-ARCOL (Tm-ARCOL) model. *In vitro* fermentations were performed with fecal samples collected from five toddlers to validate this newly-developed colonic model compared to *in vivo* data in toddlers. Results were also compared to those obtained with the classical adult M-ARCOL configuration. This new *in vitro* model was then used for our evaluation of PE MP impact on toddler gut microbiota (see Chapter IV) but will also provide a powerful platform for further gut microbiome mechanistic studies in a pediatric context.

A child is not an adult: development of a new *in vitro* model of the toddler colon

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A child is not an adult: development of a new in vitro model of the toddler colon

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Abstract

Early life is a critical period where gut ecosystem and functions are being established with significant impact on health. For regulatory, technical, and cost reasons, in vitro gut models can be used as a relevant alternative to in vivo assays. An exhaustive literature review was conducted to adapt the Mucosal Artificial Colon (M-ARCOL) to specific physicochemical (pH, transit time, and nutritional composition of ileal effluents) and microbial parameters from toddlers in the age range of 6 months–3 years, resulting in the Tm-ARCOL. In vitro fermentations were performed to validate this newly developed colonic model compared to in vivo toddler data. Results were also compared to those obtained with the classical adult configuration. Fecal samples from 5 toddlers and 4 adults were used to inoculate bioreactors, and continuous fermentations were performed for 8 days. Gut microbiota structure (lumen and mucus-associated microbiota) and functions (gas and short-chain fatty acids) were monitored. Clearly distinct microbial signatures were obtained between the two in vitro conditions, with lower α -diversity indices and higher abundances of infant-related microbial populations (e.g., *Bifidobacteriaceae*, *Enterobacteriaceae*) in toddler versus adult conditions. In accordance with in vivo data, methane was found only in adult bioreactors, while higher percentage of acetate but lower proportions of propionate and butyrate was measured in toddlers compared to adults. This new in vitro model will provide a powerful platform for gut microbiome mechanistic studies in a pediatric context, both in nutritional- (e.g., nutrients, probiotics, prebiotics) and health-related (e.g., drugs, enteric pathogens) studies.

Key points

- Development of a novel in vitro colonic model recapitulating the toddler environment.
- Specific toddler versus adult digestive conditions are preserved in vitro.
- The new model provides a powerful platform for microbiome mechanistic studies.

Keywords Colonic model · Gut microbiota · Toddler · Mucus · Nutrition

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Introduction

In the first years of life, the human gastrointestinal tract and the resident microbiota evolve together with changes in diet and progressive exposure to xenobiotics including drugs (Fournier et al. 2021). Over the first months, microorganisms colonize the infant gut with a continuous increase in terms of load and diversity, evolving until the age of 3 years towards a mature and adult-like microbiota (Odamaki et al. 2016; Derrien et al. 2019). Most available data on infant gut microbiome are collected from fecal sample analysis and focus on the bacterial fraction (Könönen 2000; Pham et al. 2016), though other communities, such as *Archaea*, fungi, or viruses, are hosted from the first days of life (Wampach et al. 2017). In adults, it is now widely acknowledged that gut

microbiota composition varies not only along the digestive tract, but also from the digestive lumen to the intestinal epithelium, where mucus offers an ecological niche for specific bacterial colonization by providing adhesion sites and nutrients (Etienne-Mesmin et al. 2019). However, to date, data on mucus-associated microbiome are not available in children. Nevertheless, it is known that the infant mucus layer is well established at birth and data collected in rodents indicate a co-maturation between mucins and gut microbiota (Rokhsfat et al. 2016). Given the importance of gut microbiota in child nutrition and health, it is essential to evaluate the impact of various ingested compounds, such as nutrients, drugs, xenobiotics, or even beneficial or pathogenic microorganisms, on gut microbiome structure and functions, not only in fecal samples but also in the colonic compartment where the highest microbial load and associated metabolites are found (Fan and Pedersen 2021).

Clinical studies with toddlers are highly challenging due to ethical, regulatory, or financial constraints and require invasive procedures when collecting microbiota from various digestive compartments (Michael et al. 2010; Auby 2012). Young rodent models can be used but their reliability is limited by major differences between animal and human digestive physiology and gut microbiota according to age (Walthall et al. 2005; Hugenholz and de Vos 2018). In addition, the 3R (Reduction, Replacement, and Refinement) European rules encourage the reduction of animal experiments and the development of *in vitro* alternative strategies (Prescott and Lidster 2017). In this context, *in vitro* systems mimicking the child digestive environment represent valuable tools for mechanistic studies given their ability to reproduce physiologically relevant digestive conditions. Yet, only a limited number of *in vitro* gut models, with various levels of complexity, have been developed to simulate the toddler colon (De Boever et al. 2001; Cinquin et al. 2004, 2006; Vamanu et al. 2013, 2015; Dostal et al. 2015; Wiese et al. 2018; Pham et al. 2019; Salli et al. 2019; Van den Abbeele et al. 2019; Bondue et al. 2020). All of them are inoculated with fecal samples from children but are still set-up with some or all physicochemical parameters derived from the adult colon conditions and are devoid of a mucosal compartment, except for the Baby Simulator of the Human Intestinal Microbial Ecosystem (SHIME) system (Van den Abbeele et al. 2019).

The present study aimed at developing a new toddler colonic model as an alternative to *in vivo* assays in animals. To this end, we first performed an exhaustive review of the literature to determine the microbial and physicochemical parameters specific of the toddler colon and accordingly set-up the Mucosal Artificial Colon (M-ARCOL) (Deschamps et al. 2020). We then carried out separate *in vitro* fermentations with fecal samples collected from five healthy toddlers and daily monitored microbiota composition and metabolic activity through measurement of main end-products (gas and

short-chain fatty acids, SCFAs). In order to validate the new system, these *in vitro* results were compared to *in vivo* data in toddlers as well as *in vitro* data obtained from M-ARCOL fermentations in classical adult set-up after inoculation with stools obtained from four adult healthy volunteers.

Materials and methods

Fecal sample collection and treatment

Infant fecal samples used to inoculate the M-ARCOL model were collected from five healthy toddlers (mean age: 20.0 ± 3.4 months): two boys aged 17 months and 2.5 years (infants #1 and #3, respectively) and three girls aged 9, 22 and 22 months (infants #2, #4, and #5, respectively) (Fig. 1a). All infants were vaginally born and breast-fed except infant #1 who was born by cesarean section and formula fed. Adult fecal samples were collected from four healthy volunteers (mean age: 34.0 ± 6.7 years): two women aged 44 and 23 years (adults #1 and #2, respectively) and two men aged 29 and 52 years (adults #3 and #4, respectively) (Fig. 1b). All donors have no history of antibiotic treatment or probiotic consumption for one month prior to sample collection. Fecal samples were anaerobically (GENbag anaer gas pack systems, Biomerieux, Marcy-l'Étoile, France) collected and transported to the laboratory within 6 h following defecation. In an anaerobic chamber (COY laboratories, Grass Lake, USA), 11 g of each fresh fecal sample was suspended into 110 mL of 30 mM sterile sodium phosphate buffer and filtered (500 μm inox sieve).

Description and set-up of M-ARCOL colonic model

The Mucosal ARTificial COLon (M-ARCOL) is a one-stage fermentation system (MiniBio, Applikon, Delft, The Netherlands), used under continuous conditions, which simulates both the physicochemical (pH, body temperature, retention time, composition of ileal effluents reproduced by continuous input of nutritive medium) and microbial (lumen and mucus-associated microbiota) conditions found in the human colon (Deschamps et al. 2020) (Fig. 1c). At the beginning of the experiment, each airtight glass vessel containing 200 mL of nutritive medium was set under anaerobiosis by flushing with O_2 -free N_2 gas before adding the fecal suspension (100 mL). Afterwards, as a main feature of the M-ARCOL model, anaerobiosis was maintained inside the bioreactors by the sole activity of resident microbiota, as occurring *in vivo*. Overproduced gases were collected daily in a sampling bag. Bioreactors were kept at 37 °C. Colonic pH and redox potential were constantly recorded (Applikon, Delft, the Netherlands), and pH was adjusted with an automatic addition of 2 M NaOH. The nutritive medium

containing various sources of carbohydrates, proteins, lipids, minerals, and vitamins was continuously introduced into the bioreactor, while the fermentation medium was automatically withdrawn, ensuring the appropriate mean retention time. Colonic mucosal environment was reproduced through bypassing the fermentation medium from the main vessel towards an airtight removable compartment maintained at 37 °C and containing an average surface of 556 cm² mucin-alginate beads. These mucin-alginate beads were obtained by dropwise gelation of a solution of 5% w/v mucin (mucin from porcine stomach type II, Sigma-Aldrich, Saint-Louis, MO, USA) and 2% w/v sodium alginate (Sigma-Aldrich, Saint-Louis, MO, USA) into a 0.2 M CaCl₂ solution, as previously described (Deschamps et al. 2020). Beads were renewed every 2 days while maintaining the external compartment under anaerobiosis through a constant CO₂ flow. M-ARCOL was set up, based on in vivo available data, to reproduce either the mean conditions found in the colon of a healthy human adult (Thévenot et al. 2015; Deschamps et al. 2020; Verdier et al. 2021) or those of a 6-month–3-year toddler in the newly developed model called the Toddler Mucosal ARCOL (Tm-ARCOL) (Fig. 1e).

Experimental design and sampling

Experimental design and sampling are summarized in Fig. 1d. Two series of fermentations were performed, with biological replicates using stools from different donors, the first one considering toddler parameters (5 donors with age ranging from 9 to 30 months) and the second one under adult conditions (4 donors from 23 to 52 years old). All fermentations were run under continuous conditions for 8 days, after a 24-h period of microbial amplification (batch fermentation). Samples from the main bioreactor, referred to as luminal microbiota, were collected daily for microbiome characterization (storage at –80 °C) and SCFA analysis (storage at –20 °C). Additional samples from the mucin-alginate bead compartment, referred to as mucus-associated microbiota, were collected every 2 days for microbiota characterization. Mucin-alginate beads were washed twice in sterile phosphate buffer saline (PBS) and stored at –80 °C for downstream analyses. Interstitial medium from mucosal compartment was also sampled. In addition, samples were harvested daily from the atmospheric phase to ascertain anaerobic conditions and determine gas composition. The daily volume of gas produced by microbial activity was also measured using a syringe connected to the sampling bag.

DNA extraction and quality controls

Genomic DNA was extracted from luminal samples and mucin-alginate bead one using the QIAamp Fast DNA

Stool Mini Kit (Qiagen, Hilden, Germany) following manufacturer's instructions with the following adjustments. Prior to DNA extraction, luminal samples were centrifuged (15,000 g, 15 min, 4 °C) and the pellet was subjected to mechanical disruption using a bead beater (5 min, 20 beats/s) with 300-mg sterile glass beads (diameter ranging from 0.1 to 0.6 mm), incubated (70 °C, 5 min) and centrifuged again (12,000 g, 1 min, 4 °C). Mucin-alginate beads were subjected to the following adjustments prior to DNA extraction: 10 min of incubation with citrate buffer at 37 °C (Capone et al. 2013), before vortexing (maximal speed, 3 min) and centrifugation (8000 g, 1 min). DNA integrity was checked by agarose gel electrophoresis and by Nanodrop 2000 analysis (Thermo Fisher Scientific, Waltham, USA). DNA quantity was evaluated using the Qubit dsDNA Broad Range Assay Kit (Invitrogen, Carlsbad, USA) with a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, USA). Samples were stored at –20 °C prior to microbiota analysis (qPCR and 16S rRNA gene metabarcoding).

Quantitative PCR

Total bacteria were quantified by qPCR using the primers BAC338R (ACTCCTACGGGAGGCAG) and BAC516F (GTATTACCGCGGCTGCTG) with an annealing temperature fixed at 58 °C (Yu et al. 2005). Real-time PCR assays were performed on a Bio-Rad CFX96TM Real-Time System (Bio-Rad Laboratories, Hercules, USA) using Takyon™ Low Rox SYBR® 2×MasterMix blue dTTP kit (Eurogentec, Liège, Belgium). Each reaction was run in duplicate in a final volume of 10 µL with 5 µL of Master Mix, 0.45 µL of each primer (10 µM), 1 µL of DNA sample (10 ng/µL), and 3.1 µL of ultra-pure water. Amplifications were carried out using the following ramping profile: 1 cycle at 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 1 min. A melting step was added to ensure primer specificity. Standard curve was generated from tenfold dilutions of bacterial DNA (extracted from a fermentation sample), allowing the calculation of DNA concentrations.

16S metabarcoding and bioinformatics analysis

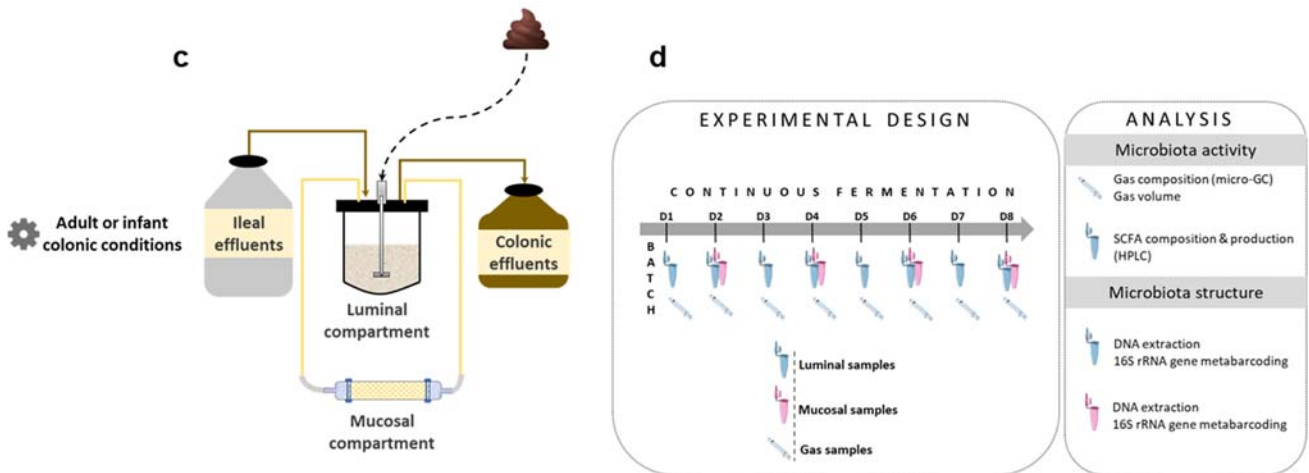
The bacterial V3–V4 region of 16S ribosomal RNA gene (rRNA) was amplified with primers V3_F357_N (CCT ACGGNGGCWGCAG) (Cordonnier et al. 2015) and V4_R805 (GACTACHVGGGTATCTAATCC) (Lane et al. 1985) for bacterial fraction and Arch349F (GYGCASCAGKCGMGAAW) and Arch806R (GGA CTACVSGGGTATCTAAT) for methanogenic *Archaea* (Zwiehler et al. 2011). Amplicons were generated using a Fluidigm Access Array followed by high-throughput sequencing on an Illumina MiSeq system performed at the

a

Donor	Gender	Age (months)	Mode of delivery	Feeding mode
Toddler 1	♂	17	Caeserean section	Formula
Toddler 2	♀	9	Vaginally	Breastfeeding
Toddler 3	♂	30	Vaginally	Breastfeeding
Toddler 4	♀	22	Vaginally	Mix breastfeeding/formula fed
Toddler 5	♀	22	Vaginally	Breastfeeding
Toddlers	/	20 ± 3.4	Vaginally	Breastfeeding

b

Donor	Gender	Age (years)
Adult 1	♀	44
Adult 2	♀	23
Adult 3	♂	29
Adult 4	♂	52
Adults	/	37 ± 6.7



e

	Parameters	Toddler model	Adult model
PHYSICO-CHEMICAL PARAMETERS	Temperature (°C)	37	37
	Retention time (h)	19	24
	pH	6.1	6.3
	Volume (mL)	300	300
	Stirring (rpm)	400	400
NUTRITIVE MEDIUM	Total carbohydrates (g/L)	7.5	7
	Lactose	1	0
	Total proteins (g/L)	3.1	12
	Mucin	4	4
	Casein	1	0
	Total lipids (g/L)	2.3	1.5
	Tween 80/tween20	0.5	1
	Total vitamins (µg/L)	28.5	28.5
	Vitamin D	10	0
	Total oligoelements (µg/L)	606.2	606.2
	CaCl ₂ (mg/L)	75	30
Total bile salts (mg/L)	150	200	
Primary/secondary bile salts	17.6	17.6	

Fig. 1 Stool donor information, sampling, experimental design, and parameters of in vitro fermentations when reproducing toddler versus adult colonic conditions in the M-ARCOL model. Two series of 8-day continuous fermentation were conducted using M-ARCOL inoculated with either adult (4 donors, a) or toddler (5 donors from 9 to 30 months, b) fecal samples. Samples from luminal, mucosal, and atmospheric phases of the bioreactors (c, d) were regularly collected to determine gut microbiota composition and metabolic activity. (e) All parameters from the M-ARCOL were adapted to specific toddler conditions according to in vivo data. Changes compared to adult set-up (as described in Deschamps et al. 2020) are indicated in bold. HPLC, high-performance liquid chromatography; micro-GC, micro-gas chromatography; SCFA, short-chain fatty acid

Carver Biotechnology Center of the University of Illinois (Urbana, IL, USA).

Bioinformatics analysis was performed by GeT-Biopuces platform (INSA/TBI, Toulouse, France) with R software version 4.1.1 (2021–08-10) and rANOMALY package (Theil and Rifa 2021). Demultiplexed raw sequence data were quality filtered and denoising process performed using DADA2 version 1.20.0 (Callahan et al. 2016). Reads with N bases or low Phred quality score (under 2) were eliminated, and reads under 100 pb length were removed. Decontamination steps were carried out to filter out sequences corresponding to PhiX DNA used as a spike-in control for MiSeq runs, and chimeric sequences were filtered out. Taxonomic affiliation of all amplicon sequence variants (ASVs) was performed with *idtaxa* function from DECIPHER package version 2.20.0 (Murali et al. 2018) using SILVA release 138 (Quast et al. 2013) and GTDB bac120_arc122 (Parks et al. 2022) databases (60% bootstrap cutoff). To improve completeness of taxonomic affiliation, alignments were carried out using BLAST (Altschul et al. 1990) (98% identity and coverage) on representative sequences of unassigned ASVs or assigned with incomplete taxonomy. A phylogenetic tree was constructed based on ASV representative sequences using functions from *phangorn* package version 2.7.1 (Schliep et al. 2017). α -diversity and β -diversity indexes were calculated using the *diversity_alpha_fun* function from rANOMALY package (Theil and Rifa 2021). The across-sample microbiota diversity was described using redundancy analysis (RDA) based on Bray–Curtis dissimilarities using *vegan* R-package version 2.5–7 (Oksanen et al. 2011). The impact of environmental factors (gas, SCFAs, mucosal or luminal samples, time and sex) on dissimilarities among groups was evaluated using PERMANOVA permutation tests (999 permutations).

Pathway profiling prediction using Tax4fun2

Computational predictions of the functional capabilities using data from 16S rRNA gene sequencing were performed using Tax4fun2 R-tool (Wemheuer et al. 2020) based on the KEGG (Kyoto Encyclopedia of Genes and Genomes)

database (Kanehisa and Goto 2000). Briefly, Tax4Fun2 picked up against the Ref99NR database into taxonomic profiles of KEGG organisms and normalize these predictions by the 16S rRNA copy number. Pearson matrix was calculated and used to display results. Statistical analysis was performed to highlight significantly different pathways between toddler and adult groups.

Gas analysis

The analysis of O₂, N₂, CO₂, CH₄, and H₂, produced during the fermentation process in the atmospheric phase of bioreactors, was performed using HP 6890 gas chromatograph (Agilent Technologies, Santa Clara, USA) coupled with a micro-TCD detector (Agilent Technologies, Santa Clara, USA). Two series columns, Molecular Sieve 5A and Porapak Q (Agilent Technologies, Santa Clara, USA), were used. Gas composition was determined using calibration curves made from ambient air (78.09% N₂, 20.95% O₂, 0.04% CO₂) and 3 gas mixtures A (5% CO₂, 5% H₂, 90% N₂), B (19.98% CO₂, 80.02% H₂), and C (19.89% CO₂, 19.88% CH₄, 20% H₂, 40.23% N₂).

Short-chain fatty acid analysis

Two milliliters of each luminal samples was centrifuged (5000 g, 15 min, 4 °C), and 900 μ L of supernatant was diluted 1/10 in H₂SO₄ 0.04 M mobile phase, vortexed, and filtered (0.22 μ m pore size). The three major SCFAs (acetate, propionate, and butyrate) were quantified by high-performance liquid chromatography (HPLC) (Elite LaChrom, Merck KGaA, Darmstadt, Germany) coupled with a diode-array detection (DAD) diode. The HPLC column (150 \times 7.8 mm) contained a negatively charged sulfonic-grafted polystyrene divinylbenzene stationary phase and carried an eluent containing acidified water. Data were obtained and analyzed by the EZChrom Elite software version 3.3.2 for Windows (Agilent, Santa Clara, CA, USA) at 205 nm. SCFA concentrations were calculated from calibration curves established from known concentration solutions of acetate, propionate, and butyrate (0, 10, 25, and 40 mM).

Statistical analysis

Statistical analyses on gut microbiota activity (gas and SCFAs) and α -diversity indexes (number of observed ASVs and Shannon index) from metabarcoding data were processed using GraphPad Prism software version 9.3.1 for Windows (GraphPad Software, San Diego, CA, USA). Differential analyses (DESeq2, metagenomeSeq, metacoder) were performed on ExploreMetabar software using rANOMALY package (Theil and Rifa 2021; Rifa and Theil 2021). Data normal distribution was verified by combining

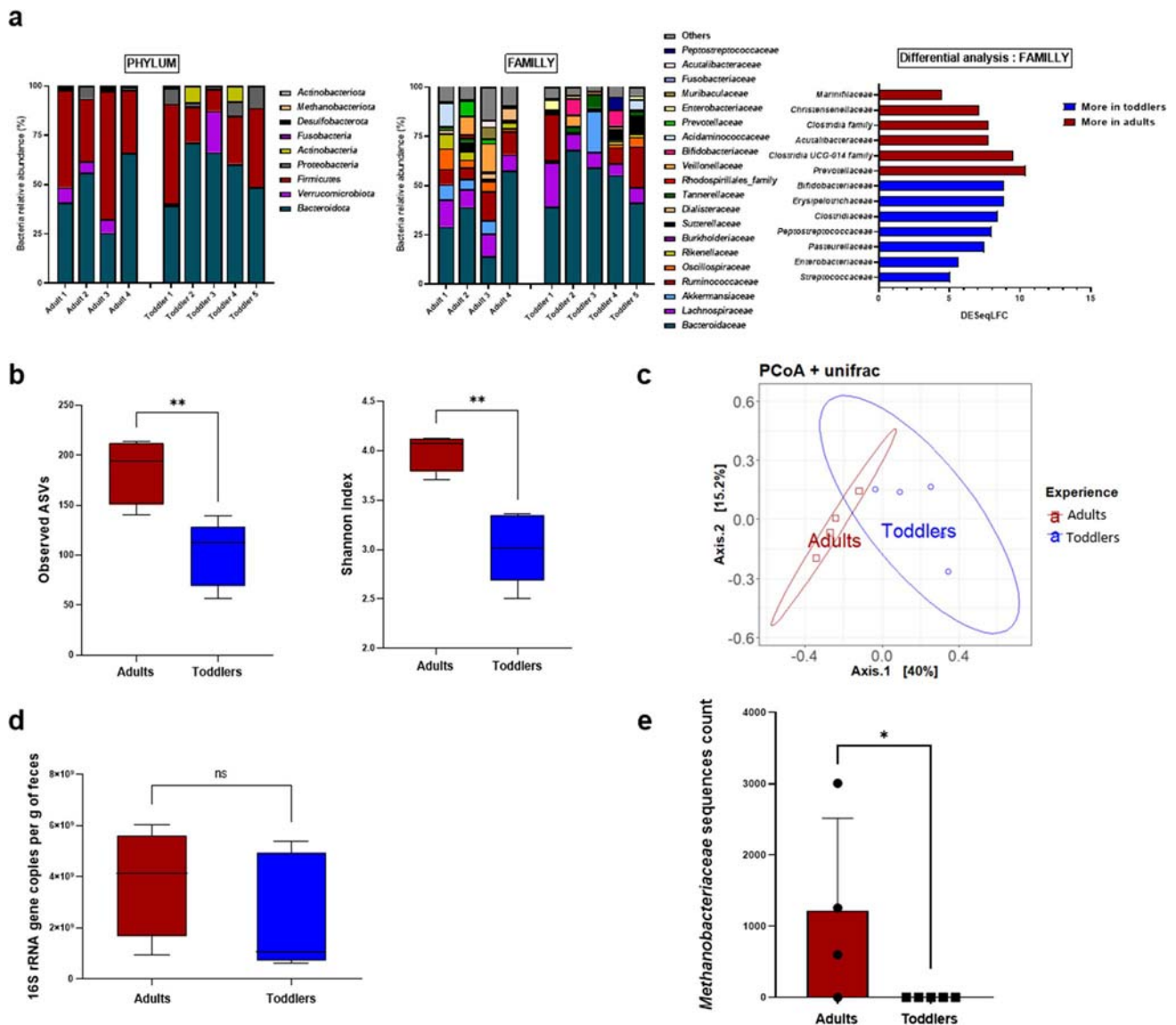


Fig. 2 Characterization of the toddler and adult initial fecal samples. The gut microbiota of toddler ($n=5$) and adult ($n=4$) stools used to inoculate M-ARCOL was characterized by 16S rRNA gene metabarcoding. (a) Relative bacterial abundances at the phylum and family levels and differential analysis (DESeq2, metagenomeSeq, meta-coder) between mean toddler and adult microbiota composition at the family level. (b) Box plot representation of observed ASVs and

Shannon α -diversity indexes (t -test, $**p < 0.01$). (c) Two-dimensional PCoA plot based on the unweighted UniFrac distance matrix illustrates bacterial β -diversity at the ASV level in adult and toddler fecal samples. (d) Box plot representation of total bacteria, determined by qPCR and expressed as 16S rRNA gene copy per gram of feces (t -test, ns). (e) Raw sequence counts of Methanobacteriaceae (Mann–Whitney, $*p < 0.05$, mean \pm SD)

Anderson–Darling, D’Agostino and Pearson, Shapiro–Wilk, and Kolmogorov–Smirnov tests, and homoscedasticity was checked using the Fisher test. Then, appropriate statistical analysis was applied (either one-way ANOVA, t -test, Mann–Whitney, or Welch’s tests). Principal coordinate analysis (PCoA) of unweight UniFrac distances was calculated, and significance between groups was assessed with a permutational multivariate analysis of variance (PERMANOVA) using ADONIS (999 permutations) with non-parametric tests.

Results

Adaptation of M-ARCOL model to the specific toddler colonic conditions

Based on the available data in the literature on toddler colonic physiology, we adapted all the physicochemical and nutritive parameters of the M-ARCOL model initially set up to reproduce digestive conditions of healthy adults, as described in Fig. 1e. Research by keywords and MESH was carried out

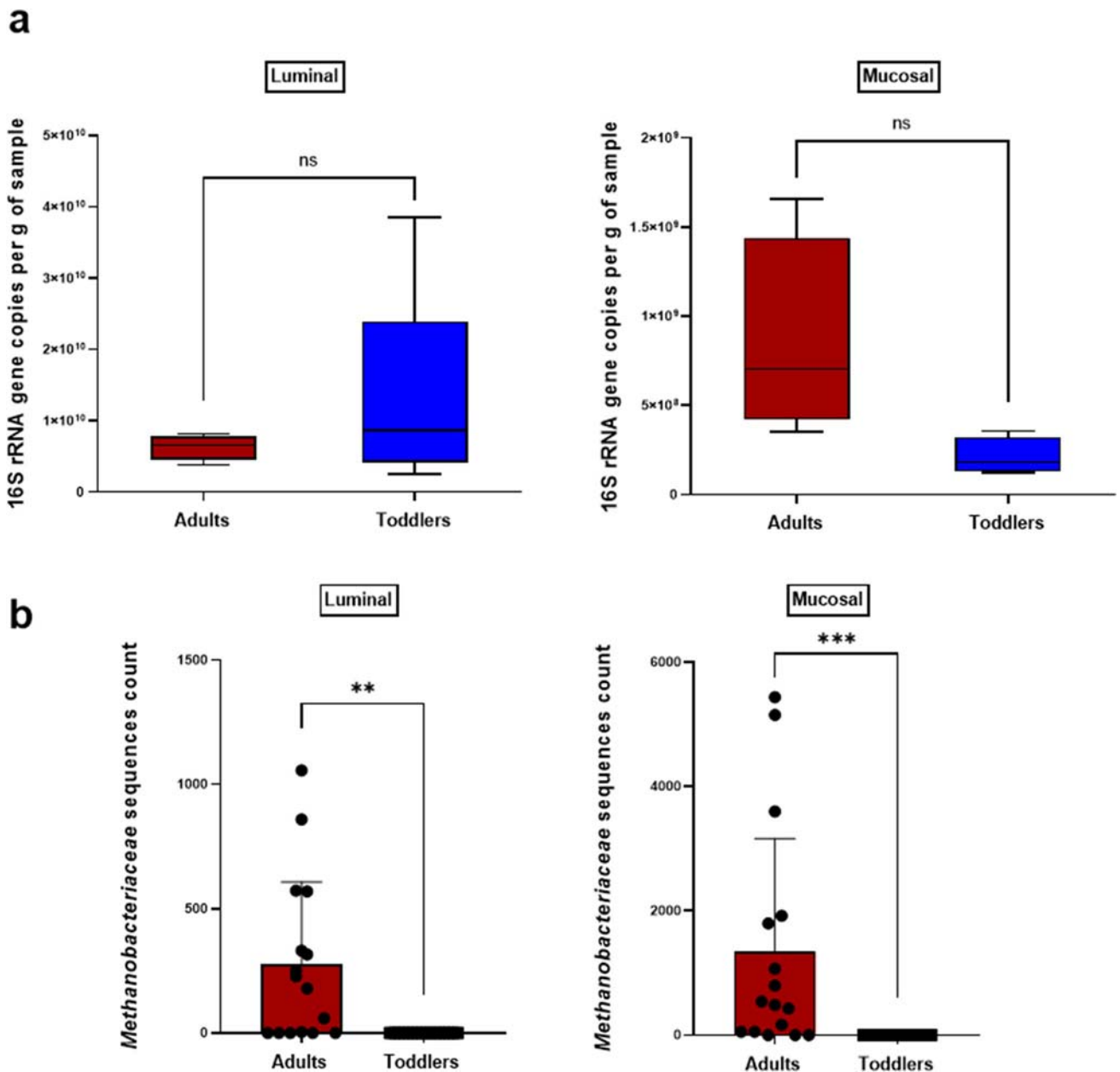


Fig. 3 Total bacteria and *Archaea* composition of the luminal and mucus-associated microbiota during toddler versus adult fermentations. Fermentations were performed in M-ARCOL during 8 days, set-up under adult ($n=4$) or toddler ($n=5$) conditions. Samples were regularly collected (days 2, 4, 6, and 8) from the luminal or mucosal compartments, and bacterial composition was determined by quan-

titative PCR and 16S metabarcoding for *Archaea* populations. (a) Box plot representation of total bacteria, determined by qPCR and expressed as 16S rRNA gene copy per gram of sample (t -test, ns). (b) Raw sequence counts of *Methanobacteriaceae* (Kolmogorov–Smirnov, $**p < 0.01$, $***p < 0.001$, mean \pm SD)

on PubMed (<https://pubmed.ncbi.nlm.nih.gov/>) and Google Scholar (<https://scholar.google.com/>) with no filter to gather in vivo data available regarding toddler gut physiology (transit time, pH), microbiota, and diet. Only publications referring to healthy children from 6 months (after the exclusive milk diet) to 3 years (maturity of the child gut) were selected. In the Tm-ARCOL model, body temperature was not modified and kept at 37 °C. Mean colonic pH was

decreased from 6.3 (in adult) to 6.1, since a slightly more acidic pH was reported in infant feces compared to adult ones (Guérin-Danan et al. 1997; Hossain et al. 2019). This difference is linked to a higher milk consumption in children, associated to an increased prevalence of lactic acid bacteria such as *Bifidobacteria* and *Bacteroidetes* strains producing organic acids (Rios-Covian et al. 2013; Laursen et al. 2021). The residence time of the bioreactor (reflecting colonic

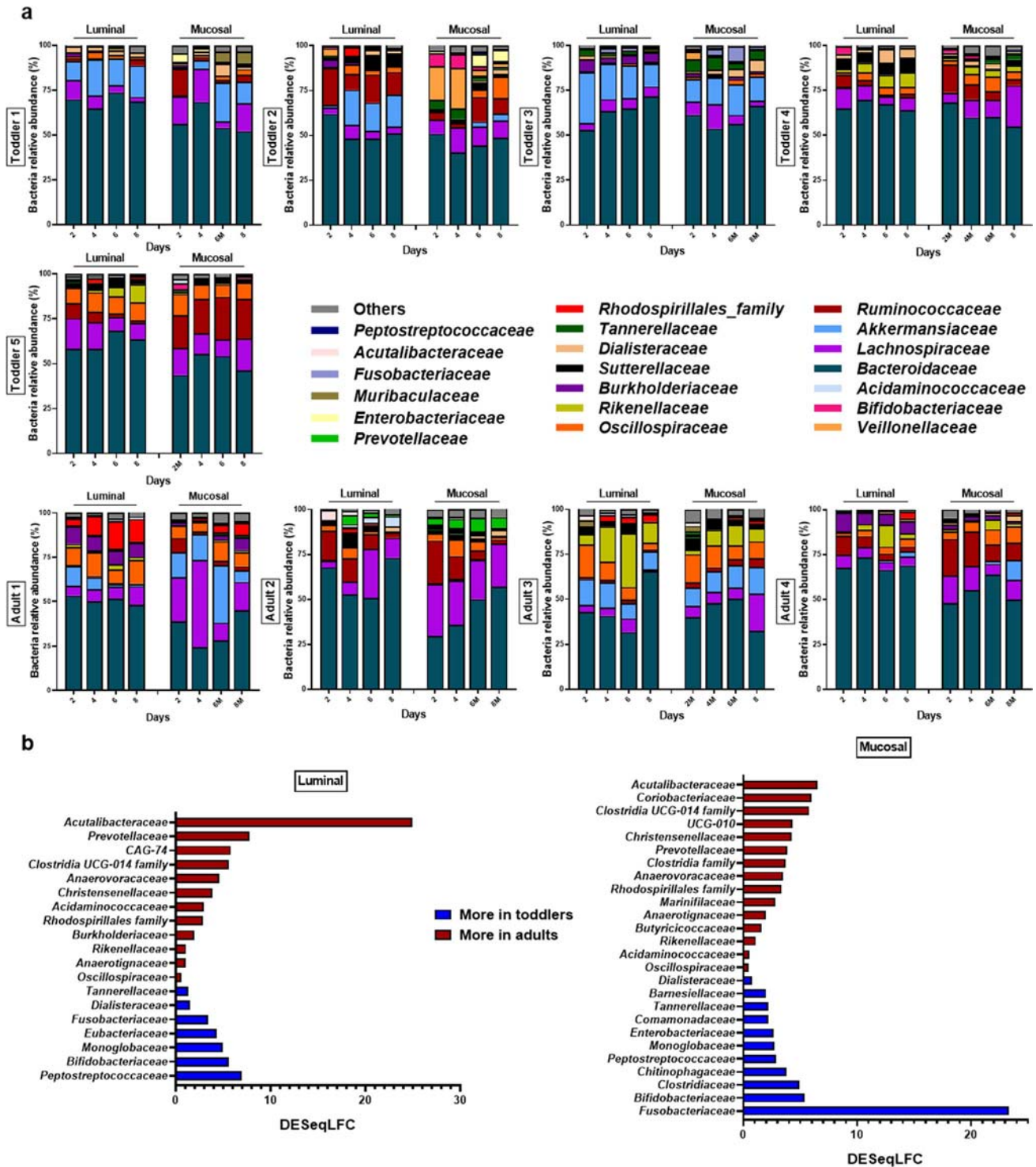


Fig. 4 Bacterial composition of the luminal and mucus-associated microbiota during toddler versus adult fermentations at the family level. Fermentations were performed in M-ARCOL for 8 days, set-up under adult ($n=4$) or toddler ($n=5$) conditions. Samples were regularly collected (days 2, 4, 6, and 8) from the luminal or mucosal compartment (from either beads or interstitial bead medium indi-

cated as M in the caption). Bacterial composition was determined by 16S rRNA gene metabarcoding. (a) Relative bacterial abundance at the family level. (b) Differential analysis (DESeq2, metagenomeSeq, metacoder) of bacterial populations at the family level between toddler and adult conditions

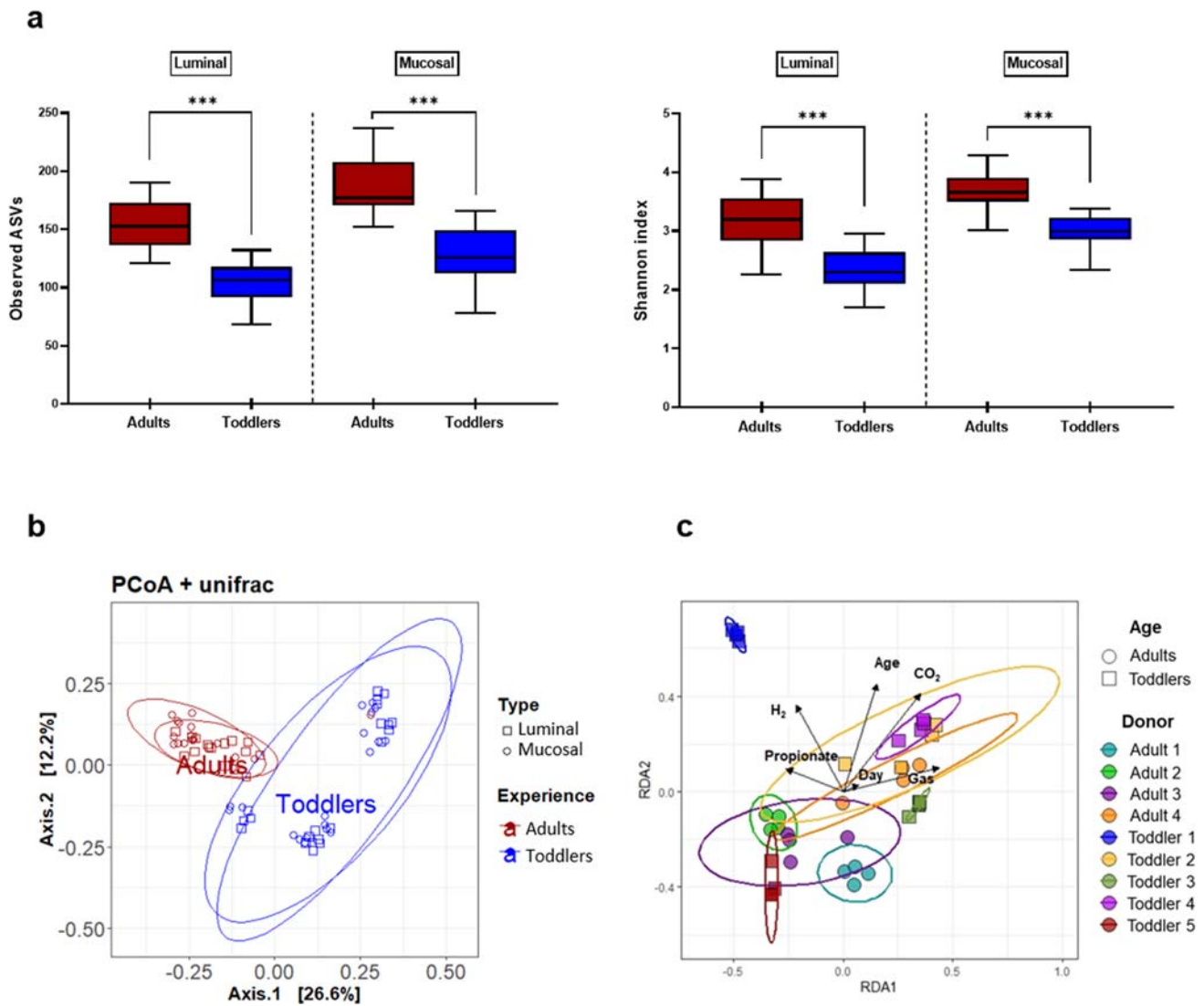


Fig. 5 α - and β -diversity indexes of luminal and mucus-associated bacterial populations during toddler versus adult fermentations. Fermentations were performed in M-ARCOL for 8 days, set-up under adult ($n=4$) or toddler ($n=5$) conditions. Luminal and mucus-associated bacterial compositions were determined by 16S rRNA gene metabarcoding for days 2, 4, 6, and 8. (a) Bacterial α -diversity

is expressed by the number of observed ASVs and Shannon index and represented by box plot (t -test, $***p < 0.001$). (b) Two-dimensional PCoA plot based on the unweighted UniFrac distance matrix illustrates bacterial β -diversity at the ASV level. (c) Distance-based redundancy analysis (RDA) of bacterial β -diversity, based on 16S data and metabolic data from the luminal phase only

transit time) was decreased from 24 h (in adult) to 19 h, since toddlers have a smaller gut than adults in terms of size and volume, they ingest a higher quantity of liquid food, and are characterized by an increased stool frequency (Walker and Walker 1985; Gutiérrez et al. 2002). The composition of the nutritive medium mimicking ileal effluents was also adapted based on differences between adults and children diet. In the age range chosen herein, children have a higher milk consumption, as introduced above but also a lower meat intake than adults (Trumbo et al. 2002; Laursen et al. 2016). Based on those food intake, total amount of proteins

under toddler condition was then divided by 3 and the ratio Bacto tryptone (issued from milk digestion)/Primatone (from meat digestion) was increased. The nutritive medium was also enriched in casein and lactose, both highly concentrated in milk. As cow milk is also rich in saturated fat, the total amount of lipids was increased by 1.5 and Tween 20 was added as a saturated fat source. Regarding micronutrients, vitamin D was included into the child nutritive medium since supplementation is present in most of infants (Leaf and RCPCH Standing Committee on Nutrition 2007). Fiber content was also decreased by one third due to a lower

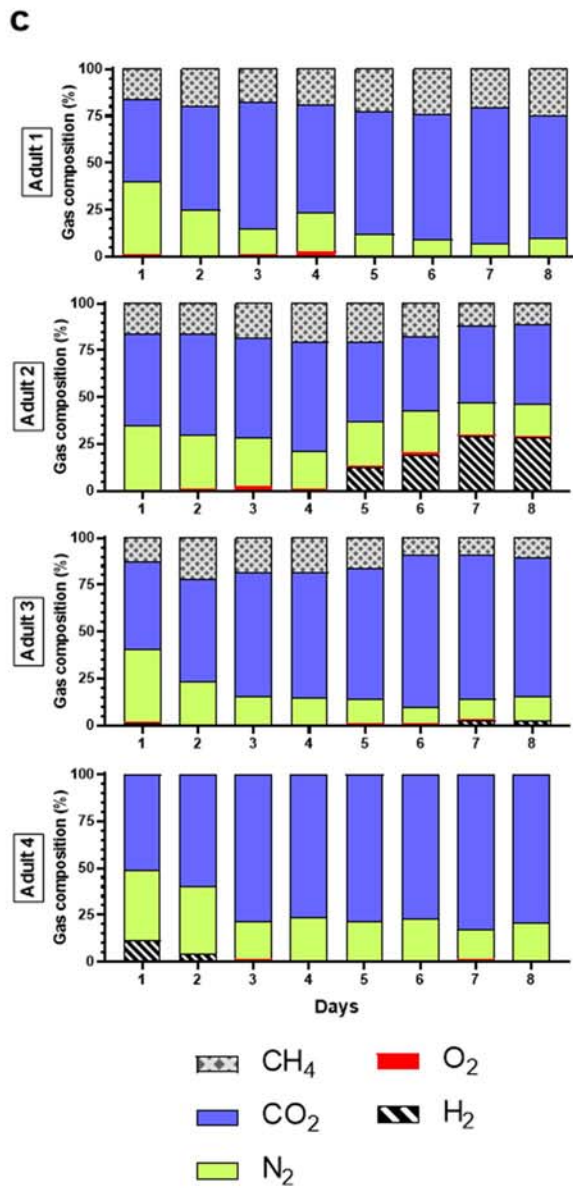
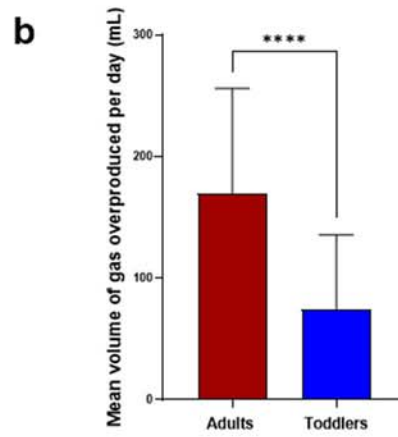
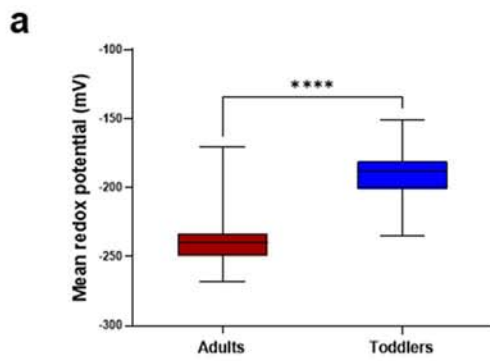


Fig. 6 Luminal microbial metabolic activity followed by gas production and redox potential measurement during toddler versus adult fermentations. Fermentations were performed in M-ARCOL under adult ($n=4$) or toddler ($n=5$) conditions. Gas composition of the atmospheric phase was analyzed daily by micro-gas chromatography, and total volume was measured with a syringe. Redox potential of the luminal phase was continuously recorded. (a) Box plot representation of redox potential in mV for days 1 to 8 (Mann–Whitney, $p < 0.0001$). (b) Daily gas production mean \pm SD in milliliters for days 1 to 8 (Mann–Whitney, $***p < 0.0001$). (c) Individual gas profiles as expressed in relative percentages for each fermentation day

consumption in children compared to adults. Furthermore, ileal effluents contain bile salts not re-absorbed in the upper gut. Infants are known to have a low bile salt pool (Heubi and Balistreri 1980). Therefore, total bile salt concentration was reduced by one fourth in the child nutritive medium. Lastly, we were not able to adjust in the Tm-ARCOL model both mucin concentration and mucin-alginate bead surface area due to the absence of human in vivo data.

Initial stool characterization

Prior to inoculation of the M-ARCOL, toddler and adult fecal microbiota composition was characterized by 16S metabarcoding (Fig. 2). Gut microbiota profiles showed clear inter-individual variations, especially at the family level (Fig. 2a). As expected, the fecal microbiota of toddlers was also significantly different from the adult one whatever the taxonomic level considered. According to DESeq2 analysis, *Bifidobacteriaceae* ($p=0.0001$), *Clostridiaceae* ($p=0.003$), *Pasteurellaceae* ($p=0.004$), *Peptostreptococcaceae* ($p=0.004$), *Erysipelotrichaceae* ($p=0.008$), and *Enterobacteriaceae* ($p=0.008$) were significantly more abundant in the toddler fecal microbiota than in the adult one (Fig. 2a). β -diversity analysis confirmed a clearly distinct clustering between adult and toddler samples (Fig. 2c). Regarding α -diversity, significantly lower number of ASVs (101 versus 186, $p=0.0065$) and Shannon index (3 versus 4, $p=0.0018$) were found for toddlers compared to adults (Fig. 2bb). Interestingly, the number of ASVs was positively correlated to toddler age (data not shown). Total bacteria also tended to be lower in toddler compared to adult stools, with 2.5×10^9 versus 3.8×10^9 16S rRNA gene copies/g of feces (Fig. 2d). Lastly, no methanogenic *Archaea* was detected in toddler fecal samples, while an average of 1215 ± 1299 *Methanobacteriaceae* raw sequences count was found in adult samples (Fig. 2e).

Luminal and mucus-associated microbiota composition in M-ARCOL in toddlers versus adults

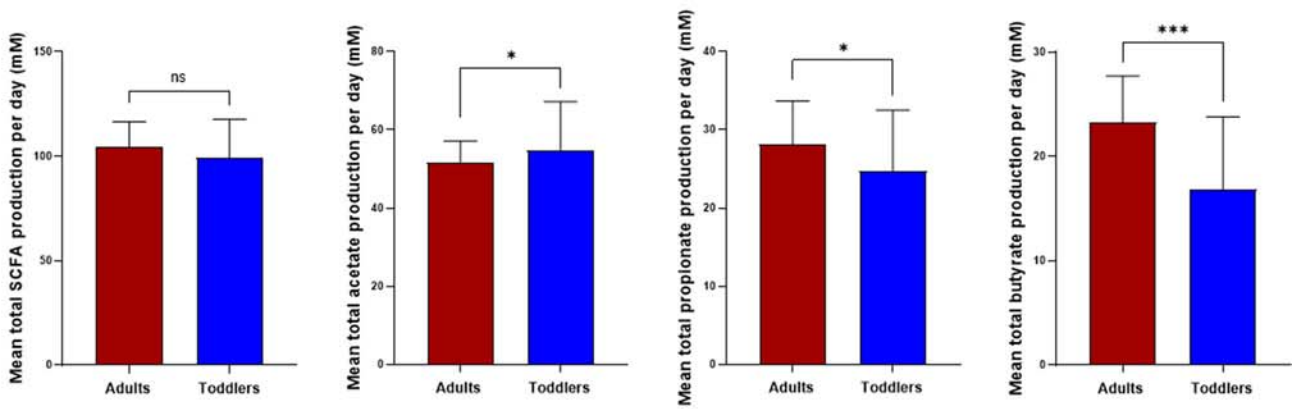
Total bacteria concentrations were first measured every two days during in vitro fermentation by quantitative PCR

(Fig. 3a). The luminal phase of M-ARCOL displayed an average of 1.3×10^{10} 16S rRNA gene copies/g of sample for toddler reactors, comparable to 6.3×10^9 16S rRNA gene copies/g of sample for adult reactors. In the mucosal phase, despite the absence of significance, total bacterial concentration seemed to be lower for toddler (2.1×10^8 16S rRNA gene copies/g of sample) conditions compared to adult (8.5×10^8 16S rRNA gene copies/g of sample). No methanogenic *Archaea* were found in toddler conditions while a *Methanobacteriaceae* raw count of 277 and 1345 was measured in adults, in the luminal and mucosal phases of M-ARCOL, respectively, leading to significant differences (Fig. 3b). The abundance data showed clearly different profiles between adults and toddlers at phylum (Supplemental Fig. S1a), family (Fig. 4a), and genus (Supplemental Fig. S1b) levels. At the phylum level, *Firmicutes*, *Bacteroidota*, *Verrucomicrobiota*, and *Proteobacteria* were found in both adults and toddlers, while *Fusobacteria* and *Actinobacteria* were toddler-specific phyla. At the family level, differential analysis between adult and toddler bacterial populations indicated that *Peptostreptococcaceae*, *Bifidobacteriaceae*, *Fusobacteriaceae*, *Tannerellaceae*, and *Dialisteraceae* were significantly more abundant in toddlers than in adults, both in luminal and mucosal phases (Fig. 4b). In addition, specifically in the mucosal environment, *Enterobacteriaceae* and *Clostridiaceae* were also more abundant in the toddler conditions. On the contrary, *Acualibacteraceae*, *Rikenellaceae*, *Christlensenellaceae*, and *Prevotellaceae* were significantly less abundant in toddler's versus adults in both mucosal and luminal phases (Fig. 4b). This clear difference between adult and toddler microbiota was confirmed by the investigation of α - and β -diversity indexes. The number of observed ASVs and Shannon index was significantly ($p < 0.001$) lower for toddlers compared to adults, whatever the compartments of M-ARCOL considered (Fig. 5a). Principal coordinate analysis (PCoA) also showed a significant clustering ($p < 0.001$) between toddler and adult bacterial communities (Fig. 5b).

Inter-individual variation in luminal and mucus-associated microbiota composition in M-ARCOL

In addition to the striking difference between toddlers and adults, sequencing analysis also highlighted a clear donor effect (Fig. 4a; Supplementary Fig. S1a and S1b). For example, in adults, at the family level, Adult #2 harbored a remarkable important abundance of *Prevotellaceae*, associated with an absence of *Akkermansiaceae*, both in the luminal and mucosal phases of M-ARCOL (Fig. 4a). Compared to other toddlers, the youngest toddler #2 (9 months) exhibited a significant higher amount of *Ruminococcaceae* in the luminal environment

a



b

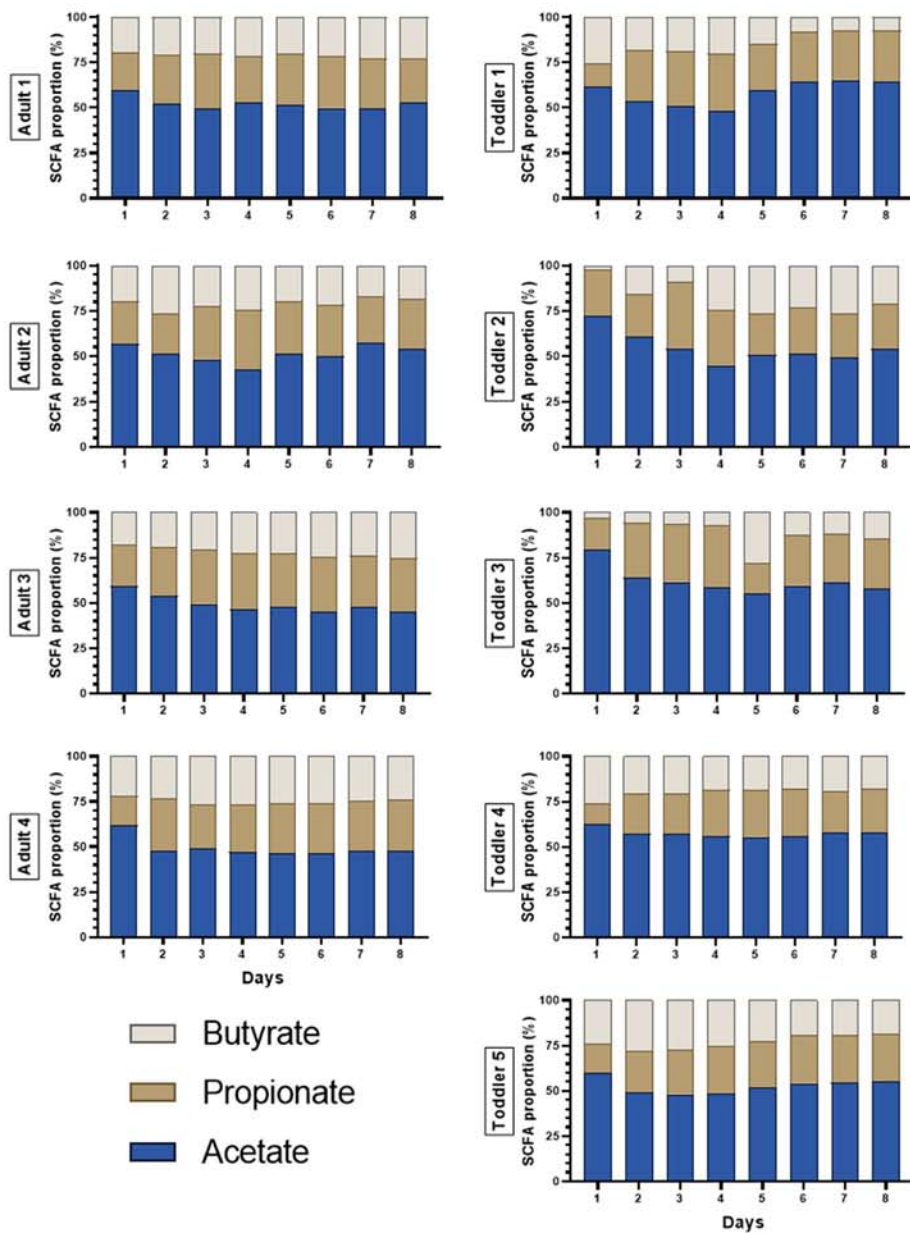


Fig. 7 Luminal microbial metabolic activity followed by short-chain fatty acid production during toddler versus adult fermentations. Fermentations were performed in M-ARCOL under adult (n=4) or toddler (n=5) conditions. Main SCFAs (acetate, propionate and butyrate) were measured daily by liquid chromatography. (a) Daily total, acetate, propionate or butyrate production mean \pm SD in mM (Mann–Whitney and *t*-test with $p < 0.05$ for significance). (b) Individual SCFA profiles as expressed in relative percentages for each fermentation day. * $p < 0.05$, *** $p < 0.001$

of Tm-ARCOL, together with larger abundances of *Veillonellaceae*, *Megasphaeraceae*, and *Eubacteriaceae* in the mucosal phase (DESeq2 analysis between toddler 2 and others; data not shown). α -diversity indexes showed significant variations in the number of observed ASVs between toddlers while no effect was evidenced between adults (Supplementary Fig. S2a). Two-dimensional PCoA plot based on the unweighted UniFrac distance matrix illustrating bacterial β -diversity confirmed a significant clustering by donors ($p < 0.001$), both in toddlers and adults (Supplementary Fig. S2b). Again, adult #2 clearly clustered far from the three other donors. Moreover, gender-dependent effect was evidenced ($p < 0.001$), especially in the toddler population (Supplementary Fig. S2c).

Gut microbiota activity in toddler versus adult in the luminal phase of M-ARCOL

The redox potential was first investigated as an indicator of fermentation activities and anaerobiosis. Values stabilized at -190 ± 14 mV under toddler conditions, which was significantly higher ($p < 0.0001$) than under adult condition that reaches -236 ± 19 mV (Fig. 6a). Gases produced by in vitro microbial fermentation were also collected daily and their volume was measured. The mean daily volume of gases was significantly ($p < 0.0001$) lower in toddler experiments (75 ± 61 mL) compared to adult ones (170 ± 86 mL) (Fig. 6b). Furthermore, gas composition was clearly different between toddlers and adults. Three out of the four adult donors exhibited methane-producer microbiota (e.g., up to 25% of total gas in adult #1), while CH_4 was never evidenced in the atmospheric phase of toddler bioreactors. H_2 was detected during the first days of fermentations in all toddler donors, but its relative percentage decreased with time to disappear in toddlers #2 and #3 (Fig. 6c). For adults, only adult #4 microbiota (non-methane-producer) showed important amounts of H_2 (25%) at initial time of fermentation. Lastly, the three main SCFAs (acetate, propionate, and butyrate) were measured in the luminal compartment of M-ARCOL. No significant difference ($p = 0.1929$) was found between total SCFA concentrations in both conditions, with 99 ± 18 mM in toddlers versus 104 ± 12 mM in adults (Fig. 7a). However, SCFA composition was different in toddlers compared to

adults (Fig. 7b). When the mean total concentrations of acetate, butyrate, and propionate were calculated per day, we observed a higher quantity of acetate (55 ± 12 mM versus 52 ± 5 mM, $p = 0.0426$) and a lower amount of butyrate (17 ± 7 mM versus 23 ± 4 mM, $p = 0.0001$) and propionate (25 ± 8 mM versus 28 ± 6 mM, $p = 0.0419$) in toddlers compared to adults (Fig. 7a).

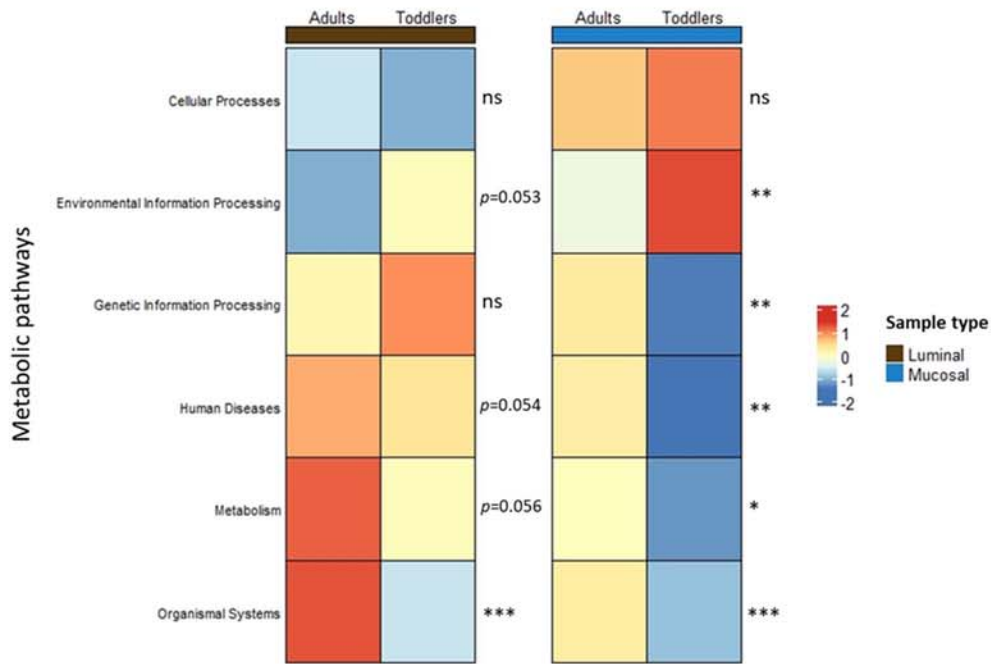
Inter-individual variations in luminal microbial activity in M-ARCOL

The mean volume of gas produced daily was highly donor-dependent, with microbiota from toddler #2 and adult #1 being high producers (Supplementary Fig. S3a). Inter-individual variability was also noticed with methane (adults #1, #2, #3) and non-methane (adult #4) producers. Interestingly, microbiota from adult #2 was the only one to produce a noteworthy amount of H_2 from days 5 to 8 (up to 29%), while CH_4 concentrations tended to decline (Fig. 6b). Regarding SCFAs, total daily production was very similar between the four adult donors, while SCFA concentrations measured for the youngest toddler #2 were significantly ($p < 0.001$) lower than that of all other toddlers, with an average value throughout fermentation of 67 mM (Supplementary Fig. S3b). Interestingly, microbiota from toddler #2 produced significantly ($p < 0.0001$) less acetate than all other donors (Fig. 7b; Supplementary Fig. S3c). RDA redundancy analyses based on ASV tables in the luminal phase and gut microbial activity showed that age and gases are the main parameters accounting for differences in β -diversity between the different individuals (Fig. 5c).

Predicted functional profiling of luminal and mucosal microbiota in toddler versus adult in M-ARCOL

Tax4Fun2 was applied to 16S rRNA gene metabarcoding results to predict functional profiles of luminal and mucosal microbiota in both toddler and adult conditions (Fig. 8). At the global level (level 3), “human diseases,” “metabolism,” and “organismal systems” pathways are more prevalent in adult compared to toddler conditions, both in luminal and mucosal microenvironments of M-ARCOL (Fig. 8a). At an intermediate level (level 2), “cell growth and death,” “transport and catabolism,” “cardiovascular diseases,” “endocrine and metabolic disease,” “glycan biosynthesis and metabolism,” “lipid metabolism,” “endocrine system,” and “immune system” were significantly ($p < 0.001$) less prevalent in toddlers, while opposite trends were observed for “membrane transport,” “cancer specific types,” and “xenobiotic biodegradation and metabolism,” again in both luminal and mucosal phases (Fig. 8b).

a



b

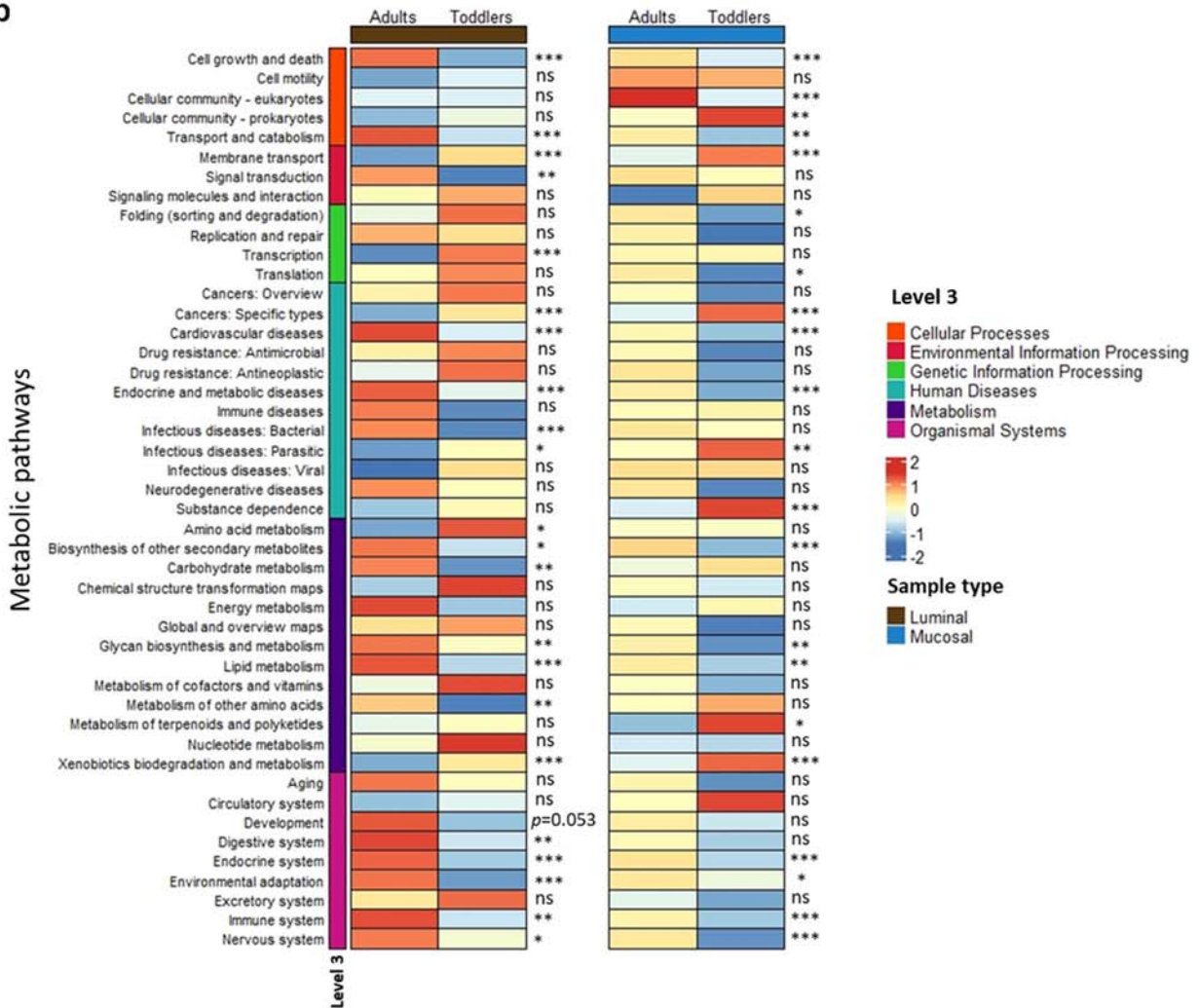


Fig. 8 Predicted functional metabolic pathways during adult and toddler fermentations. Functional pathways were predicted using Tax4fun2 R-package with ASV table made from sequencing data for days 2, 4, 6, and 8 of adult ($n=4$) and toddler ($n=5$) fermentations matched against KEGG pathway database. From the metabolic pathway prediction, the Pearson correlation matrix was calculated and results represented as heatmap. Results are shown at global level (level 3) in (a) and intermediate level (level 2) in (b). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Discussion

The main objective of the present study was to develop and validate a new in vitro model of the toddler (from 6 month- to 3-year-old) colon, named Tm-ARCOL, as an alternative to in vivo assays. The first step in Tm-ARCOL development was to adapt all parameters of the existing M-ARCOL in vitro model to toddler-specific digestive conditions. This required an important literature review, with around 100 publications analyzed regarding toddler digestive physiology, and only 10 were selected to set-up the Tm-ARCOL. When comparing the available models of the toddler colon, as summarized in Table 1 (De Boever et al. 2001; Cinquin et al. 2004, 2006; Vamanu et al. 2013, 2015; Dostal et al. 2015; Wiese et al. 2018; Pham et al. 2019; Salli et al. 2019; Van den Abbeele et al. 2019; Bondue et al. 2020), Tm-ARCOL was the only one where both physicochemical (pH, transit time) and nutritional (ileal effluents composition) parameters were adapted to toddler colonic conditions. This is particularly important since these parameters largely shape the composition of the gut microbiota and metabolic activities. Our in vitro model exhibits the unique feature to maintain anaerobiosis by the sole activity of the resident gut microbiota, allowing us to determine daily gas production and composition in the atmospheric phase of bioreactors. With baby-SHIME (Van den Abbeele et al. 2019), it is also one of the rare systems that distinguish the mucosal from the luminal microenvironments and their associated microbiota, and this adaptation has been previously performed in M-ARCOL based on the M-SHIME technology (Deschamps et al. 2020). However, due to a lack of in vivo data on mucus-associated microbiota in toddlers (in vivo data are only from stool samples), it was difficult to establish in vitro/in vivo comparison. However, Tm-ARCOL does not reproduce the three parts of the toddler colon, as do the PolyFermS, GIS, Enteromix[®], CoMiniGut, or Baby/Toddler SHIME[®] systems (De Boever et al. 2001; Cinquin et al. 2006; Vamanu et al. 2015; Wiese et al. 2018; Salli et al. 2019; Van den Abbeele et al. 2019; Bondue et al. 2020), nor integrate the upper gastro-intestinal compartments as in the M-SHIME.

A novelty in toddler colon development of our study was the comparison of data between adult and toddler conditions

as we also performed in vitro fermentations in the M-ARCOL model inoculated with adult stools and set-up to reproduce adult colonic conditions. For gaining further insights, main changes associated to toddler versus adult conditions in M-ARCOL have to be compared to existing in vivo data in humans from literature. Due to the absence of information, these in vitro/in vivo comparisons were performed using data from human stools and not from colonic samples (no data available from either the luminal or mucosal compartment). Relative changes evaluated in vivo included either evolution with age in the same individuals (longitudinal studies from birth to 2 or 3 years) or comparison between toddlers and their mothers. These correlations were established on both gut microbiota structure (Table 2a) and metabolic activities (Table 2b). Despite the inability to make some comparisons due to absence of data in humans, we established that 10 of the 18 bacterial populations followed throughout fermentation in M-ARCOL exhibit a shift from adult to toddler according to in vivo data (Table 2a). When investigating the microbiota activity, such correspondence was even higher with 5 out of 6 metabolic parameters found in agreement with in vivo data (Table 2b).

Regarding gut microbiota composition, α -diversity of microbial communities was increasing from toddler to adult experiments, in complete agreement with in vivo data where infant gut microbiota exhibits an initially low diversity, which then increases with the gradual transition from milk-based feeding to family diet, and continuous exposure to environmental microbes (Mariat et al. 2009; Hong et al. 2010; Koenig et al. 2011; Vallès et al. 2014; Bäckhed et al. 2015; Laursen et al. 2016; Bokulich et al. 2016; Hill et al. 2017; Differding et al. 2020; Appert et al. 2020; Nilsen et al. 2020; Roswall et al. 2021; Wang et al. 2021). The “first 1000 days” of life are characterized by important changes in gut microbiota profiles with a clear shift from aero-anaerobic facultative to strictly anaerobic bacteria (Arbolea et al. 2012; Nagpal et al. 2017). In particular, *Lactobacillaceae*, *Bifidobacteriaceae*, *Enterobacteriaceae*, *Fusobacteriaceae*, and *Peptostreptococcaceae* families are increasing from 6 months to 3 years while *Rikenellaceae*, *Akkermansiaceae*, *Lachnospiraceae*, and *Ruminococcaceae* are less abundant (Ahrne et al. 2005; Mariat et al. 2009; de Morais Tureli et al. 2010; Bergström et al. 2014; Laursen et al. 2016; Bokulich et al. 2016; Yassour et al. 2016; Levin et al. 2016; Hill et al. 2017; Appert et al. 2020; Nilsen et al. 2020; Wang et al. 2021). Most of these changes were also observed in vitro, except for *Lactobacillaceae* (almost not detected in Tm-ARCOL) and *Lachnospiraceae* or *Ruminococcaceae* (not significantly different between toddlers and adults in vitro). Variations with age were also observed with non-bacterial populations, such as methanogenic *Archaea*. Interestingly in Tm/M-ARCOL, archaeal populations were found in none of the toddler set-up but maintained in 3

Table 1 Comparison of Tm-ARCOL with previously developed toddler colonic in vitro models

Name	Age (months)	Upper GI part	3 colonic parts	Individual stool	Colonic physico-chemical parameters			Ileal effluent composition				Mucus-associated microbiota	Eukaryote or Archaea monitoring	References
					pH	Transit time	Anaerobiosis without flushing	Carbo hydrates	Proteins	Lipids	Bile acids			
PolyFermS	2	X	X	✓	✓	✓	X	✓	✓	X	✓	X	X	Pham et al., 2019
	4 - 6	X	✓	✓	X	X	X	✓	✓	X	✓	X	X	Cinquin et al. 2004
	30	X	X	✓	✓	✓	X	✓	✓	X	✓	X	X	Dostal et al., 2015
Three-stages PolyFermS	6	X	✓	✓	✓	✓	X	✓	✓	X	✓	X	X	Cinquin et al., 2006
GIS1	6	X	X	✓	X	X	X	✓	✓	X	✓	X	X	Vamanu et al., 2013
GIS2	6	X	✓	✓	X	X	X	✓	✓	X	✓	X	X	Vamanu et al., 2015
CoMiniGut	6	X	✓	✓	X	X	X	X	X	X	X	X	X	Wiese et al., 2018
EnteroMIX®	< 12	X	✓	✓	X	X	X	X	X	X	X	X	X	Salli et al., 2019
	3 - 12	✓	✓	X	✓	✓	X	✓	✓	✓	X	X	X	De Boever et al., 2001
Baby SHIME	6	✓	✓	✓	X	X	X	✓	✓	✓	X	✓	X	Van den Abbeele et al., 2019
Toddler SHIME®	12 - 24	✓	✓	✓	X	X	X	✓	✓	✓	X	X	X	Bondue et al., 2020
Tm-ARCOL	6 - 36	X	X	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	The present study

Red cross: the in vitro model considered does not reproduce the corresponding parameter or has not been adapted to specific toddler digestive conditions; green tick: the in vitro model considered reproduces the corresponding parameter according to toddler characteristics

GI gastro-intestinal, GIS unicameral system of in vitro colonic simulation, *PolyFermS* polyfermentor intestinal model, *SHIME* Simulator of the Human Intestinal Microbial Ecosystem, *Tm-ARCOL* Toddler mucosal ARCOL

out of 4 of the adult conditions. Accordingly, in humans, several studies have already shown that *Methanobrevibacter smithii* can be detected at birth independently of the mode of delivery (Sagheddu et al. 2017; Grine et al. 2017), with a prevalence increasing with age (Fiedorek et al. 1990; Rutili et al. 1996; Mihajlovski et al. 2010; Camara et al. 2021). Of note, among the available toddler colonic models, our system is the only one providing data on microbial populations other than bacterial ones. This can be a real contribution to the field regarding the importance of methanogenic *Archaea* in children health development, since a higher *Archaea* prevalence in fecal samples was associated to lower severe malnutrition and improved weight regulation (Camara et al. 2021).

In addition to microbial profiles, several parameters were investigated in Tm/M-ARCOL to assess gut microbial activities, namely redox potential, gas and SCFA production, as main end-products of microbial metabolism. We showed a higher redox potential and a lower gas production in vitro in toddler bioreactors compared to adult ones. Those latter indicators reflect a lower microbiota activity under toddler conditions, in line with in vivo gut microbial diversification from toddler to adult and continuous increase of metabolism capacity with time (Gosalbes et al. 2019; Turroni et al. 2020). Our results regarding gas are supported with difficulty by in vivo data, since we did not find information on toddler gas production and studies in adults are scarce (Montalto et al. 2009; Kalantar-Zadeh et al. 2019), except three studies showing that a higher proportion of toddlers was able to produce CH₄ in expired air when growing (Fiedorek et al. 1990; Hudson et al. 1993; Tormo et al. 2001). This is

in accordance with CH₄ measurement during M-ARCOL experiments (no production in toddler bioreactors) and reflective of the differences in relative abundance of archaeal populations between adult and toddler conditions. Only one study reported a higher redox potential in children older than 3 years compared to adults, similarly to our in vitro results (Million and Raoult 2018). Lastly, we found similar total SCFA production by luminal microbiota in toddler and adult bioreactors. In vivo data differ according to toddler diet, with formula-fed infants showing higher total fecal SCFA concentrations than breast-fed ones (Bridgman et al. 2017). However, total fecal SCFAs in breast-fed infants were reported to be comparable to adult levels, which corroborates our in vitro results where 4 out of the 5 toddler donors were breast-fed (Baxter et al. 2019). Changes from toddler to adult in the proportions of the three main SCFAs are notably in accordance with in vivo data, with higher acetate and lower levels of propionate and butyrate (den Besten et al. 2013; Bernal et al. 2013; Maldonado-Lobón et al. 2015; Roduit et al. 2019; Differding et al. 2020; Nilsen et al. 2020; Pekmez et al. 2020; Tsukuda et al. 2021). Again, these variations in SCFA profiles are clearly associated with gut microbial structure evolution in M-ARCOL, with for instance increase in *Bifidobacteriaceae* which are well-known acetate producers (Mattarelli and Sgorbati 2018), while butyrate producers *Lachnospiraceae* and *Clostridiaceae* were lower in toddlers (Vital et al. 2017; Appert et al. 2020). Finally, we performed metabolic pathway prediction from metabarcoding data and found changes in predicted microbial nutrient metabolism from toddler to adult that are in accordance with in vivo gut physiology, such as a lower carbohydrate and glycan

Table 2 In vitro-in vivo comparisons of relative changes in gut microbiota structure and metabolic activity between toddler and adult

a	Microbial populations	In vitro data		In vivo data consistency	In vivo references (data from stools)
		VS			
		Luminal	Mucosal		
MICROBIOTA COMPOSITION	<i>Archaea</i>	↘*	↘*	★★★	Fiedorek et al. 1990; Rutili et al. 1996; Mihajlovski et al. 2010; Roswall et al. 2021
	<i>p_Verrucomicrobia</i>	=*	↗*	★★	Koenig et al. 2011; Levin et al. 2016; Differding et al. 2020
	<i>f_Akkermansiaceae</i>	=	=	★★	Bergström et al. 2014; Yassour et al. 2016
	<i>p_Fusobacteria</i>	↗*	↗*	ND	ND
	<i>f_Fusobacteriaceae</i>	↗*	↗*	★	Laursen et al. 2016
	<i>p_Bacteroidota</i>	=	↗	★★	Hong et al. 2010; Koenig et al. 2011; Bergström et al. 2014; Laursen et al. 2016; Bokulich et al. 2016; Davis et al. 2016; Hill et al. 2017; de Muinck and Trosvik 2018; Differding et al. 2020; Wang et al. 2021
	<i>f_Prevotellaceae</i>	↘*	↘*	★	Bergström et al. 2014
	<i>f_Rikenellaceae</i>	↘*	↘*	★	Laursen et al. 2016
	<i>f_Tannerellaceae</i>	↗*	↗*	ND	ND
	<i>f_Barnesiellaceae</i>	=	↗*	ND	ND
	<i>f_Marinifilaceae</i>	=	↘*	ND	ND
	<i>f_Chitinophagaceae</i>	=	↗*	ND	ND
	<i>p_Firmicutes</i>	=	=	★	Hong et al. 2010; de Morais Tureli et al. 2010; Koenig et al. 2011; Bergström et al. 2014; Laursen et al. 2016; Bokulich et al. 2016; Davis et al. 2016; Hill et al. 2017; de Muinck and Trosvik 2018; Differding et al. 2020; Wang et al. 2021
	<i>f_Clostridia family</i>	=	↘*	ND	ND
	<i>f_Acutalibacteraceae</i>	↘*	↘*	ND	ND
	<i>f_Clostridia UCG014</i>	↘*	↘*	ND	ND
	<i>f_Clostridia CAG-74</i>	↘*	=	ND	ND
	<i>f_Christensenellaceae</i>	↘*	↘*	ND	ND
	<i>f_Anaerovoracaceae</i>	↘*	↘*	ND	ND
	<i>f_Anaerotignaceae</i>	↘*	↘*	ND	ND
	<i>f_Acidaminococcaceae</i>	↘*	↘*	ND	ND
	<i>f_Peptostreptococcaceae</i>	↗*	↗*	★★★	Levin et al. 2016; Appert et al. 2020
	<i>f_Monoglobaceae</i>	↗*	↗*	ND	ND
	<i>f_Oscillospiraceae</i>	↘*	↘*	ND	ND
	<i>f_Oscillospirales UCG-010</i>	=	↘*	ND	ND
	<i>f_Eubacteriaceae</i>	↗*	=	★	Levin et al. 2016
	<i>f_Christensenellaceae</i>	↗	↗	★	Roswall et al. 2021
	<i>f_Clostridiaceae</i>	=	↗*	★★★	Mariat et al. 2009; Bokulich et al. 2016; Yassour et al. 2016; Appert et al. 2020
	<i>f_Butyricocccaceae</i>	=	↘*	ND	ND
	<i>f_Dialisteraceae</i>	↗*	↗*	ND	ND
<i>o_Lactobacilliales</i>	NO	NO	★★★	Ahrne et al. 2005; Mariat et al. 2009; Bergström et al. 2014; Levin et al. 2016; Nilsen et al. 2020	



metabolism in toddlers (Koenig et al. 2011; Vallès et al. 2014; Bäckhed et al. 2015; Wang et al. 2021). Microbiota pathways related to digestive, endocrine, immune, and nervous systems were also lower in toddler compared to adult

conditions in M-ARCOL, probably reflecting the immaturity of toddler gut microbiome.

Taken together, our results show that the Tm-ARCOL model can capture various changes in microbial composition

Table 2 (continued)

<i>p_Proteobacteria</i>	↘	=	★★	Hong et al. 2010; de Morais Tureli et al. 2010; Koenig et al. 2011; Davis et al. 2016; Hill et al. 2017; Differding et al. 2020; Huey et al. 2020; Wang et al. 2021
<i>f_Rhodospirillales family</i>	↘*	↘*	ND	ND
<i>f_Enterobacteriaceae</i>	=	↗*	★★★	Bergström et al. 2014; Laursen et al. 2016; Bokulich et al. 2016; Yassour et al. 2016; Levin et al. 2016; Hill et al. 2017
<i>f_Comamonadaceae</i>	=	↗*	ND	ND
<i>f_Burkholderiaceae</i>	↘*	=	ND	ND
<i>p_Actinobacteria</i>	↗	↗	★★★	Hong et al. 2010; Koenig et al. 2011; Koenig et al. 2011; Davis et al. 2016; Hill et al. 2017; de Muinck and Trosvik 2018; Differding et al. 2020; Wang et al. 2021
<i>f_Bifidobacteriaceae</i>	↗*	↗*	★★★	Mariat et al. 2009; de Morais Tureli et al. 2010; Bergström et al. 2014; Laursen et al. 2016; Bokulich et al. 2016; Yassour et al. 2016; Hill et al. 2017; Nilsen et al. 2020; Wang et al. 2021
<i>p_Actinomycetota</i>	=	=	ND	ND
<i>f_Coriobacteriaceae</i>	=	↗*	★	Levin et al. 2016; Roswall et al. 2021
α-diversity	↘*	↘*	★★★	Mariat et al. 2009; Hong et al. 2010; Koenig et al. 2011; Vallès et al. 2014; Bäckhed et al. 2015; Laursen et al. 2016; Bokulich et al. 2016; Hill et al. 2017; Differding et al. 2020; Appert et al. 2020; Nilsen et al. 2020; Roswall et al. 2021; Wang et al. 2021

b	Parameters	In vitro data  VS 	In vivo data consistency	In vivo references (data from stools)
	MICROBIOTA ACTIVITY	GAS PROPORTION		
Total		↘*	ND	ND
CH ₄		↘*	★★★	Hudson et al. 1993; Tormo et al. 2001
CO ₂		↘	ND	ND
H ₂		↗	ND	ND
SHORT CHAIN FATTY ACID CONCENTRATION				
Total		=	★	Høverstad et al. 1984; Bernal et al. 2013; Bridgman et al. 2017; Baxter et al. 2019
Acetate		↗*	★★★	Bernal et al. 2013; Maldonado-Lobón et al. 2015; Roduit et al. 2019; Pekmez et al. 2020; Differding et al. 2020; Nilsen et al. 2020; Tsukuda et al. 2021
Propionate		↘*	★★★	
Butyrate		↘*	★★★	
OTHERS				
Redox potential		↗*	★	Million and Raoult 2018

Differences obtained in vitro between toddler and adult conditions for microbial populations (a) and microbiota activity (b) were compared to in vivo data changes in stools between toddler and adult. In vivo data consistency was indicated from low (★) to high (★★★) relevance. This indicator was based on the number of available studies and the consistency of results between these studies. ↗ means higher level of the considered parameter in toddler versus adult in vitro while ↘ means lower level of the considered parameter and = no difference. (*) next to arrows indicates significance of in vitro results. A color code was used to indicate the concordance between in vitro and in vivo data, from high (green) to medium (orange) and low (red) concordance

ND no data (gray)

and activity associated with toddler to adult transition. This in vitro model shows the substantial advantage to distinguish luminal from mucus-associated microbiota profiles and to retain fine-scale microbiome inter-individuality. Notably, a

gender effect was observed for in vitro gut microbiota profiles, especially marked in toddlers, even if the number of subjects in this study was too low to definitively conclude. The Tm-ARCOL also maintained clear differences between

ages, with the youngest toddler #2 (9 months) showing distinct metabolic profiles compared to the four other toddlers. In the near future, this *in vitro* model can be advantageously used as an alternative to *in vivo* assays to test various nutritional (e.g., nutrients, milk formula, prebiotic, probiotic, post-biotic) or pharmaceutical (drug including antibiotics) strategies, study enteric pathogens, or evaluate food contaminants in a pediatric context (Fournier et al., 2021). Despite some limitations, such as the lack of host interactions (e.g., no immune, hormonal, or nervous systems) and feedback mechanisms, the Tm-ARCOL emerges as a powerful platform to increase our mechanistic understanding of the interrelationships between the infant gut microbes and nutrients, drugs, xenobiotics, or pathogens. Of great interest, by capturing inter-individual variations in gut microbes, this *in vitro* model allows the possibility to shift toward personalized nutrition or medicine.

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Author contribution EF and AD performed the literature research on toddler colonic environment. EF, SD, AD, LEM, and SBD selected and designed the toddler model parameters. EF performed the experiments and analyzed the data. EF, TVdW, MA, MMB, LEM, and SBD interpreted data. LEM, MMB, and SBD supervised the work. EF, LEM, and SBD wrote the manuscript with input from co-authors. All authors critically revised and approved the manuscript.

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Data availability Raw data are available at NCBI under the Sequence Read Archive database in the BioProject no. PRJNA830641.

Declarations

Ethics approval This study is a non-interventional study with no additions to usual clinical cares. The protocol does not require approval from an ethics committee according to the French Public Health Law (CSP Art L 1121–1.1).

Conflict of interest Tom Van de Wiele is advising ProDigest as a science officer and is a member of the scientific advisory board of MRM Health. Stéphanie Blanquet-Diot is advising NexBiome as a science officer. The other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be considered as a potential conflict of interest.

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Chapter IV - Exposure to polyethylene microplastics alters immature gut microbiome in an infant *in vitro* gut model

Toxicological studies investigating the potential impact of MPs on infant health remain sparse, contrarily to the more abundant literature in adult conditions with our own results in the M-ARCOL set-up, as presented in Chapter II. However, already in the mother's womb, fetus seems to be exposed to MPs as they were detected in the placenta and meconium from newborns. Importantly, there is to date no study that has investigated the bi-directional interactions between MPs and the immature and developing infant microbiome. After birth, infants may also be exposed through dust breathing and ingestion during roaming, but also during bottle feeding, as both milk and feeding bottles contain MPs. Silicone-rubber baby teats suckling represents an additional exposure source as their steam sterilization conducts to MP release. In this context, this study investigated for the first time the interactions of virgin PE MPs with infant gut microbiota (from 6 months to 3 years, four different donors) under realistic infant colonic conditions simulated by the Tm-ARCOL model, as presented in Chapter III. Similarly to the approach implemented for adult conditions in Chapter II, potential indirect effects of gut microbe metabolites after PE MP exposure were evaluated on co-culture of Caco-2 and HT29-MTX cells, which were exposed to Tm-ARCOL luminal supernatants before and after exposure.

Exposure to polyethylene microplastics alters immature gut microbiome in an infant *in vitro* gut model

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Research Paper

Exposure to polyethylene microplastics alters immature gut microbiome in an infant *in vitro* gut model

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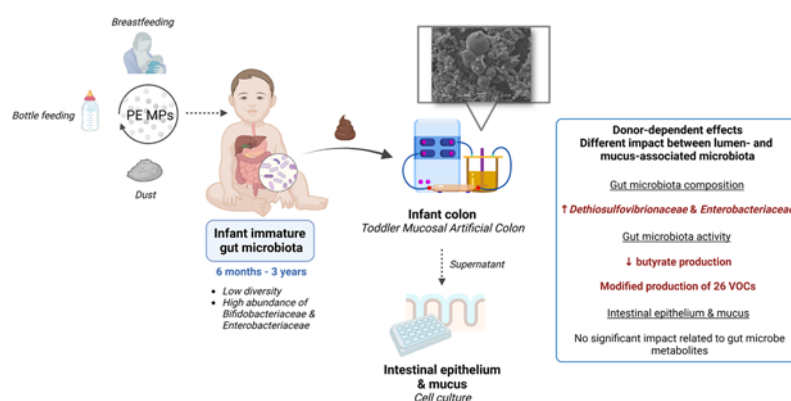
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HIGHLIGHTS

- Infants are an at-risk population for oral exposure to MPs.
- Innovative coupling of toddler *in vitro* fermentation model with intestinal cell culture.
- Chronic exposure to PE MPs induced donor-dependent shifts in infant immature microbiota.
- Potentially harmful pathobionts increased after exposure to PE MPs.
- Butyrate, a health-associated gut microbiota derived metabolite, was decreased.

GRAPHICAL ABSTRACT



Abbreviation: AhR, aryl hydrocarbon receptor; ASV, amplicon sequence variant; DSC, differential scanning calorimetry; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GC-MS, gas chromatography-mass spectrometry; HRP, horseradish peroxidase; HPLC, high-performance liquid chromatography; LDPE, low density polyethylene; LY, lucifer yellow; MP, microplastic; MUC2, mucin 2; MUC5AC, mucin 5AC; M-ARCOL, mucosal artificial colon; OCLN, occludin; Papp, apparent permeability coefficient; PE, polyethylene; PET, polyethylene terephthalate; PS, polystyrene; qPCR, quantitative polymerase chain reaction; SCFA, short chain fatty acid; SPME, solid-phase microextraction; TEER, trans-epithelial electrical resistance; TGA, thermogravimetric analysis; TJ, tight junction; Tm-ARCOL, toddler mucosal artificial colon; VOC, volatile organic compound; ZO-1, zonula occludens-1.

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ABSTRACT

Infants are characterized by an immaturity of the gut ecosystem and a high exposure to microplastics (MPs) through diet, dust and suckling. However, the bidirectional interactions between MPs and the immature infant intestinal microbiota remain unknown. Our study aims to investigate the impact of chronic exposure to polyethylene (PE) MPs on the gut microbiota and intestinal barrier of infants, using the new Toddler mucosal Artificial Colon coupled with a co-culture of epithelial and mucus-secreting cells. Gut microbiota composition was determined by 16S metabarcoding and microbial activities were evaluated by gas, short chain fatty acid and volatolomics analyses. Gut barrier integrity was assessed *via* evaluation of intestinal permeability, inflammation and mucus synthesis. Exposure to PE MPs induced gut microbial shifts increasing α -diversity and abundance of potentially harmful pathobionts, such as *Dethiosulfovibrionaceae* and *Enterobacteriaceae*. Those changes were associated to butyrate production decrease and major changes in volatile organic compounds profiles. In contrast, no significant impact of PE MPs on the gut barrier, as mediated by microbial metabolites, was reported. For the first time, this study indicates that ingestion of PE MPs can induce perturbations in the gut microbiome of infants. Next step would be to further investigate the potential vector effect of MPs.

1. Introduction

Plastic is a multifunctional material, easy to process, cheap and resistant. These valuable properties have led to massive plastic production, which began in the 1950's and reaching 367 million tons in 2020 (PlasticsEurope, 2021). Various types of plastic are produced around the world, but the most widely manufactured polymer is polyethylene (PE) (PlasticsEurope, 2021). Plastic materials are gradually accumulating in all ecological environments, which leads to major environmental threats (Wang et al., 2021a; Vázquez and Rahman, 2021; Prata et al., 2021). Subject to weathering and (bio)degradation in the environment, plastic waste releases large amounts of smaller particles that could be sorted by size as microplastics (MPs), which are particles ranging from 1 μ m to 5 mm (Frias and Nash, 2019).

The ubiquity of MPs throughout the food chain has raised concerns about their potential health effects following ingestion in humans (Lehel and Murphy, 2021; Gouin et al., 2020; van Raamsdonk et al., 2020; Provencher et al., 2020), since MPs have been detected in blood (Leslie et al., 2022), colon (Ibrahim et al., 2021) and stools (Zhang et al., 2021a, 2021b; Schwabl et al., 2019). Already in the mother's womb, the fetus seems to be exposed to MPs as they have been detected in the placenta and meconium of newborns (Ragusa et al., 2021; Braun et al., 2021). After birth, infants are highly exposed through breathing and ingesting dust during roaming (Zhu et al., 2022), but also during breastfeeding (Ragusa et al., 2022) or bottle feeding, since milk (Kutralam-Muniasamy et al., 2020; Da Costa Filho et al., 2021) and baby bottles (Li et al., 2020a, 2021) are contaminated with MPs. In addition, the suckling of silicone rubber represents a source of exposure as steam sterilization leads to the release of MPs (Su et al., 2021). Furthermore, a recent study has detected a higher amount of polyethylene terephthalate MPs in American infant stools compared to adult ones (Zhang et al., 2021b).

Despite this critical exposure, toxicological studies investigating the potential impact of MPs on infant health remain rare (Song et al., 2021; Sripada et al., 2022). The embryotoxic effect of polystyrene (PS) nano- or micro-sized plastics seems to vary depending on the model used (Bosman et al., 2005; Bojic et al., 2020; Nie et al., 2021; Hua et al., 2022). Oral exposure to PS MPs also induced fecal microbiota dysbiosis and gut barrier dysfunction in dams, while offspring showed a higher risk of metabolic disorders (Luo et al., 2019a, 2019b). Importantly, no studies have investigated to date the bidirectional interactions between ingested MPs and the immature and developing infant microbiome, despite its key role in gut homeostasis. Indeed, during the first 1000 days of life, while the digestive physiology is still under development, the infant intestinal microbiota (which mainly colonizes the colonic compartment) simultaneously undergoes significant maturation in terms of composition and metabolic activity (Yatsunencko et al., 2012; Fournier et al., 2021; Bourlieu et al., 2014). The gut microbiome acts primarily in the degradation of undigested food, resulting in the

production of secondary metabolites such as gases, short-chain fatty acids (SCFAs) (Kalantar-Zadeh et al., 2019; Morrison and Preston, 2016), volatile organic compounds (VOCs) (Raman et al., 2013) and aryl hydrocarbon receptor (AhR) ligands (Lamas et al., 2018). SCFAs (i.e., butyrate, propionate and acetate) are fermentation byproducts of non-digestible dietary fibers under the action of intestinal bacteria, whereas AhR ligands are primarily derived from tryptophan metabolism tryptophan. Both groups of metabolites are involved in maintaining the integrity of intercellular tight junctions in the intestinal epithelium, thus linking gut microbial activity to the maintenance of effective intestinal barrier function. In addition, the gut microbiota plays an important role in the formation of host immunity, in the regulation of gut endocrine and neurological functions, and together with the intestinal mucus and epithelium, it forms a protective barrier against exogenous threats such as pathogens and foreign particles (Andoh, 2016; Birchenough and Johansson, 2020; Claus et al., 2016).

In this context, as an extension of our previous study on the adult microbiota (Fournier et al., 2022a), we investigated for the first time the impact of oral exposure to PE MPs on the infant gut microbiome and the intestinal epithelium/mucus barrier. Due to major ethical constraints associated to clinical studies on young populations, but also the striking differences between human and rodent digestive physiology -including gut microbiota- (Hugenholtz and de Vos, 2018), these experiments were conducted using an innovative *in vitro* approach, combining the Toddler mucosal Artificial Colon (Tm-ARCOL, (Fournier et al., 2022b)) simulating the colonic ecosystem specific to infants (6 months to 3 years) and a co-culture of intestinal epithelial and mucus-secreting goblet cells (Caco-2 and HT29-MTX cells, respectively). After a 2-week daily exposure to PE MPs, the structure and metabolic activities of the infant microbiome were determined by 16S metabarcoding and measurements of gas/SCFAs/VOCs/AhR activity, respectively. Fecal water was then applied to co-cultures of intestinal cells to assess the impact of gut microbial metabolites on cell-related inflammatory pathways, mucin synthesis and barrier function.

2. Materials and methods

2.1. Fecal sample collection and treatment

Fecal samples were collected from four healthy infants (two boys aged 30 and 20 months -Toddlers 1 and 3, and two girls aged 25 and 22 months -Toddlers 2 and 4) were collected. All donors had no history of antibiotic treatment or probiotic consumption during the month prior sample collection. Fecal samples were processed as previously described (Fournier et al., 2022a). In an anaerobic chamber (COY laboratories, USA), 11 g of each fresh stool sample were suspended into 110 mL of 30 mM sterile sodium phosphate buffer and filtered (500 μ m stainless steel sieve).

2.2. Description and set-up of the colonic Tm-ARCOL model

The Tm-ARCOL is a one-stage fermentation system (MiniBio, Applikon, The Netherlands), operated under continuous conditions, which simulates both physicochemical (pH, retention time, ileal effluents supply and anaerobiosis) and microbial conditions (lumen and mucus associated microbiota) encountered in the colon of a healthy toddler based on *in vivo* data (Fournier et al., 2022b). This model is composed of a main bioreactor (luminal compartment) connected to an external glass compartment (mucosal compartment) containing mucin-alginate beads (Fig. 1a). Briefly, the composition of the ileal effluents was adjusted to reflect the specificities of the infant diet (Fig. 1a). The temperature of the main bioreactor and mucin-alginate bead compartment was maintained at 37 °C, the average retention time was set at 19 h and the pH was maintained at a constant value of 6.1 by addition of 2 M NaOH. After an initial N₂ flush upon introduction of the fecal inoculum, the anaerobiosis was maintained inside the bioreactor by the activity of the resident microbiota alone, in order to faithfully reproduce the *in vivo* situation.

2.3. Experimental design and sampling during *in vitro* fermentations

PE MPs (CPMS-0.96, 1–10 µm – 0.2 g; non-fluorescent particles) were purchased from Cospheric (CA, USA) and previously characterized (Fournier et al., 2022a). Four Tm-ARCOL bioreactors were first inoculated with fecal samples from the four infant donors and operated in parallel. Fermentations were then conducted under continuous conditions for 22 days, after a 24-h batch microbial amplification. An eight-day microbiota stabilization phase was applied and the three last days of this period (days 6, 7 and 8) were chosen as control. Thereafter, fourteen days of daily exposure to PE MPs (21 mg of MPs in 8 mL of a solution of deionized water containing 0.01% (w/v) Tween 80) were carried out and the three last days (days 20, 21 and 22) were chosen as exposure days. Samples from the main bioreactors, termed luminal microbiota, were collected daily for microbiome characterization (storage at –80 °C) and SCFA analysis (storage at –20 °C). Additional samples from the mucin-alginate bead compartment, termed mucosal microbiota, were collected every other day (when mucin-alginate beads were replaced) for microbiota characterization. Beads were washed twice in sterile phosphate buffer saline -PBS- (pH 7.1) and stored at –80 °C before downstream analyses. The medium surrounding mucin-alginate beads was also collected at the same times in case of insufficient DNA amounts on the mucin-alginate beads. Additional luminal fermentation media were collected on day 8 (control) and day 22 (exposure) for cell culture experiments, AhR activity measurement, scanning electron microscopy, Raman spectroscopy and volatolomic analysis. Samples were also collected daily from the atmospheric phase of the bioreactors to verify anaerobic conditions and determine gas composition. The daily extra volume of gas produced by microbial fermentation was also measured using a syringe connected to the gas bag. The experimental design and sampling are summarized in Fig. 1b.

2.4. DNA extraction

Genomic DNA was extracted from luminal samples and mucin-alginate beads (or surrounding medium) using the QIAamp Fast DNA Stool Mini Kit (12830–50, Qiagen, Germany) following the manufacturer's instructions with minor adjustments as previously described in (Fournier et al., 2022a; Capone et al., 2013). Samples were stored at –20 °C before gut microbiota analysis (qPCR and 16S metabarcoding).

2.5. Total bacteria quantification by qPCR

Total bacteria were quantified by qPCR using primers BAC338R and BAC516F with an hybridization temperature set at 58 °C (Yu et al., 2005) (Table 1). Real-time PCR assays were performed on a Biorad

CFX96TM Real-Time System (Bio-Rad Laboratories, USA) using the Takyon™ Low Rox SYBR® 2X MasterMix blue dTTP kit (B0701, Eurogentec, Belgium). Each reaction was performed in duplicate in a final volume of 10 µL with 5 µL of Master Mix, 0.45 µL of each primer (10 µM), 1 µL of DNA sample (10 ng/µL) and 3.1 µL of ultra-pure water. The amplifications were performed as follows: 1 cycle at 95 °C for 5 min, followed by 40 cycles at 95 °C for 30 s and 60 °C for 1 min. A melting step was added to ensure the specificity of the primers. The standard curve was generated from 10-fold dilutions of bacterial DNA as described in (Deschamps et al., 2020).

2.6. 16S metabarcoding and data analysis

The bacterial V3-V4 region of the 16S ribosomal DNA (rDNA) was amplified using primers V3_F357_N and V4_R805 for bacterial fraction and Arch349F and Arch806R for methanogenic Archaea. Amplicons were generated using a Fluidigm Access Array followed by high-throughput sequencing on an Illumina MiSeq system performed at the Carver Biotechnology Center of the University of Illinois (Urbana, USA).

Bioinformatics analysis was performed by GeT-Biopuces platform (INSA/Toulouse Biotechnology Institute, Toulouse, France) with R software version 4.1.1 (2021–08–10) and rANOMALY package (Theil and Rifa, 2021) as previously described in (Fournier et al., 2022a). Taxonomic affiliation of all amplicon sequence variants (ASVs) was performed with *idtaxa* function from DECIPHER package version 2.20.0 (Murali et al., 2018) using SILVA release 138 (Quast et al., 2012) and GTDB bac120_arc122 (Parks et al., 2022) databases (60% bootstrap cut-off). To improve completeness of the taxonomic affiliation, alignments were carried out using BLAST (Altschul et al., 1990) (98% identity and coverage) on representative sequences of unassigned ASVs or assigned with incomplete taxonomy. A phylogenetic tree was constructed based on the representative ASVs sequences using functions from *phangorn* package version 2.7.1 (Schliep et al., 2017). α - and β -diversity indexes were calculated using the *diversity_alpha_fun* function from rANOMALY package (Theil and Rifa, 2021). The across-sample microbiota diversity was described using redundancy analysis (RDA) based on Bray-Curtis dissimilarities using *vegan* R-package version 2.5–7 (Oksanen et al., 2011). The impact of the different parameters analyzed (total gas and SCFA production, gas and SCFA composition, luminal or mucosal sample types, time and sex of donors) on dissimilarities among groups was evaluated using PERMANOVA permutation tests (999 permutations).

2.7. Gas and short chain fatty acid analysis

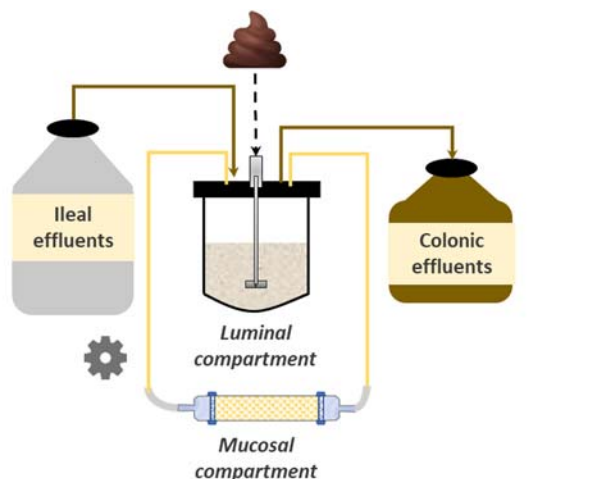
The analysis of O₂, N₂, CO₂, CH₄ and H₂ gases produced during the fermentation process in the atmospheric phase of the main bioreactor was performed using a HP 6890 gas chromatograph (Agilent Technologies, USA) coupled to a micro-TCD detector (Agilent Technologies, USA) as described in (Deschamps et al., 2020). For SCFAs, 2 mL of each luminal sample was centrifuged (5000 g, 15 min, 4 °C) and 900 µL of the supernatant was diluted at 1/10 in 0.04 M H₂SO₄ mobile phase, vortexed and filtered (pore size 0.22 µm). The three major SCFAs (acetate, propionate and butyrate) were quantified by high-performance liquid chromatography (HPLC) (Elite LaChrom, Merck HITACHI, USA) coupled to a DAD diode as described in (Deschamps et al., 2020). Data were obtained and analyzed by the EZChrom Elite software at 204 and 205 nm. SCFA concentrations were calculated from calibration curves established from solutions of known increasing concentration of acetate, propionate and butyrate (0, 10, 25 and 40 mM).

2.8. Measurement of AhR activity

The AhR activity of Tm-ARCOL luminal supernatants after exposure to PE MPs (day 22) relative to control conditions (day 8) was measured using a luciferase reporter assay method, as previously described (Lamas

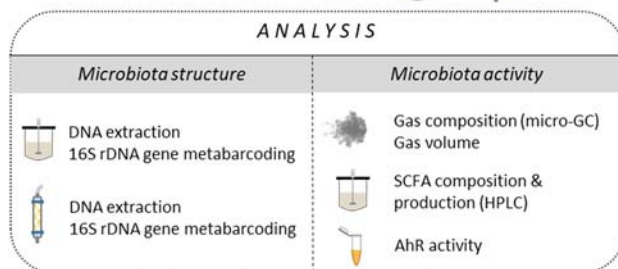
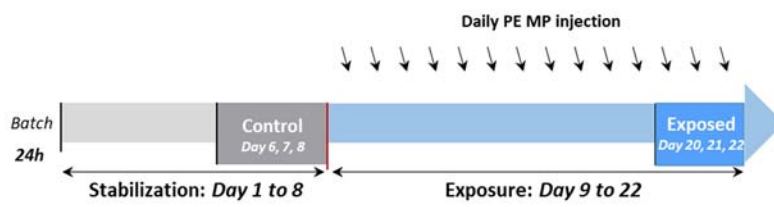
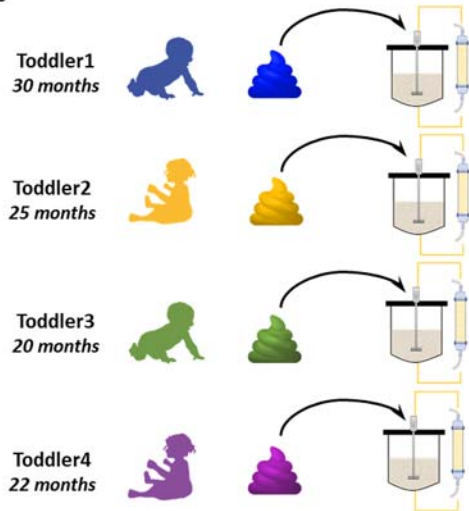
a

	Parameters	Tm-ARCOL model
PHYSICO-CHEMICAL PARAMETERS	Temperature (°C)	37
	Retention time (h)	19
	pH	6.1
	Volume (mL)	300
	Stirring speed (rpm)	400
NUTRITIVE MEDIUM	Total carbohydrates (g/L)	7.5
	Total proteins (g/L)	3.1
	Total Lipids (g/L)	2.3
	Total vitamins (µg/L)	28.5
	Total oligoelements (µg/L)	606.2
	Total bile salts (mg/L)	150



PE beads (spherical), 1-10 µm
21 mg of PE

b



c

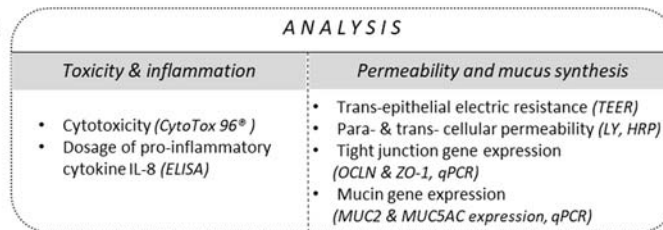
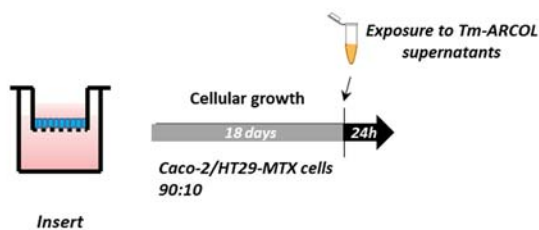


Fig. 1. Outline of *in vitro* fermentations and cell culture experiments. a) Schematic representation of the Tm-ARCOL gut model and the parameters used to reproduce the toddler colonic conditions. b) Tm-ARCOL experimental design and analysis. Four bioreactors inoculated with fecal samples from four different toddlers were run in parallel. After a stabilization phase, PE MPs were added daily into the bioreactor at a dose of 21 mg for 14 days. The last three days of the stabilization period (days 6, 7, 8) were chosen as the control while the last three days of the treatment period (days 20, 21, 22) were selected as exposed days. Atmospheric and luminal fermentation media were sampled daily while mucin-alginate beads were collected every other day for further analysis of gut microbiota structure or activity. c) Cell culture experimental design and analysis. Caco-2 and HT29-MTX intestinal cells in co-culture were seeded using inserts. After an 18-day growth period, cells were exposed during 24 h to day 8 or day 22 luminal supernatants of Tm-ARCOL and cytotoxicity, inflammation, mucin gene expression and intestinal permeability were evaluated. AhR: Aryl Hydrocarbon Receptor; ELISA: Enzyme-Linked Immunosorbent Assay; GC: Gas Chromatography; HRP: Horse Radish Peroxidase; HPLC: High-Performance Liquid Chromatography; LDH: Lactate Dehydrogenase; LY: Lucifer Yellow; MP: Microplastic; MUC2: Mucin 2; MUC5AC: Mucin 5AC; OCLN: Occludin; PE: Polyethylene; SCFA: Short Chain Fatty Acid; TEER: Trans-Epithelial Electrical Resistance; ZO-1: Zonula Occludens-1.

Table 1

Primers used for qPCR for total bacteria load, Illumina sequencing for bacteria and Archaeal populations and RT-qPCR to monitor intestinal gene expression.

Gene	Primer sequence 5' – 3'	Target	Annealing temperature (°C)	References
Primers qPCR				
16 S	F-GTATTACCGCGGCTGCTG R-ACTCCTACGGGAGGCAG	Total bacteria	58	(Yu et al., 2005)
Primers 16 S for Illumina sequencing				
16 S	F-CCTACGGGNGGCWGCAG R-GACTACHVGGGTATCTAATCC	Bacteria		(Klindworth et al., 2013)
16 S archaea	F-GYGCASCAGKCGMGAAW R-GGACTACVSGGTATCTAAT	Archaea	–	(Takai et Horikoshi, 2000)
Primers RT qPCR				
Gene	Primer sequence 5' – 3'	Target	Annealing temperature (°C)	References
<i>GAPDH</i>	F-GTGAAGGACTCATGACCACAG R-GCAGGGATGATGTTCTGGAGAG	Housekeeping gene	60	This study
<i>OCLN</i>	F-CTGAAGCAAGTGAAGGGATCTG R-CCAACCATCTTCTTGATGTGTGA	Occludin	60	This study
<i>ZO-1</i>	F-AGCACAGTGCCTAAAGCTATTCC R-AAGATGGTCATACTGTGGTGGC	Zonula occludens 1	60	This study
<i>MUC2</i>	F-CAGTGCAGCATCCTCAA R-GCAGAAGCACTCACAGTC	Mucin 2	60	This study
<i>MUC5AC</i>	F-CACACATGGGAAGCTGAG R-TGGCAGTCAAAGAACAC	Mucin 5AC	60	This study

et al., 2016). H1L1.1c2 mouse hepatocellular carcinoma cells, containing a stably integrated dioxin response element-driven firefly luciferase reporter plasmid pGudLuc1.1, were seeded in 96-well plates at 10^5 cells/well and cultured as previously described in (Fournier et al., 2022a) before stimulation with the Tm-ARCOL supernatants for 24 h. After incubation, cell lysis was performed and the luciferase activity was measured after addition of luciferase reagent (Promega, France) using a luminometer (Tecan, Switzerland). The experiments were performed using three biological replicates and three technical replicates. All values were normalized for sample cytotoxicity using the Lactate Dehydrogenase Activity Assay (Promega, USA).

2.9. Volatolomic analysis

Volatile organic compounds (VOCs) from control (day 8) and exposed (day 22) luminal samples were analyzed by solid-phase microextraction (SPME) coupled with gas chromatography-mass spectrometry (GC-MS), as previously described (Fournier et al., 2022a; Defois et al., 2017, 2018). Provisional identification of VOCs was performed on the basis of mass spectra, by comparison with mass spectra libraries (Wiley Registry 12th Edition / NIST 2020), and of retention indices (RI), by comparison with published RI values and with those of our in-house database. The peak area of the tentatively identified compounds was determined for each of the targeted molecules using a mass fragment selected for its specificity and lack from co-elution.

2.10. Scanning electron microscopy analysis

The luminal and mucosal samples of Toddlers 2 and 3, one girl and one boy, were deposited on SEMPore filters (Jeol, Japan) and analyzed using a scanning electron microscope JSM-6060LV (Jeol, Japan) at 5 kV in high vacuum mode as recently described (Fournier et al., 2022a).

2.11. Raman spectroscopy mapping

The mapping of exposed luminal samples (day 22, Toddler 3) by Raman spectroscopy was conducted using a Raman spectrometer (Alpha300R Apyron, Witec, Germany) equipped with a confocal microscope, as previously described (Fournier et al., 2022a). The recorded signal (Raman signal plus background) was integrated over a 20 cm^{-1} spectral range of centered at 1294 cm^{-1} corresponding to the CH_2 twisting vibration band of PE. The signal value was then automatically transformed by the software in a color scale (from black to bright yellow).

2.12. Intestinal cell lines and cell culture conditions

Since no intestinal epithelial cell model is available for infant conditions, we used the human colon adenocarcinoma Caco-2 cell line, obtained from the European Collection of Cell Cultures (ECACC, UK) and used at passage 58–62. The mucus-secreting colon adenocarcinoma HT29-MTX cell line was kindly provided by Dr. Thécia Lesuffleur (INSERM Lille, France) and used at passage 10–16. Both cell lines were maintained in cell culture medium and split at 80% of confluence using Tryple express, as previously described (Fournier et al., 2022a). The intestinal cells (90% Caco-2/10% HT29-MTX) were seeded in 24-well inserts with $1\text{-}\mu\text{m}$ pore size (Millipore, Merck Millipore SAS, France) and maintained in the culture medium. From day 17, Fetal Bovine Serum (FBS) of the culture medium was replaced by 1% (v/v) Insulin Transferrin Selenium (ITS) in order to limit any interference with the samples to be tested. After 18 days, Caco-2/HT29-MTX co-cultures were incubated apically for 24 h with Tm-ARCOL luminal samples collected on day 8 (control) and day 22 (exposed), centrifuged (5000 g, 15 min, $4\text{ }^\circ\text{C}$) and diluted 20-fold in cell culture medium (Fig. 1c). Experiments were performed with 2 biological replicates and 5 technical replicates for each donor.

2.13. Cell viability

In order to determine the potential cytotoxic effect of Tm-ARCOL luminal supernatants on Caco-2/HT29-MTX cells and to establish sub-toxic conditions for testing on inserts, different dilutions (1/2, 1/5, 1/10, 1/20, 1/30 and 1/50 in cell culture medium) were evaluated. Cell viability was measured in triplicate with CytoTox 96® non-radioactive cytotoxicity test (Promega, France) after 24 h of exposure, as previously described (Fournier et al., 2022a).

2.14. Intestinal permeability

Starting on day 12, trans-epithelial electrical resistance (TEER) was continuously monitored in real time using the CellZScope2 device (NanoAnalytics, Germany). In addition, after a 24-h contact time with the 20-fold diluted luminal supernatants of Tm-ARCOL, the inserts were transferred to a new 24-well plate containing 1 mL of Hank's balanced salt solution (HBSS). Lucifer yellow -LY- (0.4 mg/mL, molecular weight 457 g/mol) and horseradish peroxidase -HRP- (0.4 mg/mL, molecular weight 44,000 g/mol) were dissolved in HBSS and added to the apical compartment. After 2 h at $37\text{ }^\circ\text{C}$ under an atmosphere of 5% CO_2 /95% air, samples were taken from the apical and basolateral compartments.

For LY measurement, samples were analyzed in duplicate using a Spark multimode plate reader (Tecan, Switzerland). Excitation and emission wavelengths were 405 nm and 535 nm, respectively. Total HRP was determined by an ELISA assay as previously described (Fournier et al., 2022a). The apparent permeability coefficient (P_{app} in cm/s) was determined by the following equation: $P_{app} = C_b / (S \times C_0)$, where C_b represents the level of accumulation of LY or HRP in the basolateral compartment after 2 h, S represents the surface of the membrane (0.33 cm^2) and C_0 is the initial concentration of the marker (0.4 mg/mL -LY- or -HRP-) in the apical compartment.

2.15. Interleukin-8 level measurement

The apical and basolateral media of the co-cultures were collected after the contact time of 24 h with the 20-fold diluted luminal supernatants of Tm-ARCOL and stored at -20°C until analysis. Interleukin-8 (IL-8) levels were measured using the Quantikine Human IL-8 ELISA kit (DY208-05, R&D Systems, USA) following the manufacturer's instructions. Briefly, in Maxisorp 96-well plates (Nunc, France), biotinylated Goat polyclonal anti IL-8 was added at a concentration of 10 ng/mL before addition of HRP-conjugated streptavidin (Becton Dickinson, France) for 20 min. Absorbance was measured at 450 nm and 540 nm using a Spark microplate reader (Tecan, Switzerland).

2.16. Intestinal cell gene expression

Total RNAs were extracted from cells for each insert using the All-Prep RNA Mini kit (Qiagen, France) according to the manufacturer's instructions. Quantity and quality of extracted RNA samples were evaluated using Nanodrop (Nanophotometer Implen-Dutscher, France) and Bioanalyzer (Agilent Technologies, USA), respectively. RNAs were reverse transcribed using enzyme IScript reverse transcription supermix (Biorad, France) and amplified using a ViiA7 Real-Time PCR System (Applied Biosystems-ThermoFisher Scientific, France), as previously described (Fournier et al., 2022a). The primer sequences of the targeted genes (genes encoding tight junction (TJ) proteins (*ZO-1*, *OCLN*) and genes involved in mucus synthesis (*MUC2*, *MUC5AC*) are listed in Table 1. Raw data that passed quality control were analyzed with LinRegPCR (version 2021.2) and then normalized against the expression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), a reference housekeeping gene.

2.17. Statistical analysis

Statistical analyses on gut microbiota activity (gas, SCFAs, AhR activity), α -diversity indexes (number of observed ASVs and Shannon index) from metabarcoding data, as well as results from cell culture experiments, obtained in control and exposed groups, were performed using GraphPad Prism software version 9.3.1 for Windows (GraphPad Software, USA). Data normal distribution was verified by combining Anderson-Darling, D'Agostino & Pearson, Shapiro-Wilk and Kolmogorov-Smirnov tests and homoscedasticity was checked using the Fisher test. Then, appropriate statistical analysis was applied (either one-way ANOVA, Kruskal-Wallis or Welch's tests) and significance was considered for $p < 0.05$. For gut microbiota results, differential analyses (DESeq2, metagenomeSeq, metacoder) were performed on ExploreMetabar software using rANOMALY package (Theil and Rifa, 2021; Rifa and Theil, 2021). Principal coordinate analysis (PCoA) of unweight UniFrac distances was performed and significance between groups was assessed with a permutational multivariate analysis of variance (PERMANOVA) using ADONIS (999 permutations) with non-parametric tests. Volatolomics data were processed using the Statistica Software (v.13) (StatSoft, France). Student's t-tests ($p < 0.05$) were conducted for the VOC abundances determined in control (day 8) and exposed (day 22) groups and principal component analyses (PCA) were performed on the discriminant VOCs selected to visualize the structure of the data.

3. Results

3.1. Characteristics of PE MPs and interactions with the infant gut microbiota

As previously reported in Fournier et al., 50% of PE MPs were in a size range between 1 and $2 \mu\text{m}$, moreover differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA) analysis showed the high purity of the PE powder and characterized it as low-density PE (Fournier et al., 2022a). Scanning electron microscopy of samples collected in the luminal phase and mucosal beads in Tm-ARCOL (Fig. 2a) showed colonization of the surface of PE MPs by various bacteria. In addition, Raman spectroscopy mapping of PE MPs in the luminal medium (Fig. 2b) after exposure provided the same spectrum as the one obtained for the initial PE particles, indicating the absence of physico-chemical changes to the MP surface during fermentation in Tm-ARCOL.

3.2. Impact of exposure to PE MPs on the infant gut microbiota composition

The stool microbiota of toddlers used for Tm-ARCOL inoculation was characterized by 16S metabarcoding (Fig. S1) and showed an absence of methanogenic Archaea, and also bacterial profiles that are highly donor-dependent, particularly at the family level. Notably, the stool of Toddler 1 was characterized by a high abundance of *Tannerellaceae*, while *Enterobacteriaceae* and *Akkermansiaceae* were found mainly in Toddler 2 and *Sutterellaceae* and *Oscillospiraceae* in Toddler 4. Alpha-diversity was also donor-dependent (data not shown), with the highest values for Toddlers 1 and 4 (107 and 145 ASVs, respectively) and the lowest for Toddlers 2 and 3 (54 and 70, respectively). Some of these stool individual specificities were captured in Tm-ARCOL, such as *Tannerellaceae* in Toddler 1 and *Enterobacteriaceae* in Toddler 2 (Fig. 3a) and a clearly higher number of ASVs under control conditions for Toddler 4 relative to other toddlers (Fig. 4a). At the end of the stabilization phase in Tm-ARCOL (control), the bacterial abundance profiles at the family (Fig. 3a) and phylum (Fig. S2a) levels clearly indicated that the inter-individual variability observed in the initial donor stools was retained in the *in vitro* model, with the donor effect being the most important factor explaining luminal and mucosal β -diversity (Fig. S2b). These data also highlighted clear differences between lumen and mucus-associated microbiota and, as expected, their stability at the end of the stabilization phase. After 14 days of daily exposure to PE MPs, clear shifts in bacterial populations were observed both in the luminal and mucosal compartments, and both at the phylum (Fig. S2a) and family (Fig. 3a) levels. Those changes were again donor-dependent. For instance, exposure to PE MPs resulted in disappearance of *Verrucomicrobia* (*Akkermansiaceae*) in Toddler 2 whereas opposite trends were seen in Toddler 4, and *Synergistetes* (*Dethiosulfovibrionaceae*) increased only in Toddler 3. Exposure to PE MPs also induced in the luminal phase a disappearance in *Ruminococcaceae* in Toddlers 1 and 2, while *Rikenellaceae* and *Sutterellaceae* decreased in Toddlers 2 and 4. In addition, the population of *Tannerellaceae*, which was only present in Toddler 1, disappeared from the mucosal phase after exposure to MPs whereas *Enterobacteriaceae* increased in mucus-associated bacteria from Toddlers 2 and 4. Differential analysis of gut microbiota composition in all donors confirmed significant changes in bacterial populations. In the luminal phase, abundance of *Dethiosulfovibrionaceae*, *Planococcaceae*, *Enterobacteriaceae*, *Moraxellaceae*, *Acidaminococcaceae* and *Oscillospiraceae* increased significantly ($p < 0.05$, $n = 4$) after exposure to PE MPs, while the proportion of *Tannerellaceae*, *Rikenellaceae* and *Monoglobaceae* decreased (Fig. 3b).

Less populations were affected in the mucosal compartment, with an increased abundance of *Dethiosulfovibrionaceae*, *Planococcaceae*, *Acidaminococcaceae* and *Oscillospiraceae* but a disappearance of *Lachnospiraceae* and *Monoglobaceae* following exposure to PE MPs. The impact of such exposure on gut microbiota structure was confirmed by β -diversity

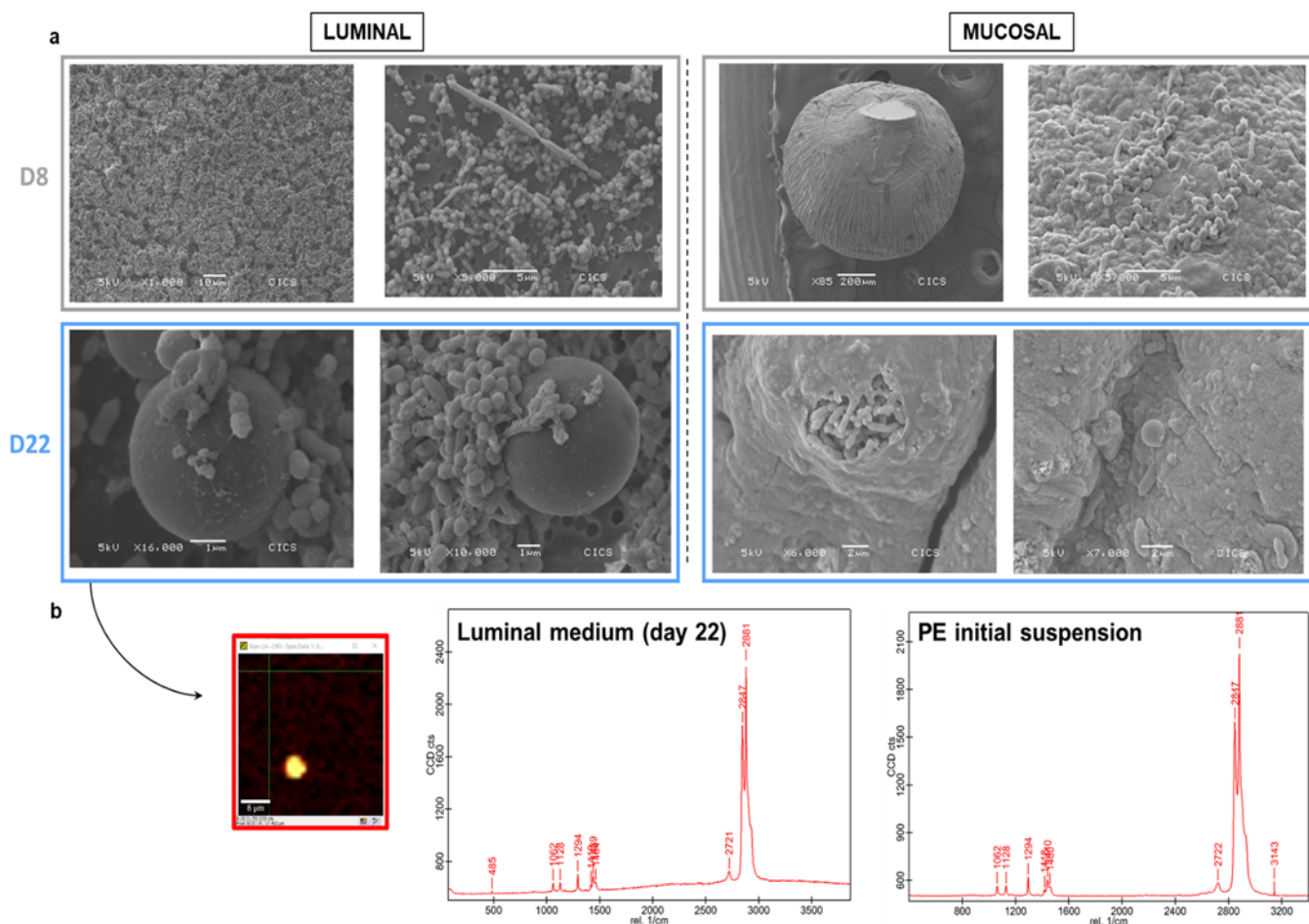


Fig. 2. Scanning electron microscopy observations of PE MPs/gut microbiota interactions in Tm-ARCOL and Raman spectroscopy analysis of PE MPs at the end of exposed period. a) Representative scanning electron microscopy images of luminal samples and mucin-alginate beads from Toddlers 2 and 3 before and after exposure to PE MPs. For the mucin-alginate beads, the analysis was performed after 48 h of colonization in the mucosal external compartment. The right panel shows a typical mucin-alginate bead and its colonization by gut microbiota (day 8). b) Example of Raman spectroscopy mapping of the surface of PE MP (the PE MP appears in yellow in the left image) at day 22 for luminal sample of Toddler 3; the Raman spectrum is compared with the one obtained with the initial suspension of PE MPs. PE: Polyethylene.

analysis, which showed clustering between control and exposed samples (Fig. 4c), with a greater impact in Toddlers 1, 2 and 3 compared to Toddler 4. Accordingly, MP treatment also resulted in a significant increase in observed ASVs ($p < 0.01$) in the luminal microbiota only for Toddlers 1, 2 and 3. Similarly, in mucosal samples, the number of observed ASVs tended to increase in Toddlers 1 and 2, and significance was reached in Toddler 3 ($p < 0.05$). Finally, RDA analysis of bacterial β -diversity, based on both 16S metabarcoding and metabolic activity data (see 3.3), indicated that exposure to PE MPs remains the most important parameter explaining variabilities.

3.3. Impact of exposure to PE MPs on the metabolic activities of human infant microbiota

In parallel to gut microbiota composition, the impact of exposure to PE MPs was also monitored *via* gut microbial activities, first by monitoring fermentation gas and SCFAs daily (Fig. S3). Similarly to the gut microbiota composition described above, this analysis showed stable gas and SCFA profiles for each donor at the end of the stabilization period, with the exception of Toddler 3 due to technical problems. Exposure to PE MPs had very limited impact on total gas and SCFA production (Figs. 5a and 5b). Regarding the production of major SCFAs (Fig. 5c), acetate tended to increase after exposure to PE MPs for Toddler 1, 2 and 3, while the opposite trend was observed for propionate. Butyrate

production dropped after exposure to PE MPs for all donors with significance reached only for Toddlers 1 ($p < 0.0001$) and 4 ($p < 0.05$), and when data were averaged ($p < 0.0001$, $n = 4$). The ability of the gut microbiota to produce ligands capable to activate AhR was also determined in Tm-ARCOL supernatants after exposure to PE MPs (day 22) and compared to control conditions (day 8) (Fig. 6). After exposure to PE MPs, luminal contents tended to have a lower ability to activate AhR in all donors except Toddler 2, suggesting a potential defect in the gut microbiota to produce metabolites known to activate this receptor which is involved in the regulation of intestinal homeostasis (Lamas et al., 2018; Agus et al., 2018). Finally, an additional analysis was performed on the microbial volatolome from the luminal phase (Fig. 7). 184 VOCs were detected in control and exposed samples (data not shown). The 26 discriminating VOCs between the two groups – PE MPs exposed *versus* control condition - (Table 2) are hydrocarbons and oxygenated compounds (alcohols, aldehydes, ketones and esters). Volatolomic profiles based on these VOCs indicated a profound effect of exposure to PE MPs (Fig. 7a). The abundances of the 8 most discriminant VOCs among these 26 VOCs ($p < 0.01$ Bonferroni corrected value) are shown in Table 2 and in Fig. 7b. In particular, the 6 hydrocarbon compounds (pentane, 3-methyl-; cyclopentane, 1,1,3-trimethyl-; 1-heptene, 2, 4-dimethyl-; heptane, 2,3-dimethyl-; methyl C9-alkane - RI 814, methyl C9-alkane - RI 821 and methyl C9-alkane - RI 861), two heptanone (2-heptanone and 2-heptanone, 4-methyl-), two alcohols

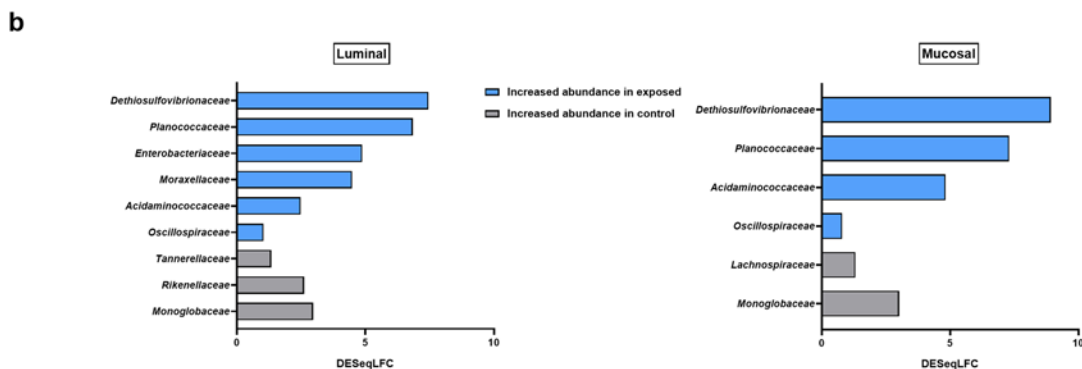
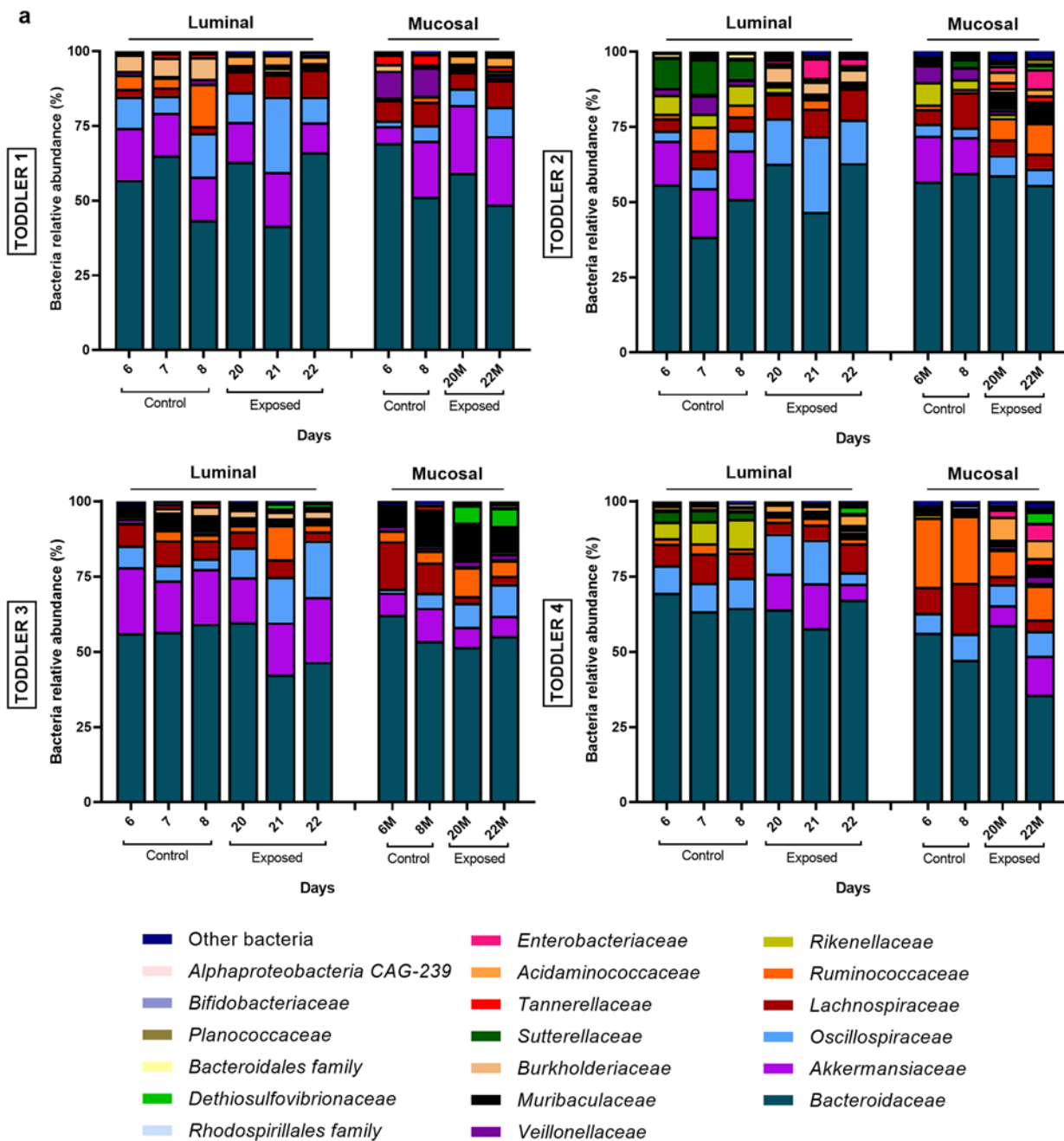


Fig. 3. Effect of chronic exposure to PE MPs on luminal and mucosal bacteria in Tm-ARCOL a) Relative abundance of the 20 main bacterial populations at the family level during the control and exposed periods for each donor. M represents the medium surrounding the mucin-alginate beads. b) Differential Analysis (DESeq2, metagenomeSeq, metacoder, $p < 0.05$) at the family level between control and exposed days ($n = 4$).

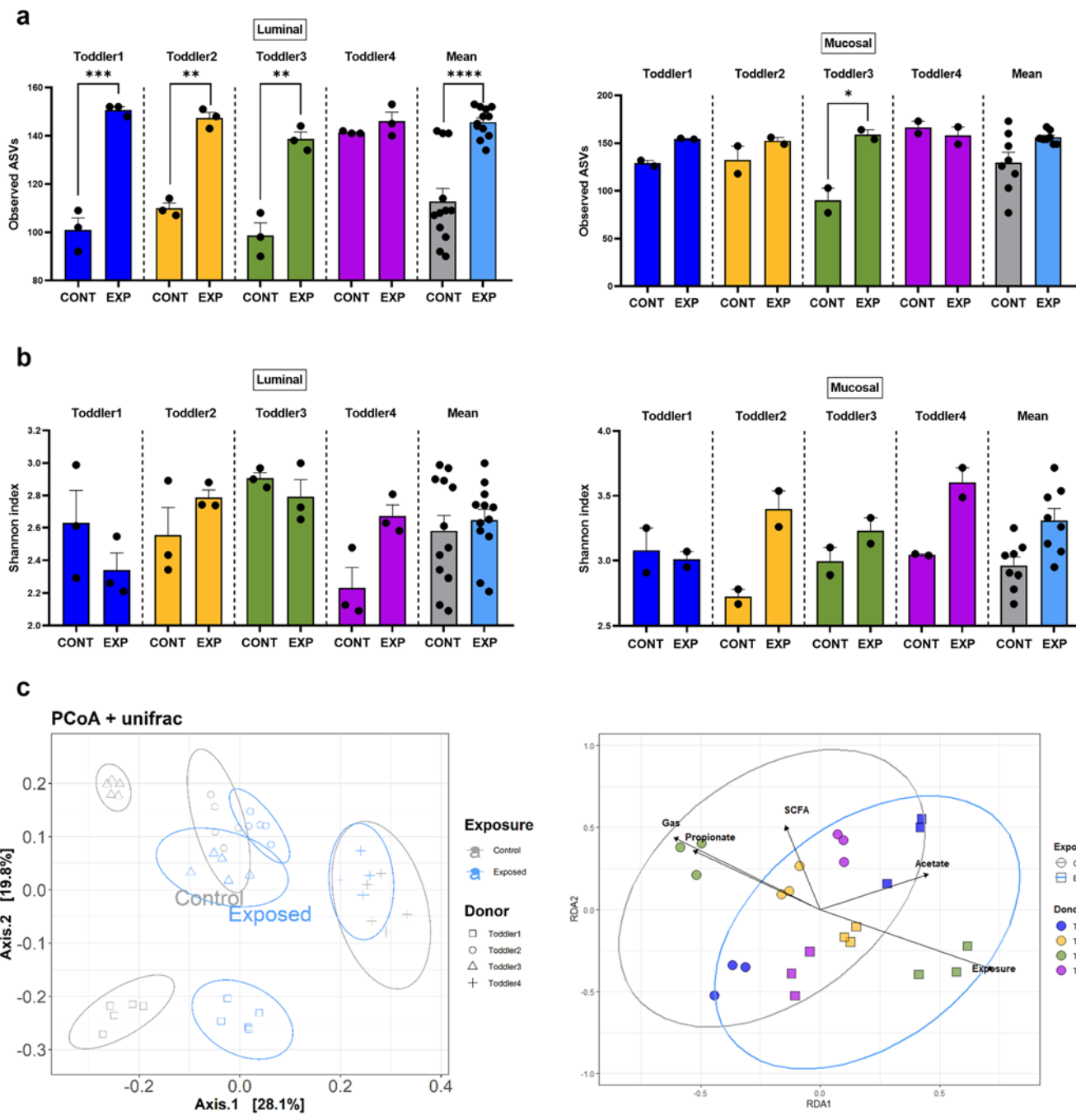


Fig. 4. Effect of chronic exposure to PE MPs on α - and β -diversity indexes of bacterial communities in Tm-ARCOL. Observed ASVs (a) and Shannon (b) indexes for each donor and on average ($n = 4$), expressed as means \pm SEM. Bacterial β -diversity (c) was also represented using a two-dimensional PCoA plot, based on the unweighted UniFrac distance matrix on luminal and mucosal samples (left) and a distance-based redundancy analysis (RDA), based on both 16S rDNA gene metabarcoding and metabolic activity data, applied to luminal samples and excluding the donor effect (right). Statistical differences were tested by one-way ANOVA with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

(1-hexanol and 1-heptanol) and two aldehydes (heptanal and octanal) were overproduced while 8 out of 12 esters (among which propanoic acid, ethyl ester and butanoic acid) were underproduced after exposure to PE MPs.

3.4. Impact of exposure to PE MPs on intestinal epithelium inflammation, permeability and mucin synthesis as mediated by gut microbiota metabolites

In order to explore whether alterations in the metabolic activities of the gut microbiota induced by exposure to PE MPs have consequences

on the intestinal barrier, we determined the impact of Tm-ARCOL luminal supernatants on Caco-2/HT29-MTX cells. The preliminary toxicity assay on intestinal cells with diluted Tm-ARCOL luminal supernatants showed no cytotoxicity for 1/20 and 1/50 dilutions (data not shown). Therefore, in the following cell experiments, the 1/20 (v/v) dilution was used. Various parameters were measured to assess the impact of Tm-ARCOL luminal supernatants on trans- and para-cellular permeability. Gene expression of tight junction proteins, *OCN* and *ZO-1*, was not significantly altered by Tm-ARCOL luminal supernatants of the exposed microbiota, although a clear decreasing trend was observed for both genes in Toddler 1 (Fig. S4a). Trans-epithelial electric

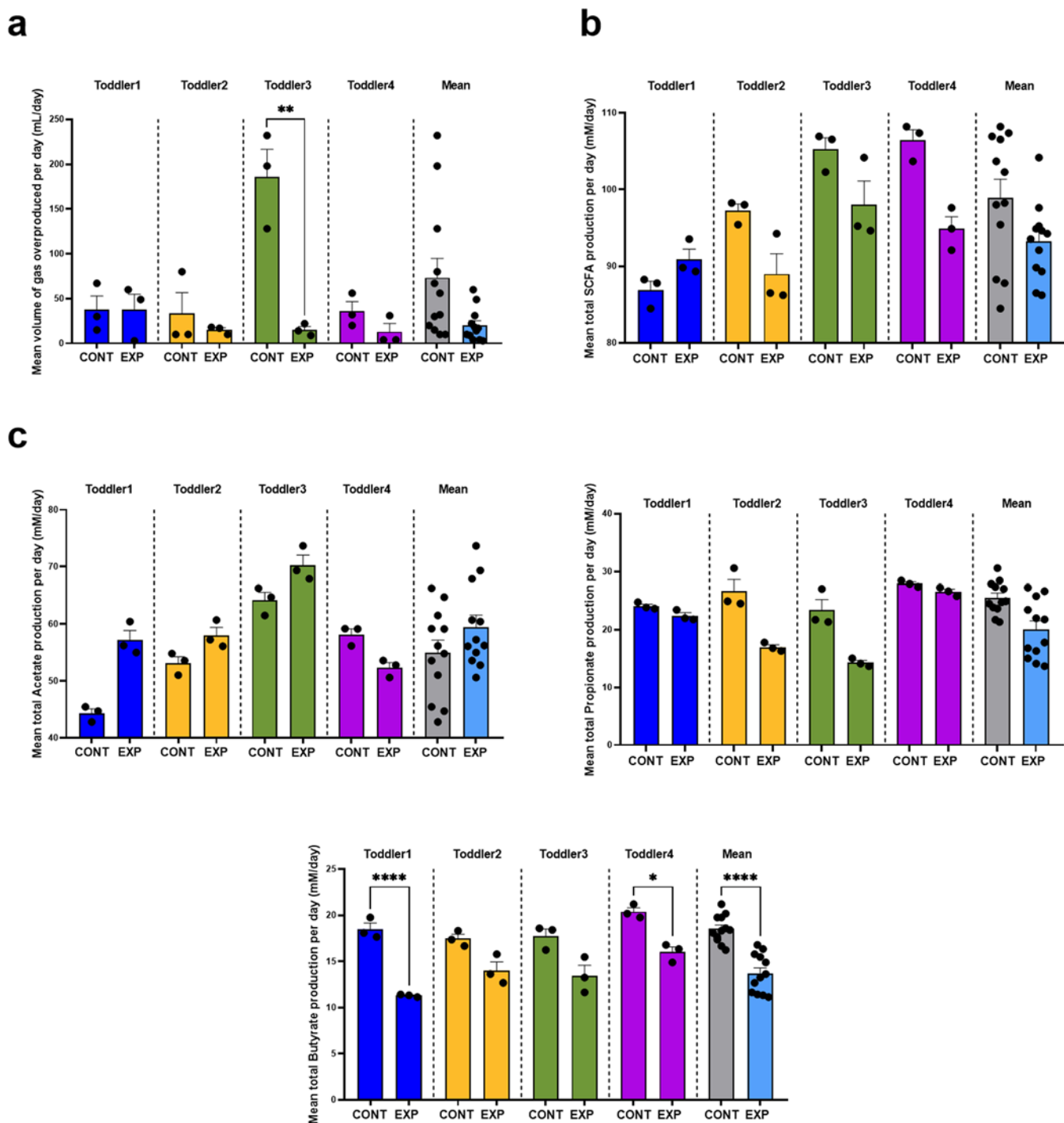


Fig. 5. Impact of chronic exposure to PE MPs on gas and short chain fatty acid production in Tm-ARCOL. a) Mean \pm SEM daily gas production in mL and (b) mean \pm SEM total SCFA production in mM, for each donor and in average ($n = 4$). c) Mean \pm SEM daily acetate, propionate and butyrate production in mM for each donor and on average ($n = 4$). Statistical differences were tested by one-way ANOVA with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$. CONT: Control; EXP: Exposed; SCFA: Short Chain Fatty Acid.

resistance (Δ TEER) (Fig. 8a) as well as the translocation of Lucifer yellow (LY) (Fig. 8b) and horseradish peroxidase (HRP) (Fig. 8b), as indicators of para- and trans-cellular permeability, respectively, were unaffected by Tm-ARCOL luminal supernatants of the exposed microbiota. Interestingly, however, HRP and LY translocation tended to increase in Toddler 1, consistent with decreased *ZO-1* and *OCN* gene expression. Finally, the expression of mucin-related genes, *MUC2* and *MUC5AC*, was measured without demonstrating any significant effect of Tm-ARCOL luminal supernatants of the exposed microbiota (Fig. S4b).

Nevertheless, *MUC2* gene expression tended to increase in 3 of the 4 donors (not detected in Toddler 4), while *MUC5AC* gene expression appeared to be slightly overexpressed in Toddlers 1 and 3 only. The inflammatory marker IL-8 (Fig. 8c) was also measured and the levels (70–100 pg/mL) detected in the apical and basolateral compartments, were not significantly impacted by the Tm-ARCOL luminal supernatants of the exposed microbiota.

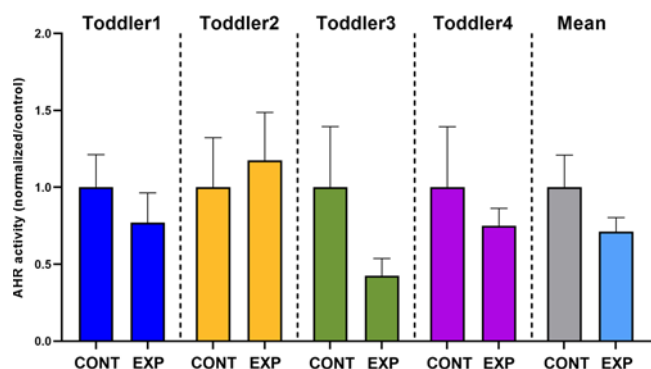


Fig. 6. Impact of chronic exposure to PE MPs on the capacity of Tm-ARCOL luminal supernatants to activate Aryl Hydrocarbon receptor. AhR activity is expressed as fold changes relative to luciferase activity of the control period and after normalisation based on sample cytotoxicity (lactate dehydrogenase activity assay). All results are given for each donor and on average ($n = 4$) and expressed as means \pm SEM. AhR: Aryl Hydrocarbon Receptor; CONT: Control; EXP: Exposed.

4. Discussion

Ingestion of MPs is increasingly recognized as a threat for human health. However, to date available data on the behavior of MPs throughout the human digestive tract are very limited, especially in the infant population. In this study, we showed for the first time, using the *in vitro* Toddler M-ARCOL colonic model (Fournier et al., 2022b), that oral exposure to PE MPs alters the composition of gut microbiota by increasing potentially pathogenic populations of *Dethiosulfovibrionaceae*, *Enterobacteriaceae*, *Oscillospiraceae* and *Moraxellaceae* while decreasing *Monoglobaceae*. These changes in microbial populations have been associated with alterations in metabolic activity, as shown by altered VOC profiles and decreased butyrate production.

As an alternative to clinical trials, most of the rare studies on the fate of MPs under human simulated digestive conditions have been conducted using *in vitro* models of the upper gastrointestinal tract, i.e. in gastric and small intestinal systems (Stock et al., 2019; Liu et al., 2020a; Krasucka et al., 2022; Tamargo et al., 2022). These studies demonstrated that artificial digestive fluids were not able to degrade MPs of five different polymers types (including PE) into smaller fragments or change their shape, size or texture, but described the formation of a biomolecular corona onto the surface of the particle surface (Stock et al., 2019; Liu et al., 2020a). Given this absence of physicochemical degradation of MPs in the upper gastrointestinal tract, in the present study we focused on interactions in the lower gut between PE MPs and gut microbiota, another major component of human gut homeostasis. Yet, only Tamargo and colleagues have studied interactions between human-derived gut microbiota and MPs, but using polyethylene terephthalate -PET- and not PE, also of different size and shape ($160 \pm 110 \mu\text{m}$, irregular morphology) (Tamargo et al., 2022). Our group also recently performed a study in the Mucosal Artificial Colon (M-ARCOL) where we assessed the impact of repeated exposure to PE MPs on the composition and activity of the adult gut microbiota (Fournier et al., 2022a). Here, as an extension of this work and in order to get as close as possible to the human situation, we have chosen to evaluate in Tm-ARCOL the effect of a physiological dose of MPs at 21 mg/day according to recent exposure data (Senathirajah et al., 2021). PE MPs were administrated daily for 14 days to reproduce chronic exposure, while Tamargo and colleagues only evaluated the effect of a single dose of 166 mg PET MPs for 72 h. Finally, their study was carried out in adult digestive conditions, while we have focused here on toddlers, because of the specificities of their digestive physiology and gut microbiota, which make them a high-risk population with regard to the ingestion of xenobiotics (Zhang et al., 2021a; Sripada et al., 2022; Ayeni et al., 2022). During the critical period of the first

1000 days, the gut microbiota of infants remains immature in terms of composition and functionalities (Fournier et al., 2021). This gut microbiota immaturity has been successfully reproduced in the Tm-ARCOL model (Fournier et al., 2022b), with lower microbial diversity, higher abundance of infant-related microbial populations (e.g. *Bifidobacteriaceae*, *Enterobacteriaceae*), higher acetate production and lower butyrate production in toddlers than in adults, consistent with *in vivo* data (Nilsen et al., 2020; Hill et al., 2017; Wang et al., 2021b). The repeated addition of PE MPs resulted in an increased α -diversity in Tm-ARCOL, as previously reported in mouse fecal microbiota by Li and colleagues after 5-week exposure to PE MPs of 10–150 μm in size (Li et al., 2020b). This could be explained by the fact that PE MPs can provide a surface for the formation of biofilm-like microbial communities, as observed here by scanning electron microscopy and as previously reported with PET MPs in another human colonic model (Tamargo et al., 2022). The presence of MPs as providers of adhesion sites but also new carbon sources for microbes has been already described in an environmental context (Zettler et al., 2013; Nanthini Devi et al., 2021; Chen et al., 2021). The second option does not seem feasible here since no degradation of MPs was observed using Raman spectroscopy analysis. Contrary to our results, Tamargo and colleagues reported a decrease in observed ASVs in the transverse and descending colonic compartments after a single addition of PET MPs (Tamargo et al., 2022), suggesting that the effect of MPs on the human gut microbiota diversity would be, in addition to the exposure scenario, plastic-dependent (i.e. polymer type, size, shape). In our previous study on the adult microbiota, no change in bacterial diversity was observed after exposure to PE MPs, suggesting that the immature infant gut microbiota of infants may be more sensitive to MPs than mature microbiota (Fournier et al., 2022a). When mice were exposed to PE MPs, the microbial shifts observed in fecal samples were study-dependent and not comparable to the changes observed in Tm-ARCOL (Li et al., 2020b; Djouina et al., 2022), certainly due to the large discrepancies between human and rodent digestion and microbiome (Hugenholz and de Vos, 2018). Interestingly, the human *in vitro* study by Tamargo and colleagues (Tamargo et al., 2022) on the exposure to PET MPs showed an increase in *Proteobacteria* and *Synergistetes* (phylum of *Dethiosulfovibrionaceae*) as observed in the present study, suggesting that these populations could be considered as biomarkers of exposure to MPs in the human gut microbiota. This hypothesis is reinforced by our recent work on the effect of PE MPs on the adult gut microbiota, which shows an increase in both luminal and mucus-associated microbiota, of *Enterobacteriaceae* and *Dethiosulfovibrionaceae* (Fournier et al., 2022a). For the first time, we also showed in Tm-ARCOL that the impact of MPs can be different between the lumen and mucus-associated microbiota. This is particularly interesting as mucosal microbes appear to play an increasing role in human health and diseases (Sauvatre et al., 2021 Etienne-Mesmin et al., 2019). In addition, the MPs size seems to have a major role on gut microbiota shifts. The use of beads of the same size as in our study (1–10 μm) but from a different polymer (PS and polyvinyl chloride -PVC-) (Chen et al., 2022; Huang et al., 2022; Wen et al., 2022) induced more similar effects on mouse fecal microbes (e.g. increase in *Enterobacteriaceae*) than larger PE particles (Li et al., 2020b; Djouina et al., 2022). Among the aforementioned cited populations associated with MP exposure, certain families, such as *Dethiosulfovibrionaceae* and *Enterobacteriaceae*, are considered to be pathobionts. The *Dethiosulfovibrionaceae* population has been associated to colorectal cancer (Liu et al., 2020b), while *Enterobacteriaceae* containing well known enteric pathogens, such as *Escherichia*, *Shigella*, *Campylobacter* or *Salmonella*, may be increased in irritable bowel syndrome (IBS-) (Pittayanon et al., 2019) and intestinal inflammatory diseases in humans (Zhu et al., 2018). Obviously, not all species in these families are pathogenic but most of them are commensal, highlighting the critical need to better characterize their potential pathogenic characteristics at a lower taxonomic level. It should be noted that the *Enterobacteriaceae* population appears to have a particular affinity for PE MPs. Indeed, *Escherichia coli* adheres

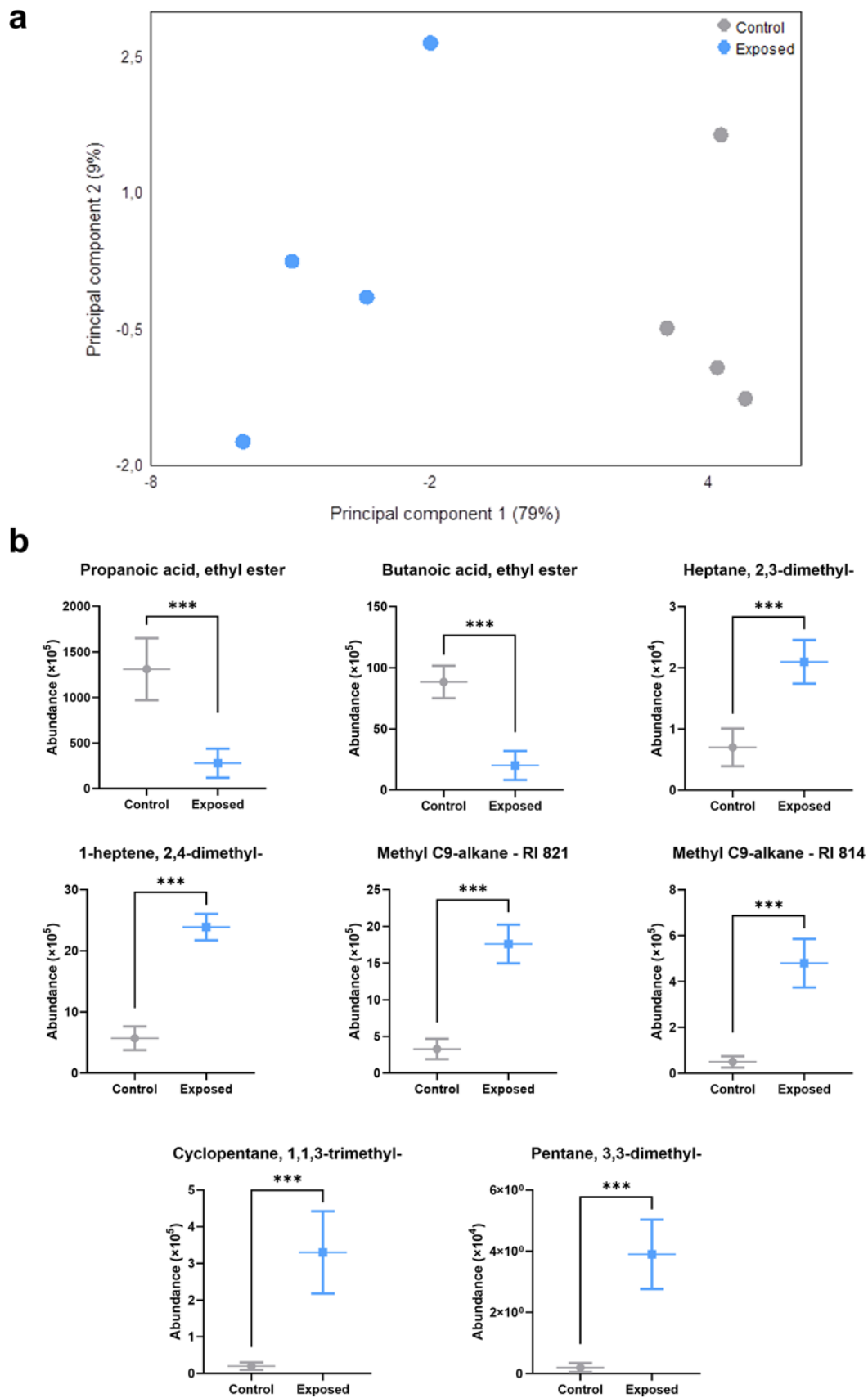


Fig. 7. Impact of chronic exposure to PE MPs on volatile organic compounds in the luminal compartment of Tm-ARCOL. a) Principal component analysis (PCA) of the control and exposed days including the 26 discriminant VOCs. b) Mean of abundance of the eight VOCs with the highest discriminating power. Abundances of selected VOCs are expressed in arbitrary units. *** $p < 0.001$ Bonferroni corrected value.

Table 2

Volatile organic compounds significantly altered by chronic exposure to PE MPs in Tm-ARCOL Values are the mean of abundances ($\times 10^5$) of each candidate marker with its standard deviation (in brackets).

Candidate markers	Tentatively identification ^a	<i>m/z</i> ^b	Exp. RI ^c	Ref. RI ^d	CAS number	<i>p</i> value	Control	Exposed	Under/over production compared to the control
Hydrocarbons									
Pentane, 3-methyl-	MS+RI	71	561	578	96–14–0	***	0.2 (74%)	3.9 (29%)	↗
Cyclopentane, 1,1,3-trimethyl-	MS	97	728	NA	4516–69–2	***	0.2 (51%)	3.3 (34%)	↗
Methyl C9-alkane - RI 814	MS	85	814	NA		***	0.5 (49%)	4.8 (22%)	↗
Methyl C9-alkane - RI 821	MS	71	821	NA		***	3.3 (42%)	17.6 (15%)	↗
1-heptene, 2,4-dimethyl-	MS+RI	70	841	855	19549–87–2	***	5.7 (34%)	23.9 (9%)	↗
Heptane, 2,3-dimethyl-	MS+RI	84	856	850	3074–71–3	***	0.7 (44%)	2.1 (17%)	↗
Methyl C9-alkane - RI 861	MS	71	861	NA		*	1.5 (59%)	5.1 (35%)	↗
Esters									
Propanoic acid, ethyl ester	MS+RI	57	710	708	105–37–3	***	1310.7 (26%)	278.9 (57%)	↘
Propanoic acid, 2-methyl-, ethyl ester	MS+RI	71	757	754	97–62–1	*	15.9 (45%)	4.9 (44%)	↘
Butanoic acid, ethyl ester	MS+RI	88	799	802	105–54–4	***	88.4 (15%)	20.1 (59%)	↘
Propanoic acid, propyl ester	MS+RI	57	807	804	106–36–5	**	203.7 (31%)	42.9 (55%)	↘
Butanoic acid, 3-methyl-, ethyl ester	MS+RI	88	851	854	108–64–5	**	1.9 (36%)	0.6 (32%)	↘
Butanoic acid, propyl ester	MS+RI	89	892	899	105–66–8	**	31.4 (37%)	8.7 (30%)	↘
Propanoic acid, butyl ester	MS+RI	75	903	908	590–01–2	**	6.0 (3%)	3.7 (29%)	↘
Hexanoic acid, methyl ester	MS+RI	74	923	924	106–70–7	*	3.3 (63%)	26.1 (64%)	↗
Pentanoic acid, propyl ester	MS	71	1006	NA	141–06–0	**	6.2 (17%)	2.5 (41%)	↘
Heptanoic acid, methyl ester	MS+RI	74	1031	1021	106–73–0	*	0.3 (44%)	10.6 (65%)	↗
Heptanoic acid, ethyl ester	MS+RI	88	1093	1097	106–30–9	*	0.5 (67%)	15.1 (74%)	↗
Butanoic acid, hexyl ester	MS+RI	71	1193	1192	2639–63–6	**	0.8 (28%)	1.6 (23%)	↗
Alcohols									
1-hexanol	MS+RI	69	866	867	111–27–3	*	759.7 (27%)	2425.3 (50%)	↗
1-heptanol	MS+RI	70	979	970	111–70–6	**	127.8 (47%)	1644.9 (36%)	↗
Aldehydes									
Heptanal	MS+RI	81	900	899	111–71–7	**	6.0 (31%)	14.0 (26%)	↗
Octanal	MS+RI	84	1017	1003	124–13–0	**	4.0 (25%)	9.1 (25%)	↗
Ketones									
2-heptanone	MS+RI	58	887	891	110–43–0	*	17.5 (51%)	75.7 (59%)	↗
2-heptanone, 4-methyl-	MS+RI	58	942	936	6137–06–0	*	2.5 (35%)	4.4 (26%)	↗
2-decanone	MS+RI	58	1197	1192	693–54–9	**	5.6 (16%)	2.5 (28%)	↘

^aMS + RI, mass spectrum and RI agree with literature data; MS, mass spectrum agrees with literature data; ^b Mass fragment used for area determination; (^{c,d}) Retention indices on a DB5 capillary column from experimental run (^c) or bibliographic data (^d); NA: Not-available; ↘: underproduction compared to the control condition; ↗: overproduction compared to the control condition; **p* < 0.05; ***p* < 0.01; ****p* < 0.001 Bonferroni corrected values

to the surface of PE MPs within 30 seconds (Tang et al., 2022). Similarly, in seawater, *Alpha* and *Gamma*proteobacteria have been identified as the core microbiome of biofilms associated with PE MPs (Tu et al., 2020).

Concerning the impact of exposure to PE MPs on the activity of the gut microbiota, apart from our recent study on the adult microbiota in M-ARCOL (Fournier et al., 2022a), no *in vitro* or *in vivo* study involving MPs has been conducted to date. Here, a decrease in butyrate production was notably observed after exposure to PE MPs. This has not been previously shown under adult colonic conditions (Fournier et al., 2022a), suggesting an infant-specific effect, which may be associated in this at-risk population with deleterious effects. In particular, the increase in butyrate production during infancy has been described as a key health milestone (Nilsen et al., 2020) as the effect of high concentrations of fecal butyrate in one-year old child has been shown to protect against allergies and asthma (Roduit et al., 2019; Depner et al., 2020). Of note, AhR activity also tended to be reduced following exposure to PE MPs, suggesting less production of AhR receptor ligands by the gut microbiota. This could be related to the decrease in abundance of some *Tannerellaceae*, like *Parabacteroides distasonis*, which are known to be producers of AhR ligands (Su et al., 2022). The ability of the gut microbiota to produce tryptophan-based AhR ligands influences the integrity of the gut epithelium as well as the development, function, production, and maintenance of several key mucosal immune cells and mediators (Lamas et al., 2018; Agus et al., 2018; Roager and Licht, 2018; Rothhammer and Quintana, 2019). A deficit in the production of AhR

ligands by the gut microbiota has been reported in inflammatory bowel disease (IBD), celiac disease and obese patients compared to healthy subjects (Lamas et al., 2016, 2020; Natividad et al., 2018). As a novel approach, the activity of the gut microbiota was finally investigated in the present study by measuring VOCs. These compounds have important roles in biological interactions between organisms and are currently used as biomarkers of various human diseases, including cancer and gastrointestinal metabolic disorders (Garner et al., 2007; Arasaradnam et al., 2014). Marked differences between the abundances of VOCs before and after exposure to PE MPs were highlighted, characterized in particular by an underproduction of 8 ester compounds and an overproduction of 7 hydrocarbons. Again, the shifts in VOC profiles under infant colonic conditions were markedly different from those previously obtained by our group under adult conditions (Fournier et al., 2022a). An increase in fecal esters has been observed in obese patients with non-alcoholic fatty liver disease (Raman et al., 2013), in IBS patients (Probert et al., 2014), but also after acute exposure *in vitro* of human faecal microbiota to benzo[*a*]pyrene (Defois et al., 2017). This suggests that overproduction of esters could be an indicator of disturbances in the gut microbiota. Moreover, when detected in the breath, the formation of hydrocarbons/alkanes has advocated as a non-invasive marker of alcoholic hepatitis but also as a measure of lipid peroxidation observed repeatedly in the pathophysiology of IBD (Probert et al., 2009). Our study also showed that among other modified VOCs, heptanone (2-heptanone and 2-heptanone, 4-methyl), two alcohols (1-hexanol and

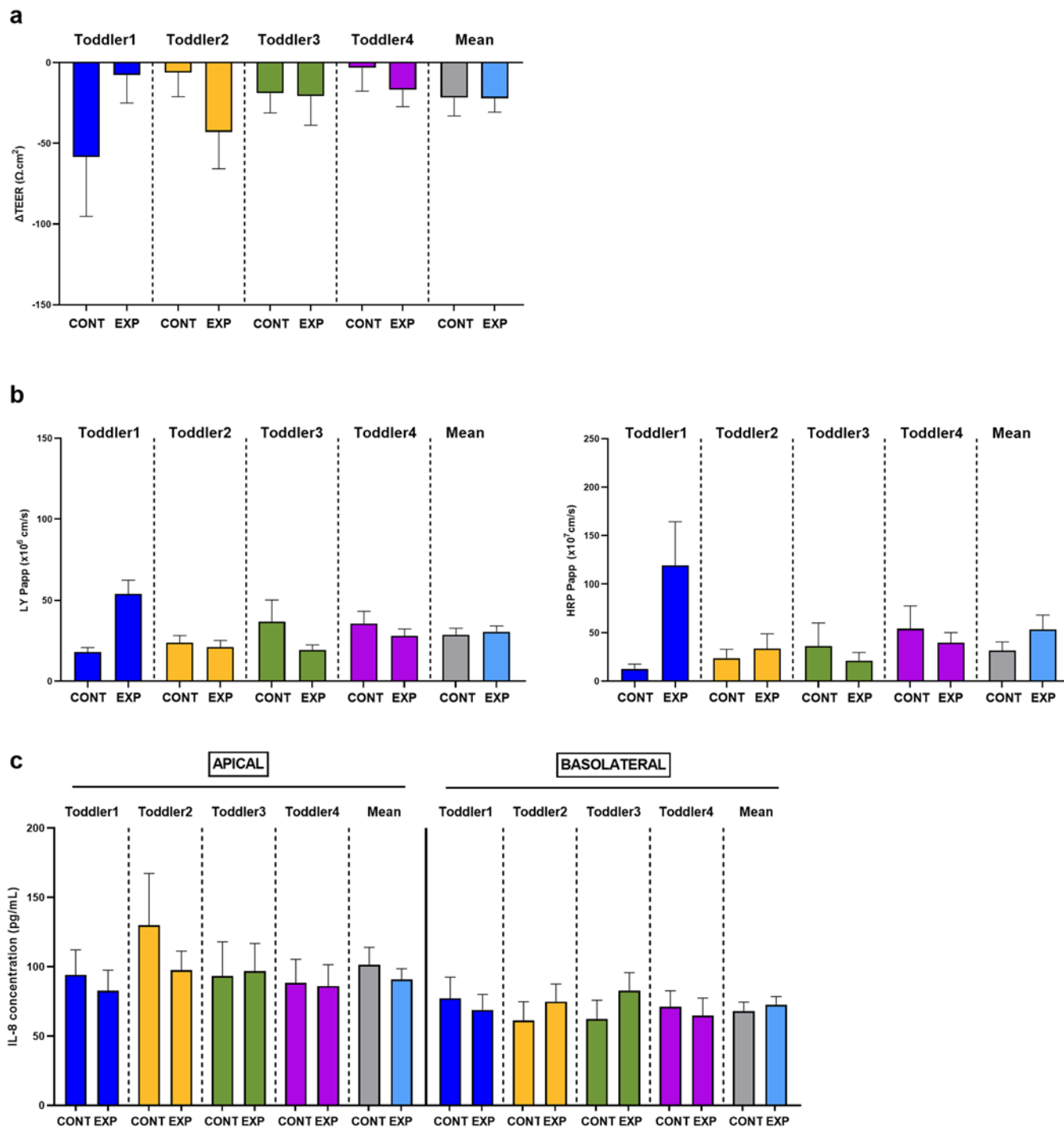


Fig. 8. Impact of Tm-ARCOL luminal supernatants before and after chronic exposure to PE MPs on inflammation and intestinal barrier integrity using a Caco-2/HT29-MTX co-culture. a) The difference between the average TEER before and after 24-h exposure to Tm-ARCOL luminal supernatants was calculated and termed Δ TEER ($\Omega \cdot \text{cm}^2$) as an indicator of paracellular permeability of the co-culture before and after exposure to MPs. b) LY transport (Papp in cm/s) and HRP Papp (in cm/s) as an indicator of paracellular and transcellular permeability, respectively. c) Mean IL-8 concentration (in pg/mL) in the apical and basolateral media of the cell co-culture. All results were given by donors, also on average ($n = 4$) and expressed as means \pm SEM. CONT: Control; EXP: Exposed; HRP: Horse Radish Peroxidase; IL-8: Interleukin-8; LY: Lucifer Yellow; Papp: Apparent Permeability coefficient; TEER: Trans-Epithelial Electrical Resistance.

1-heptanol) and two aldehydes (heptanal and octanal) were over-produced after exposure to PE MPs. These compounds were identified during low-density PE photodegradation in the environment (La Nasa et al., 2020), suggesting the possibility of PE degradation during our Tm-ARCOL fermentations, but again not consistent with Raman spectroscopy results. In light of these findings, the potential degradation of PE MPs by the human gut microbiota merits further investigation,

although the restricted contact time in the human gut during gastrointestinal transit certainly does not promote plastic degradation, compared to longer exposure to soil or water microorganisms in the environment (Akarsu et al., 2022; Tareen et al., 2022; Giangeri et al., 2022).

Considering the host response to exposure to PE MPs, we finally combined our *in vitro* colon model with co-cultured intestinal epithelial

cells. Such a coupling has already been carried out to evaluate the cellular toxicity of virgin or digested PS or PE MPs (Fournier et al., 2022a; Liu et al., 2020a) in adult but never in infant digestive conditions. Previous studies in mice orally exposed to PE MPs have reported deleterious effects on the intestinal barrier characterized by a low-grade inflammatory state (Li et al., 2020b; Sun et al., 2021) and conflicting results regarding the production of mucus (Djouina et al., 2022; Sun et al., 2021). This negative impact of MPs on the intestinal barrier was further confirmed *in vitro*. Direct exposure of human intestinal Caco-2 cells to MPs led to oxidative stress for PE MPs (Gautam et al., 2022) and increased epithelial permeability for PS MPs (Liu et al., 2020a). Here, for the first time under infant conditions, we investigated the potential indirect effect of exposure to PE MPs on human intestinal Caco-2/HT29-MTX cells, i.e. mediated by the metabolites of gut microbes. No significant effect was demonstrated, since we only observed for some toddlers a trend towards an increase in the expression of mucin genes as well as a non-significant decrease in the expression of genes encoding for tight junction proteins (occludin and ZO-1). Overall, this absence of significant deleterious effects suggests a mucus-protective effect of our co-culture model (Hesler et al., 2019) and/or a loss of the putative response triggered by gut microbial metabolites on inflammation and disruption of the intestinal barrier due to the dilution of the Tm-ARCOL luminal supernatants.

Taken together, our results suggest that chronic exposure to PE MPs induced significant perturbations in the composition and activity of toddler gut microbiota that may be related to a “dysbiotic” state of the microbiota, even though increased bacterial diversity does not fit the accepted definition of dysbiosis (Wei et al., 2021). This study also highlighted that the impact of PE MPs on the gut microbiota and the intestinal barrier *in vitro* was highly dependent on the donor. For example, exposure to MPs had no significant effect on the microbial diversity of Toddler 4 and induced greater deleterious effects on intestinal permeability in Toddler 1 than in others. Such data could help to define which infants are high and low “responders” to ingesting MPs. Our *in vitro* approach can definitively help for a better mechanistic understanding of the interactions between ingested micro- or even nano-sized plastics, the gut microbiota and mucus, considering inter-individual specificities. Further studies are needed to better investigate the effects of different plastic types, surface properties and sizes (Zhang et al., 2021b; Stock et al., 2022; Wang et al., 2022a; Völk et al., 2022) on the structure and functions of the gut microbiota. The potential of the Tm-ARCOL model should be also extended to the simulation of other specific at-risk populations, such as neonates or pathological situations associated with gut microbial dysbiosis, such as obesity in infants (Yang and Ni, 2019). Finally, since MPs may be the carrier of various chemical pollutants, heavy metals, antibiotics or pathogens (Gao et al., 2022; Budhiraja et al., 2022; Wang et al., 2022b), their bioavailability and/or their survival and biological effects could be studied using our *in vitro* approach, as well as their interactions with the human gut microbiota.

Environmental implication

Plastic production has exploded the last half-century, resulting in environmental pollution and microplastics (MPs) contamination throughout the food chain. This raised concerns for human health, particularly in the highly exposed and at-risk infant population. We investigated for the first time the effects of a chronic exposure to polyethylene MPs on the immature infant gut microbiome and intestinal barrier, using a novel approach combining a newly developed infant colonic model with co-cultures of enterocytes and goblet cells. This work provides useful data on MP interactions with infant gut microbiota and mucus, considering inter-individualities, increasing our knowledge on the health impact of MP pollution.

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CRedit authorship contribution statement

Elora Fournier: Conceptualization, Data curation, Formal analysis, Methodology; Funding acquisition, Investigation, Supervision, Writing – original draft. **Jeremy Ratel:** Formal analysis, Investigation, Validation, Writing – review & editing. **Sylvain Denis:** Formal analysis, Methodology, Validation. **Mathilde Leveque:** Data curation, Formal analysis, Investigation, Methodology, Validation. **Philippe Ruiz:** Methodology, Validation. **Carine Mazal:** Investigation. **Frederic Amiard:** Formal analysis, Methodology, Validation, Writing – review & editing. **Mathieu Edely:** Formal analysis, Methodology. **Valerie Bezirard:** Methodology. **Eric Gaultier:** Methodology, Validation. **Bruno Lamas:** Methodology, Validation, Writing – review & editing. **Eric Houdeau:** Resources. **Erwan Engel:** Formal analysis, Validation, Writing – review & editing. **Fabienne Lagarde:** Methodology, Validation, Writing – review & editing. **Lucie Etienne-Mesmin:** Conceptualization, Funding acquisition, Resources, Project administration, Supervision, Validation, Writing – review & editing. **Muriel Mercier-Bonin:** Conceptualization, Funding acquisition, Resources, Project administration, Supervision, Validation, Writing – review & editing. **Stéphanie Blanquet-Diot:** Conceptualization, Funding acquisition, Resources, Project administration, Supervision, Validation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Raw data are available at NCBI under the Sequence Read Archive database in the BioProject n° PRJNA831093.

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Financial interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be considered as a potential conflict of interest.

Ethics approval

This study is a non-interventional study with no additions to usual clinical cares. The protocol does not require approval from an ethics committee according to the French Public Health Law (CSP Art L

1121–1.1).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jhazmat.2022.130383](https://doi.org/10.1016/j.jhazmat.2022.130383).

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Section 3: Discussion

1. An original study at the heart of today's preoccupations

MPs are ubiquitous in our environment and everyone is exposed daily simply by breathing, drinking water or eating. As a first estimate of human exposure in adults, it has been published that we consume up to 5 g of MPs (the equivalent of a credit card) per week (Senathirajah et al. 2021). It should be noted that extremely sparse data are available for infants, with the exception of the Cox et al. 2019 publication presented in Section 1 Part 3. Regardless of the targeted population, little is known about the degree of exposure of the digestive tract, the potential impact of MPs on human health, and especially what happens along the digestive tract. To assess the toxicity of MP, increasing studies have been conducted in *in vivo* models such as rodents (see Section 1 Part 3), but few studies have determined the impact of MP exposure on the gut microbiota of human origin. In order to assess the interactions of MP with the human gut microbiota, clinical studies are limited by obvious ethical constraints, while studies using human *in vitro* gut models are more accessible. Especially, *in vitro* human colonic models are valuable tools allowing to maintain a complex and metabolically active human gut microbiota for several weeks under physiologically relevant conditions to investigate the fate and impact of MPs. Before this PhD work, only one study had previously evaluated the impact of a single dose of PET MPs on the luminal gut microbiota of two adult donors using the *in vitro* colonic model SIMGI® (Tamargo et al. 2022, see Section 1 Part 3).

The first originality of my PhD work, which was carried out in the framework of PLASTOX INRAE project, relies on the use of the M-ARCOL *in vitro* colonic model to study MPs in the human digestive tract. This model was previously developed to mimic adult colonic conditions and was adapted during this work to the specific digestive environment of infant (resulting in the Tm-ARCOL model), a population at risk for ingestion of xenobiotics, including MPs (Zhang et al. 2021a). M- and Tm-ARCOL have been shown to mimic the colonic microenvironment of the lumen and mucus-associated microbiota. For the first time in such complex *in vitro* models, we assessed the impact of repeated 14-day exposure to PE MP, not a single dose, on the adult and infant gut microbiota and for both the lumen and mucus-associated microbiota. As a novelty in the field, we also evaluated not only the effect of MPs on the composition of the gut microbiota but also, for the first time, its impact on the activity of the gut microbiota activity by measuring fermentation products, including gas and SCFAs, as well as AhR ligands derived from tryptophan metabolism, and the uncommon determination of VOCs. Furthermore, we used an original and complementary approach combining *in vitro* gut

models with epithelial and goblet intestinal cells in co-cultures for the evaluation of MP impact on the intestinal barrier (see Section 1 Part 2), while such approach was mostly used for nutritional studies (Déat et al. 2009; Van Rymenant et al. 2018). These researches have led to the production of 8 scientific publications during the PhD (4 original articles, 3 reviews articles and a book chapter).

My PhD work began with the evaluation of the most appropriate method of stool preservation. The aim was to preserve as much as possible the complexity and specificities of the initial gut microbiota inside the colonic *in vitro* model. We therefore investigated the effect of three preservative methods (freezing 48 h at -80°C, freezing 48 h at -80°C with glycerol as a preservative agent or lyophilization with maltodextrin/trehalose) on the kinetics of colonization of the lumen- and mucus-associated microbiota in M-ARCOL when the model was inoculated with fecal samples from two adult donors. Fresh stool was used as the positive control and we showed that inoculation with raw frozen stool was the best option among those tested (if the use of fresh stool is not possible). For this PhD work, we also decided to inoculate the colonic model with unpooled stools to retain *in vitro* the inter-individual variabilities of the gut microbiota between donors, both in adult and infant conditions. Thus, we showed that exposure to PE MP induced donor-dependent effects, on the gut microbiota of both adults and infants. In contrast, no significant impact of PE MPs on the intestinal barrier, as mediated by gut microbial metabolites changes, was evidenced during cell culture experiments performed with the luminal supernatants from adult and infant experiments (in M-ARCOL and Tm-ARCOL, respectively). These PhD results demonstrate the importance of considering the different impacts of MPs on the gut microbiota, including composition and functional activities, depending on individuals and age conditions (here infant vs adult).

2. A comparison between adults and an at-risk population, the infants

2.1 Infant and adult gut is not the same and therefore MP effects are different

Infanthood is a critical stage in the development of gut health, particularly through the maturation of the gut microbiota (see Section 1 Part 1). Major differences between the composition of the adult and infant gut microbiota have been described in the literature, such

as lower α -diversity, a higher proportion of facultative anaerobes and certain populations (e.g. *Bifidobacteriaceae*, *Enterobacteriaceae*) in the infant compared with the adult, as well as changes in the activity of gut microbiota associated with higher acetate and lower butyrate production in infant. Those differences were successfully replicated in the newly developed Tm-ARCOL (Section 2 Chapter 3).

While few data are available to date to truthfully estimate the exposure of infants to MPs, a recent study reported more MPs in infant stools than those of adults (Zhang et al. 2021f). In addition, other studies have shown greater exposure of this sensitive population to MPs through breast- (Ragusa et al. 2022) and formula feeding (Kutralam-Muniasamy et al. 2020; Da Costa Filho et al. 2021), but also through dust ingestion during roaming (Zhu et al. 2022) and when sucking on silicone rubber nipples (Su et al. 2021) (See Section 1 Part 3). This leads to a likely higher exposure of infants compared to adults, which justifies the importance of studying MP impact on the immature gut microbiota of infants.

However, existing studies only focused on the adult gut microbiota, and none of them had investigated the infant one. For the first time, we showed different effects of exposure to PE MP on both the gut microbiota of adults and infants. However, it is interesting to note that under *in vitro* conditions for both adults and infants, we observed an increase in potential pathobionts, such as *Enterobacteriaceae*, after repeated 2-week exposure to PE MP. In adult, *Desulfovibrionaceae* was increased while *Christensenellaceae* and *Akkermansiaceae*, two bacterial families associated to human health, were decreased. In infant, *Dethiosulfovibrionaceae* was increased. These bacterial changes were associated to a decrease in butyrate production and important modifications of the VOC profile (i.e. 26 differentially produced VOCs in exposed vs control conditions) in infants. Contrarywise in adults, we did not observe modification regarding butyrate production and more modest differences in VOC profiles (i.e. only 5 differentially produced VOCs), mostly characterized by a drastic increase in indole 3-methyl (or skatole).

Increased butyrate production during infancy has been described as a key milestone in digestive health (Nilsen et al. 2020) and associated with progressive colonization of the gut by butyrate-producing species (e.g. *Ruminococcaceae*, *Lachnospiraceae*, *Clostridiaceae*) (Louis and Flint 2017). In our study, we observed a loss in *Lachnospiraceae* and *Ruminococcaceae* (2 out of 4 infants) in the mucosal gut microbiota after PE MP exposure, which may partially explain the observed decrease in butyrate production. Before one-year old, infants harbor lower bacterial genera generating butyrate-production (Vital et al. 2017). This

lack of redundancy of butyrate production function in their gut microbiota may make them less resilient to environmental stresses targeting this function, such as exposure to MPs. Decreased butyrate production during this early life period, as induced in our study by MP exposure, could lead to longer-term gut health problems. In particular, high fecal butyrate concentration in 1-year old child has been associated with protective effects against allergy and asthma (Roduit et al. 2019; Depner et al. 2020). Regarding skatole, which was only increased in adults after PE MP exposure, this tryptophan-derivative compound can be produced by the human gut microbiota involving various genera or species such as *Bacteroides*, *Clostridia*, *Escherichia coli*, *Lactobacillus* or *Desulfovibrio* (Smith and Macfarlane 1997; Cook et al. 2007). The increase in *Desulfovibrionaceae* after PE MP exposure observed in our study in adults, not infants, may explain the increase in skatole production.

As aforementioned 26 VOCs (hydrocarbon compounds and oxygenated compounds as alcohols, aldehydes, ketones and esters) were differentially produced under infant conditions after PE MP exposure. Several studies have attempted to decipher the specific characteristics of respiratory of fecal VOC patterns in pediatric gastrointestinal conditions (Buijck et al. 2016; Course et al. 2021). In particular, several exhaled VOCs, such as pentane and 1-heptene also observed in our study, were overproduced in obese or overweight children with nonalcoholic fatty liver disease and nonalcoholic steatohepatitis, and certainly produced by lipid peroxidation mediated by oxidative stress (Alkhoury et al. 2014; Eng et al. 2015). Specific fecal VOC profiles have also been identified in late-onset neonatal sepsis (Berkhout et al. 2017) and necrotizing enterocolitis in preterm infants (Meij et al. 2015) but their specific identity has not been reported. The study of the volatolome is in its infancy, particularly in infants, and needs to be further investigated for a better understanding of MP exposure.

2.2 The need for more studies of at-risk populations

The higher exposure of infant together with the marked differences between infant and adult observed in this PhD work underlines the need to investigate the MP effects not only on healthy individuals, but also on other at-risk populations. In particular, to address this question, previous studies have been conducted in perturbed cellular models or in mice with disturbed gut microbiota.

Concerning *in vitro* studies, a triple and inverted culture Transwell model (combining Caco-2, HT29-MTX-E12 and THP-1 cells), simulating a healthy vs IFN- γ -induced inflamed intestine, was used to investigate the toxicity of MPs of PE (200 nm to 9.9 μ m) (Busch et al.

2021b), and nanoplastics of PS (35–75 nm) and PVC (200–350 nm) (Busch et al. 2021a) at a dose of 50 $\mu\text{g}/\text{cm}^2$ for 24 h. No significant difference was reported before and after PE MP exposure in inflamed conditions, despite a trend describing a dose-dependent increase in pro-inflammatory interleukin (IL-8 and IL-1 β) (Busch et al. 2021b). Whereas no significant difference was observed before and after PS MP exposure, exposure to PVC NPs significantly increased the release of pro-inflammatory IL-1 β in the inflamed triple culture and led to cytotoxicity of epithelial cells in a dose-dependent manner (Busch et al. 2021a). This suggests the importance of considering an impaired gut barrier when assessing the health hazard of ingested MPs. From the microbial point of view, to date, no study has investigated the consequences of MP exposure on a disturbed gut microbiota. Noteworthy, in MEDIS laboratory, the M-ARCOL model was recently adapted to simulate the specific conditions found in the colon of IBS and obese patients. Likewise, the Tm-ARCOL model could also be further adapted to mimic infant disturbed-gut microbiota.

Concerning *in vivo* studies in rodents, more experimental evidence is provided. For instance, the impact of PS MP exposure (5 μm , 0.5/5 μg per day by gavage for 7 days and 5 μm , 500 $\mu\text{g}/\text{L}$ in drinking water for 28 days) on mice with DSS-induced colitis was studied by Luo's and Liu's teams (Liu et al. 2022; Luo et al. 2022a). While minor effects of MP exposure were shown in healthy mice, both teams demonstrated increased intestinal histopathological damage and inflammation, reduced mucus secretion, and increased colonic permeability in DSS-treated mice. Huang's team also evaluated the effects of 80 mg/kg of MPs, including 20 mg/kg administered by gavage once a day for 10 weeks of PS MP exposure (5, 50, 100 and 200 μm) on mice fed a normal or a high-fat diet (Huang et al. 2022a). High fasting blood glucose and insulin level were observed in mice fed with a normal diet and exposed to PS MPs, as occurred in high-fat diet mice. An increase in IL-1 β and LPS levels in tissues after PS MP exposure was also observed with both diets. Of note, microbiota analysis in caecal contents revealed that *Enterobacteriaceae* were increased after PS MP exposure for both diets as we reported in our study for both adult and infant gut microbiota. The induction of insulin resistance in mice exposed to PS MPs was confirmed by another study where mice were given 1- μm PS MPs at a dose of 10 mg/L in drinking water for 2 weeks (Shi et al. 2022b). Fully in line with these experimental approaches, within the Next-PLASTOX INRAE project (which is a follow-up of the PLASTOX INRAE project), Toxalim is currently investigating the consequences of a 90-day oral exposure in adult mice fed a normal vs Western diet (Monsanto et al. 2016). Animals are treated with the same type of PE MPs as in my PhD work (*via* the

diet) at a dose of 10 mg/kg bw/day, adapted from the first human exposure estimate given by Senathirajah and colleagues (Senathirajah et al. 2021). In this ongoing work, the influence of PE MPs and/or diet (to reveal any exacerbating effects) is determined on both gut homeostasis and gut/liver axis disturbances. Taken together, the pioneering studies from this PhD work illustrate the importance of further evaluating MP effects in sensitive populations such as obese or IBD patients, but also in infant suffering these pathologies (Sanyaolu et al. 2019; Ye et al. 2020).

3. Microplastics: a multifaceted contaminant

MPs are complex particles, characterized by many different parameters, such as their polymer types, sizes, shapes, surface properties, the presence of adsorbed contaminants and/or the formation of a biomolecular corona, for example after digestion (see Section 1 Part 3). However, most studies to date have used virgin PS microbeads for reasons of availability and ease of handling, although this polymer type only accounts for about 6% of total plastic production (Rozman and Kalčíková 2021; PlasticsEurope 2021). Furthermore, the spherical shape is not fully representative of the ingested forms (e.g. fibers, fragments, see Section 1 Part 3), thereby highlighting the need to unravel the effects of “real-life” MPs. While virgin MPs can simulate a direct source of human exposure (e.g. from food packaging), the use of weathered particles are also needed to simulate environmental exposure as weathering leads to strong modifications of MPs due to oxidation, fragmentation and degradation (e.g. photo-oxidation, hydrolysis, mechanical abrasion, possible biodegradation by microorganisms) and surface coverage (biomolecular corona) by a complex mixture of organic and inorganic materials. Corona formation, but also sorption capacity (see section 3.4 below), may be modified by polymer oxidation and the subsequent changes in surface properties.

3.1 Various types, sizes and shapes of MPs

Human exposure to MPs is undoubtedly due to a mixture of various plastic particles in terms of size and/or size distribution, which could lead to different health impacts compared to those already assessed *in vivo* and *in vitro* using single-sized particles. Liang and colleagues investigated in mice the biodistribution and the effect on the intestinal barrier of three PS particles (5- μ m MPs vs smaller-sized particles of 50 nm and 500 nm) under single and co-exposure conditions (Liang et al. 2021a). The authors showed an enhancement of 500-nm and 5- μ m PS particle absorption and toxicity when they were co-exposed with 50-nm PS particles

for 24 h. The mixture of 50-nm and 500-nm PS particles caused more severe gut barrier dysfunctions than the particles considered individually, which was mainly due to an increase in intestinal permeability and reactive oxygen species-mediated epithelial cell apoptosis. Interestingly, 50-nm and 500-nm PS particles decreased mucus secretion in the colon while 50-nm PS particles alone stimulated mucus secretion in the duodenum, jejunum and ileum. Size-dependent effects of PS MPs (5 μm) vs smaller-sized particles (0.1 μm) were also investigated *in vitro* on human Caco-2 cells, with a special emphasis on cytotoxicity and efflux pump inhibition (Wu et al. 2019a). While both types of PS particles exhibited low effects on cell viability and oxidative stress, 5- μm PS MPs induced higher mitochondrial depolarization than 0.1- μm particles, while 0.1- μm particles led to higher inhibition of ATP-binding cassette (ABC) transporter compared to 5- μm PS MPs.

The shape of MPs is another critical factor that could influence their effects on human health. Few studies have investigated to date the different impact of plastic particles according to their shape. Conducted with PVC MPs (<5 μm) as well as small-sized PS particles (50, 100, 310 nm) and on primary human monocytes and monocyte-derived dendritic cells, Weber's team revealed a stronger release of pro-inflammatory cytokines following exposure to irregular PS or PVC particles compared to their spherical counterparts (Weber et al. 2022). More studies should be conducted on other shape of MPs but also with cocktails of various MP types, sizes and/or shapes.

3.2 MPs are not in a virgin state in the environment

As MPs found in the environment are mostly irregular and polydisperse, some studies used artificially-aged MPs and compared their effects to virgin ones. For instance, 2- μm PS MPs were weathered by irradiation for 200, 400 and 600 h, corresponding to approximately 43, 86 and 130 days of weathering in nature (Völkl et al. 2022). Their effects were evaluated in murine macrophages. The authors showed a higher toxicity and broader adverse cellular responses (e.g. metabolic activity, membrane integrity, genotoxicity and reactive oxygen species production), but a lower inflammatory response, with weathered particles compared to virgin ones. Another study evaluated the effects of photo-transformed PS MPs (1.0-1.9 μm) on intestinal Caco-2 cells (Yu et al. 2021). Photo-transformation of PS MPs led to a gradual transformation of some carbon-containing functional groups into oxygen-containing functional ones and to a rough surface with the appearance of cracks and pits. Exposure of Caco-2 cells to such chemically- and physically-modified particles also induced a progressive increase in

cytotoxicity and cell membrane damage with increasing light irradiation time and PS MP concentration. These more deleterious effects may be explained by the greater toxicity of oxygen-containing functional groups compared to carbon-containing functional ones. Lastly, an *in vivo* study, conducted with photo-transformed polyamide MPs (fragments between 5 to 50 μm in size) on zebrafish (Zhang et al. 2021e), showed higher bioaccumulation, oxidative stress, growth inhibition, intestinal injury and lipid metabolism disorder than with virgin MPs.

Although these studies are not always performed on intestinal cells or tissues, due to the paucity of data available in the literature, they support the importance of using particles that are representative of those found in the environment. Furthermore, while half of the existing studies do not characterize the MPs used (Rozman and Kalčíková 2021) and given the many factors that can influence the effects of MP in human, it is crucial to standardize the conditions of MP administration, such as the use of harmonized concentration terms (e.g. mg/L or particles/L) (Leusch and Ziajahromi 2021), as well as to develop standardized toxicity assessments. For this reason, EFSA is currently working on the field by bringing together all the scientific communities involved in MP detection and quantification methodologies, as well as health hazard and risk assessment. This will be developed in the last section below.

3.3 Modifications of MP physico-chemical properties during human digestion

3.3.1 MP physico-chemical modifications during human digestion according to the literature

It is important to unravel the physico-chemical transformations of MPs during their fate in the human gastro-intestinal tract. Few studies, described in Part 3 of the literature review, have already explored the modifications of MPs during human digestion using in simple *in vitro* models. Briefly, while some of them reported no striking alterations of PS, PET, PE, PP and PVC MPs but the formation of a biomolecular corona at the particle surface (Stock et al. 2019c; Tan et al. 2020), Tamargo and colleagues used the combination of *in vitro* models of the upper and lower gut to show a progressive amorphization of PET MP surface, especially during colonic fermentation in SIMGI[®], with an organic matter deposition on the particle surface (Tamargo et al. 2022). This study demonstrates the importance of MP characteristics, as well as the role of both biotic and abiotic factors of the human digestion.

Despite these first steps forward, the systems currently in use remain generally simplified, operated under static conditions and consist only of different vessels simulating the gut with the corresponding digestive juices adjusted for pH and temperature. Some important parameters are absent, such as dynamic digestion processes (i.e. transit between successive digestive compartments or time-effect on gastric and intestinal pH and digestive secretion concentrations and flow rates), the presence of compounds from food matrices, the influence of gut microbiota (mainly in the large intestine, but also in the small intestine), and finally interactions with intestinal epithelial, mucus-secreting and/or immune cells.

In particular, the physico-chemical transformations of MPs (e.g. formation of a biomolecular corona, particle degradation) could be investigated using the complex *in vitro* TIM-1. As presented in Section 1 Part 2, this model is currently the most complete simulator of the upper human GIT reproducing the main physico-chemical parameters of the stomach and small intestine (Cordonnier et al. 2015). Compared to static models, TIM-1 also allows a better assessment of the bioaccessibility of ingested compounds, closer to the *in vivo* situation. The uptake of small-sized MPs or molecules (such as MP-associated contaminants) can be followed in TIM-1 by using a circulating dialysis fluid through hollow fibers. In addition, TIM-1 could be used to study the influence of food matrices (including lipid-rich matrices) on the physico-chemical transformations of MPs, degradation and/or bioaccessibility of associated contaminants.

Another knowledge gap in the field is related to the absence of reliable methods to track the fate of MPs during human digestion. In fact, the detection, quantification and physico-chemical analysis of MPs in complex media, such as biological matrices, represent major analytical challenges (Toussaint et al. 2019; Brachner et al. 2020). Numerous methods are currently tested or under development but their use has not been standardized yet across studies (Yan et al. 2019; Malafaia et al. 2021). While the gold standard for MP identification involves staining with Nile Red dye (a lipophilic, metachromatic and solvatochromic dye) (Erni-Cassola et al. 2017), some methodological limits cannot be set aside. In particular, the fluorescently-labeled MPs may be easily confused with other organic residues especially for the smallest particles (Nalbone et al. 2021). Thus, an increasing number of studies has emerged to overcome this methodological issue, like the development of automated methods and label-free identification models to detect and quantify MPs in human cells and tissues (Ishmukhametov et al. 2021; Shi et al. 2022a). In this framework, we present, in the following section, some preliminary results obtained on state-of-the-art label-free methods for the detection of PE MPs

in digestive fluids using the TIM-1 gastric and small intestinal model, implemented both under adult and infant conditions.

3.3.2 PE MP digestion in the TIM-1 model and associated analysis for their detection in digestive fluids using state-of-the-art instrumentations

During my PhD work, we conducted experiments in the TIM-1 model to track the fate of PE MPs and decipher their potential physico-chemical modifications during human digestion (**Fig 4.1**). A single dose of PE MPs (21 mg), suspended in a glass of water to mimic human exposure during drinking, was digested *in vitro* in two independent duplicates under adult- and infant-specific conditions (n=2). The TIM-1 model was previously adapted in MEDIS laboratory to the infant-specific conditions (Roussel et al. 2016a) and has been described in Section 1 Part 2. Digesta samples after 1 hour of gastric digestion were collected, as well as ileal digesta samples, after 4 h of a complete digestion process across the gastric, duodenal, jejunal and ileal compartments. We then proceeded to the analysis of our samples via the use, for the first time to our knowledge, of two analytical methods, detailed below, and which involved various collaborators at the French and European levels.

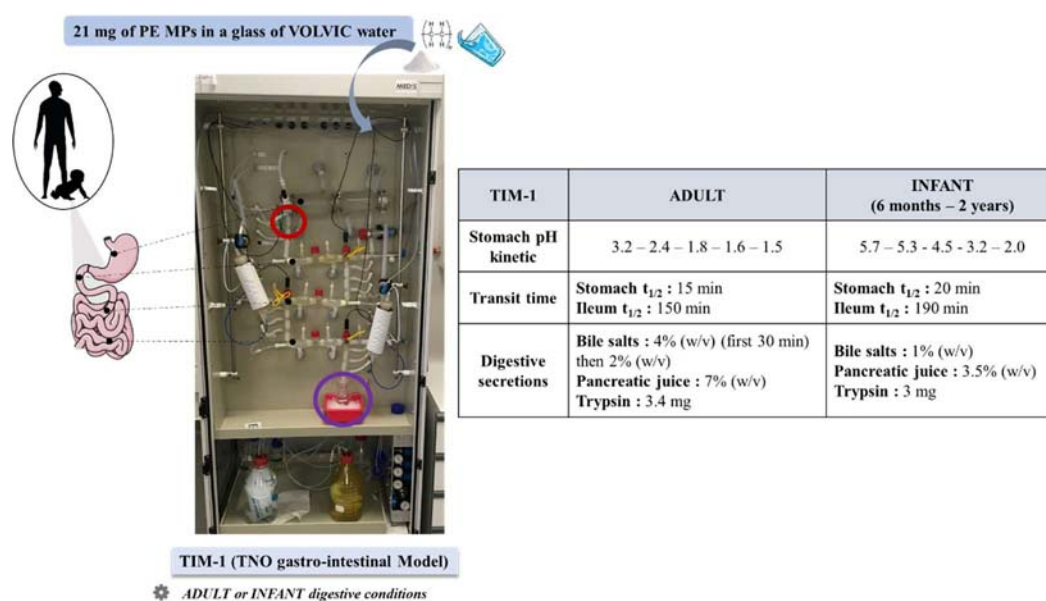


Fig 4.1. The PE MP digestion process in the TIM-1 model under adult or infant conditions

The digestion process, in the adult and infant upper digestive tract, of a glass of water containing PE MPs was carried out using the TIM-1 model. The red and violet circles indicate the localization of the gastric and intestinal samplings, respectively. The table gives the main parameters that are different between the adult and infant TIM-1 models when simulating digestive conditions of a glass of water. $t_{1/2}$: half-time of delivery

The table was modified from Roussel et al. 2016.

3.3.2.1 Synchrotron UV fluorescence imaging of PE MPs in human digestive fluids

We first used deep ultraviolet (DUV) fluorescence imaging of PE MPs after human digestion (proposal 20201456 completed in July 2021) at the DISCO beamline (Giuliani et al. 2009) of the SOLEIL synchrotron facility (**Fig 4.2**). This high-technology facility is based on electromagnetic radiation source covering a wide range of energies (from the infrared to the x-rays), coupled to a research laboratory at the cutting edge of experimental techniques dedicated to matter analysis down to the atomic scale.

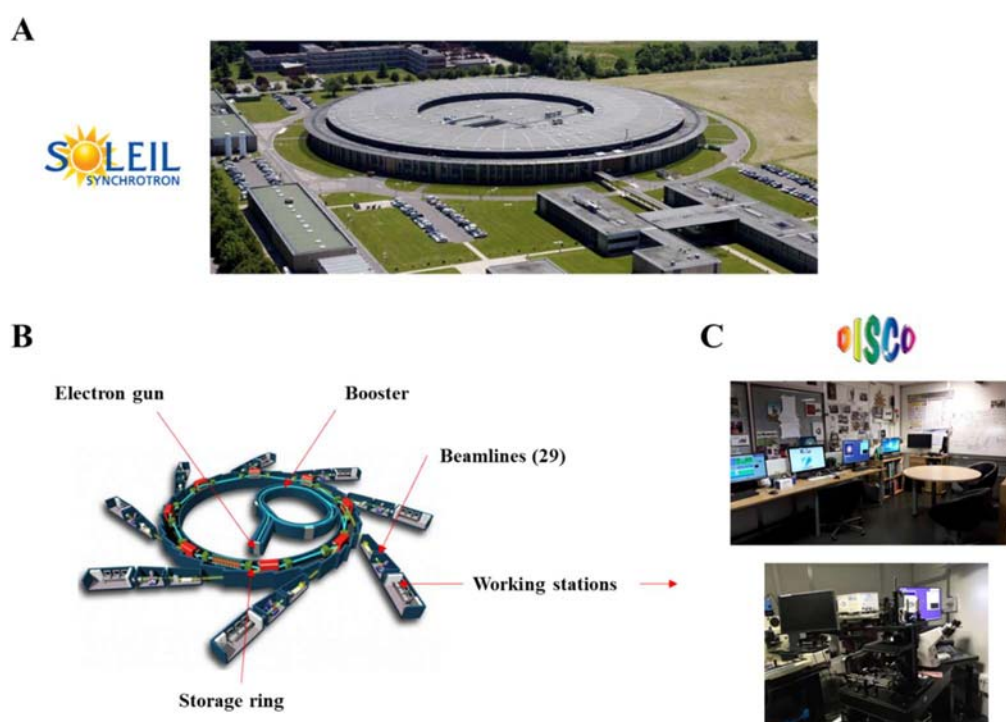


Fig 4.2. The SOLEIL synchrotron facility and the DISCO beamline

(A) General photography of the SOLEIL synchrotron facility at Gif-sur-Yvette. (B) Schematic representation of key elements of the SOLEIL synchrotron facility. The electron gun, a metal pellet, is heated and produces electrons. Then, a high-frequency electric field groups them into packets. These packets gain speed to reach almost the speed of light, increasing their energy, thanks to an electromagnetic wave injected into the accelerator sections by high-frequency generators. They are finally injected into the storage ring and turn there during hours by emitting light radiations. Specific wavelength radiations are captured by each beamline and used for various biological analyses. (C) Experimental set-up on the DISCO beamline with the Telemos microscope and monochromatized synchrotron beam as the excitation source for MPs (bottom) and the analysis station (top).

We analyzed the initial PE MP powder, 1-hour gastric and 4-hour intestinal effluents after digestion under adult and infant conditions. Examples of two clichés (PE MP initial powder and 1-hour gastric effluent samples collected during adult digestion) are presented in **Fig 4.3**.

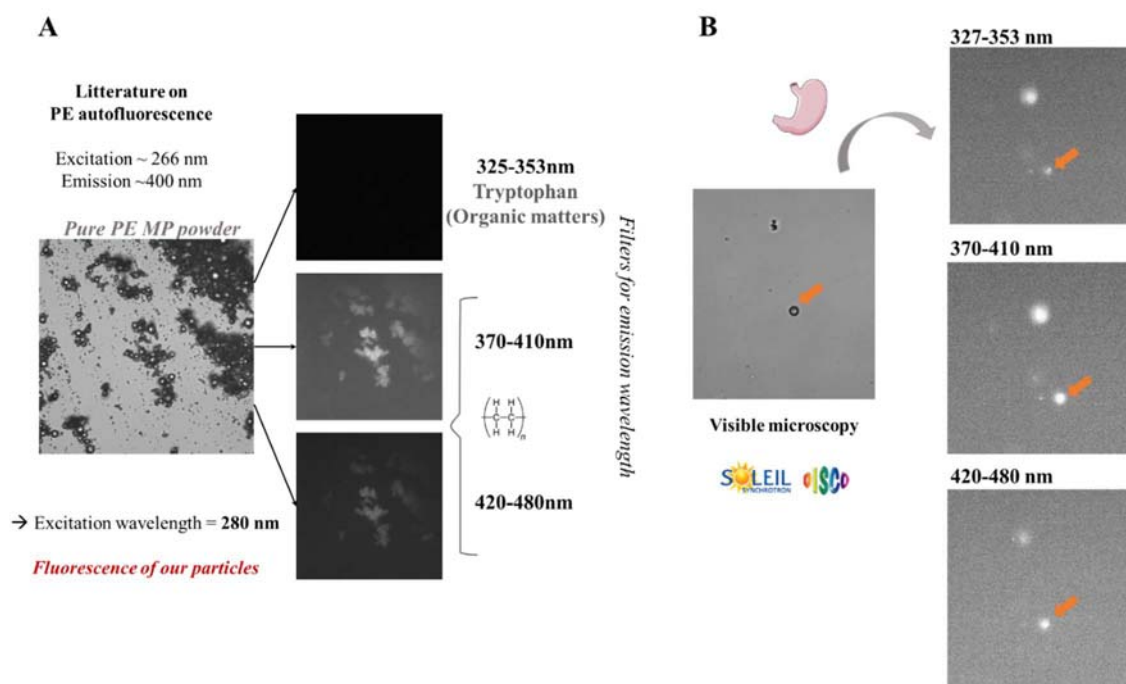


Fig 4.3. Analysis at the DISCO beamline of the initial PE MP powder and 1-hour gastric effluent in TIM-1

For mapping with DUV beam mode, 3 μL of each sample were placed on a quartz coverslip, observed using the Telemos microscope in bright field and then illuminated by a 280-nm monochromatized synchrotron beam as the excitation source, based on the study of Spizzichino and colleagues (Spizzichino et al. 2016) on the detection of different polymer-type plastic debris in “real”, albeit non biological, matrices. Fluorescent signals were collected at 327-353 nm (organic matter background) and 370-410 and 420-480 nm (auto-fluorescence of PE MPs). For each condition, two independent quartz coverslips, with at least five different locations, were investigated to validate the consistency of the observations.

(A) Visible microscopy image (on the left) and fluorescence images for the three filters (on the right) for the initial PE powder and (B) for the 1-hour gastric effluent from PE MP adult digestion (see one PE MP indicated by the orange arrow).

DUV fluorescence imaging allowed non-invasive monitoring of PE MPs in our biological samples without any artificial labeling at single-particle resolution. Indeed, using the tunable UV wavelength available on the imaging branch of the DISCO beamline, auto-fluorescence of PE-MPs with excitation at 280 nm was exploited to identify them in the initial PE powder, as well as in the 1-hour gastric effluents of TIM-1 from PE MP digestion in adult conditions (Fig 4.3) and infant ones (data not shown). However, critical technical issues appeared, like the quenching of MP fluorescence in different samples (e.g. intestinal effluents) probably due to interaction with biological compounds (e.g. bile salts and aromatic amino acids). Altogether, our results clearly underlined (i) the influence of the surrounding biological medium on MP fluorescence emission and (ii) the need of more specific MP chemical signature.

3.3.2.2 Raman Tweezer for PE MPs detection in human digestive fluids

To overcome the major issues raised during our synchrotron radiation experiments at the DISCO beamline, and beyond the on-going analytical work with M-H. Ropers (BIA, Nantes) using spectroscopic methods within the PLASTOX INRAE project, we initiated a collaboration in 2021 with P.G. Gucciardi from the Institute of Chemical and Physical Processes (IPCF, CNR, Messina, Italy) to detect and identify PE MPs in our TIM-1 digesta using Raman tweezer (RT) (Snook et al. 2009). RT, namely optical tweezer (OT) combined with Raman spectroscopy, is a very promising analytical tool for the study of MPs, and ultimately nanoplastics, in different environmental or biological matrices. OT exploits the tiny forces that light exerts on matter (Ashkin et al. 1986) to trap and manipulate micro and nanoparticles dispersed in liquid (Maragò et al. 2013). When coupled to a Raman spectrometer, OT enables chemical analysis of the trapped particles. Its application was successfully demonstrated in seawater by Gucciardi's team by showing optical trapping and chemical identification of sub-20 μm plastic particles, down to the 50 nm range (Gillibert et al. 2019). An example is given below, together with a simplified schematic RT representation, for identification of a nylon fiber and a spheroidal PP particle dispersed in seawater (Gillibert et al. 2019).

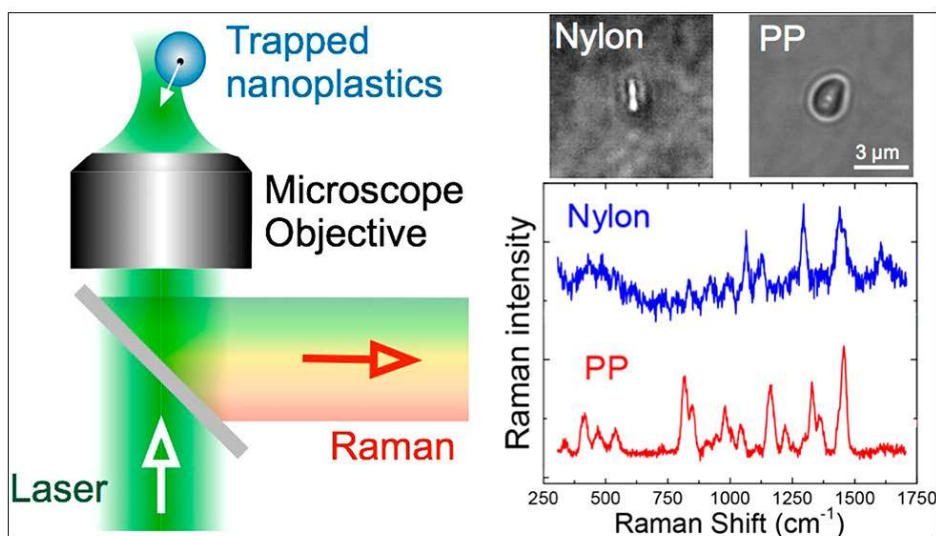


Fig 4.4. Simplified schematic representation of Raman Tweezer (on the left) and optical images (on the upper right) of a nylon fiber and a spheroidal PP particle dispersed in seawater with Raman spectra (on the lower right) of the optically trapped particles (blue and red, respectively)

Extracted from Gillibert et al. 2019

From an experimental point of view, RT is an inverted microscope equipped with a high numerical aperture (NA) objective, coupled to a Raman spectrometer (**Fig 4.4**). The high NA ensures the large intensity gradient needed for trapping and, at the same time, the power density needed to maximize the Raman signal. The set-up used for our experiments was a homemade single-beam RT in which the same laser was used for both Raman excitation and trapping. Further details can be found in the article of Gillibert and colleagues (Gillibert et al. 2019).

For the first time, we successfully demonstrated the valuable use of RT for analyzing MP-exposed human digestive fluids in adult conditions by showing optical trapping and PE chemical identification of small-sized plastic particles (of about 1-2 μm) (**Fig 4.5**). Interestingly, agglomerates containing PE MPs were detected after adult gastric digestion. However, no isolated PE MP was seen. After intestinal digestion, a deagglomeration of PE MPs was observed, which may be due to pH increase and/or influence of organic compounds (e.g. biliary acids), as previously described for other particles types as carbon, silver and hydroxyapatite nanoparticles (Walczak et al. 2013; Antonello et al. 2022). In infant digestion, the same profiles were obtained (data not shown). This work will be the subject of a forthcoming publication.

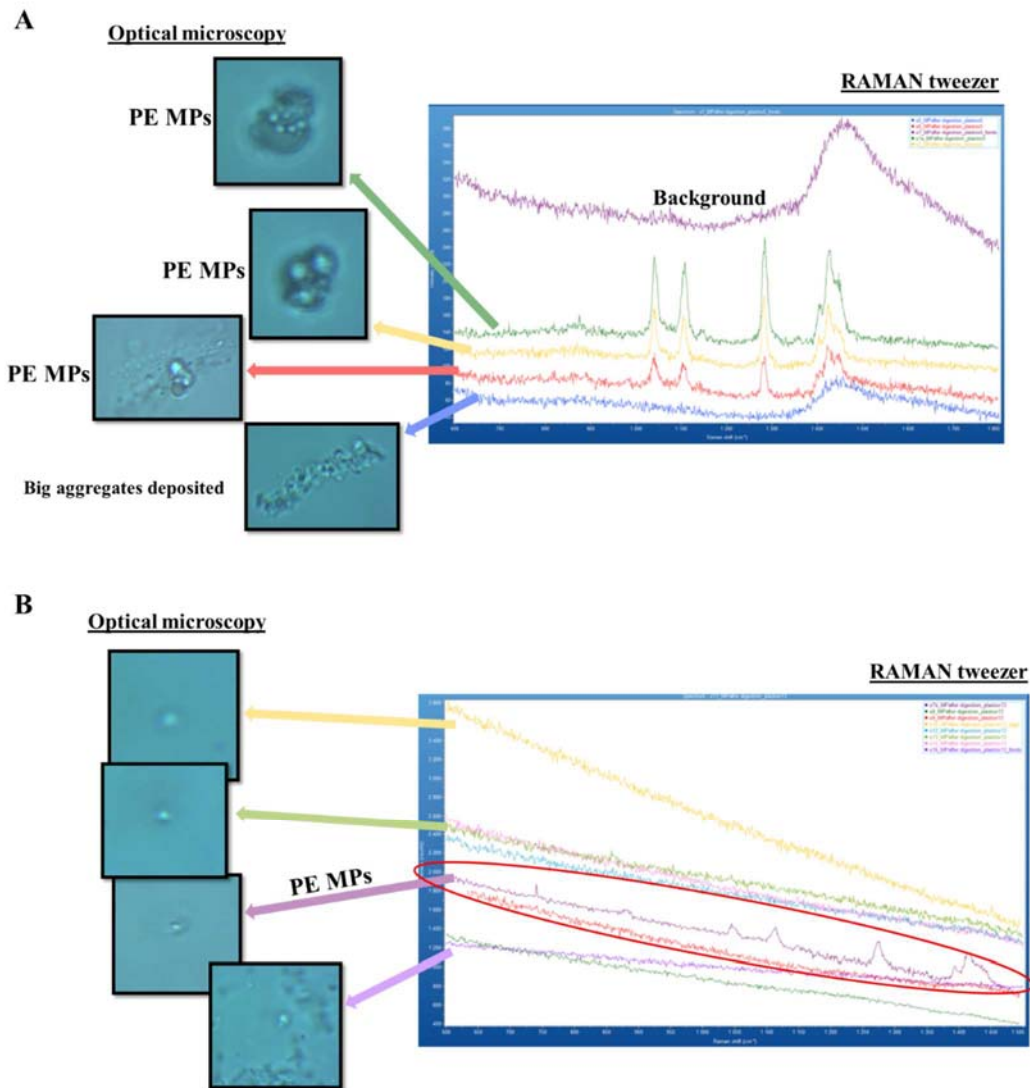


Fig 4.5. RT analysis of a 1-hour gastric (A) and 4-hour intestinal effluent (B) samples from adult digestion in TIM-1

(A) Raman spectra of PE MPs (on the right) optically trapped (images on the left) in 1-hour gastric effluent sample from adult digestion. (B) Raman spectra of the particles (on the right) optically trapped (images on the left) in 4-hour intestinal effluent sample from adult digestion. Noteworthy, no PE MP was detected in control samples (data not shown).

3.4 MPs as vector of chemicals, heavy metals and microorganisms: the “Trojan horse” hypothesis

MPs are vectors of various chemicals, heavy metals and microorganisms (see Section 1 Part 3). As an illustration, it was found *in vivo* by Deng and colleagues that co-exposure of PE MPs (45–53 μm) bound to di-(2-ethylhexyl) phthalate (DEHP) during 30 days in mice led to stronger effects than for virgin MPs. In particular, the authors described gut microbiota

composition modifications, characterized by lower β -diversity and increase in the relative abundance of Actinobacteria in the fecal content (Deng et al. 2020).

In addition, the sorption capacity of weathered MPs is significantly higher than that of pristine particles. The adsorption capacity of photo-transformed vs virgin PS and PVC MPs was evaluated toward a hydrophilic organic pollutant, the ciprofloxacin (Liu et al. 2019a). Results showed surface oxidation and appearance of microcracks on the aged MPs, leading to greater sorption capacity compared to virgin particles, which involved partitioning, electrostatic interactions and intermolecular hydrogen bonding. It should be noted that the sorption capacity of virgin MPs decreases as the degree of crystallinity increases, while the opposite trend was observed for aged MPs. As mentioned above, Tamargo and colleagues have shown a progressive amorphization of the surface of PET MP during digestion (Tamargo et al. 2022), which could lead to modification of the sorption capacity of organic pollutants. In addition, pH influences the electrostatic interactions between MPs and their associated-chemicals, and increase in salinity suppressed the adsorption of ciprofloxacin on all the tested MPs.

These assessments questioned about the potential desorption of such chemicals during human digestion. To date, *in vitro* studies are scarce and mainly focus on simple batch models exposing MPs with adsorbed chemical contaminants (only heavy metals studied so far) to simulated digestive fluids. A high desorption under acidic conditions was reported in the specific case of Cr in a human static *in vitro* digestion model, including mouth, gastric, small intestinal, and large intestinal digestive phases (Liao and Yang 2019). Cr adsorbed on various MP types was more bioaccessible in the gastric environment than in the small or large intestinal phases, due to acidic conditions favoring desorption of anionic Cr species (e.g. CrO_4^{2-} and HCrO_4^-) from the MP surface, while no release was observed in the oral phase. Differences were noted between Cr(VI) and Cr(III), characterized by a higher bioaccessibility in the gastric phase for Cr(VI). Moreover, in the different compartments of the digestion model, the Cr-loaded MPs released more Cr than in a CaCl_2 solution mimicking the freshwater environment (Liao and Yang 2019).

Importantly, different results related to the bioaccessibility of some heavy metals, previously obtained in a simple static model compared to TIM-1 (Torres-Escribano et al. 2011), clearly underline the importance to investigate the behavior of MPs, in their virgin vs contaminated forms, in the human gut through more complex *in vitro* dynamic and multicompartamental systems. The PLASTOX INRAE project has a research axis on this

specific theme. In collaboration with INRAE QuaPA (J. Ratel and E. Engel), we initially planned to investigate the fate of PE MPs with adsorbed polychlorinated biphenyls (PCBs) in the human digestive tract of adults and infants. This family of well-known pollutants was banned in the 80s but is always found in the environment and notably on MPs (see Section 1 Part 3). Our collaborators already succeeded to adsorb PCBs on PE MPs. Next step would be to investigate the potential release of PCBs in the upper digestive tract compartments during TIM-1 digestion. Then, the potential release and impact of these PCB-adsorbed PE MPs on the gut microbiota of adults and infants would be evaluated in the M-ARCOL and Tm-ARCOL models, respectively. It would also be interesting to study such potential effects on previously weathered PE MPs as the sorption profile may be different.

4. Interaction of gut microbiota, a key player in human health, with MPs

4.1 Fecal inoculum: A focus on this key component of *in vitro* fermentation

In order to reproduce and maintain the complexity and diversity of the microbial ecosystem encountered within the adult and infant colon, the M- and Tm-ARCOL models are inoculated with fecal suspensions collected from healthy volunteers. The initial inoculum preparation is an important step to consider but there is yet no consensus regarding the preparation of fecal samples.

As the collection of sufficient amounts of freshly-collected fecal samples is not always feasible, especially in infants, freezing may be a good alternative for stool conservation. Studies usually use either fresh- or frozen-fecal samples but the preservative methods may impact the kinetics of implantation of microorganisms in the *in vitro* colon models and resulting microbial diversity and metabolic activities (Chen et al. 2019b; Pérez-Burillo et al. 2021). Most previous studies have evaluated the impact of preserving methods, mainly freezing at different temperatures (4°C, -20°C or -80°C) with the addition of various cryoprotectants (ethanol, RNA later, DNA shield), on the microbial composition of the initial stool (Gorzela et al. 2015; Choo et al. 2015; Moore et al. 2017; Al et al. 2018), and only one has investigated the impact of freezing preservative techniques on the gut microbiota structure and function during 3 days in the TIM-2 model (Aguirre et al. 2015). The authors recommended the use of fresh stools as

their results showed minor differences in terms of composition and activity of gut microbiota in the TIM-2 system when using frozen stools resuspended in glycerol and stored 1 week at -80°C. Our results (see Section 2 Chapter 1) showed that inoculation of M-ARCOL with fecal frozen samples (freezing 48 h at -80°C with glycerol) led to differences in microbial composition and activity compared to fresh stools. As underlined before, an originality of our study in M-ARCOL was to investigate both lumen and mucus-associated gut microbial communities. We showed that applying preservative methods (freezing 48 h at -80°C, freezing 48 h at -80°C with glycerol as a preservative agent or lyophilization with maltodextrin/trehalose) seemed to limit the expansion of dominant populations (e.g. *Ruminococcaceae*) normally present in the mucosal phase, leaving the opportunity for Proteobacteria (e.g. *Enterobacteriaceae*) and Actinobacteria to colonize this ecological niche. With this in mind, we used only fresh stool samples as much as possible throughout the experiments conducted in this thesis.

Besides, fermentation experiments can be performed using pooled-fecal samples from several donors or each bioreactor can be inoculated with a stool from a single donor. As mentioned in Part 2 of the literature review, there is no consensus on this subject yet. Aguirre and colleagues demonstrated that the microbial diversity and metabolic activity were comparable when using pooled and unpooled human fecal samples for inoculation (Aguirre et al. 2015), while Van de Wiele and colleagues stated that “the artificially high microbial diversity in pooled inocula creates disturbances in the cross-feeding processes between microorganisms that are adapted to one another in each of the separate microbiomes”. Therefore, these last authors advised to study inter-individual variability through separate fecal experiments (Van de Wiele et al. 2015). In this objective, for all our fermentation studies, we used unpooled-fecal samples from various donors to capture inter-individual variability. Our experiments performed on both adult and infant microbiota highlighted donor-dependent effects of PE MPs, confirming the importance to use unpooled stools for MP-related fermentation studies.

Lastly, as reported in Part 3 of the literature review, human is daily exposed to MPs and these particles can be found in their stools (Schwabl et al. 2019; Zhang et al. 2021c; Ho et al. 2022). As fecal samples are used to inoculate M- or Tm-ARCOL, an important point to consider is the initial “contamination” of stool samples with MPs. In collaboration with Drs Teresa Rocha-Santos and Ana Silva from the Laboratory Centre for Environmental and Marine Studies (CESAM) in Portugal, a Nile Red coloration assay, followed by Microplastic Visual

Analysis Tool (MP-VAT) (Prata et al. 2019b), was planned to determine the initial MP contamination of our stool samples. Analyses are still on-going and could not be included in this PhD manuscript.

4.2 Further investigations of the impact of MPs on gut microbiota activity

The emergence of high-throughput technologies enables deep investigations of the human gut microbiota. Sequencing-based analyses of microbiomes have traditionally focused on addressing the question of community membership and profiling taxonomic abundance through amplicon sequencing of 16 rDNA genes, and more recently, through shotgun metagenomics (Shakya et al. 2019). However, their metabolic capability remains less characterized, as well as their influence on the host either structurally or functionally (e.g. by modulating gut permeability and metabolic pathways through bile acid signaling properties) (Holmes et al. 2012a; Ramírez-Pérez et al. 2017), even if “Who is active?” is more important than “Who is there?”. Of interest, it was shown that unrelated subjects share almost the double of metabolic pathways than species (Visconti et al. 2019).

Available studies evaluating MP impact on the gut microbiota (mainly on rodents) focus on the modulation of bacterial composition, but never on its activity. Our study is therefore the first one to investigate the effect of MP exposure on various parameters of the human gut microbiota activity (i.e. gases, SCFAs, AhR ligands, VOCs), especially in the infant at-risk population. We showed several metabolic activity impairments, which might be further investigated. A first simple and rapid way to screen the potential variations of the gut microbiota activity after MP exposure is provided by computational predictions of its functional capability (e.g. PICRUSt (Langille et al. 2013), Piphillin (Iwai et al. 2016), Faprotax (Louca et al. 2016), Tax4Fun2 (Wemheuer et al. 2020), using data from 16S rDNA gene sequencing and close genomes referenced using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. However, the main limitation of such methods is the absence of data available in the databases allowing to characterize only 10 to 50% of the total sequences. Tax4Fun2, the most recent and powerful tool among the existing predictive methods, was applied to the development of the Tm-ARCOL model (see Section 2 Chapter 3) and we identified specific pathways in infants in comparison with adults. Such method could also be applied to assess the effect of PE MP exposure on infant and adult gut microbiota.

In addition to VOC profiling, another possibility to go further into the effect of MP exposure on gut microbiota activity is the use of other omics technics, such as metatranscriptomics (gene expression), metaproteomics (protein production) and metabolomics (metabolite production). Metatranscriptomic and volatolomic analyses were already successfully used to study the effect of different persistent organic pollutants and foodborne chemicals on human gut microbiota activities in 24-h batch *in vitro* experiments (Defois et al. 2018). These omics technics have also been already applied to study the plastisphere characteristics in river and marine environments. The MP community in urban river displayed distinct and actively expressed antibiotic resistome and transcript profile compared to the river water (Wu et al. 2022). Noteworthy, *Enterobacteriaceae*, which were increased after PE MP exposure in the M- and Tm-ARCOL, were identified as bacterial host of antibiotic resistance genes in the plastisphere. An integrated metagenomics/metaproteomics approach, conducted on MPs in marine environment, also highlighted that genomic and proteomic profiles of communities associated with the structurally different PE and PS polymers were highly similar, hence polymer-unspecific (Oberbeckmann et al. 2021), while the taxonomic composition of the plastisphere was previously reported to be plastic-type dependent (Zettler et al. 2013). Although very interesting, one of the main limitations of omics techniques is the lack of adequate reference databases and the huge amount of data generated. In our study, volatolomic analyses brought first information on the impact of PE MPs on the metabolic activity of human gut microbiota. However, this technic has represented a tremendous amount of data to process and confront with literature (Shakya et al. 2019; Karaduta et al. 2021). Establishing an integrated multi-omics protocol will be ideal to fully characterize the gut microbiota activity, especially in the context of MP exposure, but the development of these technics is still at its infancy and will require collaboration between bioinformaticians, biostatisticians and biologists. MEDIS unit gathers all these expertise and may consider in the future, to develop such full-scale protocol to complete its already well-known skills on the field.

4.3 Better characterization of the MP/gut microbiota interaction

Several xenobiotic chemicals have been described to interact with the biological activity of the intestinal microbiota affecting the microbial composition and global homeostasis, with hazardous alterations for the host health (Koppel et al. 2017; Collins and Patterson 2020). The molecular mechanisms underlying these interactions are not well-known. Reciprocally, regarding xenobiotics metabolism, the gut microbiota can directly metabolize poorly-absorbed

chemicals through the action of several bacterial enzymes (such as β -glucosidases, β -glucuronidases, sulfatases, azoreductases, nitroreductases and transferases) in the large intestine (Koppel et al. 2017). Overall, the gut-microbiota-mediated metabolism potentially results in inactivation, activation or reactivation of compounds (Collins and Patterson 2020). In the case of inactivation, this could lead to xenobiotic neutralization while activation may be detrimental and increase the risk of developing cancer as stomach or colorectal cancer (Abdelsalam et al. 2020). Consequently, the maintenance of a healthy gut microbiota may be protective against xenobiotics, probably including MPs, and the occurrence of associated health impairment. Sensitive populations harboring distinct gut microbiota, and particularly disturbed microbiota, may differentially interact with MPs and these aspects need properly-designed investigations.

As already pointed out with other MP types in previous *in vitro* studies (Part 3 of the literature review), we showed during this PhD work that microorganisms, from both adult and infant gut microbiota, were able to form “biofilm-like” structures on MP surface. However, the identity of these microorganisms and the mechanisms by which they interact with MPs have not been investigated. In the environment, the core microbiome of the PE MP plastisphere is composed by *Alphaproteobacteria*, *Gammaproteobacteria* and *Bacteroidia* and the dominant bacterial families vary according with time during the biofilm formation (Tu et al. 2020). These variations highlight the importance to investigate the gut plastisphere, its composition but also its spatial and temporal variations during its presence in the digestive tract. Several technics may then be used to study the MP gut plastisphere (Bartkova et al. 2021). Those technics have been first applied to environmental samples but might be adapted for gut samples. The first step is usually to isolate MPs by filtering samples through sieves or membranes and can also include a purification process involving treatments with ethanol, acidic or alkaline solutions. This step is crucial and may be challenging according to the sample matrices. In the case of M-ARCOL nutritional medium, numerous particles, among which several have the same size range as MPs, may be encountered and complexify the isolation of the latter. Bacterial association with MPs is usually analyzed by scanning electron microscopy and fluorescence microscopy (Zettler et al. 2013; Hossain et al. 2019a). To study the biofilm matrix, in particular the extracellular polymeric substances, the use of confocal laser scanning microscopy (CLSM) is possible (Hossain et al. 2019a). Classical 16S rDNA and functional metagenomics could be finally applied to characterize the gut plastisphere composition and its activity, respectively (**Fig 4.6**).

Besides, no study has yet investigated the capacity of the gut microbiota to degrade PE MPs, but recent studies have shown that several bacteria identified in the soil (e.g. *Alcaligenes faecalis*, *Bacillus cereus*, *Anoxybacillus flavithermus*) are able to degrade PE MPs and alter their surface (Akarsu et al. 2022; Tareen et al. 2022). Of note, *Bacillus cereus* and *Bacillus paramycooides* are able to degrade in 21 days in the environment 63% and 79% of PE and PP MPs, respectively (Nanthini Devi et al. 2021). These studies confirm the existence of potential plastic-degrading bacteria, especially for our PE MPs model. However, their presence and ability to degrade PE MPs in the human gut have still to be investigated. Of note, it is also important to consider not only the bacterial fraction, but also the mycobiota since fungi from the gut microbiota of wax moth (*Galleria mellonella*) have been described as PE degraders (Zhang et al. 2020a; Lou et al. 2020). In collaboration with Fabienne Lagarde, Frederic Amiard and Mathieu Edely from the laboratory IMMM in Le Mans, we conducted Raman spectroscopy imaging on PE MP surface after M- or Tm-ARCOL fermentations to decipher potential physico-chemical modifications by the human gut microbiota. Our results indicate no modification when compared to the initial PE powder, but the analysis was carried out on PE MPs present only for 19 (infant) or 24 (adult) hours in the bioreactors (corresponding to the respective retention time). In the environment, biodegradation of MPs works in conjunction with abiotic factors (e.g. high temperature, light), not found in the human gut, which further favors accessibility to enzymatic attacks (Lear et al. 2021). While the integrity of virgin PE MPs does not seem to be impacted by the human gut plastisphere, the conclusions would likely be completely different for aged-MPs and future investigations of those more representative forms are needed.

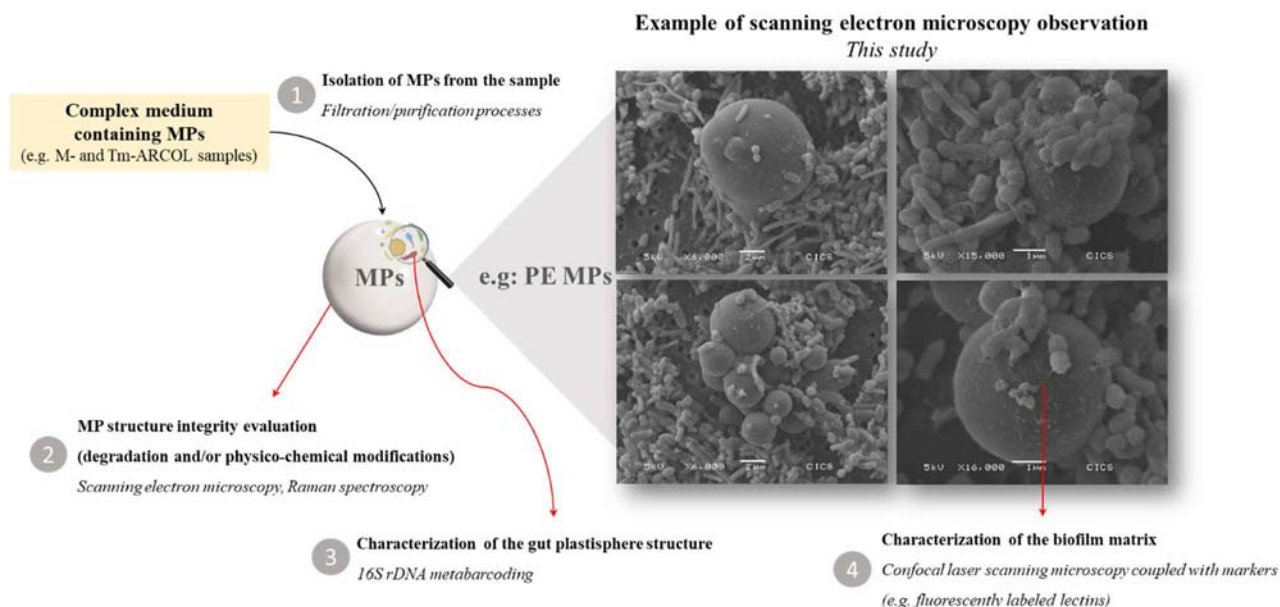


Fig 4.6. Approach and analytical methods to study the gut plastisphere

Samples from the M- or Tm-ARCOL models could be filtered for further investigation regarding the gut plastisphere. First, scanning electron microscopy would be highly valuable to visualize the interaction between MPs and the gut microbiota, as done in this work for PE MPs. Second, to characterize the composition of the gut plastisphere, 16S rDNA metabarcoding could be carried out after methodological adaptation (e.g. to overcome the limited amount of available biological material), while confocal laser scanning microscopy would enable to identify the nature of the biofilm matrix. Lastly, potential physico-chemical modifications and/or degradation of MPs could be investigated by Raman spectroscopy, as performed here.

4.4 From colonic to small intestinal microbiota

As aforementioned, interactions between colonic microbiota and MPs have been poorly studied to date, most studies relying on fecal samples. However, the colonic microbiota is not the only that potentially interact with MPs during their transit through the human GIT, and it would also be of great interest to study the interactions of MPs with the microbiota of the upper digestive tract, particularly that of the small intestine (see Section 1, part 1). Indeed, colonic and small intestinal microbiota exhibit several differences, with more facultative anaerobes in the upper gut, such as *Enterobacteriaceae* (Sundin et al. 2017). In this PhD work, both under adult and infant conditions, we showed an increased abundance of *Enterobacteriaceae* after PE MP exposure, suggesting that investigation of small intestinal compartments where a high prevalence of this family is found would be of great importance (see Section 2 Chapters 2 and 4). However, studying the small intestinal microbiota in humans is very challenging and requires the use of invasive methods such as nasoduodenal catheters or the collection of ileal

mucosa samples during colonoscopy, intestinal resection, small-bowel transplantation, or from sudden death victims (Wang et al. 2005; Baumgart et al. 2007; Ahmed et al. 2007; Willing et al. 2009; Hartman et al. 2009; Hayashi et al.).

As an alternative, to study the small intestine and its associated gut microbiota, animal models and especially rodents are often used although major differences compared to human (e.g. intestinal size, metabolic rate, dietary habits, and spatio-temporal microbial structures) (Hugenholtz and de Vos 2018). *In vitro* gut models allowing the maintenance of, at least some population, of the human small intestinal microbiota are really scarce (Cieplak et al. 2018; Roussel et al. 2020; Écale 2021). MEDIS is working on the development of different *in vitro* models reproducing the ileal microbiota. First, the M-ARCOL model is currently being adapted to reproduce the ileal human compartment, by applying physico-chemical parameters specific from the human ileum (pH, bile salts concentration, residence time, nutrient availability) to a fecal sample. Second, the ESIN model (Guerra et al. 2016) described in Section 1 part 2, is now optimized to introduce microbiota in the jejunal and ileal compartments. This can be achieved either by using fecal microbiota that should be shifted to a small intestinal profile or through a consortium of isolated strains mainly found in the jejunal or ileal compartment in humans. This newly developed model would be relevant to investigate the effects of both physico-chemical conditions and microbiota of the upper digestive tract on MP fate and impact. Interestingly, both the TIM-1 and ESIN models have been or will be optimized at short term to integrate, like in the M-ARCOL model, a mucosal compartment, for a better evaluation of MP interactions with mucus-associated microbiota (Sauvaître et al. 2022).

5. The intestinal barrier and its interactions with MPs

5.1 Cell culture models for MP-related studies: some innovation in the field

Many studies have already evaluated the effects of MPs on cell cultures by direct contact. However, due to their low density (lower than that of water), some MPs as PE MPs, when added to the surface, float upwards during the incubation period and therefore do not come in contact with the cells located at the bottom of the cell culture dish or insert. To overcome this issue, Stock's team developed a smart set-up consisting of an inverse cell culture model, which was tested on hepatic cells (Stock et al. 2019a). While exposure to PE MPs (4-198 μm) induced no cytotoxicity on cells in the regular cell culture model, in the inverted configuration, with the

same culture conditions, cytotoxicity was observed. Regarding intestinal models, Des Rieux and colleagues were among the first research groups studying particle uptake in an inverted combined Caco-2/Raji-B model (des Rieux et al. 2007). They demonstrated that the uptake of 200-nm carboxylated PS particles was significantly increased in the co-culture and the inverted model compared to the control Caco-2 monoculture or the non-inverted model through a non-specific absorption endocytosis. This methodological innovation was taken further and applied to a triple cell culture model (Caco-2, HT29-MTX-E12 and THP-1 cells) of healthy vs inflamed intestine (Busch et al. 2021b). While the inverted model exhibited comparable characteristics to the original one in terms of barrier development and inflammatory parameters, effects of exposure to different PE particle sizes, from 200 nm to 9.9 μm , at a dose of 50 $\mu\text{g}/\text{cm}^2$ for 24 h (e.g. cytotoxicity, increase in IL-8 secretion), were exclusive to the inverted configuration.

Another technical improvement was proposed by Lehner and his team to counteract this buoyant-particle issue (Lehner et al. 2020). MPs were aerosolized directly onto the cells in order to avoid particle floating in the cell culture medium, thus ensuring a better control of particles/cells interactions. This system was tested on a three-dimensional *in vitro* intestinal cell model, composed with a mixture of Caco-2 and HT29-MTX cells coupled to human blood monocyte-derived macrophages and dendritic cells, exposed to PP and PA MPs (50-500 μm) after 6, 24 and 48 h of an aerosolized exposure to a concentration of 823.5–1380.0 $\mu\text{g}/\text{cm}^2$. No significant toxicity effect was reported in terms of inflammatory response and barrier integrity disruption.

5.2 Coupling cell culture and *in vitro* gut model to study MPs

5.2.1 Assessing the direct effects of MP exposure

To date, only one study on MPs has considered the coupling of cell culture with simple *in vitro* systems (Stock et al. 2019c). Regarding more complex models, Liu and colleagues used a dynamic three-stage *in vitro* digestion system mimicking the mouth, stomach and small intestinal human conditions to investigate the differences between pristine and digested PS MPs (5 μm vs 100 nm) on Caco-2 cells (Liu et al. 2020a). The digestive process induced an increase in the average size and zeta potential of the particles, thus favoring their agglomeration. Caco-2 cells were then exposed either to the digested MPs or to the pristine ones. For undigested particles, alterations of transport function were observed with an increased epithelium permeability, reported through the para-cellular LY marker, as well as a down-regulation of

ZO-1 and *occludin* gene expression. In addition, alteration of transmembrane transport was shown by a down-regulation of *ABCC2* and *ABCG2* gene expression. However, for digested MPs, a reduction in the cytotoxicity and impairment of the transport function was obtained. Concerning the inflammatory response, an increase in the pro-inflammatory effects (interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP1)) was observed, but only for digested small-sized particles (100 nm) compared to pristine ones. These differences between digested and pristine particles could be attributed to the formation of a biomolecular corona at the particle surface (Liu et al. 2020a).

5.2.2 Assessing the indirect effects of MP exposure, mediated by the gut microbiota

These easy-to-use intestinal models are interesting to study direct effects of MP exposure but, in our case, we investigated the potential indirect impact of gut microbe metabolites after PE MP exposure on cells. For this purpose, we incubated luminal M-ARCOL supernatants before and after PE MP exposure, which did not contain MPs anymore (as verified by optical microscopy), with a co-culture of Caco-2 and HT29-MTX cells. Our study is the first one coupling *in vitro* colon model to intestinal cell culture for MP-related issues. As presented in the Part 2 of the literature review, such coupling has already been investigated for other research topics, notably for nutritional studies, but few on pollutants (Cai et al. 2017; Calatayud et al. 2018; Réquillé et al. 2018; Defois et al. 2018).

The major issue, by incubating fermentative medium with cell cultures, is the high sensibility of cells to numerous compounds present in this medium as bile acids. To avoid cytotoxic conditions for cells, we diluted fermentative supernatants from luminal M- and Tm-ARCOL samples, and a 1:20 dilution was required. Prior to exposure on intestinal cells, Defois and colleagues diluted the supernatants, collected from a simple 24-hour batch *in vitro* colonic system, per 1:4 (Defois et al. 2018). For supernatants obtained from the SHIME colonic model, Calatayud and colleagues diluted them by 1:20, the same factor as ours, prior to cell exposure (Calatayud et al. 2018). Finally, Cai and his team diluted the SHIME colonic chyme samples per 1:4, after a phase of heating at 90°C to inactivate enzymes followed by filtration through a 0.22- μ m pore size membrane (Cai et al. 2017). Given the dilution of 1:20 required before exposure of cell to M- or Tm-ARCOL luminal supernatants, it is highly plausible that bioactive compounds were also present in a very small amounts, influencing the cellular response. A striking example may be butyrate. In Tm-ARCOL, we observed a significant reduction in butyrate production (18.5 mM vs 13.7 mM in control) by the infant gut microbiota after PE MP

exposure. This compound is well-known to interact positively with the intestinal barrier (Hamer et al. 2008). In particular it promotes the integrity of the intestinal barrier at a concentration of at least 2 mM (Peng et al. 2007). Under our exposure conditions, the concentration of butyrate, averaging 0.9 and 0.7 mM for the diluted luminal supernatants of Tm-ARCOL before and after PE MP exposure, respectively, was too low to induce any remarkable effect. This observation for butyrate can certainly be extrapolated to other microbial metabolites present in the luminal supernatants from M- or Tm-ARCOL.

5.3 The importance of mucus in physiologically-relevant *in vitro* cell models

We observed an increase in *Enterobacteriaceae* in both adult and infant gut microbiota after PE MP exposure (see Section 2 Chapters 2 and 4). Members of this bacterial family, as well as most of the enteric pathogens, have to invade the mucus barrier before reaching the intestinal epithelium to promote their colonization and persistence (Sauvatre et al. 2021). This underlines the importance of mucus, its protective properties but also possible impairment, in any gut microbiota- and/or xenobiotic-related studies. Noteworthy, for MPs, the protective effect of the mucus layer has been pointed out *in vitro* (Hesler et al. 2019). The mucus-producing HT29-MTX cells have been extensively used as colonic goblet cells to study the role of mucus (Béduneau et al. 2014; Lozoya-Agullo et al. 2017). We chose to work with a co-culture of Caco-2 and HT29-MTX cells, allowing the *in vitro* simulation of an intestinal epithelium with cell repartition and permeability more representative of intestinal physiology compared to a simple monoculture of Caco-2 cells. Interestingly, Esch and colleagues used a co-culture of Caco-2 and HT29-MTX cells on a microfluidic device in order to assess the uptake of nano-sized PS particles across the intestinal barrier (Esch et al. 2014). However, some major limitations cannot be set aside. In particular, it was shown that co-cultures of Caco-2 and HT29-MTX cells generally led to the formation of mucus patches and not a regular mucus layer at the cell surface (Dorier et al. 2017; García-Rodríguez et al. 2018; Gillois et al. 2021). Furthermore, other cell types, as T84 (presented in the literature review) or LS-174T (isolated from well-differentiated primary colon adenocarcinoma) could be used as an alternative to Caco-2 and HT-29-MTX cells, respectively. It was suggested that a T84 monolayer is more representative than a Caco-2 monolayer to model colonocytes (e.g shorter microvilli, more abundant colonic markers as monocarboxylate transporter 1 -MCT1-) (Devriese et al. 2017). Moreover, LS-174T goblet cells produce a higher amount of mucus than HT29-MTX cells and with a mucin pattern

containing more MUC2 than MUC5AC, which is closer to the *in vivo* situation in the human colon (Bu et al. 2011). Recently, a human *in vitro* triple co-culture model (T84, LS-174T and THP-1 cells) was developed, also incorporating an agar-mucin layer isolated from the cell layer by a 0.4- μm pore size filter to mimic the inner mucus layer and its permeability towards ions and small molecules (Beterams et al. 2021) (Fig 4.7). This methodological innovation allows to test directly *in vitro* microorganisms-containing colonic samples on the cell culture model. Thus, no dilution is needed, which avoids masking effects described above, and the investigation of gut microbial metabolites-mediated impact on cells is possible under more realistic exposure scenarios.

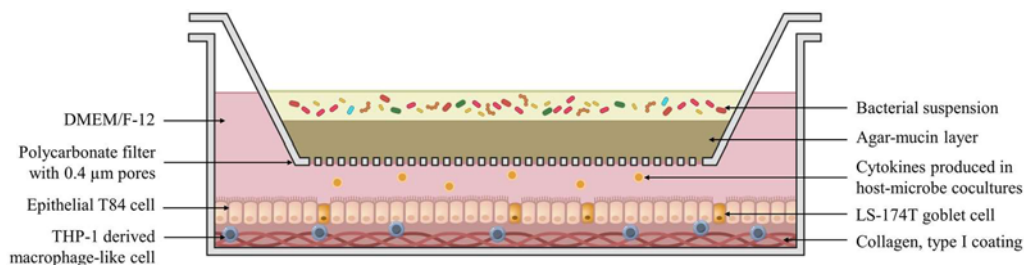


Fig 4.7. A triple co-culture model, incorporating an agar-mucin layer directly in contact with the bacterial suspension

The triple co-culture model consists of a collagen coating with confluent THP-1, LS-174T and T84 cells in DMEM/F-12 at the basolateral side, in indirect contact with apically-incubated microbes, which are adhered to a solidified agar-mucin layer covering the Transwell polycarbonate membrane filter (0.4- μm pore size). This model was developed to study host-microbe interactions in the human gut.

Extracted from Beterams et al. 2021

It is important to note that mucus acts both as a physical barrier (i.e. a selective filter depending on the size of the particles) and as a chemical barrier that preferentially retains positively-charged particles as it is itself negatively charged (Cui et al. 2020). As developed in Part 3 of the literature review, positively-charged PS particles induce in mice more deleterious impact on intestine than negatively-charged ones (Qiao et al. 2021). Regarding smaller-sized nanoplastics (i.e. size < 1 μm), the effect of cationic PS particles was also more intense than that of anionic particles on macrophage cells (Bhattacharjee et al. 2014) and splenic lymphocytes (Li et al. 2022c). However, in rats, it was shown that negatively-charged PS nanoplastics tended to accumulate in a greater amount than their positively-charged counterparts (Walczak et al. 2015). Given the paucity of data, it would be interesting to address

in vitro this surface charge-related question on MPs effects on the intestinal barrier, including mucus as a key player in MP uptake.

With this in mind, further studies on the interaction of MPs with the intestinal barrier could be performed through a more complex coupling between *in vitro* gut models and cell cultures, as previously done with arsenic in the SHIME system and subsequent exposure of food digests to Caco-2/HT29-MTX cells (Calatayud et al. 2018), or even more advanced cell models including immune cells. As a next step, as presented in Part 2 of the literature review, a relevant approach could be a continuous input of the digestive medium from *in vitro* gut models on cell cultures, as done with the HMI module, mainly adapted to be combined with the SHIME model but limited to 48 h for cytotoxic reasons (Marzorati et al. 2014).

5.4 Complex 3D models to investigate MP impact on the intestinal barrier

The intestinal cell models, in monocultures or co-cultures (with mucus-producing and/or immune cells), presented in the previous section, have shown their interest to increase the knowledge on the absorption of MP, their mode of action and the cellular responses. Nevertheless, the currently used models still suffer from critical limitations. First, almost all cells described are from cancer origin and, therefore, the observed effects on cell signalling, immune responses, oxidative stress and toxicity must be carefully examined because cancer cells have distinct characteristics, for instance with respect to basal metabolism and reactive oxygen species (ROS) levels. Furthermore, the most widely used Caco-2 cell line is a model for small intestinal enterocytes (the use of T84 cells is still limited), and thus does not account for spatial differences in intestinal mucosal morphology and function, depending on the location to the GIT, which may be of importance such as seen *in vivo* (Carr et al. 2012).

More complex 3D models could be used in the future, such as multicellular models, which are currently used for MP study (Lehner et al. 2018; Lehner et al. 2020) or intestinal organoids (Kim et al. 2020a), not explored to date. Interestingly, liver and airway organoids have already been used for MPs-related studies (Cheng et al. 2022; Winkler et al. 2022). Cheng's team set-up a new *in vitro* 3D model, namely liver organoid, generated from human pluripotent stem cells and explored the adverse effects of 1- μm PS MP exposure (0.25, 2.5 and 25 $\mu\text{g}/\text{mL}$) (Cheng et al. 2022). The authors showed that PS MP exposure induced hepatotoxicity and lipotoxicity regarding ATP production, alteration in lipid metabolism, ROS generation,

oxidative stress and inflammation response. Regarding the inhalation exposure route, an airway organoid was also developed to investigate the effect of MP fibers on the lung. Such exposure did not inhibit organoid growth but induced a significant reduction of *SCGB1A1* gene expression, related to bronchiolar exocrine cell functionality, as well as a polarized cell growth along the fibers. MP exposure did not induce inflammation or oxidative stress, but the particles were coated by a cell layer, resulting in the inclusion of fibers in the organoid. This effect may have long-term implications for lung epithelial cells undergoing repair. Taken together, all of these results underscore the importance of developing intestinal organoids for MPs-related studies. However, although they more closely mimic *in vivo* conditions, they remain very expensive. In addition to specialized expertise, they also require easy access to human biopsies and, in terms of operational constraints, high levels of oxygen (Etienne-Mesmin et al. 2019a).

Similarly, human gut bioengineering on-a-chip devices, such as HuMiX (Shah et al. 2016; Eain et al. 2017), Intestine-Chip (Kim et al., 2015) and Colon-Chip systems (Sontheimer-Phelps et al. 2020), would allow to consider the influence of mechanical forces (peristalsis, intestinal flow), and ultimately commensal microbes and mucus, on the fate and toxicity of MPs in the gut.

All of these promising *in vitro* models could be adapted to a compromised intestinal barrier, such as the leaky gut in inflamed models, to mimic the disease state of sick populations. The *in vitro* approach, which mimics human intestinal physiology with increasing complexity and reliability, is therefore undoubtedly very helpful in elucidating the fate, physico-chemical transformations, degradation, metabolism and toxicity-related mechanisms of MPs, which will be useful for future hazard and health risk assessment.

5.5 Gut and its crosstalk with other organs for further studies in MP toxicity assessments

Despite the intestinal sphere described along this PhD manuscript, it was shown in mammals or with *in vitro* cell cultures that MP exposure impacted brain cells (Shan et al. 2022; Hua et al. 2022), heart (Zhang et al. 2022d), pulmonary cells (Zhang et al. 2022a), ovary and testis (Hou et al. 2020; An et al. 2021), liver (Zheng et al. 2021) and kidneys (Wang et al. 2021d), even though the underlying mechanisms are still poorly investigated. After evaluating such impact on each organ separately, it is also important to evaluate systemic effects *via* a focus on inter-organ communication. This question has been mainly addressed by *in vivo*

studies. A recent study in mice showed that an intragastric administration of PS MPs and nanoplastics (10 μm or 5 μm and 80 nm, respectively) for 42 days, at a dose of 60 $\mu\text{g}/\text{day}$, led to gut microbiota disorder, metabolite changes, cytokine alterations and *in fine* to hematopoietic toxicity (Jing et al. 2022). A MP-perturbed gut microbiota was also linked to testicular disorder in male mice (Wen et al. 2022b) and to an exacerbation of liver injury in mice (Wen et al. 2022a). Using a multiomic approach including metabolomic and microbiome analyses, Shi and colleagues studied the gut-liver axis metabolism by exposing mice to pristine PS MPs (1 μm) at a dose of 10 mg/L (in water) for 2 weeks (Shi et al. 2022b). The authors showed an impairment of the gut-liver crosstalk, characterized by modifications of the gut microbiota and disorders of the liver metabolism and metabolic pathways, leading altogether to an increased risk of insulin resistance and diabetes. Conducting their study on zebrafish, Huang and colleagues revealed a novel mechanism by which MPs might cause behavioral toxicities *via* the brain-gut axis (Huang et al. 2022b). Finally, Yin and colleagues showed that PS MP exposure in chicken induces up-regulation of liver glutamine and glutamate synthesis, and promoted autophagy-dependent ferroptosis and apoptosis in the cerebellum through the liver-brain axis (Yin et al. 2022).

In contrast to the growing number of *in vivo* studies, only one has been conducted *in vitro* to disentangle the potential inter-organ dependent effects of MPs. In their aforementioned study (Esch et al. 2014), Esch and colleagues developed a co-culture of Caco-2 cells and HT29-MTX cells on a microfluidic device, which was also connected to the HepG2/C3A liver model to evaluate the hepatic toxicity of the absorbed particles. Thus, for inter-organ studies on MPs, the gut models, cited above in the part 5.4, could be used but also advanced cell (co)culture models involving for example intestinal, extra-digestive but also immune cells (e.g. Raji-B or THP-1 cells (Kämpfer et al. 2020)).

6. The outcomes of my research: a focus on MP risk assessment

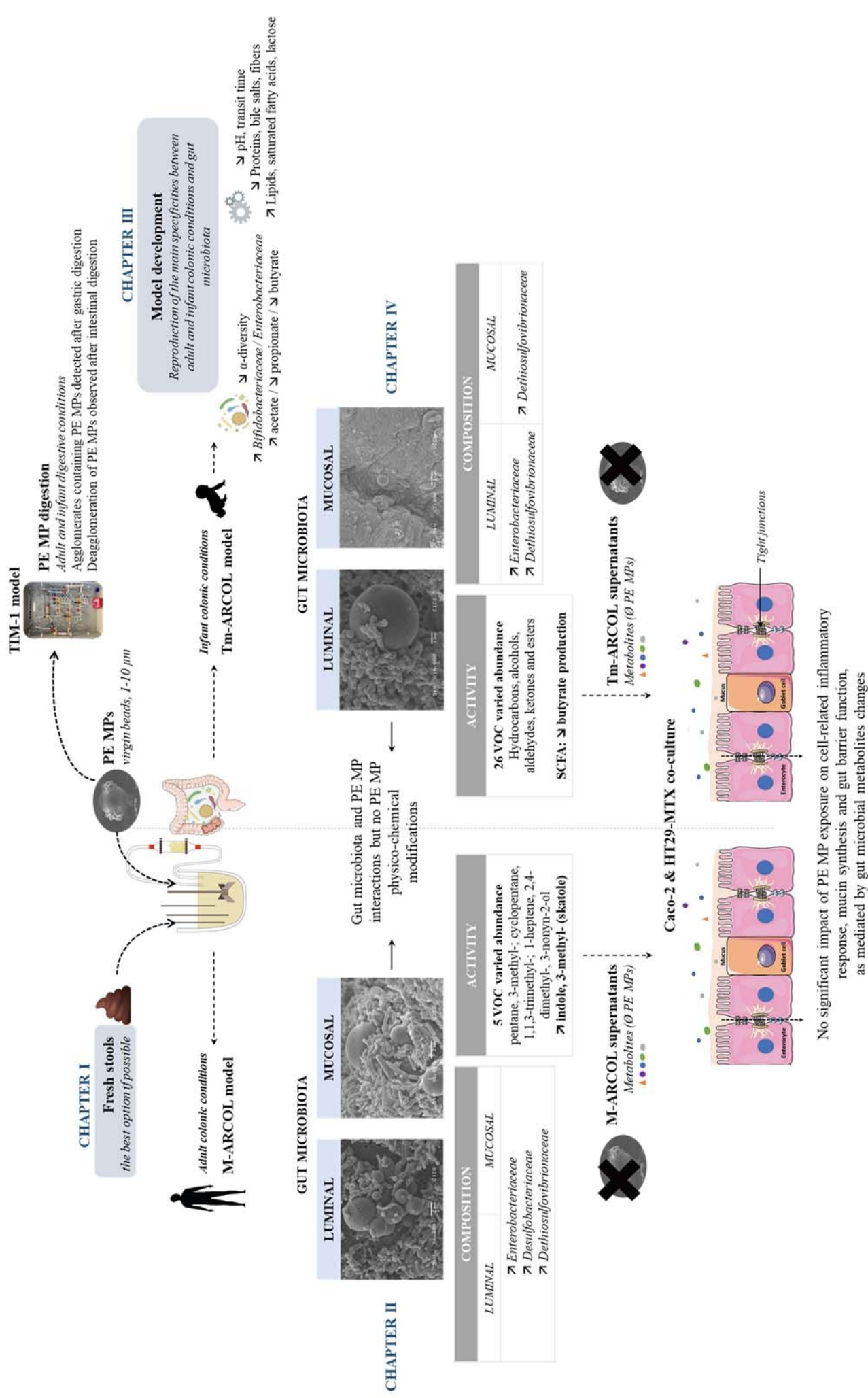
My PhD work started at the end of 2019, corresponding to the beginning of the “race” for a better understanding of human exposure to MPs and associated health risks. My research on PE MPs was a prospective study given a first overview of PE MP fate and impact in the human digestive tract of both adults and infants using relevant *in vitro* models (**Fig 4.8**), in a time period where most MP-related studies were focusing on animal models and PS MP exposure at high doses. Addressing the health hazards of MPs requires an interdisciplinary expertise gathering biologists, chemists and physicists. MPs do not easily fit within traditional risk-based regulatory frameworks because their extreme diversity (e.g. size, shape and physico-chemical properties associated with adsorbed chemicals), as well as significant gaps in analytical methods for their standardized detection and quantification in food, air, drinking water and biological matrices, result in high levels of uncertainty in hazard and exposure estimates. Due to this critical complexity, addressing the impact of MP requires open discussions between scientists, as illustrated by the various collaborations during my PhD work. Another complexity resides in the numerous sources of contamination during MP analyses in stools, tissues, packaging, food, air or water, as all the laboratory equipment’s which is essentially composed of plastic materials. For instance, gloves and clothing may lead to an overestimation of MPs (Witzig et al. 2020; Gwinnett and Miller 2021). That is the reason why during my PhD work, special care was taken to limit MP contamination, with also the systematic inclusion of control conditions in each experimental design.

My research, built upon the others, will contribute to a better health hazard assessment in human, and combined with exposure data, will favor the development of a MP risk assessment framework. This will facilitate the implementation of future regulations at the French but also European levels. I presented my work in 2021 as a short communication at the EFSA Scientific Colloquium “A coordinated approach to assess the human health risks of micro- and nanoplastics in food”. This clearly underlines the urgent need of policy measures for regulatory bodies and reference laboratories (e.g. exposure limit value for MPs) but also industry activities as in the packaging sector, for instance for the development of recycled plastics/alternative materials to plastics. A hand-in-hand work with academic research will then be required to optimize these new materials and verify their safety for human health.

Fig 4.8. Main results of the PhD work on the impact of PE MPs on the human digestive environment in adult and infant conditions

This joint PhD between MEDIS (Clermont-Ferrand) and Toxalim (Toulouse) aimed to investigate the fate of virgin PE MPs in the adult and infant gut and, notably, to decipher their interactions with the adult and infant human gut microbiota, using the M- and Tm-ARCOL models (developed during the PhD work) respectively, and finally to evaluate the potential impact on the intestinal epithelium/mucus barrier, as mediated by gut microbial metabolites, using a co-culture of Caco-2 and HT29-MTX cells exposed to luminal M- and Tm-ARCOL supernatants. While the interaction between PE MPs and the adult and infant gut microbiota was assessed by scanning electron microscopy and Raman spectroscopy, the effects of PE MP exposure on the luminal and mucosal gut microbiota composition and activity were evaluated by 16S rDNA metabarcoding, and the monitoring of gas and short chain fatty acid production and composition, AhR ligand production and volatile organic compound analysis, respectively. PE MPs were also digested in the TIM-1 system under adult and infant specific digestive conditions and their detection and potential physico-chemical modifications were investigated by Raman Tweezer instrument.

MP: microplastic; PE: polyethylene; SCFA: short chain fatty acid; VOC: volatile organic compound



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Appendix

Scientific Curriculum Vitae

SCIENTIFIC DEGREES AND TRAININGS

2019-2022

PhD student | Biology and Health

PhD school Sciences de la Vie, de la Santé, Agronomie et Environnement (SVSAE)

Supervisors: Stéphanie BLANQUET-DIOT, Lucie ETIENNE-MESMIN, Muriel MERCIER-BONIN

Host laboratories: UMR454 Microbiologie, Environnement Digestif et Santé (MEDIS), Clermont-Ferrand, Université Clermont Auvergne/INRAE & Toxalim (Research Center in Food Toxicology), Université de Toulouse, INRAE, ENVT, INP-Purpan, UPS, Toulouse

Subject: Fate of microplastics in human digestive in vitro environment and study of the dialogue between epithelium, microbiota and mucus

Funding: Ministerial scholarship

- **2020-2022 : Teaching assistant, IUT Génie Biologique, Clermont-Ferrand**

Practical courses of Microbiology for undergraduate students (1st year of technological school, IUT Clermont Auvergne), 64 hours/year

2016-2019 : Double degrees

- ❖ **Engineer Diploma in Agronomy and Food Processing** | VetAgro Sup, Clermont-Ferrand

Diploma of young entrepreneur student | Treasurer of the Junior enterprise SESTAGRI

- **From January to June 2018 - ERASMUS** in Norway, Norwegian University of Life Sciences
- **From June to August 2018 – Research internship (2 months)**

Supervisor: Ludovic Bigot

Host laboratory: UMR981 Laboratoire de recherche Gustave Roussy, Villejuif (94)

Subject: BRAF resistance in prostate cancer

- ❖ 3rd year option - **Master 2 in Biology & Health, Option Human Nutrition**

Université Clermont Auvergne

- **From January to July 2019 – Research internship | Master 2 (6 months)**

Supervisors: Stéphanie BLANQUET-DIOT, Lucie ETIENNE-MESMIN

Host laboratories: UMR454 Microbiologie, Environnement Digestif et Santé (MEDIS), Clermont-Ferrand, Université Clermont Auvergne/INRAE

Subject: Development of an *in vitro* model of the colic environment of irritable bowel syndrome patient

2014-2016

Preparatory classes for to enter French elite universities | **B.C.P.S.T**, Lycée Chaptal Paris 8^{ème}

LIST OF PUBLICATIONS

Fournier E, Ratel J, Denis S, Leveque M, Ruiz P, Mazal C, Amiard F, Edely M, Bezirard V, Gaultier E, Lamas B, Houdeau E, Engel E, Lagarde F, Etienne-Mesmin L, Blanquet-Diot S, Mercier-Bonin M (2022) Exposure to polyethylene microplastics alters immature gut microbiome in an infant *in vitro* gut model. *Journal of Hazardous Materials*. 443:130383. doi: 10.1016/j.jhazmat.2022.130383

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Fournier E, Etienne-Mesmin L, Blanquet-Diot S, Mercier-Bonin M (2022) Microplastiques dans notre alimentation: un focus sur la santé intestinale. *Les cahiers de Nutrition et de Diététique*. 57:270–283. doi: 10.1016/j.cnd.2022.03.001

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Fournier E, Etienne-Mesmin L, Grootaert C, Jelsbak L, Syberg K, Blanquet-Diot S, Mercier-Bonin M (2021) Microplastics in the human digestive environment: A focus on the potential and challenges facing *in vitro* gut model development. *Journal of Hazardous Materials* 415:125632. doi: 10.1016/j.jhazmat.2021.125632

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Deschamps C, **Fournier E**, Uriot O, Lajoie F, Verdier C, Comtet-Marre S, Thomas M, Kapel N, Cherbuy C, Alric M, Almeida M, Etienne-Mesmin L, Blanquet-Diot S (2020) Comparative methods for fecal sample storage to preserve gut microbial structure and function in an *in vitro* model of the human colon. *Appl Microbiol Biotechnol*. 104(23):10233-10247. doi: 10.1007/s00253-020-10959-4

ORAL COMMUNICATIONS

Fournier E, Denis S, Durif C, Mercier-Bonin M, Etienne-Mesmin L, Blanquet Diot S. Development of a mucin-associated *in vitro* model of the toddler gut microbiome adapted to infant specific diet and colonic environment. 7th International Conference on Food Digestion, May 2022, Cork, Ireland.

Fournier E, Etienne-Mesmin L, Mercier-Bonin M, Blanquet-Diot S. Microplastics (MPs): Fate in *in vitro* human digestive environment and study of the dialogue between epithelium. microbiota and mucus. Webinaire, groupe régional de recherche en microbiologie des Interactions (G-RREMI), July 2021, Clermont-Ferrand, France.

Fournier E, Etienne-Mesmin L, Mercier-Bonin M, Blanquet-Diot S. Microplastics (MPs): Fate in *in vitro* human digestive environment and study of the dialogue between epithelium. microbiota and mucus. Journée de l'école doctorale SVSAE, 18-19th May 2022, Clermont-Ferrand, France.

Fournier E, Etienne-Mesmin L, Mercier-Bonin M, Blanquet-Diot S. Microplastics in our diet: Complementary *in vitro* gut and epithelium models to understand their fate in the human digestive tract. EFSA 25th Scientific Colloquium, 6-7th May 2021, Lisbon, Portugal (online).

Fournier E, Etienne-Mesmin L, Mercier-Bonin M, Blanquet-Diot S. Fate and impact of microplastics (MPs) in *in vitro* human digestive environment and dialogue between gut microbiota and mucus. 12th International Symposium on Gut Microbiology (INRAE-ROWETT), 13-15 October 2021, Clermont-Ferrand, France (online)

POSTER PRESENTATIONS

Fournier E, Etienne-Mesmin L, Denis S, Verdier C, Chalancon S, Durif C, Uriot O, Mercier-Bonin M, Blanquet-Diot S. Impact of polyethylene microplastics on human gut microbiota as assessed in an *in vitro* gut model. EUROTOX 2021, 27 September- 1st October 2021, Copenhagen, Denmark (online)

Fournier E, Mercier-Bonin M, Denis S, Uriot O, Roussel Charlène, Leveque Mathilde, Arlic Monique, Van de wiele T, Blanquet-Diot S, Etienne-Mesmin L. Deciphering the influence of physicochemical and microbial parameters of the human digestive tract on orally-ingested microplastics using *in vitro* gut models. EUROTOX 2021, 27 September- 1st October 2021, Copenhagen, Denmark (online)

SCIENCE POPULARIZATION

Short communication on the impact of MPs on human health and **round table** on the « One Health » concept, Clermont Week Innovation 2022, VEGEPOLYS VALLEY, VETAGROSUP, SVSAE/UCA, 14th April 2022.

Written interview in collaboration « link between babies and plastics » for a short film, students from lycée Théophile Rousse (Saint Chély d'Apcher, Lozère), February 2022

Testimony of my career at the Proforum of former graduated engineers, 14th January 2022, VetAgro Sup

Interview, documentary project on the exposure and impact of MPs in human, October 2021 (producer: Olivier Husson)

Written interview in collaboration « Impact of microplastics on human health with a focus on the gastro-intestinal tract: some insights from our work » for a book on plastic pollution and their impacts (Matt Simon, request 2021).

TECHNICAL SKILLS

***In vitro* models**

In vitro digestion in artificial model of the upper GIT (TIM-1): adult and infant conditions

In vitro colon fermentation (M-ARCOL): adult and infant conditions

Cellular culture (CACO-2, HT-29 MTX, H1L1)

Analyses

Molecular biology: DNA/RNA/proteins and qPCR

Analytical technics: ELISA technique, HPLC to dose SCFAs, μ GC, cytotoxicity tests for cells (LDH, Lucifer Yellow, CCK-8, Alcian Blue), use the DISCO beamline (synchrotron SOLEIL, Paris-Saclay), Western Blot

Bioinformatics: Data analysis of 16S rDNA metabarcoding analysis (QIIME2 and R Studio), functional prediction (Tax4fun2), classical statistical tests (R studio, PRISM)

Software: R (basics), Git, Zotero, pack office, PRISM (GraphPad), Illustrator & Adobe Premiere Pro (basics)

Appendix 2:

Book chapter from *Handbook of Microplastics in the Environment*

Publication:

Fournier E, Etienne-Mesmin L, Blanquet-Diot S, Mercier-Bonin M (2020) Impact of Microplastics in Human Health. In: Rocha-Santos T, Costa M, Mouneyrac C (eds) *Handbook of Microplastics in the Environment*. Springer International Publishing, Cham, pp 1–25



Impact of Microplastics in Human Health

A Focus on the Gastrointestinal Tract

Elora Fournier, Lucie Etienne-Mesmin, Stéphanie Blanquet-Diot,
and Muriel Mercier-Bonin

Contents

Introduction	2
Characteristics of Microplastics Ingested by Humans	4
Presence of Microplastics in Beverages	4
Presence of Microplastics in Food	5
Potential Microplastic Contamination from Food Packaging	6
Physicochemical Transformations of Microplastics During Digestion	7
Effects of Microplastic Exposure on the Intestinal Barrier	8
Microplastics and Intestinal Epithelium	8
Microplastics and Intestinal Mucus	14
Microplastics and Intestinal Microbiota	15
Summary and Future Directions	16
More Representative Ingested Forms of Microplastics Are Needed, Probably Displaying Distinct Physico-chemistry, Behavior, and Toxicity in the Gut	18
More Physiologically Relevant Gut Models for Human Oral Exposure Should Be Investigated	18
Microplastics May Compromise Gut Barrier Homeostasis by Influencing Crosstalk Between Mucus and Microbiota	19

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Microplastics Under Realistic Oral Exposure May Impact Gut Health Across the Lifespan and Influence Interorgan Communication	20
References	20

Abstract

Because plastics are a multifunctional, resistant, easy-to-process, and affordable material, they play a central role in our daily life. However, their extensive use has become tainted by the continuous rise of plastic pollution worldwide, which generates, after slow photo, chemical, physical, and biological degradation, massive amounts of small-sized microplastics. Their ubiquitous nature in the environment but also in foodstuff and consumer packaged goods has revealed a potential threat to human health. In this chapter, a focus is given on the human gastrointestinal tract, as portal of entry but also first barrier and target for microplastics. We summarize the current state of knowledge on human oral exposure to microplastics and the characteristics of ingested forms (origin, occurrence, size, shape, polymer type, surface properties). Then, we highlight the physicochemical transformations of microplastics during digestion. Afterwards, we detail their potential impact on gut homeostasis disruption, including gut microbiota, mucus and epithelial barriers, considering *in vitro* and *in vivo* studies (rodents). Finally, this chapter points out future research directions about microplastics in the field of human intestinal health. Special emphasis is given to the critical need of developing robust *in vitro* gut models to adequately simulate human digestive physiology for better gut health risk assessment and management of microplastics.

Introduction

Plastic production has significantly increased worldwide over the last decades, from 1.7 million tons in the 1950s to over 368 million tons in 2019 (PlasticsEurope 2020). Because plastics represent a multifunctional, resistant, easy-to-process, and affordable material, they play a central role in our daily life and represent a crucial source for future innovation-driven growth. In 1957, a French intellectual, Roland Barthes, celebrated them in his book *Mythologies*. “The hierarchy of substances is abolished, and a single one replaces them all – the whole world can be plasticized and even life itself since, we are told, they are beginning to make plastic aortas.” Plastics are carbon polymers water-insoluble easily molded and shaped under heat and pressure. They are mainly derived from fossil fuel as oil, coal, or natural gas but can also be bio-based (mainly from cellulose, lignin, and polysaccharides). Today, the five major plastics produced are high- or low-density polyethylene (HDPE or LDPE), polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), and polyethylene terephthalate (PET), while 62% of global demands come from PE and PP (Wu et al. 2019a) and around 40% of all plastic production is used for packaging (PlasticsEurope 2020).

However, the extensive use of plastics has become tainted by the continuous rise of plastic pollution worldwide. Plastic wastes are transported by rivers, runoff, tides, winds, and catastrophic events but also come directly from fishing, aquaculture gears, and ships. Great Pacific Garbage Patch, or the seventh continent, in the Pacific Ocean is an evidence of the magnitude of the problem. It is a 1.6 million sq km of 79,000 tons of floating plastic garbage (more than three times the size of Spain) mainly composed (94%) of microplastics in terms of pieces number. Actually, plastics in the environment undergo slow photo, chemical, physical, and biological degradation, which leads to fragmentation into secondary microplastics, in addition with primary microplastics, which are intentionally manufactured for industrial purposes and directly entering the environment. So far, there is no internationally agreed definition of microplastics (Hartmann et al. 2019), but in general, they are considered to vary in size ranging from 0.1 to 5000 μ m (EFSA 2016). Microplastics are ubiquitous in the environment from marine to ground water, lakes, sediments, soil, and atmosphere (Wu et al. 2019a). Borrelle and colleagues recently underlined that plastic pollution is a planetary threat and extraordinary efforts to transform the global plastics economy are needed (Borrelle et al. 2020).

Facing these critical issues, there is an increasing effort from governments to implement policy measures, legislative instruments, and regulatory laws related to plastic pollution. In the European Union (EU), the European Strategy for Plastics in a Circular Economy (European Commission 2018) stressed the need to tackle the challenges related to plastics. It aims at moving from a linear “take-make-consume-discard” pattern to a closed loop in which materials are continually reused, recycled, and do not leak into the environment. Moreover, the Commission plans to ban ten single-use plastic products by 2021 targeting 70% of the marine plastic wastes (European Commission 2018). Not only EU but numerous actions have been developed all around the world to address plastic pollution, like a complete ban of using, producing, or selling plastic bags in Kenya since 2017 followed by many other countries. However, the current COVID-19 sanitary crisis, which has confirmed the essential role of plastics in our daily life, is forcing to rethink plastic problem worldwide. Klemeš and colleagues showed that demand for medical products and packaging was sharply increasing during the COVID-19 pandemic (Klemeš et al. 2020). The city of Wuhan, the COVID-19 epicenter in China, experienced a massive increase of medical waste from between 40 and 50 tons/day before the outbreak to about 247 tons/day in March 2020 (You et al. 2020). A similar situation might arise in the food industry and packaging, textiles, and other services that have decided to temporally limit reusable, delay restriction reforms on plastic products, thereby contributing to the increasing plastic pollution (Patrício Silva et al. 2021). These plastics have the potential to spread throughout earth, and under the influence of environmental conditions, to break down into microplastics. In such context, the use of disposable face masks has been recently added to the list of potential microplastic sources (Aragaw 2020).

The negative impact on the environment and organisms resulting from plastic pollution is now well-established (Bucci et al. 2019). Inevitably, plastics enter the food chain with probable effects on human gut homeostasis (Lu et al. 2019; Prata et

al. 2019). Barely mentioned 5 years ago, the emerging question of microplastics as a threat to human health is today one of the most active research topics in the field. However, only few studies are focused on human intestinal health at the forefront of oral exposure (Lu et al. 2019; Paul et al. 2020; Hirt and Body-Malapel 2020). Based on this background, we first summarize the available evidence regarding human oral exposure and the characteristics of ingested microplastics (origin, occurrence, size, shape, polymer type, surface properties). Next, we highlight the physicochemical transformations of microplastics during digestion. Afterwards, we detail the putative role of microplastics on the intestinal barrier disruption from *in vitro* and *in vivo* studies (rodents), with a special emphasis on the “orchestrated” triad epithelium/mucus/microbiota. Finally, we discuss some future research directions, in particular the potential of advanced *in vitro* gut models simulating human digestive physiology, for better gut health risk assessment and management of microplastics.

Characteristics of Microplastics Ingested by Humans

Humans daily ingest microplastics through beverages, food but also from air. A large part of inhaled particles could enter the digestive tract through the mucociliary clearance mechanisms of the lung after inhalation (Toussaint et al. 2019; Prata et al. 2019). However, as underlined by Toussaint et al. (2019), it is still difficult to assess human oral exposure to microplastics, due to the lack of a standardized methodological approach, including sampling/analytical procedures, as well as reference materials (for different polymers in different matrices). In such context, Schwabl’s team reported the presence of microplastics in stools collected from healthy volunteers worldwide. Although this study only investigated a small number of subjects ($n = 8$), microplastics were found in all stool samples, with an average of 20 particles/10 g of stool, composed of various polymer types (PP and PET being the most abundant) and sizes (between 50 and 500 μm) (Schwabl et al. 2019). To date, only one study clearly quantified human exposure to microplastics through drink and food consumption of a typical American diet, considering childhood or adulthood. A child daily consumes 106 and 113 microplastics for female and male, respectively, while an adult consumes 126 (female) and 142 (male) microplastics/day. In this study, seafood products and bottled water were identified as among the major contributors of microplastic intake; however, according to geographical localization and dietary habits, exposure levels could vary widely (Cox et al. 2019).

Presence of Microplastics in Beverages

Both tap and bottled water contain microplastics, resulting, for the latter, from bottling process and bottle degradation and, for the former, from environmental water pollution and plastic water pipe degradation (Mason et al. 2018; Vertova et al. 2019). According to the analytical protocols used, quantitative data are highly variable. Bottled water contains the highest microplastic concentration reaching

325 particles/L in average compared to tap water with 5.45 particles/L from global sources (Kosuth et al. 2018; Mason et al. 2018). In European samples, a maximum of 628 and 4889 microplastics/L was reported in tap water and bottled water, respectively. Based on a typical human water consumption profile, this exposure route could represent a yearly exposure for an adult of 458,000 microplastics with tap water and 3,569,000 microplastics with bottled water (Danopoulos et al. 2020). A lot of polymer types are encountered in drinking water but PP, PET, and PE are the most abundant (Koelmans et al. 2019; Danopoulos et al. 2020). Concerning the characteristics of microplastic particles, bottled water may contain fragments and fibers, with size ranging from 6.5 to 100 μ m (Mason et al. 2018). For tap water, the presence of fibers, with length varying from 0.1 to 5 mm, has been reported (Kosuth et al. 2018).

Water is not the only beverage contaminated with microplastics. Common soft drinks, energy drinks, cold tea and beers revealed the presence of microplastic particles for 48 samples out of 57 tested. The amount was in the range from none to 28 ± 5.29 particles/L and included particles of various polymer types (e.g., polyamide, PET), shapes (fibers and fragments), colors and sizes (0.1–3 mm) (Shruti et al. 2020).

Presence of Microplastics in Food

Food items, including vegetables, fruits but also animal products, contain microplastics. A recent report showed a contamination level ranging from 52,050 to 233,000 particles/g depending on fruit and vegetable types (Oliveri Conti et al. 2020). This microplastic contamination could be incriminated to soil pollution, which leads to root absorption and translocation of plastic particles in other tissues. Fruits were more polluted than vegetables. Among fruits, apples were the most contaminated, while among vegetables, lettuces were the least contaminated (Oliveri Conti et al. 2020). To date, 201 edible species, i.e., 200 marine species (due to marine pollution) and 1 terrestrial species (chicken), were reported to be contaminated by microplastics and/or smaller-sized nanoplastics (Toussaint et al. 2019). Seafood products were particularly affected, as bivalves (0.2–4 particles/g), shrimps (0.75 particles/g), and fish (1–7 particles/g) (Toussaint et al. 2019). In addition, honey (40–660 fibers; 0–38 fragments/kg of honey), sugar (217 ± 123 fibers and 32 ± 7 fragments/kg of sugar), salt (0–19,800 particles/kg of salt) but also canned sardines and sprats showed contamination with microplastics (Karami et al. 2018; Peixoto et al. 2019). Few studies have investigated the presence of microplastics in other commonly edible products yet. For milk products, microplastics of different sizes (0.1–5 mm), shapes (fibers and fragments), and colors were detected (6.5 ± 2.3 particles/L in average). For poultry, only one report highlighted microplastic contamination in chicken (129.8 ± 82.3 and 10.2 ± 13.8 particles/g in chicken feces and gizzards, respectively) (Huerta Lwanga et al. 2017), even though its representativeness for a real meat contamination issue was raised by others (Toussaint et al. 2019).

Potential Microplastic Contamination from Food Packaging

This contamination, which acts as a direct source of human exposure, probably originates from processing and/or food packaging steps but could also be already present in raw materials. Opening a plastic packaging (e.g., containers, bags, tapes, caps) by scissoring, tearing with hands, cutting with knives, or twisting manually may generate microplastics (0.46–250 particles/cm in average), depending on the plastic properties like size, density, stiffness, or thickness (Sobhani et al. 2020). As reported in the previous section, common soft drinks, energy drinks, cold tea, and beers contain microplastics, which seems to originate from synthetic textiles and packaging (Shruti et al. 2020). For instance, Wrinkler and colleagues demonstrated in plastic bottles release of microplastics (90% of particles $<5\mu\text{m}$) from HDPE caps and from PET bottle necks to a lesser extent on their surface after a series of bottle openings/closings, with great differences in cap abrasion between brands (Winkler et al. 2019). Bottles themselves were not a source of microplastic release after exposure to mechanical stress mimicking squeezing/crushing effects. In another study (Hernandez et al. 2019), plastic teabags were shown to release microplastics (as well as smaller-sized nanoplastics) into steeped tea. The composition of released particles matched of the ones from original teabags (nylon and PET). Furthermore, disposable paper cups, currently used for coffee and tea, are coated by a hydrophobic plastic film onto their internal surface to make it waterproof. When filled with hot water (85–90 °C), the film was deteriorated and then released microplastics. Within 15 min, 25,000 micron-sized plastic particles were found to be released for a total liquid volume of 100 mL (Ranjan et al. 2021). Infants may also be exposed through PP feeding bottles that could release 16,200,000 particles/L in average mainly during sterilization process and filling with high-temperature liquids (Li et al. 2020b). In addition, polyethersulfone and polysulfone, commonly used as membrane materials in dairy processes, were identified as the main polymer types of microplastics contaminating milk samples from various brands (Kutralam-Muniasamy et al. 2020). As for beverage packaging, microplastics from food packaging are able to migrate into food especially in high-fat products due to the hydrophobic properties of plastics. A recent study showed the presence of microplastics (ranging from 3 to 29 particles/container, fibers as the dominant shape) in take-out food containers made of PP, PS, PE, or PET. The highest abundance occurred in PS containers with a rough surface. This contamination mainly originated from flaking from container inner surfaces and/or atmospheric fallout, at an extent depending on the polymer type. This could represent an additional intake of up to 203 microplastics/person/week for people who order take-out food from 4 to 7 times/week (Du et al. 2020). In addition, canned fish contains microplastics (PET polymer and fiber shape), probably originating from the cleaning and canning processes (Akhbarizadeh et al. 2020).

Physicochemical Transformations of Microplastics During Digestion

The impact of microplastics on the intestinal barrier depends on their initial physicochemical properties (e.g., particle size and distribution, shape, surface coating, weathering, adsorbed chemical and microbial contaminants), as well as any changes in these properties as they pass through the gastrointestinal tract (GIT), which has changing pH, transit time, redox potential, and other chemical characteristics. Human digestion is a multistep process including mechanical and chemical breakdown by which ingested food is converted into nutrients that can be directly absorbed and assimilated by the human body for growth, cell maintenance, and energy (Boland 2016). The digestive process is segmented all along the GIT from the mouth to the rectum. After ingestion, the food is subjected to mechanical and chemical degradations occurring within the mouth through coupled actions of teeth and saliva enzymes, mainly lipase and amylase. Following, the food bolus reaches the stomach where an acidic digestion by pepsin and lipase enzymes and a mechanical breakdown through gastric contractions occur. Next, the chyme reaches the intestine where it undergoes the substantial degradations through the combined action of pancreatic juice (containing a cocktail of amylase, lipase, and protease enzymes, as well as bile acids produced by liver and acting on the lipid fraction only) and intestinal enzymes secreted by enterocytes. Small intestine is also the main site for nutrient absorption. Undigested nutrients then reach the colon, in particular non-digested carbohydrates, which are hydrolyzed and fermented by the resident microbial communities (see details in the sections below).

In such context, knowledge of the physicochemical transformation of microplastics during digestion is of paramount importance and could be critical in affecting gut physiology. To date, studies are scarce and mainly focused on in vitro models exposing microplastics to simulated digestive fluids. Using a three-stage in vitro system mimicking the saliva, gastric, and intestinal phases of human digestion, Stock and colleagues demonstrated that digestive fluids were not able to degrade five types of microplastics (PE, PP, PVC, PET, and PS) into smaller fragments and to modify their shape, size, or texture (Stock et al. 2019b). Likewise, in a dynamic three-stage in vitro digestion system mimicking the mouth, stomach, and small intestinal human conditions, Liu and colleagues showed no alteration of PS microplastics in terms of chemical constitution but described the formation of a biomolecular corona onto the particle surface (Liu et al. 2020). Composed of adsorbed organic compounds during the digestion process (e.g., proteins, mucins, and lipids), this biomolecular corona remains poorly investigated but is essential to better understand the microplastic behavior in the GIT as it could modify microplastic physicochemical properties, such as hydrophobicity or surface charge as shown for PS particles (Liu et al. 2020). Furthermore, a recent study (Tan et al. 2020) showed that five types of microplastics (PS, PET, PE, PVC, and poly(lactico-glycolic acid (PLGA))) reduced lipid digestion in an in vitro system reproducing the main processes of human digestion occurring in the mouth, stomach, and small intestine. PS particles exhibited the highest inhibition rate by decreasing the

bioavailability of lipid droplets via the formation of large lipid-microplastic hetero-aggregates, and by adsorbing lipase with subsequent alterations in the enzyme secondary structure and activity (Tan et al. 2020).

Effects of Microplastic Exposure on the Intestinal Barrier

The intestinal barrier on its luminal side involves epithelium, mucus, and microbiota, and their crosstalk provides, in an orchestrated manner, a physical, chemical, and biological line of defense for the host. Data dealing with the impact of microplastics on these three gut players are summarized below.

Microplastics and Intestinal Epithelium

Characteristics and Functions of Intestinal Epithelium

The primary function of the GIT is the digestion process accompanied by water and nutrient absorption from the lumen to the blood through the single-layered epithelium, which acts as a selective filter against luminal pathogens and dietary antigens (Turner 2009). The ability of intestinal epithelial cells to act as immune cells and to crosstalk with mucosal immunity is not in the scope of this chapter. The relationships between microplastics and smaller-sized nanoplastics, the intestinal mucosa, and the immune system have recently been illustrated (Hirt and Body-Malapel 2020).

Gut epithelium is composed of a single layer of columnar cells, organized into crypts and villi, which are “finger-like” structures conferring an increase in small intestinal absorptive surface. Small and large intestine share many similarities in the histological structure of the mucosa, the most striking difference being that the latter is devoid of villi. Absorptive (enterocytes/colonocytes) and differentiated (goblet cells, Paneth cells, enteroendocrine cells, and tuft cells) cell types have been described in the intestinal epithelium. Moreover, M-cells are specialized in the capture of antigens for presentation to immune cells overlying the Peyer’s patches for the induction of immune responses. The most abundant cells are the enterocytes/colonocytes, which enables nutrients and water to be transported along the small and large intestine into the internal environment, respectively. In the secretory cell lineage, the largest number is represented by goblet cells, in charge of secreting mucins, which are diverse and complex glycoproteins as a major constituent of mucus (see details in the section below). Paneth cells, which are specifically located in the small intestine, provide antimicrobial peptides and proteins to protect the host from enteric pathogen invasion, help shape the composition of the commensal microbiota, and act as a safeguard from bacterial translocation across the epithelium (Vaishnava et al. 2008). Epithelial integrity and selective permeability are regulated by a set of adhesive complexes of proteins, including Tight Junctions (TJs), desmosomes, and Adherens Junctions (AJs). TJs surround the apical ends of the lateral membranes of epithelial cells and determine the selective paracellular permeability to solutes (Tsukita et al. 2001). TJs are multiple protein complexes, composed of (i)

4 integral transmembrane proteins, including occludin, claudins (consisting of at least 24 members), junctional adhesion molecule (JAM), and tricellulin and (ii) a wide spectrum of cytosolic proteins. The intracellular domains of these transmembrane proteins interact with cytosolic scaffold proteins, such as *zonula occludens* (ZO) proteins, which subsequently anchor transmembrane proteins to the perijunctional actomyosin ring (Suzuki 2013).

Effects of Microplastics on the Gut Epithelial Barrier

Numerous studies investigating uptake, fate, and effects of microplastics on the gut epithelial barrier have been performed in vitro using human epithelial Caco-2 cell monolayers on pristine particles (generally PS model microspheres, easy to use with precise size distribution, and possible coupling with fluorescent dyes to facilitate detection). Nevertheless, there is a growing trend to use more advanced cellular models that combine multiple intestinal cell types, including immune cells (e.g., M-cells (des Rieux et al. 2007; Carr et al. 2012; Stock et al. 2019a)) and/or mucus-secreting HT29-MTX cells (see examples given in the section below). Questions concerning the pivotal role of simulated digestive fluids (e.g., physicochemical transformations of microplastics, biomolecular corona formation) are also increasingly addressed, especially the consequences on uptake and toxicity (Liu et al. 2020). Interestingly, further developments on healthy versus inflamed gut conditions have been proposed to study the effect of inflammatory processes on plastic particle toxicity (Busch et al. 2021). A summary of the effects of microplastics on Caco-2 cells is given in Table 1.

The Caco-2 cell line, originally isolated from a human adenocarcinoma, spontaneously differentiates upon confluence to a monolayer of polarized enterocytes, expressing microvilli, transporters, and brush border enzymes. Microplastics were shown to disrupt the intestinal epithelial barrier, as measured by a decreased trans-epithelial electrical resistance (TEER), with a particle transport regulated by tight and adherent junctions (Carr et al. 2012). In addition, on the same cell model, increasing the microplastic exposure concentration (from 10^{-8} to 10^{-1} mg/mL) and duration (24 and 48 h) led to a higher cytotoxicity albeit at a slight extent (maximal loss of viability of 10%) (Wu et al. 2019b). Stock and collaborators studied particle uptake in Caco-2 monoculture acutely-exposed (24 h) to PS microplastics of three different sizes (1, 4, and $10\mu\text{m}$) (Stock et al. 2019a). The highest uptake into Caco-2 cells (of about 3%) was observed for $4\mu\text{m}$ particles. Interestingly, ethanol, which acts as a “loosener” of TJs, facilitated an increased particle passage across the epithelium (Carr et al. 2012). Liu and colleagues showed that the formation of a biomolecular corona onto the PS microplastic surface was probably a player in the reduction of cytotoxicity and impairment of the transport function observed in Caco-2 cells, particularly for small-sized digested particles ($0.1\mu\text{m}$) compared to their larger counterparts ($5\mu\text{m}$) (Liu et al. 2020).

Table 1 Summary of the reviewed literature using in vitro and in vivo models (mice) to study the fate and effects of microplastics on the three protagonists of the intestinal barrier, epithelium, mucus, and microbiota

Model	Objective	Exposure conditions	Analytical methods	Main results	References
Caco-2 cells	Determine intestinal uptake of MPs	Fluorescent latex (2µm) 21-day Caco-2 cell monolayer grown on polyester inserts (3µm pore size) 5–120 min exposure Duration of ethanol exposure: 15 or 60 min	TEER measurement (paracellular permeability) MP counting (fluorescence microscopy) Caco-2 cell epithelium immuno-fluorescence labelling (confocal laser scanning microscopy)	TEER ↓ MP transport regulated by tight and adherens junctions Particle passage ↑ after ethanol exposure	Carr et al. (2012)
		Investigate the toxicity mechanisms of PS MPs	PS (5µm) 10^{-8} – 10^{-1} mg/mL (for cell viability analysis); 12.5 or 50 mg/L for gene expression analysis Cells cultured for 24–48 h 24 or 48 h exposure	Cell viability measurement (MTT assay) Illumina RNA seq	Wu et al. (2019b)
		Evaluate the cellular uptake of PS MPs by the intestinal epithelium	PS (1µm carboxylated; 4 and 10µm sulfonated) 10^8 /mL for 1 and 4µm MPs 3×10^6 /mL for 10µm MPs	Particle fluorescence measurement	Highest uptake for 4µm MPs (of about 3%)

			21-day Caco-2 cell monolayer grown on polycarbonate inserts (3µm pore size) 24 h exposure		Cell viability measurement (release of LDH) Intestinal permeability measurement using the paracellular flux of Lucifer yellow	5µm digested versus undigested MPs: no changes in cytotoxicity and intestinal permeability (for 0.1µm digested particles: reduction of cytotoxicity and impairment of intestinal barrier observed with undigested particles)	Liu et al. (2020)
	Evaluate and compare intestinal toxicity of PS MPs with and without digestion		PS (5µm versus nano-sized particles 0.1µm) 1 and 20µg/mL 21-day Caco-2 cell monolayer grown on polycarbonate inserts (0.4µm pore size) 96 h exposure Digestion by a dynamic three-stage in vitro digestive tract				
Intestinal mucus							
Caco-2/HT29-MTX cells (mucus model)	Compare the cellular uptake of PS MPs between the mucus model and the Caco-2 cell monoculture		PS (1µm carboxylated; 4 and 10µm sulfonated) 10 ⁸ /mL for 1 and 4µm MPs 3 × 10 ⁶ /mL for 10µm MPs 21-day Caco-2 cell monolayer grown on polycarbonate inserts (3µm pore size) 24 h exposure	Particle fluorescence measurement	Uptake ✓ for 1 and 4µm MPs for the mucus model compared to the monoculture No significant difference with 10µm MPs		Stock et al. (2019a)
Caco-2/HT29-MTX co-culture with human blood monocyte-derived	Evaluate cytotoxicity, release of inflammatory cytokines and barrier integrity of the co-culture		PP, PA PU (50–500µm) Cells grown on inserts for 20 days 6, 24, and 48 h of	Cell viability measurement (release of LDH) ELISA for release of IL-	No cytotoxicity No release of inflammatory cytokines		Lehner et al. (2020)

(continued)

Table 1 (continued)

Model	Objective	Exposure conditions	Analytical methods	Main results	References
macrophages and dendritic cells		aerosolized exposure (average dose of about 1000µg/cm ²)	8, IL-1β, and TNFα Cell layer integrity (TEER measurement)	No change in barrier integrity	
5-week-old ICR mice (sex not specified)	Evaluate the effects of PS MPs on mucus synthesis and secretion in the colon	PS (0.5 and 50µm) 100 and 1000µg/L in drinking water 5 weeks	AB-PAS staining (colon) <i>Muc1</i> , <i>Muc2</i> , and <i>Klf4</i> gene expression analysis (colon)	Mucus secretion ↓ in all PS MP-treated animals mRNA levels of <i>Muc1</i> and <i>Klf4</i> ↓ (depending on conditions) No significant change in the mRNA level of <i>Muc2</i>	Lu et al. (2018)
5-week-old ICR mice (sex not specified)		PS (5µm) 100 and 1000µg/L in drinking water 6 weeks	AB-PAS staining (colon) <i>Muc1</i> , <i>Muc2</i> , <i>Muc3</i> , <i>Klf4</i> , <i>Meprin-β</i> , and <i>Remlb</i> gene expression analysis (colon)	Mucus secretion ↓ in all PS MP-treated animals mRNA levels of <i>Muc1</i> and <i>Klf4</i> ↓ in all treated animals mRNA levels of <i>Muc2</i> and <i>Remlb</i> ↓ for the highest dose mRNA levels of <i>Muc3</i> and <i>Meprin-β</i> ↓ but not significantly	Jin et al. (2019)
Intestinal microbiota					
5-week-old ICR mice (sex not specified)	Evaluate the effects of PS MPs on the gut microbiota composition	PS (0.5 and 50µm) 100 and 1000µg/L in drinking water 5 weeks	Fecal and/or cecal contents: qPCR Cecal content: 16S rRNA gene sequencing (V3-V4 region)	qPCR (fecal and cecal contents) Relative abundances of Firmicutes, Actinobacteria, and α-Proteobacteria ↓ 16S analysis	Lu et al. (2018)

5-week-old ICR mice (sex not specified)		PS (5µm) 100 and 1000µg/L in drinking water 6 weeks		Amounts of Firmicutes, Bacteroidetes, and Verrucomicrobia ↘ Relative abundances of Proteobacteria and Actinobacteria ↗ Abundance of <i>Bifidobacterium</i> , <i>Prevotella</i> , and <i>Ruminococcus</i> ↗ Abundance of <i>Oscillospira</i> and <i>Anaerostipes</i> ↘	Jin et al. (2019)
5-week-old SPF grade C57BL/6 male mice	Evaluate the effects of PE MPs on the gut microbiota composition	PE (10–150µm) 6, 60, or 600µg/day in the diet 5 weeks	Fecal content: 16S rRNA gene sequencing (V4 region)	qPCR (cecal content) Relative abundance of Firmicutes, α, β, and γ-Proteobacteria ↘ 16S analysis Actinobacteria ↘ <i>Bifidobacterium</i> , <i>Prevotella</i> , and <i>Veillonella</i> ↘ <i>Coproccoccus</i> and <i>Anaeroplasmma</i> ↗ ↗ Bacterial abundance and microbiota diversity for the highest dose	Li et al. (2020a)

AB-PAS Alcian blue-periodic acid Schiff, *ICR* Institute of Cancer Research, *IL-8* interleukin-8, *IL-1β* interleukin-1β, *LDH* lactate dehydrogenase, *MPs* microplastics, *MTT* thiazolyl blue tetrazolium bromide, *PA* polyamide, *PE* polyethylene, *PP* polypropylene, *PS* polystyrene, *PU* polyurethane, *SPF* specific-pathogen-free, *TEER* transepithelial electrical resistance, *TNFα* tumor necrosis factor α

Microplastics and Intestinal Mucus

Characteristics and Functions of Intestinal Mucus

Mucus is a viscoelastic gel, notably composed of mucins (MUC2 in the human small intestine and colon), that lines and protects the intestinal epithelium, separating it from the lumen content. It functions as a dynamic barrier, permeable to gases, water, and nutrients, but impermeable to most microorganisms (Etienne-Mesmin et al. 2019). Moreover, its lubrication ability is crucial to improve intestinal transit. The small intestine presents a single and discontinuous mucus layer whereas the large bowel is generally considered as a two-layered mucus system organization with an inner layer firmly attached to the epithelium and devoid of bacteria, and a loose outer layer inhabited by bacteria (Johansson et al. 2008). The study of Kamphuis and collaborators in rodents proposed to revisit the model of mucus structure by showing that in the distal colon, the mucus layer covers the feces instead of the epithelium (Kamphuis et al. 2017). This fecal mucus layer allows to confine the microbiota to the feces and to prevent it from remaining in the empty distal colon. More recently, Bergstrom et al. (2020) found that proximal colon-derived mucus forms both a niche and a primary barrier that encapsulates fecal materials before being strengthened by a secondary layer produced in the distal colon (Bergstrom et al. 2020).

In addition, mucus can be viewed as a gut target for environmental pollutants (heavy metals, pesticides, persistent organic pollutants) and food additives (emulsifiers, nanomaterials) with the existence of bidirectional relationships (Gillois et al. 2018). Due to its size- and charge-exclusion barrier properties, mucus may trap chemicals, thereby decreasing their toxicity to the host. Conversely, toxic pollutants may affect mucus characteristics (e.g., thickness, mucin expression, and composition) all along the GIT, with a putative negative impact on intestinal health.

Bidirectional Relationships Between Microplastics and Intestinal Mucus

To date, a few studies have investigated the interactions between microplastics and intestinal mucus, as summarized in Table 1. To overcome the absence of a mucus protective barrier in Caco-2 cells, a Caco-2/HT29-MTX co-culture has been developed as a more physiologically relevant intestinal model to mimic a mucus-producing epithelium (Lozoya-Agullo et al. 2017). Considering Caco-2/HT29-MTX (mucus model) co-culture versus Caco-2 monoculture exposed to PS microplastics of three different sizes (1, 4, and 10 μ m), Stock and collaborators surprisingly found a significantly higher uptake rate for both the 1 and 4 μ m particles in the co-culture compared to the monoculture (no significant difference with the 10 μ m particles) (Stock et al. 2019a). However, it should be noted that numerous studies have highlighted that mucus in this Caco-2/HT29-MTX co-culture model does not form a uniform layer over the cell surface, but rather a patchy structure (Gillois et al. 2021) while others depicted a mucus layer completely covering the cell surface (Mahler et al. 2009). These discrepancies stress the necessity of examining mucus characteristics, and thus barrier properties, when HT29-MTX cells are used for the toxicological assessment of microplastics in vitro. A three-dimensional in vitro intestinal cell model, composed with a mixture of Caco-2 and HT29-MTX cells in combination

with immune cell types (i.e., human blood monocyte-derived macrophages and dendritic cells), was developed for testing environmentally relevant microplastics (PP, polyamide (PA) and polyurethane (PU), 50–500µm) after 6, 24, and 48 h of an aerosolized exposure, chosen to avoid particle floating in the cell culture medium (Lehner et al. 2020). Whatever microplastic type and exposure conditions, no significant deleterious effect was reported in terms of cytotoxicity, barrier integrity disruption, and (pro-)inflammatory response. In vivo, recent studies in mice highlighted the deleterious effects of microplastics on mucus homeostasis pathways. After exposure to 0.5, 5, and 50µm PS microplastics at 100 and 1000µg/L in drinking water for several weeks, mucus secretion in the colon, quantified by using Alcian blue-periodic acid Schiff staining, was significantly decreased, supported by the downregulation of genes specifically involved in mucus synthesis/secretion pathways (*Muc2*, *Muc1*, and *Klf4*) (Lu et al. 2018; Jin et al. 2019).

Microplastics and Intestinal Microbiota

Characteristics and Functions of Intestinal Microbiota

During their fate within the gut, microplastics are facing a huge community of microorganisms composed of 100 trillion bacteria, fungi, archaea, and virus-like particles forming the intestinal microbiota. These populations are spread along the intestine in a transverse and longitudinal manner according to the gut environment encountered (e.g., pH and oxygen gradients, antimicrobial peptide levels, transit time). Each individual harbors a unique gut microbiota, which is the densest in the large intestine, and predominantly composed with bacteria belonging to the phyla Bacteroidetes, Firmicutes, Actinobacteria, and to a lesser extent Proteobacteria and Verrucomicrobia (Cheng et al. 2013). This microbial ecosystem plays major functions for intestinal homeostasis and host health through the digestion and assimilation of dietary nutrients, like fibers, which leads to the production of fermentation products (e.g., short chain fatty acids and gases) acting as metabolic and immune mediators. The gut microbiota also plays a crucial role in immunity and protection against pathogens (Andoh 2016).

To date, increasing attention is given to the gut microbiota/mucus interplay. Colonic mucus is the natural habitat for commensal bacteria, due to the presence of mucin-derived carbohydrates utilized as an energy source (Sonnenburg 2005) and/or binding sites (MacKenzie et al. 2010). Numerous mucolytic bacteria have been described, including *Akkermansia muciniphila*, *Bacteroides thetaiotaomicron*, *Bacteroides fragilis*, *Ruminococcus gnavus*, and *Ruminococcus torques* (Tailford et al. 2015). Reciprocally, commensal bacteria may shape the mucus barrier properties, as shown in mice (Jakobsson et al. 2015).

In addition, gut microbiota/chemicals crosstalk is increasingly recognized as an important player in health and disease. The gut microbiota has indeed the capacity to metabolize different chemicals introduced through the diet and to modulate their pharmacokinetics and toxicity to the host; concurrently, these compounds may alter composition and/or metabolic functions of the gut microbiota (Claus et al. 2016).

Bidirectional Relationships Between Microplastics and Intestinal Microbiota

No study has been conducted yet on the potential impact of exposure to microplastics on the human gut microbiota. However, a handful of studies in mice have yielded first findings on alterations of the gut microbiota composition through cecal or fecal content analysis following microplastic exposure. A summary of the effects of microplastics on intestinal microbiota is given in Table 1. Modifications related to gut microbiota composition were reported after exposure to PS microplastics (0.5, 5, and 50 μ m) at 100 and 1000 μ g/L in drinking water for several weeks, with impaired α - and β -diversities indices characterized by a significant reduction in the cecal abundance of Firmicutes, Actinobacteria, and α , β , and γ -Proteobacteria using a PCR-based quantification assay. Metabarcoding with 16S rRNA sequencing analysis showed a decrease in the cecal relative abundance of Firmicutes, Actinobacteria, Bacteroidetes, and Verrucomicrobia while the proportion of Proteobacteria increased (Lu et al. 2018; Jin et al. 2019). At the genus level, 15 types of bacteria were modified in the cecal content. In particular, *Bifidobacterium*, *Prevotella*, and *Veillonella* decreased while *Coprococcus* and *Anaeroplasm*a increased. In another study, an increase in the fecal microbiota abundance and diversity was reported by 16S metagenomics analysis, after exposure to PE microplastics (10–150 μ m) at 6, 60, and 600 μ g/day for 5 weeks (Li et al. 2020a).

In mammals, little is known about the potential of gut microbiota to degrade microplastics. Nevertheless, some microorganisms able to degrade plastic particles from the gut microbiota of soil waxworms have been identified. Interestingly, two bacterial strains, *Enterobacter asburiae* YTI and *Bacillus* spp. YPI from the gut of the larvae *Plodia interpunctella*, were able to alter PE as shown by the formation of pits and cavities on the PE surface and the addition of carbonyl groups (Yang et al. 2014). To date, no rigorous study has been conducted on the putative capacity of the human gut microbiome to degrade plastic particles. However, the comparison between bacteria identified as able to degrade microplastics (Jacquin et al. 2019) and those found in the human gut microbiota, according to the database of Plaza Oñate and his team (Plaza Oñate et al. 2019), showed some overlap for bacteria belonging to Proteobacteria, Actinobacteria, Bacteroidetes, and Firmicutes phyla, which warrants further attention.

Summary and Future Directions

The potentially deleterious effects of microplastics on the structure and function of GIT compartments, albeit of uppermost importance for host health, remain largely unknown. Fig. 1 illustrates a schematic representation of microplastic-related issues, from their occurrence in food items, drinking water, beverages, and consumer packaged goods to their fate within the GIT, i.e., digestion and interactions with the gut barrier protagonists. A special focus is given to epithelium, mucus, and gut microbiota. Main findings from the literature on the characteristics and effects of

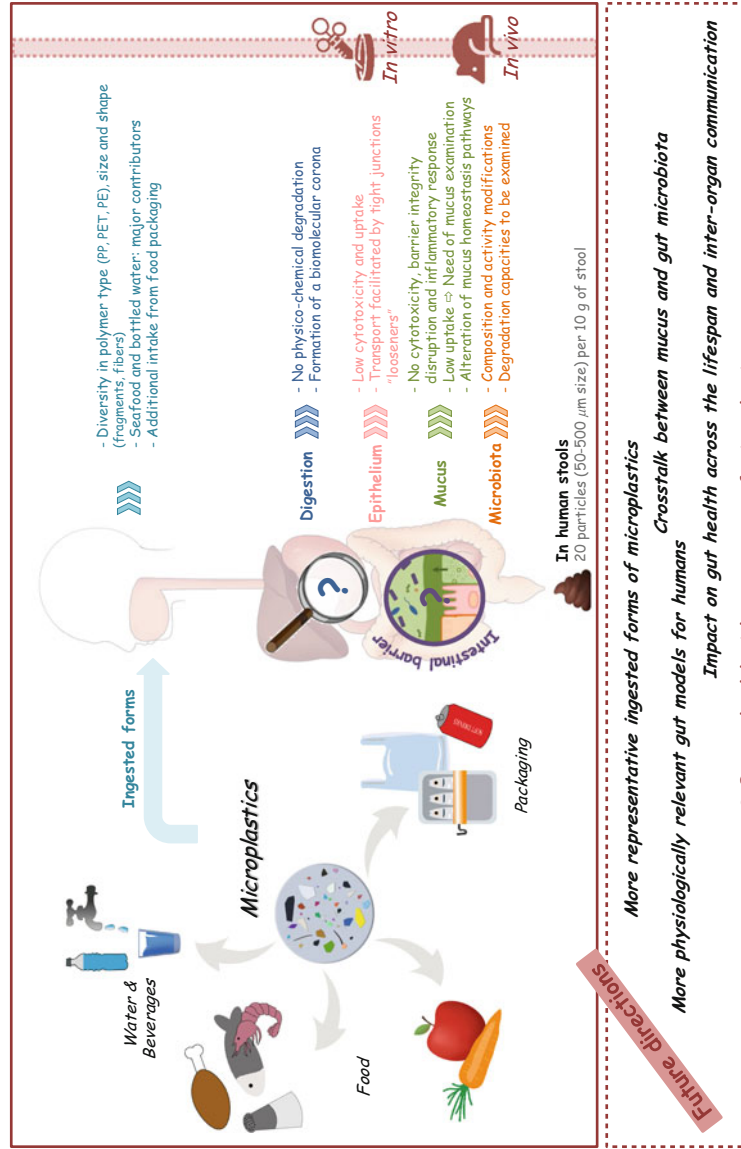


Fig. 1 Schematic representation of the impact of microplastics on intestinal health, from their occurrence in food items, drinking water, beverages, and consumer packaged goods to their fate within the gastrointestinal tract after oral exposure, i.e., digestion and interactions with the gut barrier protagonists (epithelium, mucus, and microbiota). Main findings from the literature on the characteristics and effects of microplastics are summarized, and research directions for the future given

microplastics are summarized. Some future research directions are also proposed and detailed below.

More Representative Ingested Forms of Microplastics Are Needed, Probably Displaying Distinct Physico-chemistry, Behavior, and Toxicity in the Gut

Most studies conducted so far have used pristine PS microbeads as a matter of availability and easy handling, although this polymer type accounts for only about 6% of total plastic production (PlasticsEurope 2020). Furthermore, the spherical shape is not fully representative of the ingested forms (e.g., fibers, fragments), thereby highlighting the need to unravel the effects of “real-life” microplastics exhibiting specific physicochemical properties (polymer type, shape, size distribution, etc.). This should be accompanied by the development of standardized methods and methodologies (including sampling and sample processing) to detect and quantify microplastics (and especially of small-size and ultimately nanoplastics) in complex media, in particular biological matrices and tissues, which still represents a major knowledge gap (Toussaint et al. 2019; Brachner et al. 2020). Pristine microplastics can simulate a direct source of human exposure. However, weathered particles are also needed to simulate environmental exposure as weathering leads to strong modifications of microplastics due to oxidation, fragmentation, and degradation (e.g., photooxidation, hydrolysis, mechanical abrasion, possible biodegradation by microorganisms) and surface coverage (namely biomolecular corona) by a complex mixture of organic and inorganic materials. Corona formation, but also sorption capacity, may be modified by the oxidation of the polymers and the subsequent changes in surface properties. The sorption capacity of weathered microplastics is significantly higher than the one of virgin material (Liu et al. 2019). In such context, the fact that microplastics might be carriers of chemical and microbial contaminants (e.g., bacterial pathogens), according to the “vector effect” hypothesis (Syberg et al. 2015), should be considered.

More Physiologically Relevant Gut Models for Human Oral Exposure Should Be Investigated

Since studies in humans remain hampered by ethical, regulatory, and cost reasons, current research dealing with the impact of microplastics on intestinal health relies on mice as preclinical models. However, *in vivo* studies in mammals cannot address all the questions raised, due to major gut characteristics differences depending on species. Some *in vitro* human gut models have thus been developed, including cell cultures and simplified digestion systems. For the latter, the systems currently used operate in general under static conditions and are only composed of different gut-simulating vessels with the corresponding digestive juices adjusted for pH and composition of digestive secretions. More physiologically relevant *in vitro* gut

models could be helpful for a better understanding of the fate and effects of microplastics in the human digestive environment. The TNO gastrointestinal system 1 (TIM-1) currently represents the most complete simulator of the upper human GIT reproducing the main physicochemical parameters of the stomach and small intestine, i.e., body temperature, kinetics of gastric and intestinal pH, transit time, gastric, pancreatic and biliary digestive secretions, and passive absorption of nutrients and water (Cordonnier et al. 2015). TIM-1 could be used to investigate the influence of food matrices (including lipid-rich matrices) on the spatiotemporal microplastic physicochemical transformations, degradation and/or bioaccessibility of associated contaminants, and survival/virulence of associated pathogens. In addition, *in vitro* lower GIT models would be highly valuable to decipher microplastic/gut microbiota bidirectional interactions with a gut microbiota from human origin (Lambrecht et al. 2019). The impact can be monitored at the level of microbiota composition and metabolic activity (e.g., production of short chain fatty acids, gases). Conversely, it is possible to investigate the gut microbiota capacity to degrade and metabolize microplastics, in particular through activation of specific microbial metabolic pathways. Interestingly, the influence of interindividual variability can be assessed by inoculating the system with fecal samples originating from different donors (e.g., sex, age).

Microplastics May Compromise Gut Barrier Homeostasis by Influencing Crosstalk Between Mucus and Microbiota

Another key challenge for understanding microplastic gut toxicity is to go beyond microplastics/mucus or microplastics/microbiota bidirectional relations by taking into account crosstalk between mucus and microbiota, which are increasingly recognized as tightly connected gut protagonists for ensuring intestinal homeostasis. To date, mucus/microbiota interplay has not been considered in the context of microplastic interactions and transformations. To address this question, studies could be performed using *in vitro* lower GIT models considering the mucus environment. These studies can be envisioned in a mono-compartmental model simulating the mean physicochemical and microbial parameters of the human colon, such as the Mucus Artificial Colon model (M-ARCOL) that integrates the mucus-associated microbiota in a specific compartment containing mucin alginate beads (Deschamps et al. 2020). To gain into complexity, a multi-compartment system simulating the entire GIT (stomach, small intestine, and three parts of the colon), the Mucosal Simulator of the Human Intestinal Microbial Ecosystem (M-SHIME) incorporating microcosms coated with mucin-agar, could be used (Lambrecht et al. 2019).

Microplastics Under Realistic Oral Exposure May Impact Gut Health Across the Lifespan and Influence Interorgan Communication

Further microplastic-driven gut research is needed for better human health hazard and risk assessment under relevant exposure scenarios (acute versus chronic exposure effects, no microplastic overload situation) (Brachner et al. 2020). It would also be highly valuable to evaluate the toxicological consequences on at-risk populations (e.g., children, elderly) and the use of dedicated in vitro gut models would allow to rule out some ethical and technical constraints (Denis et al. 2016; Van den Abbeele et al. 2019). Pathophysiological conditions (e.g., obese patients, inflammatory bowel disease patients) could ultimately been simulated. Interestingly, these “sensitized” people exhibit already compromised mucus/epithelial barriers and unbalanced gut microbiota (Boulangé et al. 2016). In addition, with these gut models, crosstalk with immune system, as well as interorgan communication in humans (e.g., gut-liver axis, gut-lung axis), via the coupling of GIT samples with intestinal/immune/extra-digestive cells, could be investigated in advanced cell (co)culture models.

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Appendix 3:

Review in Journal of Hazardous Materials

Publication:

Fournier E, Etienne-Mesmin L, Grootaert C, Jelsbak L, Syberg K, Blanquet-Diot S, Mercier-Bonin M (2021a) Microplastics in the human digestive environment: A focus on the potential and challenges facing in vitro gut model development. *Journal of Hazardous Materials* 415:125632. <https://doi.org/10.1016/j.jhazmat.2021.125632>



Microplastics in the human digestive environment: A focus on the potential and challenges facing *in vitro* gut model development

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ABSTRACT

Plastic pollution is a major issue worldwide, generating massive amounts of smaller plastic particles, including microplastics (MPs). Their ubiquitous nature in the environment but also in foodstuff and consumer packaged goods has revealed potential threats to humans who can be contaminated mainly through air, food and water consumption. In this review, the current literature on human exposure to MPs is summarized with a focus on the gastrointestinal tract as portal of entry. Then, we discuss the vector effect of MPs, in their pristine versus weathered forms, with well-known contaminants as heavy metals and chemicals, or more emerging ones as antibiotics or microbial pathogens, like *Pseudomonas* spp., *Vibrio* spp., *Campylobacter* spp. and *Escherichia coli*. Comprehensive knowledge on MP fate in the gastrointestinal tract and their potential impact on gut homeostasis disruption, including gut microbiota, mucus and epithelial barrier, is reported *in vitro* and *in vivo* in mammals. Special emphasis is given on the crucial need of developing robust *in vitro* gut models to adequately simulate human digestive physiology and absorption processes. Finally, this review points out future research directions on MPs in human intestinal health.

1. Introduction

Plastic production has significantly increased worldwide over the last decades, from 1.7 million tons in the 1950s to over 368 million tons in 2019 (PlasticsEurope, 2020), because of its success as a multifunctional, resistant, easy-to-process and affordable material. Due to these unique properties, plastics are central to modern living and constitute a vital source for innovation-driven growth. However, negative impacts related to their end-of-life cannot be set aside. Plastic wastes have different fates: they can be reused, recycled, energy recovered (burned), deposited at dumping sites or lost in the environment (European Parliament, 2008). Plastic debris can be classified according to their size. Concerning microplastics (MPs), the most frequently used definition considers all plastic particles < 5 mm in diameter (Arthur et al.,

2009) even though no international consensus has been reached yet (Hartmann et al., 2019). The possible mechanism for generation of a majority of MPs is the *in situ* weathering breakdown of plastic debris. In the marine environment, it is primarily the UV-B radiation in sunlight that leads to photo-oxidative degradation of plastics and once initiated, the degradation can proceed through thermooxidation. Other types of degradation processes, like biodegradation, are orders of magnitude slower compared to light-induced oxidative degradation and hydrolysis is usually not a significant mechanism in seawater (Andrady, 2011; Mattsson et al., 2015). MPs are then found in all environmental media from marine to ground water, lakes, sediments, soil and even in the atmosphere (Miranda et al., 2019; Wu et al., 2019a). Moreover, MPs may be directly released from domestic and industrial sources (Weinstein et al., 2016), as well as from plastic products used for food

Abbreviation: Cr, Chromium; GIT, Gastro-Intestinal Tract; M-ARCOL, Mucus Artificial COLon model; MPs, microplastics; PA, polyamide; PE, polyethylene; PET, polyethylene terephthalate; PLA, polylactic acid; PLGA, poly(lactic-co-glycolic acid); PP, polypropylene; PS, polystyrene; PVC, polyvinyl chloride; M-SHIME, Mucosal-Simulator of the Human Intestinal Microbial Ecosystem; TIM-1, TNO Gastro-Intestinal Model 1.

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packaging, fast-food delivery, and water consumption (Fadare et al., 2020; Hernandez et al., 2019). Negative impact on the environment and organisms resulting from plastic pollution is well-established (Bucci et al., 2019). Each organism is exposed, from fish or worms to mammals (Toussaint et al., 2019). Inevitably, plastics enter the food chain and drive contaminants, probably impacting gut homeostasis (Lu et al., 2019). While more and more studies evaluate the toxicological issue of MP exposure on various organisms, a debate remains on whether the associated risk is real or overrated (Backhaus and Wagner, 2020). Even though humans are also exposed through inhalation and dermal contact, food ingestion remains the main exposure source (Galloway, 2015). Increasing works and reviews have reported the human health issues of MP exposure but only few are focused on human intestinal health at the forefront of this exposure (Hirt and Body-Malapel, 2020; Lu et al., 2019; Paul et al., 2020). Based on this background, we first summarize the available evidence regarding the degree of human exposure to MPs via oral route. Next, we highlight the vector effect of MPs for well-known contaminants as heavy metals and chemicals, and more emerging ones as antibiotics or microbial pathogens. Afterwards, we detail the fate and impact of MPs on the digestive tract of mammals and humans, with a special emphasis on *in vitro* approaches to determine the physico-chemical transformations of MPs during digestion and their putative role on intestinal barrier disruption. Finally, we will discuss the potentialities of advanced *in vitro* gut models simulating human digestive physiology for unravelling toxic and health effects of MPs.

2. Oral exposure sources to MPs in humans

2.1. Drinking water and beverages

MP contamination has been reported in various beverages including bottled and tap water. In the study reported by Mason and colleagues (Mason et al., 2018), samples from bottled water were processed by Nile red staining, 1.5 μm filtration and analyzed using an optical microscope; they were then classified in two size ranges: larger than 100 μm (polymer identity further confirmed with Fourier transform infrared spectroscopy (FTIR)) and between 6.5 and 100 μm . In the study of Kosuth and collaborators (Kosuth et al., 2018), samples from tap water were processed by a 2.5 μm filtration, analyzed using a dissection microscope and only particles not stained by the Rose Bengal were counted; no confirmation by FTIR was performed. Under these conditions of sample analysis, bottled water seemed to have the highest particle concentration (ranging from 0 to 10,000 particles/L), compared to tap water (ranging from 0 to 61 particles/L) (Kosuth et al., 2018; Mason et al., 2018). These differences in contamination level have also been found by Danopoulos and colleagues, with concentrations of 628 MPs/L and 4889 MPs/L for tap and bottled water, respectively (Danopoulos et al., 2020). After tap water analysis from fourteen different countries, the most abundant shapes reported were for 98.3% fibers being between 0.1 and 5 mm in size (Kosuth et al., 2018). For bottled water from various brands in nine different countries, fragments (66%) and fibers (13%) were predominant, with 95% of particles being between 6.5 and 100 μm in size. Polypropylene (PP), polyethylene terephthalate (PET) and polyethylene (PE) are the most abundant polymers (Danopoulos et al., 2020; Koelmans et al., 2019). MP contamination in tap water can derive from environmental water pollution but also from the degradation of plastic water pipes, amplified by water disinfectants commonly used as chlorine dioxide (Vertova et al., 2019). For bottled water, the presence of MPs results from bottle degradation, mainly through ultraviolet radiations or during bottling process (Mason et al., 2018). Other studies have shown the contamination of beer (from 2 to 79 fibers/L, from 12 to 109 fragments/L and from 2 to 66 granules/L), milk but also refreshments (ranging from 10 to 100 MPs/L) and white wines (2563–5857 particles/L) (Diaz-Basantes et al., 2020; Liebezeit and Liebezeit, 2014; Prata

et al., 2020). Despite the small number of studies available to date, results are highly variable and depend on the analytical methods and experimental protocols used, notably for the minimal size of particles to be extracted and analyzed.

2.2. Air

Numerous studies have reported air contamination by MPs: PE, PP, polystyrene (PS) and PET are generally the major polymers found, with fibers as the predominant shape (more than 90%). Synthetic textiles are the main source of airborne MPs even though other sources may occur, such as from degradation of larger plastics in landfills or from incineration, from traffic or re-suspended dust. Meteorological conditions like wind, rain and snow events, and human activities affect the distribution and deposition of airborne MPs (Chen et al., 2019). Indoor air of different apartments was investigated and all were contaminated with MPs (1.7–16.2 particles/ m^3) (Vianello et al., 2019). MP fibers can be ingested by eating air-dust exposed food but also by dust ingestion particularly for young children (Dris et al., 2017). This route of exposure is important for infants with a median daily intake of inhaled PET-based MPs estimated between 4000 and 150,000 ng/kg of body weight (bw) per day (from 360 to 150,000 ng/kg bw/day in adults depending on their living area) (Zhang et al., 2020). In fact, adults consume MPs through fiber deposition from dust fallout in a house during a meal (13, 731–68,415 particles/year/capita), which may represent a higher exposure than after mussel consumption (from 123 to 4620 MPs/year/capita) (Catarino et al., 2018). A large part of those particles could enter the digestive tract through the lung mucociliary clearance mechanisms after inhalation.

2.3. Food

In 2016, the European Food Safety Authority (EFSA) published an overview on the presence of MPs (and also smaller-sized nanoplastics) in food, with a focus on seafood (EFSA, 2016). Up to now, 201 edible species of which only one terrestrial species, chicken, have been reported to be contaminated (Toussaint et al., 2019). MPs in bivalves, shrimps and fish reach average concentrations of 0.2–4 particles/g, 0.75 particles/g and 1–7 particles/g, respectively. In addition, contamination in honey (40–660 fibers; 0–38 fragments/kg of honey), sugar (217 ± 123 fibers and 32 ± 7 fragments/kg of sugar), salt (from 0 to 19,800 particles/kg of salt), canned sardines and sprats was reported (Bouwmeester et al., 2015; Karami et al., 2018; Peixoto et al., 2019). Currently, it remains unclear whether this contamination is already present in raw materials or comes from processing and/or food packaging steps. A recent study has reported the important release of MPs from PP feeding bottles (16,200,000 particles/L) especially during sterilization process and exposure to high-temperature water, highlighting the urgent and critical need of risk assessment in infants (Li et al., 2020b). Another study reported MP pollution ranging from 52,050 to 233,000 particles/g depending on fruit and vegetable types, with fruits being more contaminated than vegetables. Among each group, apples were the most polluted while lettuces represented the least contaminated. This MP pollution seems to originate from soil pollution leading to root absorption and translocation in other tissues (Oliveri Conti et al., 2020). It should be underlined that an insufficient number of studies has yet investigated the presence of such particles in other very commonly consumed food items including beef, poultry, dairy and cereals.

2.4. Human consumption

Humans are exposed to numerous MP sources (Fig. 1) (Rist et al., 2018) but to date, only one study clearly quantifies human exposure to MPs integrating dietary and inhalation sources. A child with a typical

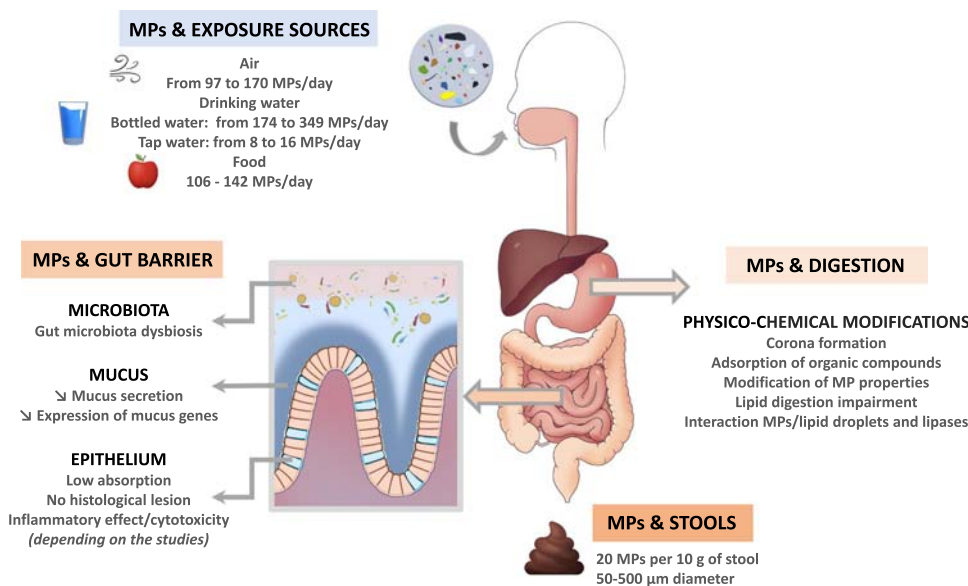


Fig. 1. MPs and the human gastrointestinal tract: an overview. Humans are primarily exposed to MPs by the gastrointestinal tract through the air inhaled, the food and the water consumed. After ingestion, some physico-chemical transformations, with notably the formation of a biomolecular corona, occur triggered by the gut environment; inhibitory effect on lipid digestion may be observed. Disruption of intestinal homeostasis affects gut microbiota, mucus and epithelial barriers. *MPs: microplastics.*

American diet consumes per day 106 (female) and 113 (male) MPs and inhales 97 (female) to 110 (male) of these particles. Regarding adults with a typical American diet, they consume 126 (female) to 142 (male) MPs and inhale 132 (female) to 170 (male) particles per day. However, Cox and colleagues pointed out that, according to the geographical localization and dietary habits, MP exposure could vary (Cox et al., 2019). For instance, when daily water intake recommendations are achieved through ingestion of bottled water, MP intake is increased by 22-fold compared to tap water consumption (Cox et al., 2019). In addition, seafood products are among the major food contributors for MP intake known to date. As illustrated in Japanese, known to be one of the largest consumers of fish and seafood products, they could ingest up to 154 MPs per day only through seafood products. However, data from other food groups consumed daily are now required for identifying additional sources of MP exposure in humans. Schwabl and his team reported in a pilot study the presence of MPs in stool samples from healthy volunteers living in eight countries worldwide to be representative of different geographic regions and dietary habits. Several plastics with in average 20 MPs per 10 g of stool and between 50 and 500 μm in size were found in all fecal samples. Each sample had 3–7 different types of plastics with a relative frequency of 62.8% of PP, 17% of PET, 11.2% of PS and 4.8% of PE (Schwabl et al., 2019). Although this study only investigated MPs larger than 50 μm for a small number of subjects ($n = 8$, 3 males, 5 females, aged 33–65 years) with only 1 stool sample provided per participant, it is still striking that MPs are found in all analyzed samples. Further studies on larger populations should now be performed to correlate types and amounts of fecal MPs to geographic area, dietary habits, other potential sources of MP ingestion, and health impacts. Recently, Senathirajah and colleagues used the current knowledge to estimate the global average rate of MP ingestion in humans, which was in the range 0.1–5 g of MPs per week (Senathirajah et al., 2021). Beyond differences in experimental conditions and analytical methods used across studies, their analysis was limited by a scarcity of data for certain food categories (honey, sugar and fish: limited available data; pasta, oil, milk, bread, rice, meat and wheat: absence of data at the time of analysis), which led to only investigate four types of consumables with the most robust data (drinking water, salt, beer and shellfish). Similarly, the probable contribution of MPs from plastic utensils/food packaging was excluded due to data limitations (Senathirajah et al., 2021). Nevertheless, this pioneering study constitutes a first reliable step towards health risk assessment in humans.

3. MPs as vectors for contaminants and microorganisms: consequences on the physico-chemical transformations of MPs and contaminants during human digestion

3.1. Sorption/desorption of chemical contaminants onto/from MPs

3.1.1. Sorption of chemical pollutants and heavy metals onto MPs

One major concern about MP health effects is related to their association with different chemicals (Fig. 2), and notably pollutants adsorbed from the surrounding environment and thereby having their environmental fate altered (Hahladakis et al., 2018; Wang et al., 2018). This “vector effect” is a potential concern facing the extent of MP pollution (Syberg et al., 2015) and little is known regarding the consequences on intestinal health.

Sorption of chemical pollutants (e.g. organochloride pesticides and polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) and other benzene-ring derivatives) onto MPs has been widely investigated, as recently reviewed (Yu et al., 2019). Sorption is highly governed by physico-chemical characteristics (e.g. polymer matrix structure combining highly ordered crystalline regions and less structured amorphous regions, particle size, surface roughness) as well as environmental conditions (Velez et al., 2018). Lowering pH protonates the surface of MPs, leading to enhanced sorption of anionic pollutants. Likewise, when salinity increases, cation competition increases, inducing sorption or desorption of contaminants (Yu et al., 2019). The presence of ionic surfactant may also influence adsorption of hydrophilic pollutants. Indeed, MP adsorption capacity could increase from three to twenty-six times according to the co-existing surfactants (Xia et al., 2020). Adsorption typically occurs in the crystalline regions whereas internal partition is more likely to happen in the amorphous regions (Velez et al., 2018). Pristine MPs that means in their original conditions adsorb and concentrate contaminants such as polycyclic aromatic hydrocarbons, organochloride pesticides, polychlorinated biphenyls, perfluoroalkylated substances (PFASs) and several types of antibiotics, but also heavy metals as chromium (Cr), zinc or lead (Godoy et al., 2019; Guo et al., 2019; Heskett et al., 2012; Li et al., 2018; Razanajatovo et al., 2018; Rochman et al., 2013; Wang et al., 2015; Yu et al., 2020).

Weathering of MPs is furthermore important for the association of pollutants. During fragmentation, chemicals are susceptible to migrate to the plastic surface and then be released (Schränk et al., 2019). The migration rate closely depends on the molecular weight, initial

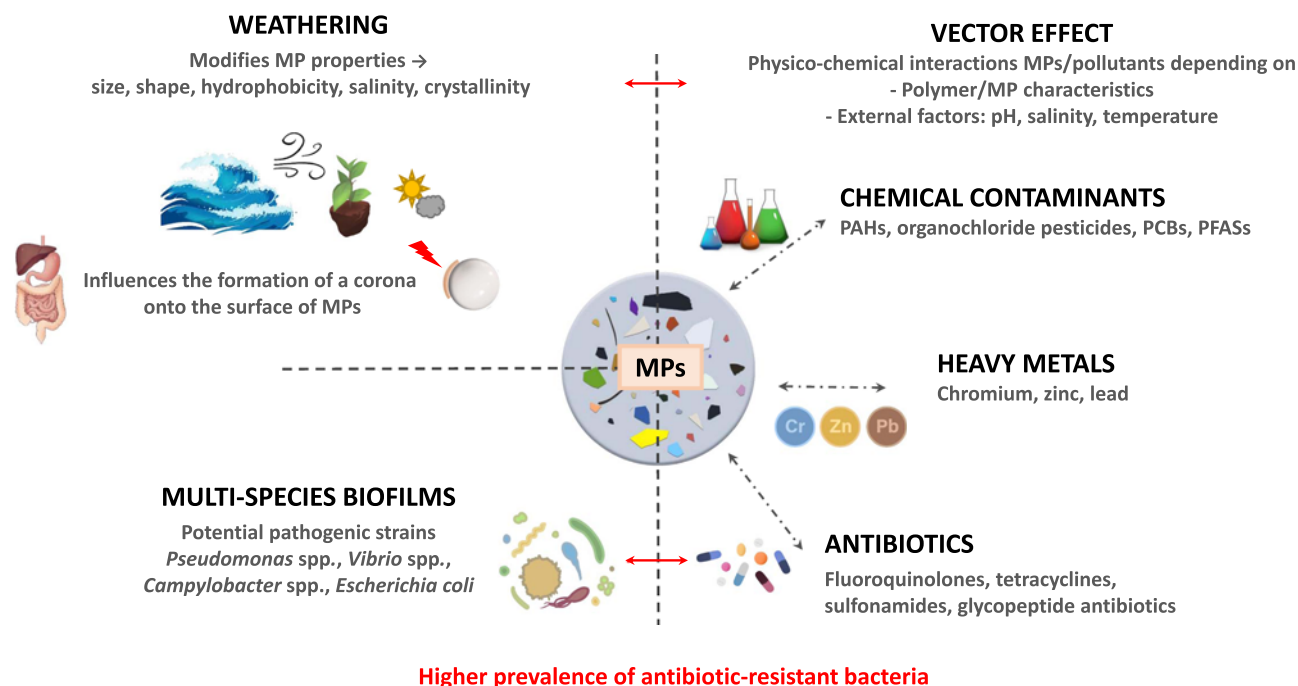


Fig. 2. MPs: vectors for contaminants and microorganisms. MPs, in their pristine and weathered forms, interact with various compounds as chemicals, heavy metals, antibiotics and even microorganisms. MPs act then as vectors that could favour the appearance of antibiotic-resistant bacteria by concentrating antibiotics and bacteria onto their surfaces. *MPs*: microplastics; *spp*: species; *PAHs*: polycyclic aromatic hydrocarbons; *PCBs*: polychlorinated biphenyls; *PFASs*: per-fluoroalkylated substances.

concentration of the chemical substance present in the plastic, as well as the thickness, crystallinity and surface structure of the plastic (Hahladakis et al., 2018; Teuten et al., 2009). Weathering affects the physico-chemical properties of MPs as size, crystallinity or oxygen-containing groups (Fig. 2) and, thus, change contaminant sorption profile onto MPs (Liu et al., 2020a). For example, weathered MPs contain more surface oxygen-containing groups than pristine ones, leading to greater contaminant sorption especially for the more hydrophilic ones. Furthermore, after etching and/or UV radiation, specific surface area and pore volume of MPs are increased, by formations of nanovoids and larger cracks in the surface, which also favors contaminant adsorption process (Li et al., 2018; Velez et al., 2018).

Sorption of heavy metals onto MPs is generally due to electrostatic interactions between metal ions and polar sites on the plastic surface. As for other contaminants, weathered MPs contain more heavy metals than their pristine counterparts (Holmes et al., 2012; Vedolin et al., 2018; Wang et al., 2019). Adsorption is enhanced in waters with high chemical and biological oxygen demands as urban wastewater and irrigation water. However, after maximum adsorption, metals tend to slowly desorb from MPs (Godoy et al., 2019). Acidic conditions, like those encountered in gastric juice during digestion (see Section 3.1.2), may promote the leaching of metals bound to MPs (Teuten et al., 2009).

The environmental importance of the “vector effect” is still debated. A study of the interaction between biota and silver-contaminated MPs in zebrafish showed that MPs altered the pollutant fate but not necessarily by increasing its uptake, illustrating that plastics can bind contaminants, which are further less available for uptake (Khan et al., 2015). Similarly, Kleinteich and colleagues found that contaminant bioavailability in soil decreased as a function of binding to MPs (Kleinteich et al., 2018). MPs can thus both serve as a source and a sink for other contaminants, in a case-by-case specific manner. The process is complex and, as described above, depends on the pollutant, the composition and state of the MPs, as well as the conditions surrounding the particles, such as pH in the gut. Koelmans and colleagues argued that the vector effect might be insignificant in an environmental context, due to the ratio of MPs to other particles in the environment such as natural particles (Koelmans et al.,

2016). Concentrations of MPs are numbers of magnitude lower than natural particles, which implies that the MP vector effect might not have large importance on a global scale. However, efforts to quantify MPs have been limited by sampling techniques and especially smaller particles, with higher surface to volume ratio, are typically underestimated. This implies that data are still needed in order to determine the impact of the vector effect, especially in “hot spot” areas where MP concentrations are significantly higher than the average.

3.1.2. Desorption of heavy metals from MPs during their *in vitro* human digestion and physico-chemical transformations

To date, studies on *in vitro* human digestion are scarce and mainly focused on simple batch models exposing MPs, alone or with adsorbed chemical contaminants (only heavy metals studied so far), to simulated digestive fluids. The main characteristics of currently used models, including their advantages and limitations, are given in Table 1. A high desorption under acidic conditions was reported in the specific case of Chromium in a human static *in vitro* digestion model, including mouth, gastric, small intestinal, and large intestinal digestive phases (Liao and Yang, 2019). The digestion was performed at 37 °C on glass tubes for each compartment independently with adapted juices composition, digestion time and pH. The large intestine phase lasting 18 h only included some enzymes in its juice, but no microbiota was implemented. Bioaccessibility was then determined as the percentage of bioaccessible Cr content in digested MPs to the total Cr content of the Cr-loaded MPs. Cr adsorbed on various MP types (PE, PP, PVC, PS and PLA, 150 µm) was more bioaccessible in the gastric environment than in the small or large intestinal phases, due to acidic conditions favoring desorption of anionic Cr species (e.g. CrO_4^{2-} and HCrO_4^-) from the MP surface, while no release was observed in the oral phase. Differences were noted between Cr(VI) and Cr(III), characterized by a higher bioaccessibility in the gastric phase for Cr(VI). Moreover, PLA exhibited the highest oral bioaccessibility of Cr(VI) and Cr(III) in comparison to other polymer types, probably due to its degradation enhanced by the action of enzymes present in simulated digestive juices (Liao and Yang, 2019).

Table 1
In vitro gut models already used for studies on MPs.

Models	Simulated compartment	Characteristics	Applications	Results	References
Cellular culture					
Caco-2 cells	Gut epithelium (enterocytes) barrier	Expressed microvilli, transporters and brush border enzymes No mucus layer, only one cell type and no other cell interactions	Epithelium barrier integrity (TEER) and uptake Cytotoxicity	PS MPs (5.9×10^5 non-ionic yellow/green fluorescent microspheres, 2 μm in diameter)Transport regulated by tight and adherent junctions Decrease in TEER Ethanol impact on tight junction integrity and increase in MP transport through Caco-2 cells	Carr et al. (2012) Wu et al. (2019b)
Co-culture of Caco-2/HT29-MTX cells	Gut epithelium (enterocytes) barrier + mucus layer	Expressed microvilli, transporters and brush border enzymes + mucus layer Very thin mucus layer, only two types of cells	Cytotoxicity	Carboxy modified PS MPs (500 nm) and 50 nm particles: no significant cytotoxicity unless applied at very high concentrations (higher than 50 $\mu\text{g}/\text{mL}$) Protective effect of mucus	Hesler et al. (2019)
Co-culture Caco-2/HT29-MTX cells coupled to human blood monocyte-derived macrophages and dendritic cells	Gut epithelium (enterocytes) barrier + mucus layer + immune cells	Expressed microvilli, transporters and brush border enzymes + mucus layer + immune cells	Cytotoxicity	PP and PA, 50–500 μm , 6, 24 and 48 h exposure period, concentration of 823.5–1380.0 $\mu\text{g}/\text{cm}^2$ No significant cytotoxicity, inflammatory response (release of IL-8, IL-1 β , TNF α) and barrier integrity disruption	Lehner et al. (2020)
Inverted insert culture system combining Caco-2/Raji-B cells	Gut epithelium (enterocytes) barrier and interaction with immune cells	Expressed microvilli, transporters and brush border enzymes Interaction of enterocytes with B lymphocytes Inserts inverted to make the Caco-2 cell monolayer more accessible to B lymphocytes leading to Caco-2 differentiation in M-cells (15–30% of the Caco-2 cells)	Uptake	Uptake of 200 nm carboxylated PS MPs (4.5×10^9 particles/mL) significantly increased in the co-culture and inverted model compared to the control Caco-2 monoculture or the non- inverted model through a non-specific absorption endocytosis	Des Rieux et al. (2007)
Caco-2/Raji-B cells leading to Caco-2 differentiation in M-cells		Expressed microvilli, transporters and brush border enzymes Interaction of enterocytes with B lymphocytes Raji-B cells leading to Caco-2 differentiation in M-cells	Uptake	No difference in absorption of PS MPs (2 μm) between Caco-2 and Caco-2/M-cell models	Carr et al. (2012)
Caco-2/HT29-MTX cells (mucus co-culture model) or Caco-2/Raji-B cells (M-cell model)	Gut epithelium (enterocytes) barrier and interaction with mucus or immune cells	Expressed microvilli, transporters and brush border enzymes Interaction of enterocytes with mucus or B lymphocytes	Uptake	Higher uptake rate of PS MPs (1 μm and 4 μm ($1 \times 10^8/\text{mL}$)) but not PS MPs (10 μm ($3 \times 10^6/\text{mL}$)) with both co-cultures compared to Caco-2 cells only No differences between the M-cell and the mucus models for the 4 μm MPs Higher uptake in the M-cell model compared to the mucus model for 1 μm MPs	Stock et al. (2019a)
In vitro gut models					
Batch model of upper GIT	3 digestive compartments mouth, stomach and intestine	Only one vessel Simple models easy to use reproducing digestive juices (pepsin, trypsin, pancreatin, bile extract), pH and temperature Batch conditions Lack of digestion dynamics, microbiota in the large intestine, interactions with epithelial cells and/or mucus Various tubes Transfer of digesta from a tube to another after the digestion step ended	Impact of the gastrointestinal passage on the physico-chemical MP characteristics	High stability of all MPs (100 mg/mL (PE 90.1 μm , PET 60 μm , PVC 136.5 μm), 50 mg/mL (PP 67.1 μm) or 10 mg/mL (PS 3.8 μm)) in artificial digestive juices No degradation of MPsAdsorption of organic compounds on MP surface (corona)	Stock et al. (2019b)
Whole digestive system <i>in-vitro</i> method (WDSM)	Mouth, gastric, small intestinal, and large	3 vessels for mouth, the stomach and the intestine compartments respectively		150 μm MPs (PE, PP, PVC, PS, PLA) Highest Cr adsorption capacity for PS	Liao and Yang (2019)

(continued on next page)

Table 1 (continued)

Models	Simulated compartment	Characteristics	Applications	Results	References
	intestinal digestive phases			Weakest Cr adsorption capacity for PLA Intermediate Cr adsorption capacity for PE, PP and PVC Highest bioavailability of Cr(VI) for PLA No Cr release from MPs in the mouth phase In gastric phase: bioaccessibility of Cr (VI) > Cr(III) but = in the intestinal phases.	
Batch model of upper GIT			Impact of MP-lipid co-ingestion on lipid digestion	PE (10 μm), PET (50 μm), PVC (1 μm), PLGA (10 μm) and PS MPs (10 μm versus 1 μm and 50 nm) Inhibitory effect on lipid digestion (high inhibition for PS MPs: size-independent but concentration-dependent) Interaction of MPs with both lipid droplets and lipases (hetero-aggregates) Decrease in the bioaccessibility of lipid droplets and reduced activity of lipases by changes in their secondary structure	Tan et al. (2020)
Dynamic and multi-compartmented model of upper GIT	3 digestive compartments mouth, stomach and intestine		Influence of the digestive process on intestinal toxicity of MPs	5 μm PS MPs lower intestinal toxicity than 100 nm PS particles Digestive treatment: alleviation of cytotoxicity and transport function disorder of the Caco-2 monolayer induced by the non-digested PS MPs Combined toxicities of PS MPs and arsenic decreased by digestive treatment <i>In vitro</i> digestion of 100 nm PS particles: increase in their proinflammatory effects Formation of a corona on the PS-MP surface during digestion: changes in size, Zeta potential, and adsorbed compounds	Liu et al. (2020b)

Numerous cell models of the intestinal barrier are used, from monocultures to co-cultures of various cell types. Limited simple batch *in vitro* systems reproducing the digestive environment are also utilized.

Cr: chromium; GIT: gastrointestinal tract; IL-1 β : interleukin 1 β ; IL-8: interleukin 8; MAPK: mitogen-activated protein kinases MPs: microplastics; NF- κ B: nuclear factor- κ B; PA: polyamide; PE: polyethylene; PET: polyethylene terephthalate; PLA: polylactic acid; PLGA: poly(lactic-co-glycolic acid); PP: polypropylene; PS: polystyrene; PVC: polyvinyl chloride; TEER: Transepithelial electrical resistance, TNF α : tumor necrosis factor α .

3.1.3. Physico-chemical transformations of MPs during *in vitro* human digestion

To support the aforementioned differences in desorption of heavy metals, and more generally chemical contaminants, during *in vitro* human digestion, it is important to unravel the physico-chemical transformations of MPs themselves (size, shape, surface properties, formation of a biomolecular corona etc.). The study of Stock and colleagues revealed no striking alteration of the physico-chemical characteristics of five types of MPs (PE, PP, PVC, PET and PS) by artificial digestive juices, mimicking the saliva, gastric, and intestinal phases of human digestion (Stock et al., 2019a). All digestion steps were successive in the same vessel. MPs were digested during 5 min at 37 °C in presence of synthetic saliva, containing α -amylase, mucin and various electrolytes, under agitation at pH 6.7. Gastric juice, composed of pepsin, mucin, electrolytes and hydrochloride acid, was then added, and the pH adjusted at 2 for the gastric phase for 2 h. Finally, the intestinal digestion began by addition of artificial intestinal juice, containing trypsin, pancreatin, bile extract and electrolytes, with pH value set at 7.5 for 2 h. The same authors emphasized the importance to consider corona formation onto MPs surface due to the adsorption of organic compounds such as proteins, mucins and lipids during digestion process (Fig. 1). Owing to its biological impact (Monopoli et al., 2012), the characteristics of the biomolecular corona formed around the MPs during their

transit in the digestive tract should be more deeply studied to determine the behavior of the MPs in the gut, and particularly their uptake rate.

3.2. Pathogenic bacteria and antibiotics on MPs, potential role in spread of antimicrobial resistance in the gut

Microorganisms, including bacteria, are able to aggregate and adhere to biological or non-biological surfaces, in complex communities commonly referred to as biofilms (Lopez et al., 2010). Common environmental surfaces for biofilm formation include rocks, weeds and woods, but also plastic particles as they offer a suitable support for the formation of biofilm communities. In contrast to other floatable materials, like wood and weeds, plastic particles provide a long-lasting surface preserving their inhabitants from influence of environmental variations (De Tender et al., 2017; Lobelle and Cunliffe, 2011). Accordingly, in recent years, marine plastic particles have been reported to harbor diverse microbial species associated in dense multi-species biofilm structures (Kirstein et al., 2016; Zettler et al., 2013) (Fig. 2). The microbial inhabitants associated with MPs include potentially pathogenic bacterial species, like *Pseudomonas* spp., *Vibrio* spp., *Campylobacter* spp. and *Escherichia coli* (Bryant et al., 2016; Curren and Leong, 2019; Kirstein et al., 2016; McCormick et al., 2014; Wu et al., 2019a; Zettler et al., 2013). These observations have led to the

hypothesis that MPs colonized by enteric pathogens may constitute a novel vector for transmission of infectious diseases, possibly indirectly through contamination of food and drinks, although the dose transmitted by this route would most likely not reach infectious levels. A more concerning aspect is the association of antibiotics on MPs as mentioned in the above section. For some antibiotics, concentrations selecting for resistance have been reported. Specifically, levofloxacin (fluoroquinolone) can be adsorbed in concentrations up to 1.2 mg/g (Yu et al., 2020) onto PVC, whereas ciprofloxacin (fluoroquinolone), amoxicillin (penicillin), and tetracycline can be adsorbed to polyamide (PA) in concentrations ranging from 1 to 3 mg/g (Li et al., 2018). Finally, sulfamethoxazole was found to be strongly adsorbed onto PA and PE MPs in concentrations of 0.4 and 0.1 mg/g, respectively (Guo et al., 2019; Razanajatovo et al., 2018). These results point out that some of the most common MP particles are not only vectors for potential pathogenic microbes, but also favour hitch-hiking of microbes that are resistant to antimicrobials. In support of this, recent studies have shown a significantly higher prevalence of antimicrobial resistance genes in microbiomes isolated from MPs than from seawater (Wang et al., 2020; Xue et al., 2020; Yu et al., 2020) whereas other studies have highlighted the ability of MPs to act as habitats for increased gene exchange. Thus, high concentrations antibiotics and bacteria on MP surfaces could drive the spread and sharing of antimicrobial resistance genes (Arias-Andres et al., 2018; Wang et al., 2020; Xiang et al., 2019; Yang et al., 2019). Accordingly, apart from enhancing the development of antibiotic resistant bacteria, ingestion of MPs may facilitate transfer of antimicrobial resistance genes to the intestinal microbiome. However, to our knowledge, no study has been performed so far to unravel the consequences of oral exposure to pathogen- and/or antibiotic resistance gene-contaminated MPs during *in vitro* human digestion or *in vivo* in mammals.

4. Impact of MPs in the digestive tract: lessons from mammalian model organism

4.1. Uptake and tissue accumulation

As a first site for MPs after ingestion, the gastro-intestinal tract (GIT) is of utmost importance as the gatekeeper for their bioavailability. The first studies investigating the fate of MPs in the GIT upon ingestion in mammals (rats and mice) have used (fluorescent) PS beads, usually 2 µm diameter size, as they are convenient for detection through diverse microscopy techniques, such as scanning electron microscopy, transmission electron microscopy or fluorescence/confocal microscopy (Carr et al., 2012). After administration to rats, it was demonstrated that MP uptake is a fast process, as particle absorption in intestinal tissue already took place after 5 min of ingestion, and significantly higher amounts of MPs were found in the epithelia of the proximal part of the small intestine compared to the distal one (Carr et al., 2012). Accumulation of these MPs was visible at the level of the microvilli of enterocytes, in the intercellular spaces, in the epithelial-stromal boundary and in the blood vessels. Interestingly, accumulation in Peyer's patches was much lower. Nevertheless, 5 weeks after a single-dose ingestion, the few MPs that were monitored in this region persisted while at the level of enterocytes, MPs had disappeared. In mice, Stock and co-workers reported that only few MPs were detected by fluorescence microscopy in the intestinal walls of animals after oral exposure three times per week for 28 days by oral gavage with a mixture of 1 µm, 4 µm and 10 µm PS MPs, corresponding to an administered dose of 1.25 mg/kg bw, 25 mg/kg bw, and 34 mg/kg bw, respectively (Stock et al., 2019b). No histological lesion nor inflammatory response was observed. In contrast, in mice fed 600 µg/day (corresponding to 30 µg/kg bw/day for a mouse weight of approximately 20 g) of PE MPs (10–150 µm) for 5 consecutive weeks, the histological scores of colon and duodenum were significantly higher than the ones determined for non-treated animals (Li et al., 2020a). In mammals, it is thus not clear whether MP exposure leads to gut

inflammation. Results depicted yet are highly dependent on treatment conditions (duration, doses) but also on the polymer type and size of MPs and potentially on their shape and biomolecular corona. In addition, doses administered *in vivo* have to be compared to the human daily intake between 0.2 and 10 mg/kg bw/day (considering an adult human body weight of 70 kg), derived from the values recently reported by Senathirajah and colleagues (Senathirajah et al., 2021), even though it is clearly a first estimation of the amount of potentially ingested MPs, which will serve as a basis for future investigations in humans.

There are different routes of MP uptake in the GIT due to their size range, as reviewed by EFSA (EFSA, 2016). Cells involved in microparticle (and also nanoparticle) transport are notably specialized M-cells overlying Peyer's patches, which belong to the mucosa-associated lymphoid tissues, and transport large structures, amongst which antigens, bacteria and viruses, to the immune system. MP particles of less than 10 µm could be trapped into the intestine by M-cells according to the adherence on the mucus, high adherence to the loosely adherent mucus layer leading to a quick clearance and fewer absorption time (Ensign et al., 2012). A marked decrease in uptake efficiency by M-cells of particles between 200 and 500 nm diameter was reported compared to smaller sizes (Powell et al., 2010). Generally, small-sized particles seem to better translocate across the gut than larger ones (Wright and Kelly, 2017). For MPs between 5 and 110 µm diameter, persorption, a paracellular transport, especially in desquamation zones and between the villi, appears to be the most important uptake mechanism (Wright and Kelly, 2017). Not only the size, but also the hydrophobicity, surface functionalization and charge, and biomolecular corona, influence MP uptake ability. The role of mucus as a first barrier, with size- and charge-exclusion properties, is also of uppermost importance, as reviewed by Gillois and colleagues (Gillois et al., 2018). Mucus may trap MPs, thereby decreasing their toxicity to the host. However, it is possible that MPs more easily cross the mucus layer because of their physico-chemical transformations after contact with the intestinal content, as reported for dietary microparticles (Powell et al., 2007). Conversely, MPs may affect mucus characteristics all along the GIT. Recent studies in mice highlighted the impact of MPs on mucus homeostasis pathways (Fig. 1). After exposure to 0.5, 5 and 50 µm PS MPs at 100 µg/L and 1000 µg/L for 6 weeks, mucus secretion in the colon was significantly decreased as shown by Alcian blue-periodic acid Schiff staining, which was supported by the down regulation of genes involved in mucus synthesis/secretion pathways (*Muc2*, *Muc1*, *Retnlb* and *Klf4*) in the colon (Jin et al., 2019; Lu et al., 2018). This dysregulation of the protective mucus barrier function, probably acting in concert with an impaired epithelial barrier and a gut microbiota dysbiosis (see Section 4.2), was already depicted for pesticides; persistent organic pollutants and food additives (Gillois et al., 2018), even though the underlying molecular mechanisms remain to be fully unveiled.

Most studies highlight MP presence in the gut and other tissues of many species, including rodents, in a MP size-dependent manner (Lu et al., 2019). In rats, 6% of MPs (PS, 870 nm) were found in the circulation 15 min after oral administration (Eyles et al., 1995) while nano-sized particles (PS, 50 nm) led to 34% of absorption, with a main accumulation in the liver (Jani et al., 1990). Following a 4-week exposure period, 20 µm and 5 µm PS MPs accumulated in the gut, liver and kidney of mice in a MP size-dependent localization. The biggest particles were consistently distributed in the three tissues while the smallest ones accumulated more in the gut of exposed mice (Deng et al., 2017). However, the relevance of this work has been recently discussed by others (Braeuning, 2019), notably due to the fact that the quantity of particles detected in the organs massively exceeded the quantity of particles administered during the study.

4.2. Bidirectional relationship with gut microbiota

The intestinal microbiota, *i.e.* the huge community of microorganisms present in the GIT, raises an increasing interest the past few years,

with a recent focus on the potential impact of MPs on this ecosystem (Fig. 1) (Fackelmann and Sommer, 2019). To date, no study has been carried out on the human gut microbiota. However, studies already conducted on the cecal or fecal contents of mice orally exposed to MPs revealed microbiota composition modifications (Jin et al., 2019; Lu et al., 2018). Mice exposed to PS MPs (0.5 μm and 50 μm) in drinking water at two different concentrations (100 $\mu\text{g/L}$ or 1000 $\mu\text{g/L}$) during 5 weeks exhibited microbiota alterations. Impaired α and β -diversities and a significant reduction in the abundance of Firmicutes and α , β and γ -Proteobacteria were reported in the cecal content of treated mice in all conditions using a PCR-based quantification assay. The Actinobacteria abundance was reduced only after an exposure to 1000 $\mu\text{g/L}$ of PS MPs (Jin et al., 2019; Lu et al., 2018). Analysis of 16S rRNA sequencing of cecal content demonstrated a decrease in the relative abundance of Firmicutes, Bacteroidetes and Verrucomicrobia at the highest dose for both MP sizes (Lu et al., 2018) and of Actinobacteria after exposure to 5 μm PS MPs at a concentration of 1000 $\mu\text{g/L}$ (Jin et al., 2019) while Proteobacteria proportion increased (Jin et al., 2019; Lu et al., 2018). In addition, at the genus level, 15 types of bacteria were significantly affected; in particular, *Bifidobacterium*, *Prevotella* and *Veillonella* decreased while *Coprococcus* and *Anaeroplasm*a increased in the cecal content following exposure with 1000 $\mu\text{g/L}$ of PS MPs (Jin et al., 2019). After exposure to 6, 60, and 600 $\mu\text{g/day}$ of PE MPs present in the diet during 5 weeks in mice, an increase in gut microbial abundance and diversity from fecal samples was reported by 16S metagenomics data. Treated-mice showed a higher abundance of *Staphylococcus* as well as a reduction in *Parabacteroides* abundance compared to untreated animals (Li et al., 2020a). Finally, co-exposure of PE MPs (45–53 μm) bound to di-(2-ethylhexyl) phthalate (DEHP) during 30 days led to gut microbiota composition modification in mice, characterized by a lower β -diversity and an increase in the relative abundance of Actinobacteria in the fecal content (Deng et al., 2020). Such effect was stronger for the di-(2-ethylhexyl) phthalate-contaminated MPs than for the virgin MPs.

Concerning the bidirectional relationship between MPs and gut microbiota, little is known about the microbial degradation ability of MPs in mammals. However, some microorganisms from the gut microbiota of soil waxworms are able to degrade MPs. The gut of the larvae *Plodia interpunctella* contains two bacterial strains, *Enterobacter asburiae* YT1 and *Bacillus* sp. YP1, able to degrade PE. Indeed, researchers observed the formation of pits and cavities on the PE surface and addition of carbonyl groups (Yang et al., 2014). Bacteria extracted from the gut of *Lumbricus terrestris*, members of the phyla Actinobacteria (*Microbacterium awajjense*, *Rhodococcus jostii*, *Mycobacterium vanbaalenii* and *Streptomyces fulvissimus*) and Firmicutes (*Bacillus simplex* and *Bacillus* spp), were able to reduce the size of low density PE MPs (from 150 μm to an average of 53.1–41.3 μm) in a gamma sterilized soil culture after 21 days (Huerta Lwanga et al., 2018).

According to the huge and diverse microbial communities in the human gut, it seems plausible that some members could harbour this degradation ability. Indeed, after comparison between bacteria identified as able to degrade MPs (Jacquin et al., 2019) and bacteria found in the human gut microbiota, according to the database of Plaza Oñate and his team (Plaza Oñate et al., 2019), bacteria belonging to Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes phyla were present in both; however, to date, no rigorous study has been carried out on the possible plastic degradation metabolism by the human gut microbiome.

As presented above, the few existing studies focused on the bacterial fraction but none investigated the impact of MP exposure on the composition and activity of the fungal population (“mycobiome”) in the gut (Sánchez, 2019), which is increasingly recognised as a key player in intestinal health and disease (Richard and Sokol, 2019). Furthermore, a limitation for many of studies reported for gut bacteria is that they mostly rely on 16S rRNA gene sequence analysis, which does not allow species level resolution. To fully resolve microbial issues, including the prevalence of pathogenic bacteria attached to MPs and the risks associated with this (see Section 3.2), there is a need to go beyond

community profiling by employing high-resolution molecular analysis of species genotypes in combination with cultivation dependent phenotypic characterizations.

5. *In vitro* gut models: current uses and new developments for addressing the fate and toxicity of MPs

Research on the fate, transformation and effects of MPs upon human intake remains limited, in part due to challenges of system complexity, analytical methods and varied forms of ingested plastic particles (size, shape, polymer type). Studies in humans, mainly through clinical studies, would obviously be the ideal strategy but remain also hampered by ethical, regulatory and cost reasons. *In vivo* studies in mammals cannot address to all the questions raised, due to major gut characteristics differences depending on species (Hugenholtz and de Vos, 2018). Furthermore, animal experiments are acknowledged by the 3R principle rules, which widely encourage the development of alternative *in vitro* approaches to reduce the number of animals used in research. Then, *in vitro* models of the digestive tract are of uppermost importance for human gut research. A wide range of *in vitro* gut models has been already developed, including cellular cultures and digestion systems, from simple static mono-compartmental models to the most complex dynamic multi-compartmental ones. All those models, and their coupling, have a huge potential for toxicological investigations related to MPs, and current developments and achievements are detailed below. Future directions are also proposed.

5.1. *In vitro* upper gut models

Complementarily to the studies conducted by Liao and Yang (Liao and Yang, 2019) and by Stock and colleagues (Stock et al., 2019a) (see Sections 3.1.2 and 3.1.3), Tan and co-workers recently conducted an *in vitro* experiment in a gastrointestinal system reproducing the main phases of the mouth, stomach and small intestinal human digestion (Table 1). Briefly, lipid-MP emulsion was incubated during 10 min at pH 6.8 at 37 °C in simulated saliva. Then, the oral digesta was mixed with simulated gastric fluid at pH 2.5 for 2 h. Finally, the gastric digesta was added to simulated intestinal fluids for 2 h at pH 7. Five MP types (PE, PET, PVC, PLGA and PS) were tested and showed an inhibitory effect on lipid digestion, especially for PS, due to an interaction of these hydrophobic particles with both lipid droplets and lipases. Laser Scanning Confocal Microscopical Imaging and Nile Red staining were used to identify the PS MPs, the lipid droplets and the digested lipid. The formation of such heteroaggregates decreased the bioavailability of lipids droplets by reducing the lipid availability to be digested by lipases but also reduced the activity of lipases by changing their secondary structure (Tan et al., 2020). Despite these first steps forward, the systems currently used remain simplified, operating under static conditions and are only composed of different gut-simulating vessels with the corresponding digestive juices adjusted for pH and temperature. Some important parameters are absent, such as dynamic digestion processes (*i. e.* transit between successive digestive compartments or time-effect on gastric and intestinal pH and digestive secretion concentrations and flow rates), the presence of compounds from food matrices, the influence of gut microbiota (mainly in the large intestine, but also in the small intestine) (see Section 5.2), and lastly interactions with intestinal epithelial, immune cells and/or mucus (see Section 5.4). More physiologically relevant *in vitro* gut models could be highly valuable for a better understanding of the fate and effects of MPs in the human digestive environment. The main characteristics of these models, including advantages but also potential limitations, are given in Table 2. In particular, the physico-chemical transformations of MPs (*e.g.* formation of biomolecular corona, particle degradation), as well as the potential desorption of particle-adsorbed pollutants in the upper part of the digestive tract, could be investigated by using the complex *in vitro* TNO gastrointestinal system 1 (TIM-1). This model is currently the most

Table 2
Potential of *in vitro* gut models for future studies on MPs.

Model	Simulated compartment	Advantages	Limits	Potential applications for MPs	References
Complex cellular culture models					
3D models	Gut epithelium (culture of Caco-2 cells, HT29-MTX cells, human Raji-B lymphocytes and immune cells)	Under development		Impact of MPs on a complex gut barrier	Lehner et al. (2018)
Intestinal organoids	Gut wall (enteroids = derived from adult stem cells isolated from the crypt of small intestine; colonoids = derived from adult stem cells isolated from colonic tissue)	- 3D organization- Cells derived from human biopsies- Personalization (e.g. infant/ adult/elderly)	GIT microenvironment not fully taken into account (e. g. pH, anaerobic, intestinal media, physical constraints or microbiota interactions) Difficult access to the lumen Highly expensive, required specialized expertise and lack of standardization	Impact of MPs on a complex gut barrier	Kim et al. (2020)
Microfluidic systems					
HuMiX (Human–microbial crosstalk) organ on a chip	Gut epithelium	- Culture of human cell lines (Caco-2) with or without bacteria (trial with <i>Lactobacillus rhamnosus</i> GG) –Conditions representative of the human GI–microbe interface (micro-anaerobic environment, oxygen gradient, epithelium absorption)	DMEM medium used to support Caco-2 cell and microbial growth (not representative of intestinal media) Lack of several cellular types characteristic of the gut epithelium (e.g. goblet cells, M-cells) and 3D structure Limited range of flow rates (low) No peristalsis reproduced, no complete mucus layer	Impact of MPs on the gut epithelium and specific species of gut microbiota	Shah et al. (2016)
Intestine-chip	Gut epithelium	- 3D structure of the gut epithelium with mechanical forces (peristalsis, intestinal flow)- Co-culture of human cell lines (Caco-2; HT29-MTX, endothelial cells, M-cells) with or without bacteria (trial with <i>Lactobacillus rhamnosus</i> GG) - Reproduced passive absorption- Formation of local anoxic microenvironment- Possibility to analyze contributions of individual cellular, chemical, and physical control parameters one-at-a-time	Limited range of flow rates (low) No complete mucus layer No true anaerobic conditions or oxygen gradient Missing muscle and neuronal system cells Possible adsorption of hydrophobic molecules by PDMS matrix	Impact of MPs on the gut epithelium and specific species of gut microbiota	Kim et al. (2015) , Bein et al., 2018
Colon Chip	Colon epithelium	Primary human colonic epithelial cells including spontaneously differentiated goblet cells and a complete mucus layer corresponding to that reported in humans	Expensive and required dedicated expertise and instrumentation Stem cell differentiation difficult to achieve No input from immune and nervous system No reproduction of the full complexity of the human gut microbiota	Impact of MPs on the colonic epithelium, the mucus layer	Sontheimer-Phelps et al. (2020)
In vitro gut models					
TNO Gastrointestinal model (TIM-1)	Upper GIT (stomach & small intestine)	- Most complex model of the human upper GIT- Main physico-chemical parameters of the stomach and small intestine (temperature, pH kinetics, transit time, digestive secretions and passive absorption)- Adapted to simulate infant, adult or elderly digestive conditions	No interaction with the intestinal epithelial cells, no oral phase, no intestinal microbiota, no mucus, no active absorption	Physico-chemical transformations/degradation of MPs in the upper GIT Biomolecular corona evolution during the fate of MPs in the upper GIT Effect of food matrices	Cordonnier et al. (2015) ; Roussel et al. (2016) ; Denis et al. (2016) Denis et al. (2016)
Mucus Artificial COLon model (M-ARCOL)	Lower GIT (colon)	- Main physico-chemical but also microbial (luminal and mucus-associated microbiota) parameters of the human colon - Anaerobiosis maintained by the sole activity of gut microbiota-	Average colonic conditions simulated (not the three compartments) No epithelium interaction Expensive and required specific expertise and instrumentation	Bidirectional interactions of MPs with human luminal and mucosal gut microbiota (composition and activity)	Deschamps et al. (2020)

(continued on next page)

Table 2 (continued)

Model	Simulated compartment	Advantages	Limits	Potential applications for MPs	References
Mucosal Simulator of the Human Intestinal Microbial Ecosystem (M-SHIME)	Physico-chemical parameters of the stomach, small intestine and the three parts of the colon and presence of gut microbiota in the colon	Possible long-term experiments (chronic exposure) - The only <i>in vitro</i> model simulating the entire human GIT. Reproduction of the three parts of the colon including gut microbiota and mucus specific microenvironment - Adapted to simulate infant or adult digestive conditions- Possible long-term experiments (chronic exposure)- Possible coupling with cell culture and HMI module)	Upper GIT less complex than in the TIM-1 model No epithelium interaction, continuous flow of N ₂ to maintain anaerobic environment Expensive and required specific expertise and instrumentation	Physico-chemical transformations/degradation of MPs in the entire GIT, biomolecular corona modification and luminal and mucosal microbiota interactions (composition and activity)	Lambrecht et al. (2019); De Boever et al. (2001); Van den Abbeele et al. (2019)
Coupling <i>in vitro</i> gut models with cellular culture models					
Culture of intestinal cells exposed to supernatant from M-SHIME or M-ARCOL	Gut epithelium	- Easy way for prospective studies on the crosstalk between digested MPs /microbiota/ epithelium	No direct interaction of microbiota with the intestinal epithelium No complete intestinal cell wall represented	Impact of digested MPs on the crosstalk between gut microbiota metabolites and epithelium	/
Host-Microbiota Interaction (HMI) module	Gut epithelium and mucus layer (first adapted for the SHIME model)	- Simulation of bacterial adhesion to the gut wall - Reproduction of physiological shear forces and microaerobiosis - Continuous coupling with the M-SHIME	No all the cell types encountered in the intestinal wall (immune cells, M-cells...). Due to cytotoxicity, studies restricted to a 48-h period.	Effects of chronic exposure to digested MPs on the gut epithelium and mucus layer	Marzorati et al. (2014)

These *in vitro* models include dynamic multi-compartmental models coupled or not to cellular cultures, 3D models like intestinal organoids and gut-on-a-chip microfluidic devices.

GIT: gastrointestinal tract; HMI: Host-Microbiota Interaction module; HuMiX: human-microbial crosstalk; M-ARCOL: Mucus Artificial COLon; MPs: microplastics; M-SHIME: Mucosal- Simulator of the Human Intestinal Microbial Ecosystem; PDMS: poly(dimethyl siloxane) polymer; TIM-1: TNO gastrointestinal system 1.

complete simulator of the upper human GIT reproducing the main physico-chemical parameters of the stomach and small intestine, *i.e.* body temperature, kinetics of gastric and intestinal pHs, transit time, gastric, pancreatic and biliary digestive secretions and passive absorption of nutrients and water (Cordonnier et al., 2015). Compared to static model, TIM-1 also enables a better evaluation of bioaccessibility, closer from *in vivo* situation. Absorption of small molecules (such as particle-associated contaminants) is modelled in TIM-1 *via* the use of circulating dialysis fluid through hollow fibers. Moreover, TIM-1 can be used to investigate the influence of food matrices (including lipid-rich matrices) on MP physico-chemical transformations, degradation and/or bioaccessibility of associated contaminants (Helbig et al., 2013; Larsson et al., 2016; Minekus et al., 2014; Miszczycza et al., 2014). Different results related to the bioaccessibility of some heavy metals, previously obtained in a simple static model compared to the dynamic TIM-1 (Torres-Escribano et al., 2011), clearly underline the importance to investigate the behavior of MPs, in their virgin *versus* contaminated (pollutants, heavy metals, antibiotics, pathogens *etc.*) forms, in the human gut through more complex *in vitro* systems.

5.2. *In vitro* lower gut models – interactions with gut microbiota

No study on the fate of MPs in the lower GIT, and in particular their potential bidirectional interactions with a gut microbiota from human origin, has been reported so far using *in vitro* models of the small intestine and colon (Table 2). As already shown for pollutant bioaccessibility (Reygner et al., 2016), effect of antibiotics on gut microbiota (El Hage et al., 2019; Ichim et al., 2018; Marzorati et al., 2020), pathogen survival and virulence (Roussel et al., 2020), and transfer of resistance genes to human gut microbiota (Lambrecht et al., 2019), these studies, including the role of mucus as an ecological niche for the gut microbiota, could be performed in a mono-compartmental model simulating the mean physico-chemical and microbial parameters of the human colon, such as the Mucus Artificial COLon model (M-ARCOL) (Deschamps et al., 2020) or in a more complex system

simulating the entire GIT (stomach, small intestine and three parts of the colon), the Mucosal Simulator of the Human Intestinal Microbial Ecosystem (M-SHIME) (Lambrecht et al., 2019). Impact of MPs and associated-contaminants on human gut microbiota can be monitored both at the level of microbiota composition and metabolic activity (*e.g.* production of short chain fatty acids, gases). Inversely, it is possible to investigate the gut microbiota capacity to degrade and metabolize MPs, in particular specific microbial metabolic pathways activated by these particles. Interestingly, inter-individual variabilities in gut microbiota structure and function can be envisioned *in vitro* by inoculating the models with fecal samples issued from different donors. Of interest, all the aforementioned *in vitro* models of the upper and lower GIT have already been adapted to simulate the digestive conditions found in different aged populations, such as infant, adult or elderly people (De Boever et al., 2001; Denis et al., 2016; Roussel et al., 2016; Van den Abbeele et al., 2019). This could allow to decipher if the fate and effects of MPs would be affected in specific populations compared to adults.

5.3. Models for uptake, fate and effects of MPs on the intestinal barrier

Complementarily to *in vitro* models of the upper and lower GIT, cell models, like Caco-2 cell monolayers, are useful to investigate uptake, fate and effects of MPs on the gut barrier (Table 1). The majority of studies reported so far are focused on virgin particles without taking into account the impact of the digestive processes and/or the influence of chemical or microbial contaminants. The Caco-2 cell line was originally isolated from a human adenocarcinoma, and spontaneously differentiates upon confluence to a monolayer of polarized enterocytes, expressing microvilli, transporters and brush border enzymes. Exposure to MPs was shown to disturb the intestinal epithelial barrier, as measured by a decreased transepithelial electrical resistance (TEER), and transport of MPs through the Caco-2 cell model was regulated by tight and adherent junctions (Carr et al., 2012). Wu and colleagues showed an up-regulation of the cell inflammatory pathways, such as NF- κ B and MAPK signalling, and a downregulation of proliferation pathways in a

concentration- (from 10^{-8} to 10^{-1} mg/mL) and time- (24 and 48 h) dependent manner on Caco-2 cells (Wu et al., 2019b). In addition, on the same cell line, increasing the MP exposure concentration (12.5, 25, 50 mg/L) and duration (24 and 48 h) led to an increased cytotoxicity. Limitations of the Caco-2 cell model are the lack of a mucus barrier and the simulation of a single cell type. Hesler et al. (2019) studied the impact of carboxy-modified PS MPs (500 nm), as well as smaller-sized particles (50 nm) on a coculture of the Caco-2 cell line with mucus-producing HT29-MTX-E12 cells, but found no significant cytotoxicity unless applied at very high concentrations, thereby pointing out a possible protective effect of the mucus layer (Hesler et al., 2019). Des Rieux and co-workers were one of the first research groups studying particle uptake in an inverted combined Caco-2/Raji-B model (des Rieux et al., 2007). They demonstrated that the uptake of 200 nm carboxylated PS particles was significantly increased in the co-culture and inverted model compared to the control Caco-2 monoculture or the non-inverted model through a non-specific absorption endocytosis. Interestingly, in another study where larger PS MPs (2 μ m) were applied to Caco-2 and Caco-2/Raji-B models, no difference in absorption was observed, thereby highlighting the importance of microplastic size in the assessment of bioavailability and toxicity (Carr et al., 2012). Monocytic THP-1 cells were also used in combination with Caco-2 cells for the study of MP toxicity (Carr et al., 2012), and the intestinal barrier function was found to decrease upon co-culture (Carr et al., 2012). Considering Caco-2/Raji-B (M-cell model) and Caco-2/HT29-MTX (mucus model) co-cultures versus Caco-2 monoculture exposed to PS MPs of three different sizes (1, 4 and 10 μ m), a significantly higher uptake rate was observed for both the 1 μ m and 4 μ m particles in the co-cultures compared to the monoculture (no significant differences with the 10 μ m particles). Furthermore, no differences were depicted between the M-cell and the mucus models for the 4 μ m MPs whereas for the 1 μ m MPs a higher uptake was found in the M-cell model compared to the other co-culture (Stock et al., 2019b). A three-dimensional *in vitro* intestinal cell model, composed with a mixture of human intestinal epithelial Caco-2 and HT29-MTX cells coupled to human blood monocyte-derived macrophages and dendritic cells, was designed to evaluate MP cytotoxicity (PP and PA, 50–500 μ m) after 6, 24 and 48 h of an aerosolized exposure to a concentration of 823.5–1380.0 μ g/cm² (Lehner et al., 2020). Interestingly, MPs were aerosolized directly onto the cells in order to avoid floating of those hydrophobic particles in the cell culture medium, thus ensuring a better control of particles interacting with cells. Contrary to previous studies, no significant toxicity effects were reported in terms of inflammatory response and barrier integrity disruption. Technical improvements were also introduced by Stock and colleagues to counteract the problem of floating particles in the cellular culture system by developing an inverse cell culture model, which was successfully tested on liver HepG2 cells (Stock et al., 2019c). MP impact on gut cells thus highly depends on MP characteristics (size, polymer type), concentration applied and duration of exposure. In addition, cell line used, and experimental design are important to consider when investigating MP uptake and impact on the gut in regard to the discrepancies of results previously described.

Overall, these cell models have shown merit in increasing knowledge on the mode-of-action of MP uptake and cellular responses. Nevertheless, the current models still suffer from limitations. First, almost all described cell cultures are from cancer origin, and therefore, effects on cell signalisation, immune responses, oxidative stress and toxicity need to be carefully interpreted as cancer cells show distinct differences in basal metabolism and reactive oxygen species (ROS) levels for instance. Furthermore, the Caco-2 cell line is a model for small intestinal enterocytes, and therefore doesn't take into account the spatial differences of the intestinal mucosal morphology and functioning depending on the location to the GIT, which may be of importance such as seen *in vivo* (Carr et al., 2012). In addition to this, colonic models – despite the longer residence time compared to small intestine – were, as far as we know, not applied yet for MP research.

5.4. Coupling *in vitro* gut models with cell cultures

To date, the very few existing studies on MPs have only considered simple systems. In particular, Liu and colleagues used a dynamic three-stage *in vitro* digestion system mimicking the mouth, stomach and small intestinal human conditions to investigate the differences between pristine and digested MPs (PS, 5 μ m versus 100 nm) on Caco-2 cells (Liu et al., 2020b) (Table 1). Each simulated digestive juice was reproduced by mixing salt solutions, organic compounds, enzymes and proteins. The pH was set at 6.8 for the saliva, 2.5 for the gastric compartment and 6.5 for the simulated small intestine. During the oral phase, the salivary digestion was reproduced as well as a mastication process *via* vibration for 5 min. The digestive treatment induced an increase in the average size of the particles and of their zeta potential, thus favoring particle agglomeration. Caco-2 cells were exposed either to the digested MPs or to pristine ones. For undigested particles, alterations of transport function were observed with an increased epithelium permeability reported through the paracellular marker Lucifer Yellow (LY) and a down-regulation of gene expression associated with the intestinal barrier functions (*zonula occludens* ZO-1 and occludin). In addition, alteration of transmembrane transport through the intestinal epithelium was shown by a down-regulation of *ABCC2* and *ABCG2* gene expression. However, for digested MPs, a reduction in the cytotoxicity and impairment of the transport function was obtained. On the contrary, an increase in the pro-inflammatory effects was observed but only for digested small-sized particles (100 nm) compared to pristine ones by cytokine release assay targeting interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP1). Those differences between digested and pristine particles could be attributed to the formation of a biomolecular corona (Liu et al., 2020b).

Further studies on interaction of MPs with the intestinal barrier could be performed by a more complex coupling between *in vitro* gut models and cell cultures (Table 2), as previously done with arsenic in the SHIME system and subsequent exposure of food digests to Caco-2/HT29-MTX cells (Calatayud et al., 2018), or even more advanced cell models including immune cells. As a next step, a relevant approach could be a continuous input of the digestive medium from *in vitro* gut models on cell cultures, as done with the Host-Microbiota Interaction (HMI™) module, primarily adapted to be coupled to the SHIME model (Marzorati et al., 2014). Of interest, this HMI module integrates Caco-2 cells combined with a mucus layer, maintained under relevant shear forces and microaerophilic conditions that can impact both commensal microbes and pathogens (De Weirdt and Van de Wiele, 2015; Marzorati et al., 2011). Other more 3D complex models could be used in the future (Table 2), such as multicellular models which are currently developed for MPs (Lehner et al., 2018, 2020) or intestinal organoids (Kim et al., 2020), not explored yet. Likewise, bioengineered human gut-on-chip devices, such as HuMiX (Shah et al., 2016), Intestine-Chip (Kim et al., 2015) and Colon-Chip systems (Sontheimer-Phelps et al., 2020), would enable to take into account the influence of mechanical forces (peristalsis, intestinal flow), and ultimately commensal microbes and mucus, on MP fate and toxicity in the gut. All these combined models mimicking human intestinal physiology *in vitro* are undoubtedly highly helpful to unravel fate, physico-chemical transformations, degradation, metabolism and toxicity-related mechanisms, which would be useful for future hazard and risk assessment in the context of MPs.

6. Conclusions

This review reports the different sources of oral exposure to MPs and focuses on the gastrointestinal route and the potential adverse effects on human intestinal health, with a special emphasis on the relevance of *in vitro* gut models for an in-depth study of associated mechanisms. In all environments, the presence of MPs increases drastically, and to tackle this critical issue shared science, policy, economy and society, governments are increasingly implementing regulatory laws related to plastic

pollution. In the European Union (EU), the European Strategy for Plastics in a Circular Economy (European Commission, 2018) stressed the need to tackle the challenges related to plastics, which have been identified as one of the five priority areas addressed by the EU Action Plan for the Circular Economy. In such context, the current COVID-19 sanitary crisis is forcing to rethink plastic concern worldwide. With the pandemic contributing to increased plastic use in healthcare, and large volumes of waste being unfit for recycling due to potential bio-hazards, medical plastic waste could grow at an unprecedented scale. A similar situation might arise in the food industry and packaging, textiles and other services that have decided to temporarily limit reusable. Recently, the use of disposable masks has been added to the list of MP sources (Aragaw, 2020). All these practices will contribute to the increasing plastic pollution and MP issues (Patrício Silva et al., 2021),

More and more studies on human health are regularly published but due to the lack of standardized detection and quantification methods for complex matrices and the self-contamination during sampling and experimentation due to plastic devices, results are often hardly comparable. An adaptation of the experimental set-ups, consumables and practices seems crucial to provide firm assessments on the potential health impact of MPs. Up to now, studies have shown that oral intake is a major route of human exposure. Drinking water, especially from plastic bottles, and seafood are among the greatest sources identified to date even though MP contamination is increasingly reported in many other food items, including milk, white wines, fruits and vegetables. Increasing attention is then paid to the GIT as the first barrier but also portal of entry and target for MPs. Complementarily to the action of virgin particles, the effects of adsorbed contaminants, including but not restricted to chemical pollutants, have to be considered in view of the vector effect hypothesis. In particular, MPs harbor a unique microbiome, which is shaped by polymer type and environmental factors. MPs then provide a stable support for widespread and specific microbial hitchhiking. Furthermore, the findings of several human pathogenic microbial species associated with MPs represent a potential risk for human exposure to these pathogens, either directly through ingestion of MPs or indirectly by cross-contamination of food or water. In addition, ingestion of MPs may enhance the release of antibiotics and the development of antibiotic-resistant bacteria in the human gut, act as vehicle for transfer of antimicrobial resistance genes to the intestinal microbiome and favour human infection related to resistant bacteria. From the physico-chemical point of view, particle weathering is also a critical, albeit poorly explored, player in the field; it leads to strong modifications of MPs due to oxidation, fragmentation and degradation and surface coverage by a complex mixture of organic and inorganic materials, thus leading to different sorption and toxicity profiles.

Current studies on the impact of MPs on intestinal health are mainly based on rodents (mice) and cellular cultures and physiologically-relevant *in vitro* models of the human gut, allowing to study the influence of digestive physico-chemical processes (pH, digestive secretions), mechanical forces (peristalsis, intestinal flow), as well as mucus and gut commensals in a regionalized manner, are needed to better evaluate the impact of such exposure on disruption of the human gut homeostasis. As endpoints of adverse effects, oxidative stress, genotoxicity, mitochondrial function and alterations in gene expression linked to inflammatory process and oxidative stress, as well as cross-talks with immune system and other organs such as liver and brain should be further studied (Yong et al., 2020). Due to its growing role in health and disease (Mathieu et al., 2018), the gut-lung axis could also be simulated.

In conclusion, as the adverse effects on the gut may strongly depend on the shape, size and surface properties of MPs, more representative particles, including weathered and/or contaminant-associated ones, than pristine PS microbeads as currently used, as well as dedicated analytical methods should be investigated in the future. Based on the first estimations of the amount of MPs potentially ingested by humans, more realistic exposure scenarios (*i.e.* relevant MP doses rather than overload conditions, chronic long-term exposure) should also be

considered for better health hazard and risk assessment. Reliable *in vitro* gut models could then be of highly relevance to mimic such exposure scenarios taking also into account the consequences on at-risk populations (*e.g.* children, elderly) and ultimately pathophysiological conditions (*e.g.* obese patients, Inflammatory Bowel Disease patients).

CRediT authorship contribution statement

Elora Fournier: Writing - original draft, Writing - review & editing, Investigation, Visualization, Methodology, Conceptualization; **Lucie Etienne-Mesmin:** Writing - review & editing, Methodology, Validation, Conceptualization, Investigation; **Charlotte Grootaert:** Writing - review & editing, Validation, Investigation; **Lotte Jelsbak:** Writing - review & editing, Investigation; **Kristian Syberg:** Writing - review & editing, Investigation; **Stephanie Blanquet-Diot:** Writing - review & editing, Methodology, Validation, Conceptualization, Investigation; **Muriel Mercier-Bonin:** Writing - review & editing, Project administration, Methodology, Funding acquisition, Supervision, Validation, Conceptualization, Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix 4:

Review in Biotechnology Advances

Publication:

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Research review paper

In vitro models of gut digestion across childhood: current developments, challenges and future trends

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ABSTRACT

The human digestion is a multi-step and multi-compartment process essential for human health, at the heart of many issues raised by academics, the medical world and industrials from the food, nutrition and pharma fields. In the first years of life, major dietary changes occur and are concomitant with an evolution of the whole child digestive tract anatomy and physiology, including colonization of gut microbiota. All these phenomena are influenced by child exposure to environmental compounds, such as drugs (especially antibiotics) and food pollutants, but also childhood infections. Due to obvious ethical, regulatory and technical limitations, *in vivo* approaches in animal and human are more and more restricted to favor complementary *in vitro* approaches. This review summarizes current knowledge on the evolution of child gut physiology from birth to 3 years old regarding physicochemical, mechanical and microbial parameters. Then, all the available *in vitro* models of the child digestive tract are described, ranging from the simplest static mono-compartmental systems to the most sophisticated dynamic and multi-compartmental models, and mimicking from the oral phase to the colon compartment. Lastly, we detail the main applications of child gut models in nutritional, pharmaceutical and microbiological studies and discuss the limitations and challenges facing this field of research.

1. Introduction

The human digestion is a multi-step and regionalized process essential for health. In early life, the development of digestive functions constitutes a considerable challenge. Digestion evolves intimately with

changes in feeding from birth to childhood, marked by a transition from liquid to solid diet, with a progressive qualitative and quantitative inclusion of nutrients (Buddington, 2011). The World Health Organization recommends exclusive milk intake during the first six months of life (World Health Organization, 2005). Milk consumption can continue

Abbreviations: CoMiniGut, Copenhagen MiniGut; DGM, Dynamic Gastric Model; DIDGI, Dynamic Gastrointestinal Digester; GI, gastrointestinal; GIS1, unicameral system of *in vitro* colonic simulation version 1; GIS2, unicameral system of *in vitro* colonic simulation version 2; INRAE, French National Research Institute for Agriculture, Food and Environment; PolyfermS, Polyfermentor Intestinal Model; SCFA, Short Chain Fatty Acids; SHIME, Simulator of the Human Intestinal Microbial Ecosystem; TIM-1, TNO gastrointestinal system 1.

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until 2 years old but thereafter represents a minor part of the child's energy needs. From 6 months old, pureed and mashed foods are gradually implemented up to 1 year of age, when infant diet becomes similar to the adult's one in terms of food consistency and variety (Fig. 1). While infants are immediately able to absorb nutrients required for their growth and development, anatomical (e.g. teeth number, organ size), mechanical (e.g. chewing or gut motility) and physicochemical functions (e.g. salivary, gastric, pancreatic and biliary secretions), as well as gut microbiota composition and function, are not instantaneously mature and evolve in a child up to 3 years of age (Figs. 2 and 3).

Due to the diversity of physiological processes occurring within the human gastrointestinal (GI) tract and their complex evolution from birth to childhood, many questions are still raised by researchers, medical doctors and industrialists to better integrate the central role of digestion in the field of child related nutrition and health. What are the fate, bioaccessibility and bioavailability of nutrients, drugs or food pollutants in the child digestive tract? How do beneficial (probiotic strains) and pathogenic (food-borne pathogens) microbes survive in the child gut? How does the large variety of ingested compounds (nutrients, drugs, pollutants and microorganisms) interact with gut microbiota? Is there any link with child health or disease? The golden approach to address these questions remains human clinical trials. However, clinical investigations in newborns, infants, and toddlers are more challenging than those involving adults due to important ethical, regulatory and financial restrictions in this subset of the general population (Auby, 2012; Michael et al., 2010). In addition, such assays require invasive procedures that are restricted to end-point measurements, with difficulties to access the different segments of the GI tract (from stomach to proximal colon). Young rodent models can be used as an *in vivo* alternative to clinical studies but are also limited by major differences between animal and human diet, digestive physiology, including resident microbiota and susceptibility to infection by pathogenic microorganisms, and by dynamic differences in the first stages of life (Hugenholz

and de Vos, 2018; Walthall et al., 2005). Alternatives to animal experiments are also widely acknowledged by the 3R (Reduction, Replacement and Refinement) European rules aiming to reduce animal experiments and favor *in vitro* alternative strategies. Among them, *in vitro* systems mimicking the child digestive environment may gain further insights into the behavior of ingested compounds throughout their GI tract. A large number of *in vitro* digestion models have been developed the last decades, ranging from mono to multi-compartmental systems and from static to dynamic ones (Cordonnier et al., 2015; Dupont et al., 2019; Guerra et al., 2012; Payne et al., 2012; Pham and Mohajeri, 2018). Nevertheless, only few of them have already been adapted to the specific conditions found in the gut of newborns (0–28 days), infants (1 month - 1 year old) or toddlers (1–3 years old).

This review describes the specificities and evolution of the healthy full-term child digestive physiology from newborn to toddlers of 3 years old, regarding anatomical, physicochemical, mechanical and microbial parameters. Then, current *in vitro* models of the child gut are described, as well as their main applications in the fields of nutrition and health. Lastly, the limitations and further challenges facing child model development are discussed.

2. Gut physiology evolution from newborns to toddlers

2.1. Digestive anatomy and functions

Changes in feeding from newborns to 3 years old toddlers require the evolution and adaptation of the whole child digestive tract anatomy allowing a more complex breakdown capacity (Figs. 1, 2 and 3).

2.1.1. Oral cavity

At birth, the orofacial and cervical anatomy is adapted to the sucking-swallowing function, with the tongue being proportionally larger and in higher position than in older children (Delaney and

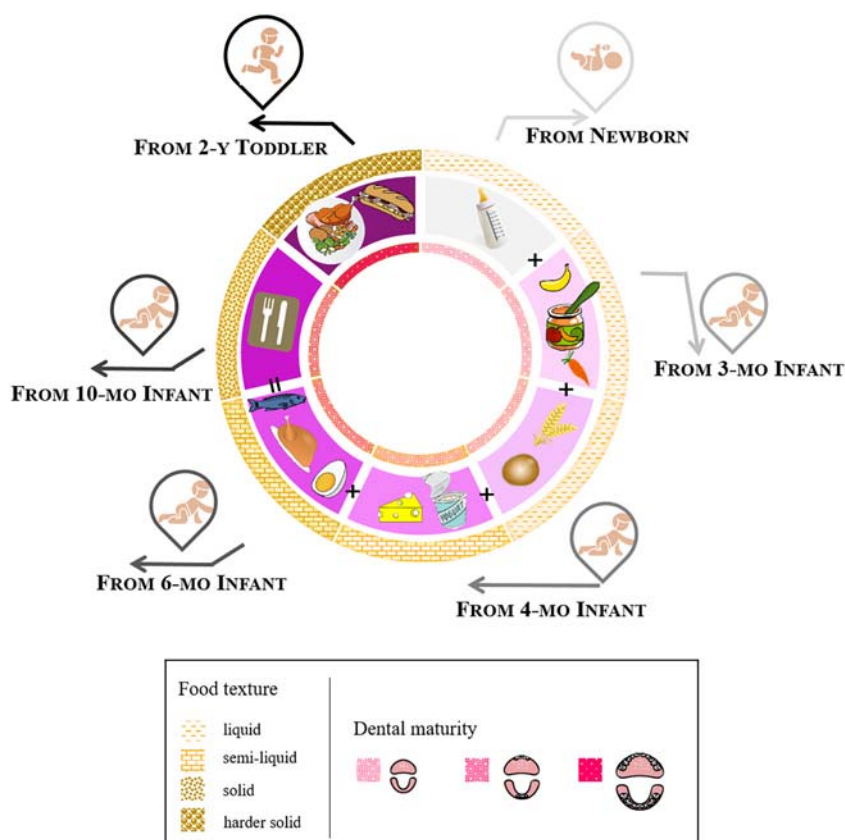


Fig. 1. Food intake and texture exposure across childhood. The three doughnut layers represent exposure from outside to inside, the gradual exposure and transitioning to food textures, respectively, from birth up till 2 years of age. Food intake transitioning ends after 10 months of age, while exposure to complex textured food starts at that age. Mo = months; Y = years.

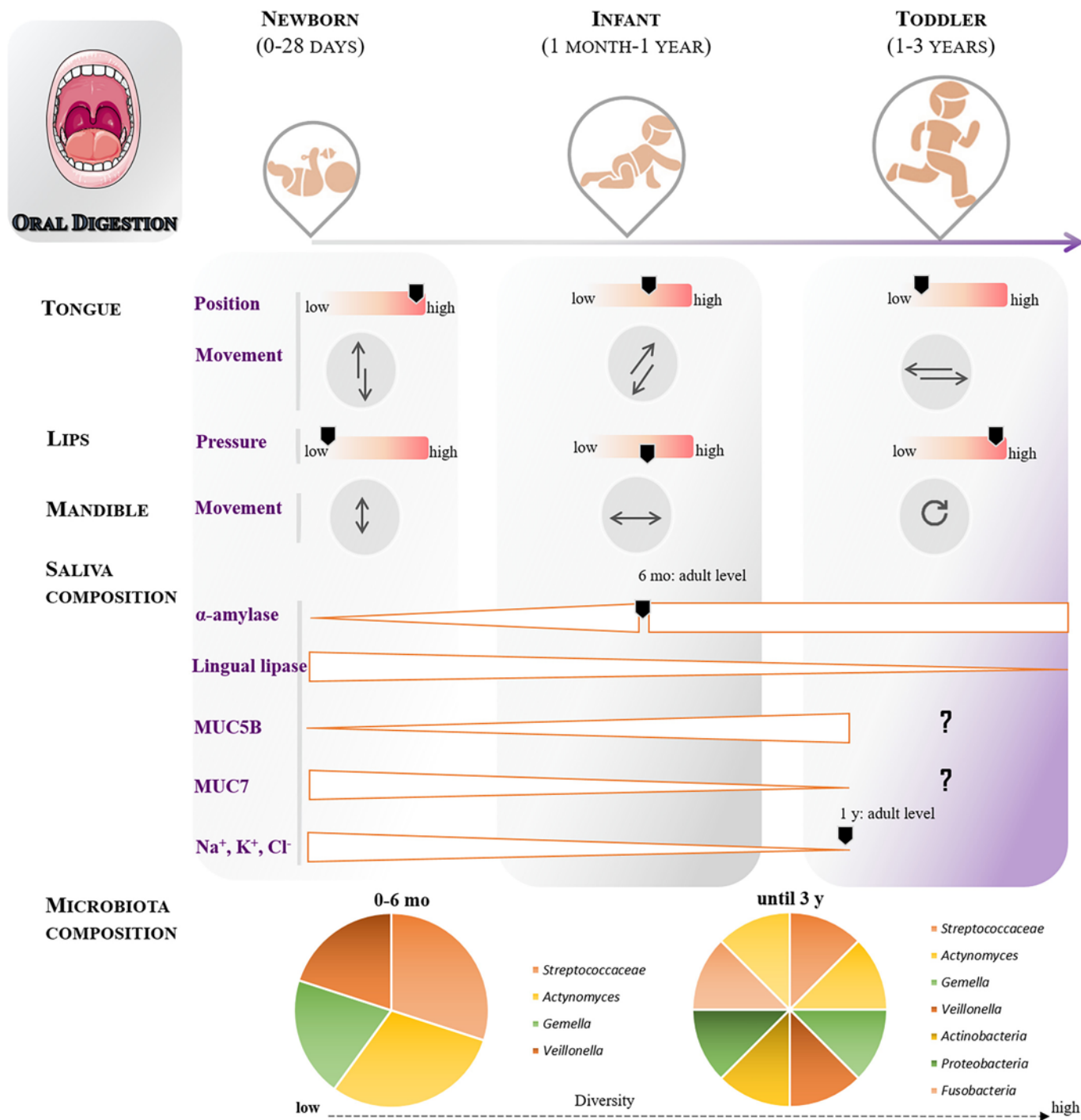


Fig. 2. Oral digestion development across childhood. Key parameters of the oral phase are summarized according to the available literature data from birth up till 3 years of age. Lack of data are represented by “?”. Mo = months; Y = years.

Arvedson, 2008; Silva et al., 2016). During child development, the tongue gets into a lower position as the pharynx grows, promoting the initiation of tongue’s lateral movements and allowing mastication maturation (Le Révérend et al., 2014). Moreover, maxilla and mandible growth increases the oral cavity space to fracture larger pieces of food and supports increased bite and masticatory forces. The eruption of anterior primary teeth between the age of 6–8 and 18 months is an important step of oral function evolution (Fig. 1). Around 3 years old, the complete occlusion contacts between primary molars allow the mandible to stabilize (Le Révérend et al., 2014). Finally, human salivary

gland development continues across childhood, and is accompanied by several quantitative and qualitative changes in terms of saliva flow, composition and physicochemical properties (Bellavia et al., 1979; Ben-Aryeh et al., 1984).

2.1.2. Stomach

At birth, the average stomach length is 7 cm (Osemlak et al., 1982), that increases with age to finally reach 25 cm (the adult length) at around 23 years old (Begum et al., 2013). During the first days of life, the increased number of feedings induces gains in stomach compliance,

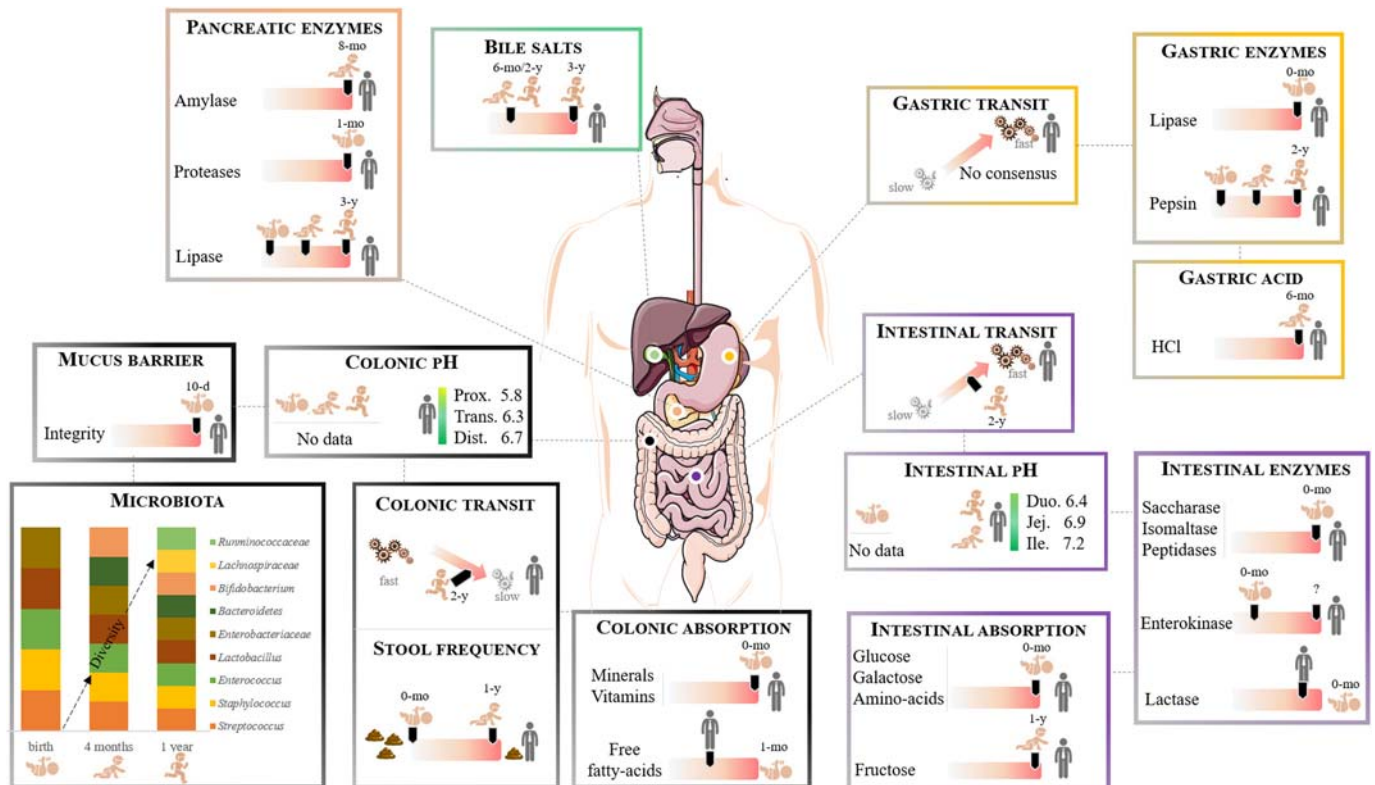


Fig. 3. Gastro-intestinal development across childhood.

Key parameters of the gastric, intestinal and colonic digestion/fermentation are summarized according to the available literature data from birth up until 3 years of age. Lack of data are represented by “?”

D = days; Dist = distal colon; Duo = duodenum; HCl = Hydrochloric acid; Jej = jejunum; Ile = ileum; Mo = months; Prox = proximal colon; Trans = transverse colon; Y = years.

followed by an increase in stomach relaxation capacity allowing a greater content (Zangen et al., 2001). At birth, parietal and mucous neck cells from gastric mucosa are well-defined, while there are few chief cells when compared to the adult stomach (Deren, 1971). In full term newborns, the number of glands and crypts remains constant until 2 and 6 months old, respectively and rises right after to reach adult values at around 1.5 years and 5 years, respectively.

2.1.3. Small and large intestine

The length of the small intestine is increasing from birth (275 cm) to reach 400 cm at 4 years old and then 350–500 cm in adults (Gondolesi et al., 2012; Weaver et al., 1991). All intestinal cell types are fully developed at birth (Moxey and Trier, 1978). There is no available data on how absorption surface area and villus length evolves in the first years of age in child. After birth, 6 months old children exhibit a colon of 568 mm \pm 27 mm in average, reaching 1224 mm \pm 57 mm in average at 4 years old. The elongation process stops around 5 years old with a length of around 1300 mm (ranging from 1100 to 2108 mm), similarly to adults (Wozniak et al., 2019). The mean relative length of the different colonic segments (ascending, transverse and distal) shortly varies compared to the total colonic length after birth (Mirjalili et al., 2017).

2.2. Mechanical digestion and transit time

2.2.1. Food mastication

To date, food fragmentation has been poorly studied in children (Barrera et al., 2011; Gavião et al., 2007), and the bolus state at the time of swallowing before 3 years old is unknown. Lips pressure increases over age (Fig. 2), particularly between 5 months and 3 years old (Chigira et al., 1994). Food is usually mashed by the tongue in upward/

downward movements at 4–6 months old (Ayano et al., 2000). At 10 months, children start to move solid food from one side of the mouth to the other using tongue lateral movement (Stolovitz and Gisel, 1991). Following introduction of hard consistencies food, horizontal jaw motion during chewing becomes more observable at 2 years old. Rotational movements typical of a mature chewing can be observed at 2.5 years old (Arvedson and Lefton-Greif, 1996; Ayano et al., 2000; Takada et al., 1994; Wilson et al., 2012). Muscle activity during mastication of different food matrices in infant increases between 8 and 22 months, to reach a level similar to that observed in adults (Green et al., 1997; Steeve and Price, 2010).

2.2.2. Gastric digestion

Mixing waves begin in the stomach following food entry and fasting antral motor patterns do not differ between adults and infants (Brousard, 1995). Gastric emptying time of newborns seems longer compared to adults even if there is no absolute consensus. In newborns, it extends from 10 to 100 min following milk consumption with a mean half-time emptying of 87 min versus 65 min in healthy adults (Signer, 1975). Besides, formula-fed infants displayed a longer gastric emptying time compared to breast-fed infants (78 versus 48 min), due to a greater amount of casein in formula (Cavell, 1979). Yet, a meta-analysis showed contrasting data with no observed difference in gastric emptying rate and time between different postnatal ages (i.e. birth–1 month, birth–23 months, 2–5 years, 6–12 years, 13–18 years), even for a variety of food types (Bonner et al., 2015).

2.2.3. Intestinal digestion

All the intestinal muscles and neural structures appear before birth, while neuroendocrine transmission and integration are not completely achieved at that time and continue to mature during the first year of life

(Berseth, 2006). Endoscopic analysis performed in a cohort of infants ranging from 10 months to 9 years displayed a median small bowel transit time of 401 min (ranging from 264 to 734 min), while in adults with mean age of 59 years old, a reduced transit time of 283 min (from 91 to 416 min) was reported (Oikawa-Kawamoto et al., 2013; Velayos Jiménez et al., 2005). Between birth and 2 years old, colonic transit time and stool frequency decrease, within a mean of 4 stools per day at 1 week old, 2.2 after 1 month and 1.7 stools after 1 year (Lemoh and Brooke, 1979). After 2 years old, the mean colonic transit time is not significantly different from adults (mean 31.78 h from various studies) as measured with radio-opaque plastic markers according to a meta-analysis (Southwell et al., 2009). In addition, stool frequency differs with diet, breast-fed infant producing daily less dry feces weight than formula-fed infant (Sievers et al., 1993).

2.3. Digestive secretions and pH

Digestive secretions also undergo deep maturation closely related to feeding evolution during childhood (Fig. 3).

2.3.1. Saliva

During infancy, saliva undergoes manifold changes closely dependent on tooth eruption and food transition from milk to progressive solid food (Fig. 2). Sodium, potassium and chloride quantities decrease from birth and during the first year of life (Ben-Aryeh et al., 1984). Among a marked steadiness in the overall pattern of salivary proteins during child growth, mucin secretion displays variations in terms of types of molecule with age, with a shift in predominance from MUC7 to MUC5B (Ruhl et al., 2005). Very low levels of α -amylase activity were reported at birth (Ben-Aryeh et al., 1984; Rossiter et al., 1974), from 6 to 9 times lower compared to adults (Bellavia et al., 1979). However, a rapid and strong increase in salivary α -amylase activity was reported, reaching adult levels at 6 months (Ben-Aryeh et al., 1990, 1984; Rossiter et al., 1974). Milk and high-fat diet of newborns require an adequate lipolytic activity. Due to a low pancreatic lipase activity in newborns and infants, salivary lingual lipase has probably a specific role in digestion of fat from milk (Fredrikzon et al., 1982; Hamosh, 1979; Hamosh et al., 1981). With growth, a progressive shift from lingual lipase activity toward an increasing role of pancreatic lipase has been reported (Lebenthal et al., 1983). Saliva in babies under 1 month is highly viscous with a very low flow rate that increases with age to reach 0.37 mL/min in adults (Sinor et al., 2009). Saliva is also slightly more acidic at birth (pH 6) than in adults (pH 6.33) (Ben-Aryeh et al., 1984).

2.3.2. Gastric secretions

Lipid digestion in the stomach is a critical step for newborn to offset the lack of pancreatic lipases and bile acid production (Hamosh et al., 1981). At birth, gastric lipase activity already reaches adult level (Lindquist and Hernell, 2010) and fat gastric digestion leads in newborn, similarly to adults, to the hydrolysis of 10 to 30% of total esterified fatty acids (Mu and Høy, 2004). On the contrary, protein digestion is impaired in newborns due to the combined effect of low pepsin secretion (at 3 months pepsin concentration represents half of the adult one) and activity (only 13% of adult level at birth) and higher post-prandial gastric pH compared to adults (Agunod et al., 1969; Bourlieu et al., 2014). Despite the absence of studies related to mucus secretion and characteristics in child, newborns seem more vulnerable to gastric acidity, which could be linked to a lower gastric mucosa thickness during the 3 first weeks of life (Bourlieu et al., 2014). To favor protein and lipid digestion by gastric pepsin and lipase, the stomach secretes hydrochloric acid to maintain a low pH. Before meal ingestion, between 5- and 13-days full-term infants have a mean pH of 3.5 (ranging from 2.0 to 6.1). Gastric pH increases in average to reach 6.4 after breast milk ingestion and remains elevated up to 60 min following meal ingestion, to decrease progressively to reach the pre-feed state pH (Mason, 1962). Therefore, like in adults, gastric pH increases after food ingestion in

child, but remains higher for a longer period (Hamosh, 1996). Accordingly, this high pH range could be explained by a low acid production by infant parietal cells, reaching adult value at 6 months, combined with the high buffering capacity of milk (Dallas et al., 2012).

2.3.3. Pancreatic, liver and intestinal secretions

Pancreas and liver, together with the small intestine, release digestive secretions in order to degrade remaining proteins, lipids and carbohydrates into small polymers and monomers ready for absorption (Fig. 3).

Pancreatic amylase is not detectable before the first month of life and reaches adult levels by 8 months old (Otsuki et al., 1977). On the contrary, intestinal saccharase-isomaltase is already present at birth (Triadou and Zweibaum, 1985). In response to milk feeding, intestinal lactase reaches its maximal activity at birth and thereafter decreases to 5–10% of its initial activity during childhood and adolescence (Drozowski et al., 2010; Montgomery et al., 1999). The expression of pancreatic proteolytic enzymes reaches adult levels few weeks after birth. Duodenal enterokinase, the enzyme activating trypsinogen into trypsin, which in turn activates pancreatic proteases, shows at birth 20% of the activity found in infants and toddlers (1–4 years old) (Antonowicz and Lebenthal, 1977). The brush borders peptidases, aminopeptidase and dipeptidyl-peptidase IV, already reach adult activity levels at term (Auricchio et al., 1981; Lacroix et al., 1984). Pancreatic lipase displays an activity that remains low at birth to increase from 10 weeks of life and reach the adult one by 3 years of age (Cleghorn et al., 1988).

At birth, bile salts concentrations in duodenal juice remain very low, representing in average 1.8 mmol/L under 2 days of age. From 10 days to 7 months, it rises up to reach 9.8 mmol/L. Bile composition switches from mainly taurine-conjugated bile salts at 7 days to glycine-conjugated bile salts after 10 days (Challacombe et al., 1975). This low bile acids pool could explain fat malabsorption in young infants leading to a greater fat delivery into the colon (Murphy and Signer, 1974; Watkins et al., 1974). Primary bile salts are released into the duodenum, and following their action on lipid emulsification, they reach the ileum to be re-absorbed. During the first week of life, cholic acid is the major primary bile acid with a 2 to 5:1 ratio with chenodesoxycholic acid, the other main primary bile acid. However, during the first month, this proportion falls to 1–2:1, which is also the ratio found in adults (Murphy and Signer, 1974). Formation of secondary bile salts requires enzymatic modifications (deconjugation and dehydroxylation) of primary bile salts by gut microbiota. Of note, no secondary bile salt is extracted from the feces of newborns (Sharp et al., 1971) and the ratio between primary and secondary bile acids changes in the colon along with gut microbiota development (Neal-Kluever et al., 2019).

2.3.4. Colonic secretions

There is no available data on the evolution of mucus composition in the colon in the early age. The intestinal mucin genes are expressed early before birth but a fully developed and protective mucus barrier is formed a couple of days after birth (Pelaseyed et al., 2014). Regarding infant colonic pH, no data is available and only fecal pH value was reported. Fecal pH varies a lot with age after birth and between individuals according to the evolution of both diet and gut microbiota composition and activity (Bullen et al., 1977; Henrick et al., 2018). It gradually increases with age, with an average fecal pH at 3 days around 5.3 ± 0.6 , reaching 5.7 ± 0.7 for 6-month infant (George et al., 1996), then 5.8 ± 1.1 between 12 and 18 months (Edwards et al., 1994), to be ultimately similar to that of healthy adults with a value around 6.6 ± 0.3 (Osuka et al., 2012).

2.4. Intestinal absorption of nutrients and water

In the small intestine, glucose and galactose released from lactose digestion are absorbed by enterocyte transporters that are already expressed in newborns (Fig. 3). On the contrary, fructose-specific

transporter is only expressed from 1 year after birth. All amino-acid transporters are functional at birth (Drozdowski et al., 2010; Malo, 1991). Similarly, the predominant apolipoprotein 48, forming chylomicrons with phospholipids and triglycerides and involved in lipid absorption, is early expressed during gestation (Glickman et al., 1986). At birth, all vitamin and mineral absorption mechanisms are mature (Abrams et al., 1997). Nevertheless, vitamin K and D storages are very low at birth, as well as sunlight exposure and gut microbiota activity, making necessary vitamin supplementation (Salle et al., 2005). Moreover, due to the weak bile salt pool during the first 2 months, absorption of fatty acids and liposoluble vitamins can be impaired (Hamosh et al., 1981). One of the main functions of the colon is to absorb water, electrolytes and short chain fatty acids (SCFA) resulting from microbial fermentation, but also to maintain normal fluid homeostasis, in particular in newborns still displaying immature small intestinal absorption functions (Pácha, 2000). In particular, neonates have a greater nutrient absorptive capacity in the colon than adults, due to the presence of apical brush border hydrolases in the colonic mucosa during the first week of life (Lacroix et al., 1984; Pácha, 2000).

3. Oral and intestinal microbiota evolution: from birth to 3 years

3.1. Colonization and composition of infant microbiota

Development of resident communities and their metabolic outcomes are crucial not only for early life maturation, but also for long-term health in children, and to a later extent in adults. Gut microbiota is also mandatory for immunologic programming of infants (Dzidic et al., 2018; Wiertsema et al., 2021) and exerts a key barrier effect against pathogens (Vazquez-Gutierrez et al., 2016).

Microorganisms rapidly colonize infants over the first months or years of life. Currently, no consensus exists concerning the time for microbiota maturity but most studies converged on the time period of 1000 days, i.e. three first years of life (Derrien et al., 2019; Odamaki et al., 2016). Of note, the only available data on resident microbiota in the first years of life are related to oral and fecal microorganisms and high individual variations in microbiota composition and functions have been reported (Könönen, 2000; Pham et al., 2016). Also, the bacterial fraction has been extensively investigated, while little is currently known on the other microbial communities, i.e. fungi, Archaea and viruses (Korpela and de Vos, 2018; Zaura et al., 2009). Gut microbiota of at term, vaginally born and exclusively breast-fed infant is recognized as healthy infant microbiota (Arbolea et al., 2015).

The colonization process occurring in infants is characterized by a continuous increase in load and diversity of bacteria during the first year of life, and even later on, up to the weaning time (Cilieborg et al., 2012; Pham et al., 2017, 2016; Sulyanto et al., 2019). A wide range of species have been identified in the oral microbiota of child below 3 years old, most of these species being more frequently present on the tongue in babies below 18 months old (Tanner et al., 2002). In early childhood, infants share 85% of their oral microbiota with their mother, pointing out the importance of maternal oral microbiota (Mason et al., 2018). Microbiota personalization begins with diet changes and teeth eruption that creates new microenvironments (Kennedy et al., 2019; Könönen, 2000; Tanner et al., 2002). From birth to 6 months, Firmicutes and especially *Streptococcaceae* dominate the oral community, as well as the genera *Actinomyces*, *Gemella*, and *Veillonella* (Könönen, 2000; Lif Holgerson et al., 2011; Pearce et al., 1995; Sulyanto et al., 2019) (Fig. 3). During the following years until 3 years old, abundances of Firmicutes (*Streptococcaceae* and *Veillonellaceae* families) and Actinobacteria decrease, while Proteobacteria, Fusobacteria and Bacteroidetes increase (Crielaard et al., 2011). The only available study on other non-bacterial populations reports a poor fungal colonization during the first months of life with *Candida parapsilosis*, *Saccharomyces cerevisiae* and *Candida albicans* (Ward et al., 2018). Regarding intestinal microbiota, aerobic

and facultative anaerobes are the first colonizers in the neonatal gut due to the presence of oxygen, which limits the colonization of strict anaerobes (Fig. 3). Fecal microbiota mainly consists of *Streptococcus*, *Staphylococcus*, *Enterococcus*, *Lactobacillus* and *Enterobacteriaceae* (mainly *Escherichia coli* and related species). Those facultative anaerobe bacteria contribute to the establishment of a reduced environment that is mandatory for strict anaerobes colonization. This important shift seems to occur at 4 months old (Bäckhed et al., 2015) and colonization process of anaerobic microbes belonging to the predominant phyla Bacteroidetes, Firmicutes and Actinobacteria occurs then very quickly. The presence of *Bacteroides* and/or *Bifidobacterium* species that outnumber facultative anaerobes and reach adult-like population densities within the first week of life is even reported from fecal sample analysis (Jost et al., 2012). Both *Bacteroides* and *Bifidobacterium* species are well-known for using most of the substrates available in the neonatal gut from infant milk-based diet (proteins, lactose but also human milk oligosaccharides) and host-produced components such as mucin glycoproteins (Jost et al., 2015), which could explain this rapid establishment. Another stage of intestinal colonization occurs after several weeks or months, together with an increase in microbial diversity. Some species belonging to Firmicutes (mainly *Lachnospiraceae* and *Ruminococceae*) significantly increase over the first year of life in stool samples. Increase in microbial diversity is further associated to diet changes after weaning (Yassour et al., 2016) and for several years. Available reports on fecal samples suggest an early colonization of bacteriophages, but a delayed colonization of fungi, and especially *Archaea* even beyond the weaning time (Korpela and de Vos, 2018; Vanderhaeghen et al., 2015).

3.2. Impact of external factors on oral and intestinal microbiota

Gestational age can strongly affect gut microbiota development. A decrease in gestational age leads to a delayed colonization of strict anaerobes and a lower diversity, together with an increase in potential pathogenic and facultative anaerobic bacteria (Henderickx et al., 2019). Direct comparison of gut microbiota composition of preterm and term infants showed that *Enterobacter*, *Enterococcus*, *Escherichia* and *Klebsiella* populations, containing well-known opportunistic pathogens, were predominantly present in preterm infants (Arbolea et al., 2012; Henderickx et al., 2019). Environmental and host conditions can partially explain these microbial differences in colonization, together with specific feeding regimen and the frequent use of antibiotics associated with prematurity (Henderickx et al., 2019).

Consistent evidence also indicates that the delivery mode has a significant impact on infant oral and gut microbiota colonization profiles. A higher number of bacterial taxa was reported in the oral microbiota of vaginally born compared to caesarian section born infants, with two distinct colonization patterns, even if these differences attenuate after 1 month (Hurley et al., 2019). Oral microbiome is dominated by *Slackia exigua* (periodontitis-associated species), *Streptococci mutans* and *Lactobacillus Cluster I* in caesarian born infant, while *Haemophilus parainfluenzae* is predominant in vaginally born individuals (Li et al., 2005; Lif Holgerson et al., 2011). Regarding intestinal microbiota, the primary gut colonizers of babies born vaginally show a high microbial similarity with their mother's vaginal microbiota dominated by *Lactobacillus* and *Prevotella* species. By contrast, this natural exposure is disrupted in caesarean born babies and the primary colonizer species are mostly *Staphylococcus* and *Propionibacterium*. This suggests an initial colonization with microbes found in the hospital environment and later from skin contact with mother as origin (Dominguez-Bello et al., 2010).

The feeding mode also strongly affects infant oral and gut microbiota colonization. The comparison between breast-fed and formula-fed babies was largely investigated over the past decades. At 3 months, breast-fed infants harbor a high amount of *Streptococcus* in their oral microbiota while *Actinomyces* and *Prevotella* prevail in formula-fed infants. Later, the implantation of solid food in the infant diet leads to a greater oral bacterial richness compared to only breast-fed or mixed (breast and

formula fed) diet, while no difference was reported for the fungal population (Oba et al., 2020). At last, feeding mode in the first months seems to have long-term effects as some differences still persist in bacterial diversity and profiles in teenagers (Eshriqui et al., 2020). Feeding mode also impacts intestinal microbiota as *Lactobacillus* and *Bifidobacterium* species are more prevalent in breast-fed infants than in formula-fed infants, associated with a decrease in their fecal pH (5.9 in breast-fed compared 6.8 in formula-fed infants) 2 weeks after birth (Simhon et al., 1982). By contrast, a more diverse gut microbiota was observed in formula-fed infants with higher level of *Bacteroides*, *Clostridium* and *Enterobacteriaceae* compared to breast-fed infants (Zhuang et al., 2019).

Besides, drugs and especially antibiotics can also profoundly influence infant gut microbiome. Impact of antibiotics on infant gut and oral microbiota obviously depends on the spectrum, dose, duration and route of treatment (Wang et al., 2016). Maternal intrapartum antibiotic treatments lead to a shift in infant oral and gut microbiota, with a loss of beneficial commensal populations (e.g. *Streptococcaceae* and *Bifidobacteriaceae*) for the benefit of *Proteobacteria* (Gomez-Arango et al., 2017; Zimmermann and Curtis, 2020). Similarly, oral use of antibiotics in infants resulted in decreased numbers of gut anaerobic communities, particularly *Bifidobacterium* and *Bacteroides* species (Penders et al., 2006) and/or increased numbers in *Proteobacteria* such as *Enterobacteriaceae* (Arboleya et al., 2015). Intestinal communities in antibiotic-exposed infants are also less mature compared to those of unexposed ones (Bokulich et al., 2016). Lastly, excessive hygiene conditions during early life reduce the continuous microbial exposure which is however essential for the development of a rich gut microbiota in infants (Neu and Rushing, 2011; Schmidt et al., 2011).

3.3. Gut microbiota activity

The infant gut microbiome metabolizes the complex substrates of milk-based diet, such as proteins, lipids and carbohydrates (especially human milk oligosaccharides) into key metabolites such as SCFAs. The only available data on SCFA production have been obtained from child fecal samples. Whatever the feeding status, total SCFA concentrations found in infant feces between 3 and 5 months were 142 $\mu\text{mol/g}$ (101.6–203.8) in average with a majority of acetate (80%) and less propionate (10%), butyrate (5%) and valerate (2%) (Bridgman et al., 2017). Another more recent study measuring only the three main SCFAs reports a median total concentration of 84.6 $\mu\text{mol/g}$ of feces in 1-year old children, allocated in 72% acetate, 16% propionate and 12% butyrate (Roduit et al., 2019). These authors underline that fecal SCFA concentrations deeply depend of the infant diet. In adults, total SCFA concentration represents an average ranging from 64 to 77 $\mu\text{mol/g}$ of feces depending on the study (Baxter et al., 2019; Høverstad et al., 1984), with percentages of around 60%, 20% and 20% for acetate, butyrate and propionate, respectively. Relative percentages of fecal acetate/propionate/butyrate in child are therefore far from the ideal adult ratio of 3/1/1 (den Besten et al., 2013). Breast-fed infants have a lower concentration of fecal SCFAs (99.4 versus 177.8 $\mu\text{mol/g}$) and a higher relative proportion of acetate (86.7 versus 77.3%) and lactate (7.2 versus 2.3 $\mu\text{mol/g}$) compared to formula-fed infants at 1 month. This is probably due to the more efficient capacity of breast-fed infant microbiota to metabolize human milk substrate (Bridgman et al., 2017) and the greater concentration of oligosaccharides in breastmilk compared to formula (Bode, 2015).

4. Current *in vitro* models of the child gut

Even though *in vitro* gut models cannot obviously integrate nervous or endocrinal systems, they can reproduce all or part of the following physicochemical and microbial parameters of the child's digestive tract when simulating a liquid or solid food intake: temperature, pH, transit time, mechanical constraints, supply of digestive enzymes and bile salts, passive absorption of nutrients and water, condition of oxygenation

(especially anaerobiosis in colonic compartments) and complex, metabolically active and compartmentalized resident microbiota from human origin (Fig. 4). The main child *in vitro* digestion models currently available (from birth to 3 years old) are described in the next sections and summarized in Table 1, their applications being further explained in Section 5.

4.1. Models of the oral phase

Despite its importance in child digestion physiology, very few *in vitro* models are reproducing the oral cavity. These models have been mainly designed for pharmacological studies.

Gerrard's team from University of Cambridge in UK developed an *in vitro* model mimicking typical suction rates and feed volume in breast-feeding for infant under 6 months of age (Gerrard et al., 2013). A silicone human nipple mimic (HNM) was constructed with ducts to simulate dynamically the breast milk flow, as induced by infant suction. The model has been adapted to the testing of tablets containing a highly soluble dispersive formulation. In practice, heated human milk is pumped from a reservoir into the silicone human nipple mimic model, where the tablet releases the tested component into milk, then removed with a vacuum pump. The suction pulse rate, milk flow rate, milk composition, as well as tablet formulation, can be adapted according to various scientific and/or application-driven questions. More recently, another *in vitro* model, the Tongue Mimic System (TMS), reproducing 1 to 3 months old infant peristaltic tongue motion and pressures during breastfeeding, was also developed by University of Cambridge (Scheuerle et al., 2017). In this model, heated fluids (water or milk) are pumped from a reservoir to undergo tongue movements simulated by a peristaltic pump. Tongue motions can be adjusted by modifying the shaft's rotation rate through variations of the pump speed. A movable metal plate also reproduces the hard palate of infant mouth. As described for the HNM system, this model can be loaded with a tablet.

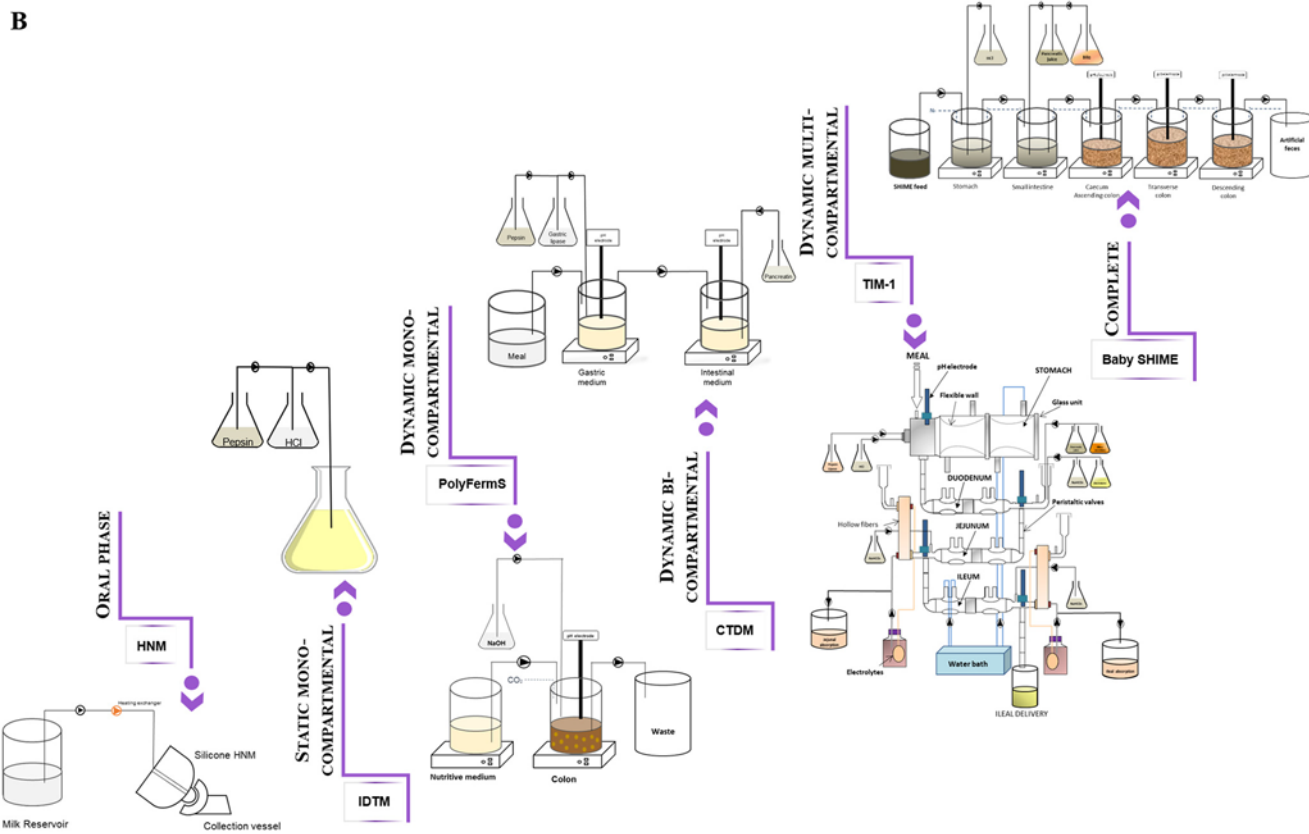
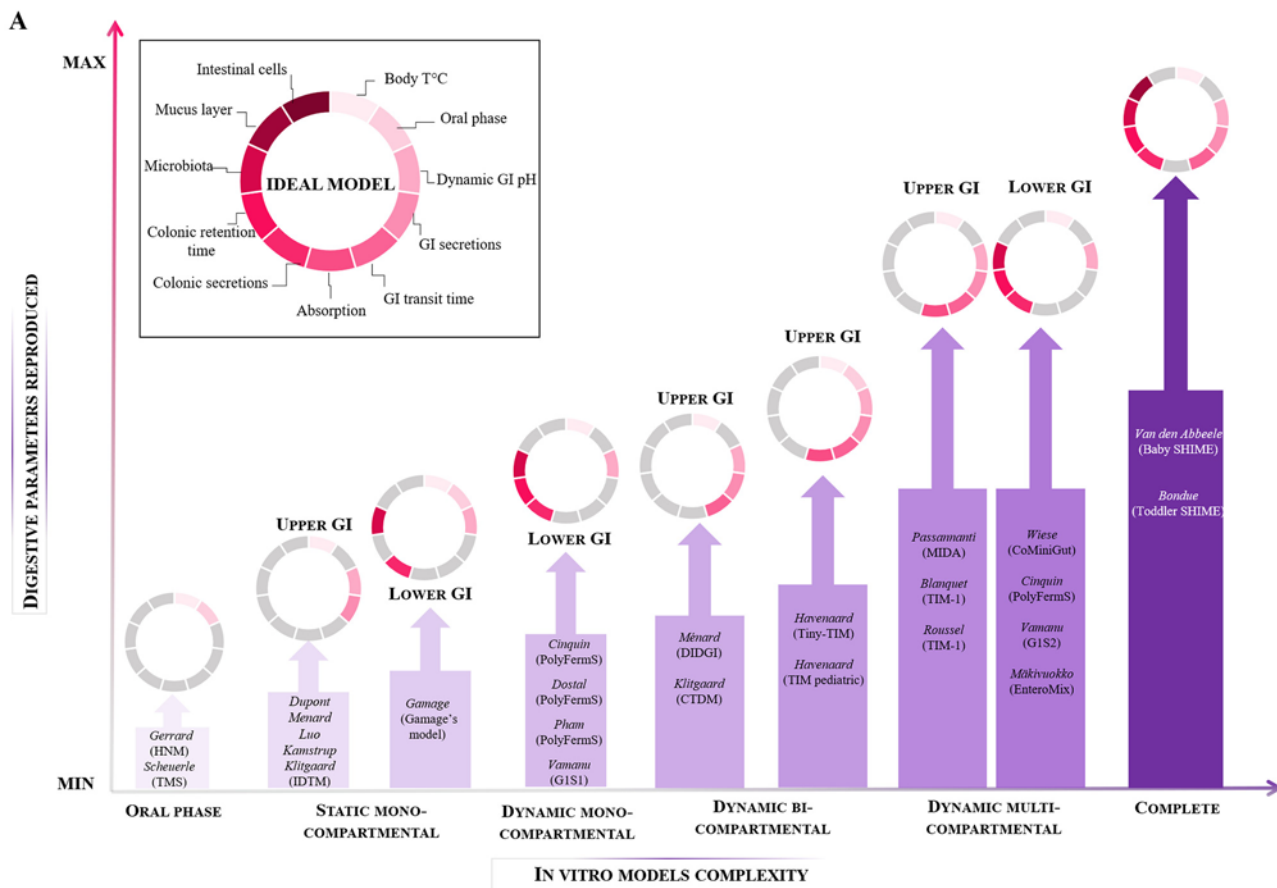
4.2. Gastric and small intestinal models

Some of the gastric and small intestinal models firstly developed for adult conditions have been adapted to child physiology. In the following section, these models of increasing complexity are described from static mono-compartmental to dynamic multi-compartmental devices.

4.2.1. Static mono-compartmental models

A large number of simple static models of the child stomach and small intestine have been developed yet. In these models, the successive steps of digestion are reproduced in a single vessel by changing pH and enzymes/bile conditions between gastric and intestinal phases, mostly without taking into account the time effect on these parameters.

The first static child model was developed in 2010 by Dupont and colleagues from the National Research Institute for Agriculture, Food and Environment (INRAE) in France (Dupont et al., 2010). The model was adapted to 2 weeks - 3 months year old infants. During the gastric phase, pH was adjusted at 3 instead of 2.5 for adult protocol and pepsin concentration was decreased 8-fold. In the intestinal phase, bile salts amount was reduced 4-fold and phosphatidylcholin, trypsin and chymotrypsin concentrations were decreased by 10-fold compared to adult conditions. Then, the adult international consensus protocol of *in vitro* static digestion (Minekus et al., 2014) was adapted several times to specific child conditions (Cattaneo et al., 2017; Ménard et al., 2018). In particular, Dupont's team set up a new static *in vitro* model for full-term newborns (28 days of life) (Ménard et al., 2018). In this model, both gastric and intestinal phases lasted 60 min, as for adult conditions. However, pH values were adapted, with a gastric pH increased to 5.3 (versus 3 in adult) while intestinal pH was reduced to 6.6 (versus 7 in adult). Gastric pepsin was largely reduced (268 U/mL versus 2000 U/mL) while gastric lipase remained equivalent to adult concentration and only slightly adapted to infant body weight (19 U/mL versus 21 U/mL).



(caption on next page)

Fig. 4. Decipher the complexity levels of the *in vitro* childhood digestive models according to the parameters reproduced.

A. On top left, the pink gradient doughnut represents an “ideal model”, as legend. In this model, all the key parameters of the digestion and fermentation are reproduced dynamically and gradually. The small doughnuts represent each class of *in vitro* models (listed according to first author publishing the original article related to the model, the model name is listed in brackets if available). The grey items mean that the parameters are not reproduced, while the pink items means that the parameter are reproduced, according to the legend on top left. **B.** Schematic representation of key *in vitro* models from static mono-compartmental to complete gastro-intestinal systems.

CoMiniGut = colon mini gut; CTDM = Continuous Transfer Digestion Model; DIDGI = gastro-intestinal dynamic digestion system; GIT = gastro-intestinal tract; GIS1 = unicameral system of *in vitro* colonic simulation version 1; HNM = Human Nipple Mimic; ITDM = Immediate Transfer Digestion Model; MIDA = Model of an Infant Digestive Apparatus; Poly FermS = Polyfermentor Intestinal Model; SHIME = Simulator of the Human Intestinal

Microbial Ecosystem; TIM = TNO gastrointestinal system 1; TMS = Tongue Mimic System. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Intestinal juices reproduced by porcine pancreatin and bovine bile were largely reduced, with a 22-fold and 3-fold reduction compared to adult levels, respectively.

Very recently, another gut model was implemented by Hunan Agricultural University in China, for infants ranging from birth to 6 months old (Luo et al., 2019), based on the publication from Bourlieu and colleagues (Bourlieu et al., 2014). Of note, this model integrates kinetics of gastric pH (with four steps: pH 6.5 from 0 to 30 min, 6.0 from 30 to 60 min, 5.5 from 60 to 90 min and 5.0 from 90 to 120 min), allowing to mimic the slower pH decrease in infant compared to adult. Intestinal digestion was set up at pH 7 as in other models. The composition of simulated gastric and intestinal fluids was also adapted to infant conditions. The gastric phase was mimicked with 0.4 mg/mL pepsin and 0.87 mg/mL lipase, both added at the beginning of each pH steps. During the intestinal phase, 5 mg/mL of porcine bile extract and 1.6 mg/mL of porcine pancreatin and porcine pancreatic lipase were added.

Kamstrup's team, from University of Copenhagen in Denmark, also provided advices to design an improved gut model for neonates under 2 months old (Kamstrup et al., 2017). This model aims to integrate various parameters, such as volume, pH, osmolality, gastric pepsin and lipase, intestinal lipase and bile salts, and distinguishes fasted state from fed state. During fasted state, gastric phase pH was set at 2.9, while a pH kinetic was considered during the fed state increasing to 6.4, 30 min after feeding and decreasing to 3.9, after 50-min digestion. For the intestinal phase (2 h), the pH was maintained at 6.5 during fed state compared to 7.0 for fasted state. For the fasted state, an osmolality of 298 and 308 mosmol/kg of intestinal content was recommended for the gastric and intestinal phases respectively, compared to 291 and 296 mosmol/kg for the fed state. In gastric fluids, lipase activity was set up at 100 tributyrin units (TBU)/mL while pepsin activity was 63 U/mL/kg of body weight. Lastly, in intestinal fluids, lipase activity was set at 50 TBU/mL and bile salts concentration at 1 mM and 4.7 mM for fed and fasted states, respectively.

From Kamstrup's work, Klitgaard and collaborators developed the immediate transfer digestion model (ITDM) reproducing the stomach and small intestine of 0 to 2 months newborns (Klitgaard et al., 2017). They used the same parameters, as previously described (Kamstrup et al., 2017), except for the gastric pH (adjusted at 6.4), and the use of pancreatic extract instead of intestinal lipase.

Finally, a dissolution model was developed and adapted from the existing dissolution paddle apparatus, the United States Pharmacopeia (USP) apparatus III of adults to simulate pH conditions and fluid volumes of the stomach, small intestine and proximal colon of infant (Karkossa et al., 2017). Intriguingly, the simulated age was not mentioned in this study. Two heated-controlled vessels were filled with the infant adapted dissolution media. The gastric phase was reproduced in one vessel during 30 min. At the end of this phase, all the gastric vessel content was transferred into a second vessel for the intestinal and proximal colonic phase by adding the specific intestinal media (lasting 4 h for the small intestine and 8 h for the proximal colon). Gastric pH was either set at 1.8 or 4 in two different experiments to test the gastric pH range reported *in vivo*, while the small intestinal and proximal colonic pH were maintained both at 6.8. For the gastric medium, a simulated

gastric fluid without pepsin (SGFsp), at pH 1.8 was considered. The intestinal medium is reproduced by a pH 6.8 bicarbonate based simulated intestinal fluid (Carbonate-SIF, CarbSIF) containing 120 mM sodium chloride and 5 mM potassium.

To summarize, an increase in gastric pH use of lower digestive volumes and a decrease in pepsin and intestinal lipase activities and bile salts concentrations, are the marked parameters changed in static infant models compared to adult conditions. These simple models can be used for large screening of active compounds due to their easy manipulation and low cost, but digestive conditions that are reproduced are far from mimicking the *in vivo* complexity of the infant/child digestive tract.

4.2.2. Dynamic mono- or multi-compartmental models

Compared to static models, dynamic systems integrate the evolution of digestive parameters over time and/or chyme transit between successive compartments.

4.2.2.1. Mono-compartmental models. Israel Institute of Technology developed a gastric model for infant under 2 years of age (Shani Levi et al., 2013). A unique vessel is water-jacketed, temperature-controlled, continuously stirred and filled with a gastric medium containing sodium chloride, hydrochloric acid and pepsin. Time of gastric digestion was increased in infant compared to adult (4 versus 2 h). pH kinetics during the gastric digestion was monitored and adapted to infant digestive conditions: 6.5 (0–30 min), 6.5 (30–150 min), 4.5 (150–240 min) and 3.5 at 240 min. Lastly, pepsin concentration was reduced compared to adult level (210 versus 240 U/mg protein).

4.2.2.2. Bi-compartmental models. The first bi-compartmental model set-up for infant conditions (birth to 6 months neonate) was adapted from that developed by INRAE in France for adult and named DIDGI® for Dynamic Gastrointestinal Digester (Ménard et al., 2015, 2014). Two consecutive temperature-controlled compartments are reproducing the stomach and small intestine. Interestingly, a Teflon membrane (2-mm holes) reproduces the pylorus sieving effect. Several sensors allow the control of temperature, pH and redox potential and variable-speed pumps ensure meal flow monitoring, compartment emptying, and supply of hydrochloric acid and sodium carbonate, bile and digestive enzymes. The volume and flow rates of secretions were adapted from another multi-compartmental infant *in vitro* model -described later in this review- (Blanquet et al., 2004) or from piglet *in vivo* data, with 1250 U/mL pepsin (0.25 mL/min) and 60 U/mL lipase (0.25 mL/min) in the stomach compartment, and 1% porcine bile (0.5 mL/min) and 10% porcine pancreatin (0.25 mL/min) in the small intestinal compartment. In the gastric compartment, half time gastric emptying was set at 70 min based on human *in vivo* studies (Bourlieu et al., 2014), while the pH followed an acidification profile based on piglet data. The intestinal half time emptying was set at 200 min according to human data (Bourlieu et al., 2014) and pH was maintained at 6.5. It should be emphasized that the validation of the infant model based on protein digestibility was performed based on *in vivo* piglet studies and not humans' ones.

Klitgaard and collaborators also improved their immediate transfer digestion model (see Section 4.2.1) into a dynamic system simulating

Table 1
Main characteristics of the current *in vitro* models mimicking the infant gastro intestinal tract.

MODELS	Age	Oral phase	pH (time (min))						Food	Digestive secretions Nutritive medium	Abs	Microbiota	Mucosal phase	References
			Stomach		Small intestine		Large intestine							
			Des	Jej	Ile	Asc	Trans	Des						
Static mono-compartmental models														
In vitro infant digestion model (INRAE France)	2 weeks - 3 months	X	pH=3 / 30 min TT = N/A	pH=6.5 / 30 min TT = N/A		X		Milk	Pepsin concentration reduced 5-fold* Bile salts concentration reduced 4-54* Phospholipase, lipase and chrysothrypsin concentrations reduced 10-fold*	X	X	X	Dupont et al., 2010	
	28 days	X	pH=5.3 / 60 min TT = N/A	pH=6.8 / 60 min TT = N/A		X		Liquid infant formula + SGF + SF	Pepsin concentration reduced 7-fold* Pepsin activity : 368 U/ml Bile salts concentration reduced 5.5-fold* Pancreatic lipase reduced 22.6-fold* Bile salts reduced 3.5-fold*	X	X	X	Ménard et al., 2018	
In vitro infant gastric digestion (Xinan Agricultural Univ., China)	-	X	pH=5.0 / 30-60 min pH=5.0 / 60-90 min pH=5.0 / 90-120 min TT = N/A	pH=7 min / 120 min TT = N/A		X		X	Pepsin : 0.4 mg/ml Lipase : 0.8667 mg/ml Pancreatin : 1.6 mg/ml Bile salts : 5 mg/ml	X	X	X	Luo et al., 2019	
In Vitro Model Simulating Gastro-Intestinal Digestion (Utrecht University, Denmark)	Neonates <2 months old	X	Fasted pH=9 / 50 min Fed pH=6.4 / 50 min TT = N/A	Fasted pH=7 / 120 min Fed pH=6.5 / 120 min TT = N/A		X		X	Gastric lipase activity : 100 TBUM Lipase activity : 65 U/ml/kg of BW Intestinal lipase activity : 50 TBUM Bile salts : 50 mg/ml	X	X	X	Kamstrup et al., 2017	
ITDM - Immotile Transfer Digestion Model (Utrecht University, Denmark)	0-2 months	X	Fasted pH=8 / 30 min Fed pH=6.4 / 50 min TT = N/A	Fasted pH=7 / 120 min Fed pH=6.5 / 120 min TT = N/A		X		Infant formula	Gastric lipase activity : 100 TBUM Pepsin activity : 741 U/L Pancreatic lipase : 250 TBUM Gastric extract Bile salts : 50 mM (sodium lauroylsulfate) Sialinase Gastric lipase activity : 17 TBUM Pepsin activity : 150 U/ml	X	X	X	Klitgaard et al., 2017	
New pediatric two-stage digestion model (Utrecht University, Denmark)	-	X	pH=1.8-4 / 30 min TT = N/A	pH=8 / 240 min TT = N/A		pH=8.9 / 480 min TT = N/A		Water Apple juice / sauce Toughnut, Pudding SGF - SF	SGF sim. pepsin CaseinSF with 150 mM sodium chloride, 5 mM potassium carbonate, and 16 mM sodium bicarbonate	X	X	X	Karkossa et al., 2017	
Dynamic mono-compartmental models														
In vitro infant gastric digestion (Israel Inst Technol, Israel)	< 2 years	X	pH=5.0 / 30 min pH=5.0 / 30-150 min pH=5.1 / 150-240 min pH=5.1 / 240 min TT = N/A	X		X		-	Sodium chloride Hydrochloric acid Pepsin	X	X	X	Shari-Levi et al., 2013	
Dynamic bi-compartmental models														
Upper GI tract models	DIOGF - Dynamic Digester (INRAE France)	-	X	pH (acidification curve) T _{1/2} = 70 min β=1.23	pH=5.0 T _{1/2} = 200 min β=2.2		X		Milk protein, dairy meal, vegetable, multi-enzymatic	Pepsin : 1250 U/ml, 0.25 mL/min Lipase : 60 U/ml, 0.25 mL/min Bile salt activity (1 %): 0.5 mL/min Pancreatic lipase : pancreatic solution (10%): 0.5 mL/min	X	X	X	Ménard et al., 2014
		0-2 months	X	pH=6.4 / 30 min pH=5.1 / 30-150 min T _{1/2} = 60 min β=1.5	Fasted pH=7 / 120 min Fed pH=6.5 / 120 min 0-10 min: 0.34 mL/min 10-20 min: 0.25 mL/min 20-180 min: 0.14 mL/min		X		Infant formula	Gastric lipase activity : 17 TBUM Pepsin activity : 150 U/ml Pancreatic lipase : 50 TBUM Fasted state Gastric lipase activity : 100 TBUM Pepsin activity : 741 U/L Pancreatic lipase : 250 TBUM Pancreatic extract Bile salts : 50 mM (sodium lauroylsulfate)	X	X	X	Klitgaard et al., 2017
	Try TM - TNO gastrointestinal system 1 (TNO, Netherlands)	6-18 months	Yes	pH 6.5 to 4.5 / 0-150 min TT = N/A	pH=6.5 TT = N/A		X		Light meal Pepsin Pancreatin	Gastric lipase Bile Pepsin Pancreatin	Passive (Jij + Ile)	X	X	Havenaar et al., 2013
	TNI pediatric - TNO gastrointestinal system 1 (TNO, Netherlands)	0-1 month	Yes	pH=6.7 to 4 / 0-100 min pH=6.7 to 2 / 100-200 min T _{1/2} = 60 min β=1.5	pH 6.5 TT = N/A		X		Formula milk Amylase Gastric lipase Pepsin Pancreatin	Gastric lipase Pepsin Pancreatin	Passive (Jij + Ile)	X	X	Havenaar et al., 2013
1-6 months	Yes	pH=6.7 to 3.2 / 0-180 min pH=3.9 to 2 / 180-300 min T _{1/2} = 60 min β=1.5	pH=6.5 TT = N/A		X		Formula milk or mixed fruit sauce	Amylase Gastric lipase Pepsin Pancreatin	Gastric lipase Pepsin Pancreatin	Passive (Jij + Ile)	X	X	Havenaar et al., 2013	
6-24 months	Yes	pH=6.7 to 2.4 / 0-180 min pH=3.9 to 2 / 180-300 min T _{1/2} = 70 min β=1.5	pH=6.5 TT = N/A		X		Milk, juice and cereals	Amylase Gastric lipase Pepsin Pancreatin	Gastric lipase Pepsin Pancreatin	Passive (Jij + Ile)	X	X	Havenaar et al., 2013	
Dynamic multi-compartmental models														
MIDA - Model of an Infant Digestive Apparatus (Utrecht University, Italy)	6 months	pH=7 / 2 min TT = N/A	pH=3 / 120 min T _{1/2} = 200 min β=2.2	pH=6.5 / 120 min TT = N/A		X		Rice starch-based food, rice-cereal based food SSF - SGF - SF	α-amylase in SSF and SF Pepsin in SGF Glucosylase in SF Pancreatic lipase in SF	X	X	X	Passananti et al., 2017	
	6 months - 3 years	X	pH=5.0 / 30 min pH=5.0 / 30-150 min pH=4.5 / 150-240 min pH=4.5 / 240 min T _{1/2} = 70 min β=1.23	pH=6.5	pH=6.8	pH=6.8	X	Milk formula	Pepsin 30 U/ml Lipase 30 U/ml Pancreatin 2% Bile salts 1%	Passive (Jij + Ile)	X	X	Blangsted et al., 2004	
	6 months - 2 years	X	pH=5.0 / 30 min pH=5.0 / 30-150 min pH=4.5 / 150-240 min pH=4.5 / 240 min T _{1/2} = 70 min β=1.23	pH=6.4	pH=6.9	pH=7.2	X	Water	Pepsin 150 U/ml Lipase 5 U/ml Pancreatin 3.5% Bile salts 2% Trypsin	Passive (Jij + Ile)	X	X	Roussel et al., 2016	
Static mono-compartmental models														
Gamaga's model (Macquarie Univ., Australia)	2-11 months	Yes	pH=3 RT = N/A	pH=7 RT = N/A		X		Cereals	α-amylase 150 U/ml Gastric lipase 312 U/ml Bile salt 2.5 to 50 mg Lipase 2000 U/ml, Colipase 4000 U/ml, Amylase 20 U/ml, Adult fecal effluents medium (from Macfarlane adult medium)	X	Child fecal sample (fresh) n=6	X	Gamaga et al., 2017	
Dynamic mono-compartmental models														
Lower GI tract models	4-6 months	X	X	X	pH=5.7 RT = 4 hours	pH=6.2 RT = 8 hours	pH=6.8 RT = 12.5 hours	X	Derived from Macfarlane adult medium and adapted to infant digestion	X	Child fecal sample (fresh) n=4 + galactan-behavior gel beads	Mucin in medium (porcine gastric type II)	Cinquin et al., 2004	
	2.5 years old	X	X	X	pH=5.7 RT = 8 hours	X	X	-	Derived from Macfarlane adult medium and adapted to infant digestion	X	Child fecal sample (fresh) n=1 + galactan-behavior gel beads	Mucin in medium (porcine gastric type II)	Dostal et al., 2015	
	2 months	X	X	X	Various pH RT = 5 hours	X	X	-	From Cinquin infant medium (8 months old)	X	Child fecal sample (fresh) n=2 + galactan-behavior gel beads	Mucin in medium	Pham et al., 2019	
GS1 - In Vitro Colonic Fermentation Model (Utrecht University, Romania)	6 months	X	X	X	pH=6.5-5.9 RT = 3 hours	pH=6.2-6.5 RT = 3 hours	pH=6.6-6.9 RT = 3 hours	Standard infant formula	Pepsin 150 U/ml Lipase 5 U/ml Pancreatin 3.5% Bile salts 2% Trypsin	X	Pool of child fecal samples (fresh) n=1 Infant (covered days of fecal collection)	Mucin in medium	Vamariu et al., 2013	
Dynamic multi-compartmental models														
ColoGut - Copenhagen MiniGut (Utrecht University, Denmark)	6 months	X	X	X	pH=5.7-6 RT = 8 hours	pH = 6-6.5 RT = 8 hours	pH=6.5-6.9	-	Sigmacell colon medium for adult	X	Child fecal sample n=2 (from 14 M PBS/20% glycerol n=1)	-	Weise et al., 2018	
Three-stage PolyForm® - Polymerenter Intestinal Model (ETH Zurich, Switzerland)	6 months	X	X	X	pH=5.9 RT = 4 hours	pH=6.2 RT = 5 hours	pH=6.7 RT = 4 hours	-	From Cinquin medium	X	Child fecal sample (fresh) n=2 + galactan-behavior gel beads	Mucin in medium (porcine gastric type II)	Cinquin et al., 2006	
GS2 - In Vitro Colonic Fermentation Model (Utrecht University, Romania)	6 months	X	X	X	pH=5.5 RT = 3 hours	pH=6.2 RT = 3 hours	pH=6.3	-	From Vamariu medium	X	Child fecal sample (fresh) n=1	Mucin in medium (porcine gastric type II)	Vamariu et al., 2015	
EuterKO06 - Danisco (Framing)	< 1 year	X	X	X	pH=5.5 RT = 3 hours	pH=6 RT = 3 hours	pH=6.5 (des) (des) (des) RT = 3 hours (des)	-	From Macfarlane adult medium	X	Child fecal sample (fresh) n=2	Mucin in medium (porcine gastric type II)	Mäkitavola et al., 2005	
Complete gastro-intestinal models														
Upper GI tract models	3-12 months	X	pH=3-8.4 / 180 min RT = N/A	pH=5.6-6 / 180 min RT = N/A	pH=5.0-6 / 120 min RT = 12 hours		pH=5.6-6.3 RT = 8 hours	pH=6.3-6.5	-	NutritionPlus® medium	X	Pool of child fecal samples (fresh) n=10	Mucin in medium	De Boever et al., 2001
	6 months	N/A	pH=3-8.4 / 90 min RT = 32 hours	pH=5.6-6 RT = 32 hours	pH=5.6-6.3 RT = 32 hours	X	X	-	From De Boever et al., 2001	X	Pool of child fecal samples (fresh) n=3	Multi-agar beads (porcine gastric type II)	Van den Abbeele et al., 2019	
	1-2 years old	N/A	pH=3-8.4 RT = 72 hours	pH=5.6-6.1 RT = 72 hours	pH=5.4-5.8 RT = 72 hours	pH=6.3 RT = 72 hours	pH=6.3-6.5	-	Medium PD-N8005 from Pro-Digest	X	Pool of child fecal samples (fresh) n=3	-	Bondue et al., 2020	

Abs: absorption; Asc: ascending colon; Duo: duodenum; Des: descending colon; Jej: jejunum; Ile: ileum; SSF: Simulated Salivary Fluid; SGF: Simulated Gastric Fluid; SI: small intestine; SIF: Simulated Intestinal Fluid; Stom: stomach; RT: residence time; T°C: temperature; Trans: transverse colon; N/A: not applicable; (-): information not available; X: not simulated in the model.

* when compared to adult condition.

infant stomach and small intestine (0 to 2 months) called the Continuous Transfer Digestion Model (CTDM) (Klitgaard et al., 2017). The same parameters as previously described were implemented but a continuous transfer of GI fluids was set up between two distinct vessels reproducing the gastric and small intestinal compartments. Gastric and intestinal digestions started together and the transfer rate between the two vessels was adjusted to mimic gastric emptying of neonates. The GI transfer ends after the complete emptying of the gastric vessel (around 150 min) and intestinal digestion is stopped later at 180 min. Interestingly, the

CTDM was adapted to incorporate the gastric pH drop following meal ingestion based on an old report from Mason (Mason, 1962): gastric pH is maintained at 6.4 during the first 30 min and then undergoes a gradual drop to 5.1 (30–120 min).

4.2.2.3. Multi-compartmental models. The digestive complexity was further improved in the Model of an Infant Digestive Apparatus (MIDA) developed by University of Naples in Italy (Passananti et al., 2017). The four compartments reproduce the esophagus, stomach, pyloric sphincter

and small intestine of 6-month infants. Each of them, except for the pyloric sphincter, is represented by two concentric tubes, the inner containing the digestive medium while the outer holds a temperature-controlled water flow to maintain a constant body temperature. As an elegant development, relevant stomach and small intestinal dimensions are used and adapted to 6-month-old infant conditions. The small intestinal compartment is made of five tubular sections continuously squeezed during digestion in order to reproduce peristaltic and segmentation motions. The overall digestion lasts 248 min with the following sequence: oral phase (8 min), gastric phase (2 h), intestinal phase (2 h). The oral (esophagus), gastric and intestinal compartments remain static and maintained at pH 7, 3 and 6, respectively. The oral pH is adjusted according to *in vivo* data, the static gastric pH is chosen to represent mean data observed between the first months to second year of life as no data specific for 6 months infant was found. The intestinal pH used corresponds to adult value. The α -amylase used in the oral phase is added based on *in vivo* data in adults at a concentration of 75 U/mL. Every 10 min, 10 mL of the incubated food bolus are injected into the esophageal compartment, together with gastric fluids composed of electrolytes and pepsin (if tested foods contain proteins). Surprisingly, a high pepsin concentration was used in this study (1000 U/mL), compared to other child models and was based on data from adult models whereas *in vivo* data in child are available (Tavares et al., 2014). During the gastric phase, the stomach is hand squeezed every 15 min after sampling, which is not relevant compared to *in vivo* data. At the end of gastric phase, the pyloric sphincter, opening and closing in a physiologically relevant manner (4 times per min in infant), allows the release in the intestinal compartment of a relevant chyme volume, depending on digested food. Gastric emptying is derived from a power exponential mathematical equation with parameters adapted to infant conditions and half-time emptying calculated according to food caloric density. During the intestinal phase, α -amylase, glucoamylase and porcine pancreatic lipase are added depending on the digested food.

Another well-validated multi-compartmental model of the upper human gut is the TNO gastrointestinal system 1 (TIM-1), developed by Netherland Organization for Applied Scientific Research. This model has been first described in 1995 (Minekus et al., 1995) under adult digestive conditions and thereafter adapted to child ones, as related in several publications from TNO (Havenaar et al., 2013b, 2013a) and Université Clermont Auvergne in France (Blanquet et al., 2004; Roussel et al., 2016). TIM-1 contains four serial compartments reproducing the stomach and three parts of the small intestine, i.e. duodenum, jejunum and ileum. Each compartment is made of a glass jacket containing a flexible wall inside and connected to the others by peristaltic valves that regulate chyme transit. Body temperature and peristaltic mixing are achieved thanks to circulating heated water. Each compartment is characterized by specific pH kinetics (especially a gradual gastric pH drop), sequential delivery of secretions (gastric lipase and pepsin, porcine pancreatic juice and porcine bile salts), and transit time. This is the only model adapted to child digestion that reproduces passive absorption of small molecules and water. TIM-1 was adapted to simulate different age ranges and meal conditions: 6 months to 3 years old child ingesting a milk formula (Blanquet et al., 2004), 6 months to 2 years child drinking a glass of water (Roussel et al., 2016), 6 to 18 months child ingesting a light meal (Havenaar et al., 2013b), and term-neonates (0–1 month), infants (1–6 months) or toddlers (6–24 months) ingesting formula milk, formula milk or mixed fruit sauce and milk, juice and cereals, respectively (Havenaar et al., 2013a). In the seminal study from Blanquet et al. (2004), half-time chyme delivery in both gastric (80 min *versus* 30 min -fast GI passage- or 70 min -slow GI passage-) and ileal (200 *versus* 160 min) compartments was increased in child compared to adult. While intestinal pH values of duodenal, jejunal and ileal compartments remained similar, gastric pH slope was less pronounced in child compared to adult (from 6.5 to 3.5 *versus* 4.5 to 1.5 -fast GI passage- or 5.5 to 1.7 -slow GI passage-). In this study, all digestive secretions were reduced in child compared to adult conditions: gastric pepsin (525 *versus* 600 U/mL) and lipase (30 *versus*

37.5 U/mL) activities, as well as porcine bile salts (1% *versus* 4% from 0 to 30 min and 2% from 30 to 360 min) and pancreatin (2 *versus* 7%) concentrations. Since absorption mechanisms seem already mature at birth, the same dialysis conditions were applied in the jejunal and ileal compartments in child conditions as in adult conditions. Recently, Roussel et al. (2016) adapted the TIM-1 to similar child age for the simulation of drinking a glass of water (instead of a milk formula). Half-time of chyme delivery was also increased both in gastric (20 min *versus* 15 min) and ileal compartments (190 min *versus* 150 min) compared to adult conditions. Gastric pH remained higher in child than in adult model, even if the pH set-up value was lower with water due to the absence of milk buffering effect. In this study, pepsin and lipase activities were not modified compared to adult condition, while pancreatic juice and porcine bile salts concentrations were reduced as previously described in Blanquet et al. (2004). In the publications from TNO (Havenaar et al., 2013b, 2013a), a special configuration of the TIM-1 was used (Tiny-TIM), where a single compartment is used to reproduce the child small intestine. As already described in many other child models, the main distinct parameter compared to adult was a less pronounced gastric pH drop, while maintaining the same intestinal pH (6.5). For 6–18 months child, the gastric pH drops from 6.5 to 4.5 within 150 min digestion *versus* 5.5 to 1.7 in adult (Havenaar et al., 2013b). This was further adjusted from 6.7 to either 4, 3.2 or 2.4 for neonate, infant and toddlers, respectively (Havenaar et al., 2013a). The gastric emptying time was similar for neonate and infant (half time emptying of 60 min) but longer for toddler (70 min), with a total digestion time of 6 h for the three kid categories. Digestive secretions were adapted to the amount of protein intake from food matrix and not the age (Havenaar et al., 2013b).

Taken together, available data indicate that scientists reached a consensus when developing *in vitro* models by setting-up a less pronounced gastric pH drop in child compared to adults, while maintaining the same intestinal pH. Developments that are more controversial are observed regarding GI secretions where broad range of concentrations and enzyme activities are used between *in vitro* models. Likewise, many discordances persist for gastric and intestinal transit times depending on studies. This discrepancy can be explained by the paucity of *in vivo* data in healthy neonates, infants and toddlers due to their invasive character. Also, the presence of the food matrix is poorly considered in most studies. The age simulated is varying widely between studies, with models set-up on age range rather than a precise age. Lastly, it is important to underline that none of these models reproduces the microbial component of the child intestinal environment, focusing mainly on digestive mechanical and physicochemical parameters.

4.3. Colon models

4.3.1. Static mono-compartmental models

In batch systems, fecal microbiota is diluted in a nutritive medium constantly stirred (estimated fecal concentration of 10% *w/v*) and cultured during 24 to 48 h in temperature-controlled vessel maintained under anaerobiosis (by flushing with nitrogen and/or carbon dioxide). Upon inoculation, the batch remains static with no further medium input or output. Available batch models of the infant (average) colon were set up by using fecal samples from infants of 5 to 11 months in the model of Gamage' team from Macquarie University in Australia (Gamage et al., 2017), and 1 to 5 months by Ahlborn and colleagues from Grassland Research Center in Australia (Ahlborn et al., 2020). Ahlborn's team uses pre-digested raw sheep and cow milk as nutritive medium according to the simple and static method proposed by Minekus (Minekus et al., 2014). The suspension is mixed with a cysteine solution to reduce the medium and baby feces diluted in phosphate buffer. The mixture is incubated at 37 °C with constant flushing with carbon dioxide and fermentation lasts up to 24 h. Gamage and colleagues use pre-digested cereal products according to an *in vitro* digestion protocol adapted to infant conditions, but after perform fermentation under

anaerobic conditions for a 48-h period without any adaptation of the basal medium simulating large intestinal conditions (Gamage et al., 2017).

4.3.2. Dynamic mono- or multi-compartmental models

Dynamic colon models rely on continuous or semi-continuous (and not batch) fermentation systems, with regular input of nutritive medium and regular output of fermentation medium, this allows to define for each bioreactor (for multi-compartmental models) a specific residence time that can be extrapolated to *in vivo* transit time. Most of the culture media used to simulate the composition of ileal effluents are derived from the initial one described by Macfarlane and colleagues from University of Reading in UK for adult colon models (Macfarlane et al., 1989). As for batch systems, bioreactors are temperature-controlled and maintained under anaerobiosis. Colonic pH is continuously monitored and controlled, and redox potential can be measured in real time in some of the models. Human colonic conditions can be reproduced by a single vessel or by multiple bioreactors in series representing different anatomical parts of the colon (ascending, transverse and descending colon). As for batch models, intermediate or end-products of microbial fermentation such as gas and SCFA can be measured in the fermentation media or atmospheric phase of bioreactors and microbiota composition is determined.

4.3.2.1. Dynamic Mono-compartmental models. The first dynamic colon model adapted to child conditions was the single stage culture system called Polyfermentor Intestinal Model (PolyFermS), set-up by Lacroix's team from ETH Zurich in Switzerland (Cinquin et al., 2004). In this study, the continuous *in vitro* model reproduces the infant colon from 4 to 6 months old. The nutritive medium was made according to Macfarlane's adult medium and adapted to infant digestion of a standard formula, considering infant digestibility indices. In particular, protein fraction was reduced, and whey proteins, starch, maltodextrin and lactose were added to replace complex fibers. Bile salts, which are less concentrated in the GI tract of infant, were also reduced compared to adult (0.05 g/L versus 0.4 g/L, respectively). Three different successive residence time and pH values were applied in order to reproduce the three colonic parts in a single bioreactor: 4 h/5.7, 8 h/6.2 and 12.5 h/6.8 (residence time/pH). Residence time and colonic pH were not modified compared to the adult model. Of note, an innovative microbiota immobilization technique on mixed gellan-xanthan gel beads was implemented to inoculate PolyFermS fermenters for mimicking planktonic and sessile cell growth. This immobilization procedure allows modeling infant gut microbiota even with a very low amount of fecal sample is available and ensures recovery and viability of dominant cultivable bacteria from infant feces such as *Bifidobacteria* (Cinquin et al., 2004; Pham et al., 2019).

A very close 6-month colonic model was developed by University of Agronomic Science and Veterinary Medicine in Romania, the mono-compartmented system of *in vitro* colonic simulation (GIS1), using the same operating parameters and medium composition as described by Cinquin (Vamanu et al., 2013). The only notable change is the addition of twenty 10-mm diameter glass balls to better simulate the peristalsis motion. Other publications from ETH Zurich in Switzerland reported further adaptations of the PolyFermS model. First, Dostal and colleagues simplified the model to simulate only the proximal colon of 2.5 years old child with the increase of retention time to 8 h at pH 5.7 (Dostal et al., 2015). In many studies, the selected retention time relies on a general rule stating that proximal colon transit time is estimated to be about one-third of the total colonic transit time. The infant adapted medium used was developed previously (Le Blay et al., 2009) and mainly relies on the reduction of biliary salts concentrations from 0.4 to 0.05 g/L

(Macfarlane et al., 1998). Then, Pham and collaborators further adapted PolyFermS for 2-month-old formula-fed infant (Pham et al., 2019). Different pH values were tested in order to represent the proximal colonic pH of breast-fed (pH 5) or formula-fed infant (pH 7), as well as different retention times (5 h or 10 h). The nutritive medium was adapted to younger infant according to Cinquin's study, by removing maltodextrin, increasing lactose (6.4 versus 5 g/L) and whey proteins (8.1 versus 7.2 g/L) and decreasing casein concentrations (0.5 versus 1.2 g/L) (Cinquin et al., 2004). Lastly, Doo and collaborators again extended potentialities of the proximal colon PolyFermS for 6-month-old formula-fed infant (pH 6, retention time 7.4 h) and 8-month-old African infant living in poor environmental conditions and fed with a mixed diet (pH 5.8, retention time 6.1 h) (Doo et al., 2017).

The Copenhagen MiniGut (CoMiniGut), developed by University of Copenhagen in Denmark, initially aims to reproduce the adult colon, and was further used to mimic 6-month infant conditions (Wiese et al., 2018). The model, inoculated with frozen stool, is composed of five small vessels in parallel (final volume of 5 mL), stirred, oxygen-free and pH controlled. Notably, except the use of infant stool, none of the operating parameters was adapted to a 6-month infant physiology. Besides, the microbiota profiles reported in this work suggest that the system is not well-controlled (maybe due to very small reactor volumes) and microbiota composition is abnormal for infant with dominance of Proteobacteria, low Firmicutes and almost no Actinobacteria.

4.3.2.2. Dynamic Multi-compartmental models. ETH Zurich in Switzerland also provided an update of the previously-mentioned PolyFermS system by developing a multi-compartmental model of the child colon simulating the three parts of the colon using three serial reactors (Cinquin et al., 2006). All the parameters were adapted to 6-months infant based on *in vivo* data. The total mean retention time is set at 13 h and fixed in each compartment at 4, 5 and 4 h, respectively. pH is only controlled in the two first reactors and set at 5.9 (proximal) and 6.2 (transverse), and naturally stabilized between 6.6 and 6.7 in the last reactor (distal). A similar system was developed by University of Agronomic Science and Veterinary Medicine, the multi-compartmental GIS2, an adaptation of the GIS1 composed by three reactors in series (Vamanu et al., 2014a).

The EnteroMix, initially developed by Danisco in Finland for adult studies (Mäkiyuokko et al., 2005) was used for infant gut research by inoculating the model with infant stools under 1 year old (Salli et al., 2019). To generate enough fecal inoculum, infant stools produced within a week were frozen, pooled, diluted in the nutritive medium, and amplified prior to inoculation. Anymore, none of the other parameters has been adapted to the specific infant colon conditions. This complex model reproduces from the ascending colon to the sigmoid/rectum area, with four glass vessels and ensures for each of them a control of pH (5.5, 6.0, 6.5 and 7.0, in the ascending, transverse, descending and sigmoid/rectum parts, respectively). Each vessel is inoculated with the amplified fecal inoculum and after 3 h, the fermentation begins with the addition of 3 mL of nutritive medium to the first vessel. Every 3-h, 3 mL are transferred from one vessel to the following one. Finally, 12 h later, 3 mL are discarded from the last compartment. The entire experiment lasts 48 h.

Altogether, compared to batch models, dynamic semi-continuous and continuous *in vitro* models of the child colon allow a better simulation of *in vivo* conditions, even if they require more trained manipulators and are more expensive and time-consuming. Adaptation of critical parameters, such as pH and retention time, that widely influence gut microbiota composition and activity, is a key feature of these models (Duncan et al., 2009; Tottey et al., 2017). Unfortunately, in some of the current developments, these parameters have not been adapted to

specific child colon conditions, adult parameters remaining the standard. This probably results from paucity of *in vivo* data or high individual variability, especially when all differentiated colon compartments are considered.

4.4. Whole gastro-intestinal models

Currently, the only available *in vitro* model reproducing the whole human gut is the Simulator of the Human Intestinal Microbial Ecosystem (SHIME), developed by Ghent University in Belgium (Molly et al., 1993). This set-up was adapted from 4 to 18-month child conditions in the Baby SHIME (De Boever et al., 2001). Baby SHIME is a semi-continuous model, composed of six temperature-controlled vessels reproducing the stomach, duodenum, jejunum/ileum and ascending, transverse and descending parts of the colon. The colon compartments are inoculated with a pooled of three feces collected from 3 to 12 months infants. pH values are not adapted to child conditions but only maintained between fixed limits ranging for stomach from 3.8 to 4.1 and for small intestine from 5.5 and 6, as previously set for adult conditions (Molly et al., 1993). The pH values of ascending (between 5.5 and 6.0) and transversal colon (between 6.0 and 6.3) compartments were also set up based on adult data, while the last vessel corresponding to the descending colon was maintained from 6.3 to 6.5 based on 10–18 months infant stool pH. For each GI compartment, the transit time is set at 3, 3, 2, 8, 12 and 8 h, respectively, as inferred from infant whole gut transit time reported in *in vivo* studies on 6-week old infants (McClure and Newell, 1999) and 1 to 4 years toddlers (Walker and Walker, 1985; Weaver and Steiner, 1984). The nutritive medium used, based on a milk formula intended for babies aged 4 to 18 months, is added to the first gastric vessel, three times per day. The SHIME system was also very recently adapted to mimic toddler digestion (Bondue et al., 2020). Colonic compartments were inoculated with fecal samples collected from healthy formula-fed children aged between 1 and 2 years. Apart from using feces from toddlers, the model was further adapted by changing the composition of the nutritive medium even if there is no information about it in the publication. pH and retention time parameters were based on adult data.

5. *In vitro* child gut models: Main application studies

Digestive *in vitro* systems represent a valuable alternative and complementary approach to *in vivo* studies in nutrition and health and their related fields of research. The selection of an *in vitro* model primarily depends on the research question to be answered (in particular interactions with physicochemical or microbial digestive parameters?), the targeted GI compartments and the number of tested compounds (Fig. 4). Dynamic and multi-compartmental models allow an in-depth control and parameter investigations, under *in vitro* conditions closely related to the *in vivo* situation, but are not always best suited, e.g. for screening studies, as there are heavy to operate and let a low number of independent repetitions. This section summarizes the main applications of child *in vitro* gut models in nutritional, pharmaceutical and microbiological studies (Table 2, Fig. 4). Currently, there is no published work regarding the use of these models in toxicological studies, even if many scientific questions raised in this field of research could be addressed with such approaches and tools.

5.1. Nutritional studies

5.1.1. Nutrient digestibility

In vitro child models developed by Dupont's team at INRAE in France were used to evaluate milk protein or lipid digestibility. First, static

models were used to compare the stability of food proteins toward infant (2 weeks to 3 months) and adult digestion. Results demonstrated that bovine β -lactoglobulin was more degraded in the infant model, while β -casein and hen egg ovalbumin were more slowly digested under infant conditions compared to adult ones (Dupont et al., 2010). Digestive kinetics of a commercial infant formula was also investigated under 28 days old infant (Ménard et al., 2018) and adult static digestion protocols (Minekus et al., 2014). As observed *in vivo*, gastric proteolysis was slower in infant conditions compared to adult, while lipolysis remained similar between the two sub-populations. Then, the bi-compartmental model DIDGI® was further employed to evaluate protein hydrolysis kinetics from infant formula and exhibited a robust similarity with *in vivo* data derived from piglets (Ménard et al., 2014). Results reported an extensive and rapid casein hydrolysis while α -lactalbumin and β -lactoglobulin remained stable during gastric digestion. α -lactalbumin, β -lactoglobulin and whey proteins were rapidly hydrolyzed during intestinal digestion. Of note, those *in vitro* results were not compared to *in vivo* data in infant. Such *in vitro-in vivo* correlations were conducted in the Dynamic Gastric Model (DGM) gastric model where digestion of β -lactoglobulin and lactoferrin in terms of proteolysis and emulsion characteristics under infant conditions (birth to 6 months) were correlated with *in vivo* datasets (Shani Levi et al., 2013). Lastly, digestibility of different types of casein (transglutaminase cross-linked caseinate and native caseinate) from various food matrices was studied in the Tiny-TIM model adapted to simulate GI infant (6–18 months) conditions (Havenaar et al., 2013b). This study showed no difference in digestion efficiency for all types of caseins between adult and infant conditions, but highlighted the impact of food matrix on protein digestion rate in adults. This model was further operated several times to evaluate overall milk protein digestion from different infant formulas (Maathuis et al., 2017).



Very recently, Luo and collaborators used their static model of the infant (from birth to 6 months) gastric and intestinal phases to evaluate the impact of a surface-active component commonly used as emulsifier in infant food products (milk fat globule membrane polar lipids) on lipid digestion (Luo et al., 2019). The authors analyzed the digestion process of different sizes of lipid droplets covered or not with this emulsifier. Results showed that even if fat droplets covered with milk fat globules (as emulsifier) are less digested during the beginning of gastric phase compared to those associated with caseins (control), they released a higher amount of free fatty acids later during digestion.

Some published works report the impact of infant formula sterilization on protein digestibility. The impact of glycation and cross-linking on protein breakdown and release of β -casomorphins was investigated *in vitro* using the infant (2 weeks - 3 months) static GI model developed by Cattaneo's team (Cattaneo et al., 2017). The authors revealed a clear hindered effect of sugar glycation (i.e. lactose and maltodextrins present in infant formula) occurring during sterilization on the digestion of caseins and whey proteins. Similarly, the impact of various sterilization methods (classical sterilization or high-temperature short-time treatment) on starch digestibility from different infant foods (rice starch mixed with water, rice cream with or without an aliquot of rice flour fermented by a strain of *Lactobacillus paracasei*) was studied in the dynamic multi-compartmental MIDA model, reproducing 6-month infant digestion (Passannanti et al., 2017). Results indicated a greater starch digestibility for rice cream with the fermented aliquot and a greater release of D-glucose from starch after the high-temperature short-time treatment compared to classical sterilization.

5.1.2. Impact of food nutrients on child gut microbiota

The PolyFermS colon model was applied to assess the impact of various prebiotic compounds found in baby formula on the gut

Table 2
Application studies of the different infant *in vitro* models in the fields of nutrition, pharmacology and microbiology.

Models	Applications		Aim of the study	Reference	
	Nutrition	Pharmacology			
Models of the oral phase 	HNM - Human nipple mimic (Univ Cambridge, UK)	+	Impact of milk composition on the release of sulforhodamine B dye into mother milk Impact of formulation (tablet) according to suction rate Impact of milk composition on the release of sulforhodamine B dye into mother milk Impact of formulation (tablet) according to tongue compression / rotation	Gerrard et al., 2013 Scheutle et al., 2017	
	TMS - Tongue Mimic System (Univ Cambridge, UK)	+			
Static mono-compartmental					
Upper GI tract models 	<i>In vitro</i> infant digestion model (INRAE, France)	+	Digestibility of milk proteins (β -lactoglobulin, β -casein and hen's egg ovalbumin) Kinetics of lipolysis/ proteolysis of commercial formula Impact of emulsifier in infant foods on lipid digestion Effect of glycation and cross-linking on protein breakdown from infant formula Release of β -casomorphins from formula Screening drug release of a pediatric form (valproate-extended release formulation)	Dupont et al., 2010 Ménard et al., 2018 Luo et al., 2019 Bourlieu et al., 2014 Cattaneo et al., 2017 Karkossa et al., 2017	
	<i>In vitro</i> infant gastric digestion (Hunan Agricult Univ, China)	+			
	Cattaneo's model (Univ Milano, Italy)	+			
	Pediatric two-stage dissolution (Univ Greifswald, Germany)	+			
	<i>In vitro</i> digestion model (adaptation of Dupont et al., 2010)	+		Digestibility of beef puree	Dupont et al., 2010; Lee et al., 2020
Dynamic mono-compartmental					
<i>In vitro</i> infant gastric digestion (Israel Inst Technol, Israel)	+		Evaluation of proteolysis/emulsion of β -lactoglobulin and lactoferrin	Shami Levi et al., 2013	
Dynamic bi-compartmental					

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Table 2 (continued)

Models	Applications		Aim of the study	Reference		
	Nutrition	Pharmacology				
DIDGI® - Dynamic Digester (INRAE, France)	+		Kinetics of protein hydrolysis from infant formula Comparison with <i>in vivo</i> data in piglets Evaluation of oral performance of furosemide in infant and young children Bioaccessibility of paracetamol and diclofenac with co-medication (esomeprazole) Impact of food matrices	Ménaud et al., 2014		
		+			Kinetics of protein digestion and indispensable amino acids Impact of food matrices (goat, cow and human milk)	Klitgaard et al., 2017
		+				
Tiny TIM - TNO gastrointestinal system 1 (TNO, Netherlands)	+			Maathuis et al., 2017		
Dynamic multi-compartmental						
MIDA - Model of Infant Digestive Apparatus (Univ Naples, Italy)	+		Impact of various sterilization methods on starch digestibility from infant foods Pharmacokinetics of paracetamol (powder or sustained-release tablet) Impact of food matrices (glass water, complete breakfast) Impact of age conditions (adult versus infant) Survival of lyophilized Lactobacillus LY/SA 1 strain	Passananti et al., 2017		
		+			Impact of food matrices (glass water, milk) Impact of age conditions (adult versus infant) Evaluation of lipid digestion Impact of food matrices (human breast milk, Similact™ infant formulas) Survival and virulence of the foodborne	Blanquet et al., 2004
TIM1 - TNO gastrointestinal system 1 (TNO, Netherlands)		+		Blanquet et al., 2004		
	+			Fondaco et al., 2014		
		+		Roussel et al., 2016		

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Table 2 (continued)

Models	Applications			Aim of the study	Reference
	Nutrition	Pharmacology	Microbiology		
Static mono-compartmental					
Gamage's model (Macquarie Univ, Australia)	+		+	Impact of commercially available cereal products (wheat, sorghum, rice or oats) on infant gut microbiota	Gamage et al., 2017
Batch system	+		+	Evaluation of human milk oligosaccharides on fermentation	Vester Boter et al., 2013
Dynamic mono-compartmental					
Lower GI tract models					
PolyFermS - Polyfermentor Intestinal Model (ETH Zurich, Switzerland)	+		+	Impact of various prebiotics (oligofructose, oligofructose-enriched inulin and inulin) from formula on gut microbiota	Le Blay et al., 2010
	+		+	Impact of nucleotides/nucleosides from infant formula on gut microbiota	Doo et al., 2017
	+		+	Impact of iron availability on butyrate production by child gut microbiota	Dostal et al., 2015
			+	Impact of amoxicillin on Salmonella survival and gut microbiota composition/activity	Le Blay et al., 2009
			+	Impact of pH and retention time on lactate metabolism and lactate-utilizing bacteria	Pham et al., 2019
	+		+	Impact of symbiotic product (lactulose + <i>L. paracasei</i> + <i>E. faecium</i>) on infant gut microbiota	Vamanu et al., 2013
GIS1 - <i>In Vitro</i> Colonic Fermentation Model (Univ Agronom Sci Vet Med, Romania) ¹¹			+	Impact of lyophilized lactic acid bacteria strains on child gut microbiota	Moroeanu et al., 2015
Dynamic multi-compartmental					
3-stage PolyFermS - Polyfermentor Intestinal			+	Interaction of <i>Salmonella</i> serovar Typhimurium with	Cinquin et al., 2006; Zihler et al., 2011

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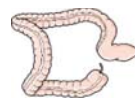
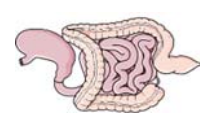


Table 2 (continued)

Models	Applications			Reference
	Nutrition	Pharmacology	Microbiology	
Model (ETH Zurich, Switzerland)			child gut microbiota Antagonistic properties of <i>E. coli</i> L1000 and <i>B. thermophilum</i> RBL67	
CoMiniGut - Copenhagen MiniGut (Univ Copenhagen, Denmark)	+		Impact of different human milk oligosaccharides (3-Fucosyllactose, 3-Sialyllactose, 6-Fructooligosaccharide) on infant gut microbiota	Wiese et al., 2018
GIS2 - <i>In Vitro</i> Colonic Fermentation Model (Univ Agronom Sci Vet Med, Romania)			Impact of two formulas containing probiotic on infant gut microbiota	Vamanu et al., 2014a
EnteromIX® - Danisco (Finland)	+		Impact of human milk oligosaccharide (2'-fucosyllactose), galactooligosaccharides and lactose on infant gut microbiota	Salli et al., 2019
Upper + lower GI tract models				
Complete gastro-intestinal				
Baby SHIME - Simulator of the Human Intestinal Microbial Ecosystem (Ghent Univ, Belgium)	+		Impact of prebiotic (2'-fucosyllactose) from formula on infant gut microbiota	Van den Abbeele et al., 2019
Toddler SHIME® - Simulator of the Human Intestinal Microbial Ecosystem (Univ Liege, Belgium)	+		Impact of symbiotic product (3-sialyllactose + <i>B. crudilactis</i> on young child gut microbiota	Bondue et al., 2020



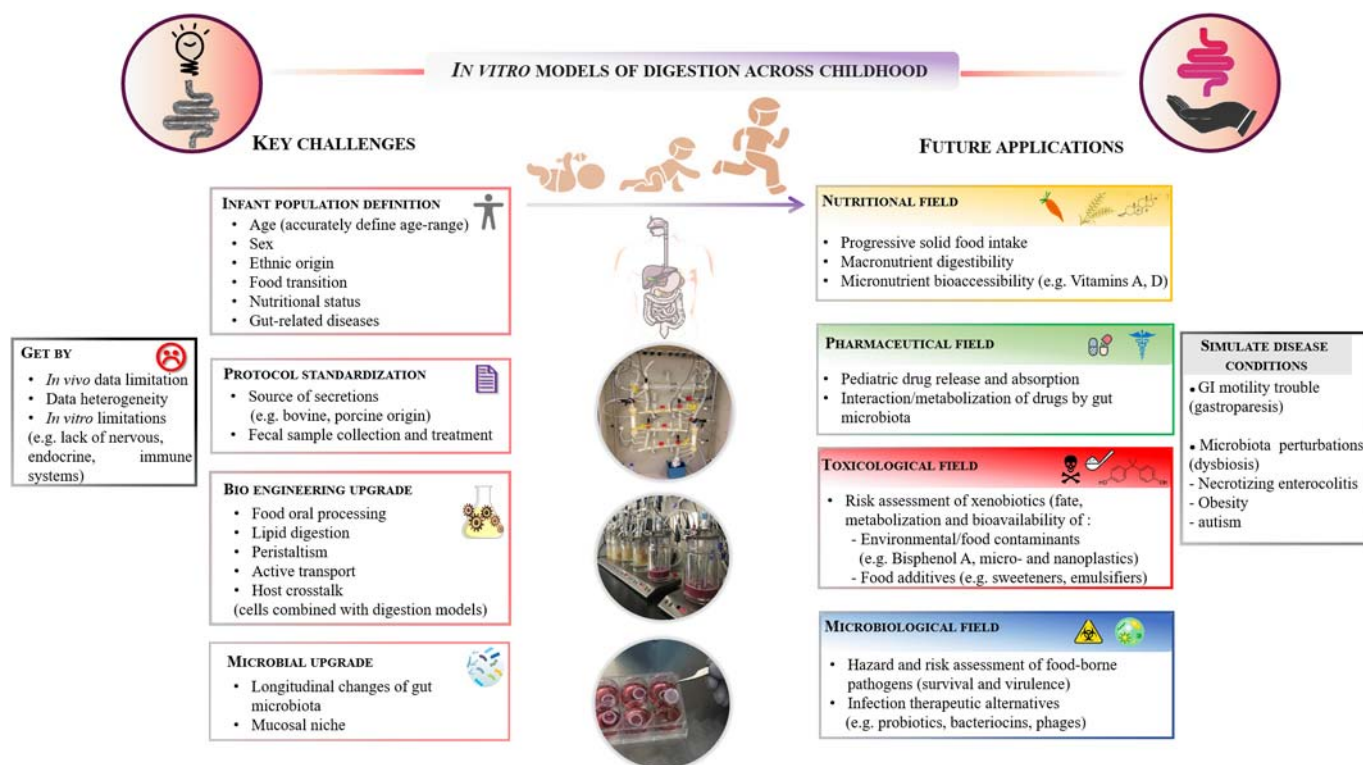


Fig. 5. Challenges and future applications of the *in vitro* models of digestion across childhood.

The key challenges in the conception, innovation and/or improvement of the current *in vitro* digestion models are categorized. Four main future applications ranging from nutritional, pharmaceutical, toxicological to microbiological fields are described.

microbiota of a 4-month old baby (Le Blay et al., 2010). The prebiotics tested were oligofructose, oligofructose-enriched inulin and high solubility inulin. All the prebiotic supplementations induced an increase in total SCFA production associated with an increase in *Bifidobacteria* and a decrease in *Bacteroides* and *Clostridia*. In another study, Wiese's team used the CoMiniGut colon model inoculated with fecal samples from 6-month infant to evaluate the impact of different human milk oligosaccharides on gut microbiota composition and activity (Wiese et al., 2018). The strongest bifidogenic effect was obtained with 3-Fucosyllactose and a higher production of butyrate was measured following addition of 3-Sialyllactose or 6-Sialyllactose compared to 3-Fucosyllactose. In order to evaluate the impact of commercially available cereal products on infant gut microbiota, Gamage's team conducted an oral, gastric and small intestinal static digestion of the products prior to exposure to its static mono-compartmental colon model inoculated with 2–11 months infant feces (Gamage et al., 2017). Addition of digested cereal products led to changes in gut microbiota composition by increasing abundance of bacterial families related to fiber degradation but decreased *Enterobacteriaceae* abundance. A higher concentration of acetate was observed whatever the cereal product, while production of propionate and butyrate fluctuated according to the tested compounds.

Besides, PolyFermS model was also used to evaluate the effect of other food nutrients on infant gut microbiota. First, Doo and collaborators studied the impact of nucleotides and nucleosides commonly added to infant formula on gut microbiota (Doo et al., 2017). Such supplementation enhanced microbiota metabolic activity, as evidenced by an increase in total SCFA production, and modulated the expression of genes involved in the two major butyrate synthesis pathways, iron acquisition, nucleotide and sulfur metabolism, but also co-factor and vitamin biosynthesis. Amino acid user populations and some butyrate producers increased while the enteric pathogen *Salmonella* decreased. Second, Dostal and colleagues used PolyFermS to investigate the impact of iron availability on butyrate production by 2.5 years old child gut

microbiota (Dostal et al., 2015). Results showed that while a low or moderate iron deficiency favored butyrate production, a strong deficiency induced a decrease in butyrate production, consistent with a loss in butyrate-producing bacteria compared to a normal iron status.

5.2. Pharmaceutical studies

The static immediate transfer digestion model was used to evaluate the performance of the furosemide drug in neonates and young infants (Klitgaard et al., 2017). The amount of furosemide solubilized in the aqueous phase during *in vitro* digestion was used to estimate the amount of drug available for absorption *in vivo*. The results pointed out an increase in drug solubilization with food intake (frequent feeding reproduced), while performance was not influenced by GI digestion of this food (addition or not of digestive enzymes). In addition, the drug dosage form (immediate-release tablet) did not affect furosemide solubilization as compared to a non-formulated powder. The dynamic bi-compartmental Tiny-TIM model was used under child conditions to evaluate the bio-accessibility of different dosage forms of two drugs (paracetamol and diclofenac) under realistic conditions including co-medication (with esomeprazole) and ingestion of food. Paracetamol availability for intestinal absorption was not influenced by the different GI conditions associated with the three-age groups (neonate, infant and toddlers), or by the tested dosage form, food matrix and co-medication. However, diclofenac had a greater bioaccessibility when administered with a food matrix than with water, but was not influenced by co-medication (Havenaar et al., 2013a). Lastly, the whole TIM-1 model was used under child or adult digestive conditions to evaluate the pharmacokinetics of paracetamol when administered under two oral dosage forms (powder or sustained-release tablet), with a glass of water or a complete breakfast (Blanquet et al., 2004). Paracetamol absorption was greater when ingested together with a standard breakfast compared to when co-ingested with water, and a slow GI transit associated with the

complete meal digestion resulted in a delay in absorption. The authors also evidenced that paracetamol was more rapidly absorbed as a free powder form comparatively to the sustained-release tablet form. These *in vitro* data were in accordance with *in vivo* data, indicating that TIM-1 is a suitable tool for pharmacological studies in child.

The silicone human nipple mimic model (Gerrard et al., 2013) was used to assess the impact of infant suction pulse rate, flow rate and fat milk content on the release from a tablet of the sulforhodamine B dye into the mother milk. Results highlighted an increase in the tested compound release with increased suction pulse rate occurring during breastfeeding, while milk fat content did not play a significant role. In addition, the release of sulforhodamine B from those tablets was tested in the tongue mimic system. The authors demonstrated an increase in compound release when the tongue strength, in particular the compression increased (Scheuerle et al., 2017).

5.3. Microbiological studies

The impact of the PROBAC product, containing both prebiotic (lactulose) and probiotics (*Lactobacillus paracasei* YR and *Enterococcus faecium* VL47 strains), on the 6-month infant gut microbiota was studied in the colonic GIS1 model (Vamanu et al., 2013). As expected, the administration of PROBAC induced an increase in *lactobacilli* and *bifidobacteria* as the products contain lactulose and stimulate lactase synthesis by those bacteria. This model was further used to evaluate the impact of lactic acid bacteria strains on 7-year old child gut microbiota (Moroeanu et al., 2015). *Lactobacillus rhamnosus* E4.2 and *Weissella paramesenteroides* FTa1 which synthesize more efficiently exopolysaccharides, had a better probiotic potential than other tested strains, as assessed by their positive influence on beneficial strains, their ability to reduce potentially pathogenic microorganisms and their effect on gut microbial activity. The multi-compartmental gastric and small intestinal TIM-1 model was used to evaluate the survival of lyophilized *Lactobacillus* LY/SA 1 strain under child and adult conditions, when administered in milk and water, respectively (Blanquet et al., 2004). In both gastric and ileal effluents, *Lactobacillus* LY/SA 1 survival rate was higher under child than adult digestive conditions. This result could be explained by a higher gastric pH and a lower bile salt concentration in child, but also by the potential protective effect of milk (as compared to water). Such *in vitro* data are consistent with *in vivo* data obtained however with other *Lactobacillus* strains.

Apart from probiotics, *in vitro* child models have been applied to study pathogen survival and virulence gene expression, as well as their interactions with gut microbiota. Using TIM-1 model, Roussel and colleagues evaluated the survival and virulence of the foodborne pathogen *Enterohemorrhagic Escherichia coli* O157:H7 under 6-months to 2-years infant digestive conditions versus adults (Roussel et al., 2016). After GI passage, a significantly higher percentage of viable pathogenic cells was recovered in ileal effluents under child compared to adult set. Expression levels of virulence genes were increased by a 125-fold in infant versus adult condition, and O157:H7 Shiga-toxins, responsible for systemic complications in patients, were only detected in child ileal effluents. These results suggest that differences in digestive physicochemical parameters may already partially explain the highest susceptibility of child to *Enterohemorrhagic Escherichia coli* infection compared to adults. In another study, infection with *Salmonella enterica* serovar Typhimurium M557 in 4 to 6-month child proximal colon was simulated in the Poly-FermS model. Amoxicillin treatment, a broad-spectrum antibiotic, was tested with two different concentrations, on pathogen survival and gut microbiota composition/activity (Le Blay et al., 2009). Addition of gel beads colonized with *Salmonella* led to a high and stable colonization of the strain in the bioreactor within 7 days, associated with an increase in *Enterobacteriaceae*, *Clostridium coccooides*-*Eubacterium rectale* and *Atopobium* populations and a decrease in *Bifidobacteria*. SCFA production was slightly impacted by *Salmonella*, with an increase in butyrate concentration and butyrate producers' group during infection. Antibiotic

administration led to a decrease in *Salmonella* concentrations but also impacted commensal bacteria, especially *Clostridium coccooides* - *Eubacterium rectale* decreased, while *Enterobacteriaceae* increased. The stable and high level of *Salmonella* serovar Typhimurium reached, as well as the impact of the antibiotic assessed in this *in vitro* model, were correlated with previous *in vivo* reports (Le Blay et al., 2009).

6. *In vitro* models of the child gut: Future developments and perspectives

6.1. Main scientific challenges and technological limitations to be addressed

6.1.1. Dealing with paucity of *in vivo* data and high individual variability

First, information regarding the complex development of digestive functions in children from birth to 3 years is still missing (Fig. 5). This absence of detailed observations and mechanisms obviously hampers the development of fully reliable *in vitro* models related to pediatric digestion. Indeed, validation of *in vitro* models is always restricted to factors for which *in vivo* data are available. Even if major scientific and technical advances have already been investigated in this field during the past decades, current *in vitro* models still show some cognitive and methodological gaps. Especially, a large number of differences between models have been emphasized, such as highly heterogeneous enzyme or bile concentrations, pH and transit time, even if the same age period was applied. Part- but certainly not all- of these differences are likely due to different conceptualization, validation and/or applications of the models. The second main limitation is the targeted age, which is rather a period of several months/years than a specific age; even if it is now well acknowledged that several modifications may occur during this time-frame. Facing *in vivo* data limitations, some parameters from many *in vitro* models are still set-up under adult rather than child conditions. *In vivo* extrapolation of *in vitro* results obtained under adult conditions can then be hazardous and may lead to overdosing or adverse side effects in child. Lastly, every model development has to face with high individual variability in terms of digestive parameters. Most of current models are based on average values but extreme conditions could be reproduced to consider for instance high/low metabolizers.

6.1.2. Considering the importance of the oral phase

To date, none of the available models of the oral phase has been adapted to progressive solid food chewing (only milk diet), nor incorporates saliva modifications in the first years of life. In addition, GI models, even the most sophisticated, only handle liquid foods and cannot incorporate solid particles even if children from 6 months to 1 year of age ingest the latter. There is therefore a crucial need to more accurately simulate the oral step of food breakdown by mastication and its role in the subsequent steps of digestion (Peyron et al., 2019). *In vitro* models of the chewing process already developed for adult, such as the Artificial Mastication Advanced Machine (AM₂) developed by Université Clermont Auvergne in France (Woda et al., 2010), the Chewing Robot set-up by Massey University in New Zealand (Sun, 2012) or the Dento-Munch by Bristol University in UK (Alemzadeh and Raabe, 2007), could be adapted to child conditions. These models reproduce both biomechanical and physicochemical aspects of the masticatory process. To go further, the ideal situation would be to combine these masticator models with advanced dynamic GI systems, such as the Engineered Stomach and Small Intestine (ESIN) from Université Clermont Auvergne, which has been specifically designed to handle both liquids and real-size food particles (Guerra et al., 2016).

6.1.3. Better reproducing lipid digestion

Most of current *in vitro* studies focus on the digestibility of milk protein fraction where distinct profiles between adult and infant are clearly described (Gan et al., 2018; Nguyen et al., 2015). On the contrary, lipid digestion has not been in-depth investigated during *in vitro*

child digestion (Abrahamse et al., 2012; Poquet and Wooster, 2016). However, it is clearly known that the underdeveloped digestive tract of infants can influence their ability to digest and absorb lipids, whereas they represent a major source of energy and essential components for growth and brain/retina cells development. Between 3 and 6 months of age, compared to adults, the digestive tract of infants exhibits lower concentrations of pancreatic triacylglycerol lipase, different lipolytic enzyme activities, lower concentrations of bile salts and different bile salt conjugation/speciation (Abrahamse et al., 2012; Poquet and Wooster, 2016). Simulating digestion of lipids then remains a major challenge, further increased by the inconsistency in lipase and bile salt concentrations used among reported *in vitro* studies. In addition, most *in vitro* models fail to adequately reproduce gastric lipolysis since they do not contain or make use of incorrect gastric lipases (e.g. fungal lipase). The source of gastric lipase commercially available is a growing concern and some companies are now trying to develop recombinant lipase or lipase from animal origin, like from rabbit (Bakala-N'Goma et al., 2015; Poquet and Wooster, 2016). In addition, most of *in vitro* studies use bile salts from bovine or porcine origin that are clearly different from human ones and evolution of bile salt conjugation and speciation with age is never considered.

6.1.4. Towards a better integration of host motility and absorption phenomena

Another main limitation of current *in vitro* child models is their inability to accurately mimic peristalsis, even if it is of great importance since dysregulations of GI motility (e.g. gastroparesis, dysphagia, gastroesophageal reflux) are commonly observed in infants (Chumpitazi and Nurko, 2008). In most of *in vitro* child models, peristaltic mixing is reproduced *via* the use of magnetic stirrers, far from *in vivo* configuration. The only two child models that more closely reproduce peristaltic movements are the gastric DGM and the gastric and small intestinal TIM-1 system, through gentle mixing with pressurized water jackets. Efforts should be pursued to more accurately simulate mechanical deformations resulting from peristalsis, as already made for the stomach compartment in the Human Gastric Model (HGM) developed at Massey University in New Zealand, so far only set-up for adult condition (Ferrua and Singh, 2015). Nevertheless, technical improvement will be limited since evaluation of motility in children is very challenging due to developmental maturation, as well as technical and cognitive issues in this population.

Regarding water and nutrient absorption, all *in vitro* child gut models are not designed to reproduce this phenomenon, except TIM-1 system that incorporates dialysis modules in the small intestinal compartments through addition of hollow fibers. This dialysis device allows mimicking passive absorption of water and small molecules (e.g. fatty acids, oligo- and monosaccharides, small peptides and amino acids, drugs and chemicals), but also ensures bile reabsorption in the distal intestine, as occurring *in vivo* (Minekus et al., 1995). Such dialysis system has even been adapted to absorption of lipophilic compounds (Minekus et al., 2005) and could be easily extended to other *in vitro* models of the upper gut, since no specific adaptation is required for simulating child digestion, according to *in vivo* data (Bourlieu et al., 2014). To further reproduce absorption phenomenon and integrate active transport, *in vitro* child systems should be coupled with human intestinal epithelial cells, as previously done with TIM-1 and Caco-2 cells (Bahrami et al., 2011; Déat et al., 2009), or even organoids to move forward an increased complexity.

6.1.5. Challenge and technical improvement to mimic the gut microbiota

Most of the available systems integrating the host microbial component are modeling the colonic microbiota, through inoculation with fecal samples. Of note, there is a crucial lack of child *in vitro* models incorporating the buccal or gastric microbiota. One main advantage of *in vitro* child gut models is the possibility to easily consider microbiota

associated with each segment of the small intestine and/or colon, and to follow changes in gut-derived metabolites, such as SCFA that are important modulators of host physiology (De Paepe et al., 2020; Poeker et al., 2018). In this endeavor, they represent a powerful platform for mechanistic studies on the role of infant gut microbiota without environmental disruptive factors and by discriminating host effects. However, some limitations have been raised from published studies. First, a very low amount of fecal sample is usually available to inoculate bioreactors and techniques allowing microbiota immobilization as developed by Lacroix's team in Switzerland can be an efficient answer (Cinquin et al., 2004). The treatment of fecal samples is also much debated as preservative method (e.g. freezing), and feces dilution or amplification may change microbial profiles and further impact metabolic activities. Besides, using pooled feces obviously removes individuality. Second, fecal samples are generally collected from child with age discrepancies, which can highly affect the results. There is also a crucial need to increase the number of volunteers to better take into account gut microbiota inter-individual variability and/or sex/gender differences. In most of available studies, *in vitro* colon models are inoculated with fecal samples from infant, but remained set-up with adult parameters (e.g. pH, transit time, composition of ileal effluents, bile salts), which could differentially shape gut microbiota structure and activity (Payne et al., 2012; Tottey et al., 2017). This limitation is still heightened by the paucity of *in vivo* data on child colonic microbiota composition (different from fecal microbiota), making difficult any *in vitro-in vivo* correlations. Third, if published *in vitro* studies consider longitudinal variations in child gut microbiota (from small intestine to distal parts of colon), none of them integrates radial changes from lumen to epithelium. Nonetheless, it is now acknowledged that in adults mucus-associated microbiota is clearly distinct from its luminal counterpart due to changes in nutrient availability and oxygen gradient (Etienne-Mesmin et al., 2019; Sauvatre et al., 2021). If similar data are not available in child, we may speculate that such difference could also be found earlier in life (Donaldson et al., 2016; Rokhsafat et al., 2016). Integrating the mucosal compartment in various pediatric *in vitro* gut models would thus help to obtain a more realistic view of the spatial organization of child gut. This optimization can be performed based on work previously described in adult, especially in the Mucosal-SHIME (M-SHIME) from Ghent University in Belgium (Van den Abbeele et al., 2012) or other colon models (Deschamps et al., 2020; Probert and Gibson, 2004). These models use mucin beads to create a mucosal microenvironment preferentially colonized by mucin-degraders and mucin-adherent bacteria. Lastly, to further investigate the mechanistic host-microbiome crosstalk, *in vitro* child gut models can be coupled to cell culture assays, as previously described (Bahrami et al., 2011; Chassaing et al., 2017a; Defois et al., 2018; Geirnaert et al., 2017; Marzorati et al., 2014; Tovaglieri et al., 2019). Specific developments from Nickerson's group at Arizona State University consist in cell cultures in rotating wall vessels to mimic the microfluidics near to the mucosa (Nickerson et al., 2003). Even more complex units have been developed, such as the Host Microbiota Interaction Module (HMI) at Ghent University (Marzorati et al., 2014) or recent microfluidic systems like HuMiX (Human-microbial crosstalk) (Shah et al., 2016) or gut-on-a-chip devices (Jalili-Firoozinezhad et al., 2019; Shin et al., 2019). Such models integrate oxygen gradient microenvironment and host cells, but can also reproduce relevant shear forces, as intestinal flow and peristalsis, that all impact gut microbiota.

6.2. Future applications

As their parameters can be adjusted in terms of target group (age-range), we can consider unlimited applications of *in vitro* child gut models (Fig. 5). To date, these models have been mostly applied to nutritional applications, focusing on digestibility of infant milk formula. Next step would be to reproduce child digestive conditions when

ingesting solid and not liquid foods. This will imply a complete adaptation of all physicochemical parameters that widely change depending on meal intake and food texture (Guerra et al., 2012; Singh et al., 2015). Apart from macronutrient digestibility, potentialities of *in vitro* models can be dedicated to the investigation of micronutrient bioaccessibility, such as vitamins A and D or calcium that are playing a key role during child growth. Adaptation of *in vitro* models to the digestion of solid food would also represent a powerful platform for pharmaceutical studies, since food matrices widely impact oral formulation disintegration, and subsequent drug release and absorption, so called food-drug interactions (Blanquet et al., 2004; Bushra et al., 2011; Havenaar et al., 2013a; Schmidt and Dalhoff, 2002). Current applications in pharmaceutical studies are restricted to *in vitro* models of the upper gut. Another main challenge that could be addressed by child gut models is deciphering how pediatric drugs interact with gut microbiota, i.e. if drugs can be metabolized by resident microbiota and conversely if they can impact gut microbiota structure and/or metabolic activity (Lucafò et al., 2020; Swanson, 2015).

If *in vitro* systems are a relevant complement to *in vivo* studies in nutritional and pharmaceutical applications, they are becoming irreplaceable tools in microbiological and toxicological studies, when investigating pathogenic microorganisms or environmental/food contaminants. In these fields of research, *in vitro* digestion models are likely to become increasingly important for comprehensive health hazard and risk assessment in infant at-risk population, but also represent valuable instruments to develop and evaluate remediation strategies. Few microbiology studies have been yet performed using child *in vitro* models, opening up a large field of investigations. *In vitro* digestion models, and particularly dynamic and multi-compartmental systems, could then be advantageously used to assess the effect of successive conditions encountered by pathogenic microorganisms within the GI tract (including physicochemical factors and host microbes) on their survival and virulence. This may help scientists to unravel microbes' pathogenesis and particularly decipher whether differences in digestive physicochemical parameters or gut microbiota related to age conditions are crucial factors in the onset of disease, as previously shown by Roussel and colleagues (Roussel et al., 2016) for an *Escherichia coli* pathotype. Moreover, those *in vitro* models could provide an efficient platform to apply facets of infection therapeutics, such as antibiotics that are the most prescribed medications in neonatal and pediatric populations and have long-term deleterious effects on their gut microbiome (Gibson et al., 2015). They would also be helpful in designing novel remediation strategies such as bacteriocins (Zihler et al., 2010), phages (Breitbart et al., 2008; Endersen et al., 2017) but also probiotics (Cordonnier et al., 2015; Etienne-Mesmin et al., 2011; Roussel et al., 2016; Thévenot et al., 2013; Vamanu et al., 2013), prebiotics (Sauvatre et al., 2021) and their symbiotic combinations. Of note, like for pathogenic strains, multi-compartmental models are extremely useful to investigate probiotic survival and metabolic activity (especially bacteriocin production) in the child digestive tract, in a temporal-spatial manner (Chaikham et al., 2013; Cinquin et al., 2006; Cordonnier et al., 2015; Martinez et al., 2013; Molly et al., 1996; Rehman et al., 2012; Van den Abbeele et al., 2016; Zihler et al., 2010). Regarding toxicological studies, up to date, there is no published study on the fate of environmental pollutants (e.g. bisphenol A, organic pollutants, heavy metals, emerging contaminants like micro and nanoplastics...) in child *in vitro* gut systems. Infant models can help in assessing the fate of such pollutants to predict the internal exposure and thereby contribute to a more accurate risk assessment (Vrijheid et al., 2016). Indeed, *in vitro* upper and lower GI systems would help investigating the interactions of food chemical contaminants with main physicochemical parameters of the child digestive tract (low pH, enzymes, bile...), as well as resident gut microbiota (so called toxicomicromicrobiomics) (Abdelsalam et al., 2020; Sharma et al., 2017; Tralau et al., 2015). They would thus give useful information on the fate, bioaccessibility and metabolization (if any, especially by gut microbiota) of those compounds in the child gut, when

ingested with different food matrices, taking also into account the role of soil that is frequently swallowed by infants (Defois et al., 2018, 2017; Joly et al., 2013; Liu et al., 2018; Yin et al., 2016). Lastly, all the potentialities of child *in vitro* models applied to food pollutants may be extended to food additives, e.g. sweeteners, flavor enhancers, food coloring, preservatives or emulsifiers (Cao et al., 2020; Chassaing et al., 2017b; Gerasimidis et al., 2020; Rinninella et al., 2019; Roca-Saavedra et al., 2018).

To date, all the available *in vitro* child models have been developed to simulate healthy conditions. Especially, digestive conditions of rich-country infants have been mostly mimicked, and simulating the digestive tract of children living in poor nutritional and sanitary conditions would be a first task (Doo et al., 2017; Toe et al., 2020). Another major scientific and technical challenge would be to optimize *in vitro* upper and lower GI models to reproduce child-diseased conditions, especially those associated with trouble in GI motility (such as gastroparesis, gastric reflux or pyloric stenosis) or gut microbiota perturbations (so called intestinal dysbiosis) described in necrotizing enterocolitis, obesity or mental disorders (Koleva et al., 2015; Moossavi and Azad, 2019; Underwood et al., 2020). In these two last situations, small intestinal and/or colonic models could be inoculated with feces collected from infant patients, and the main objective would be to maintain gut microbiota dysbiosis (considered as a typical feature of these pathologies) in the bioreactor for a sufficient period of time in order to test remediation strategies, such as probiotics.

7. Conclusion

Digestion is an essential but sophisticated process for human health widely evolving within the first years of life. This paper offers a complete and well-organized state of the art on child digestive physiology including interplay between morphological, physicochemical, mechanical and microbial features. The review also compiles and describes the main *in vitro* models simulating child digestion processes, from the simplest to the most sophisticated systems. It addresses to all scientists or industrials who aims to set up *in vitro* infant protocols or want to select the best models for developing or testing their compounds. The technical developments of child gut models are currently impeded by the paucity of *in vivo* data for validation and the detrimental lack of consensus between studies on major GI parameters reproduced. The recent developments of non-invasive methods to follow these digestive parameters in humans open new avenues and may help in a near future to fill these scientific and technological gaps. Developing *in vitro* child models that are fully validated will enlarge even more their range of applications in food and pharmaceutical fields and boost the development of new child-specific products in food, nutrition and health.

Declaration of Competing Interest

Tom Van de Wiele is advising ProDigest as a science officer and is a member of the scientific advisory board of MRM Health. The other authors declare no conflict of interest.

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Appendix 5:

Review in Cahiers de Nutrition et de Diététique

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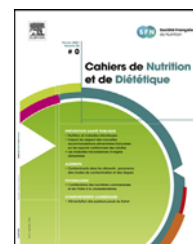


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TOXICOLOGIE

Microplastiques dans notre alimentation: un focus sur la santé intestinale



Microplastics in our diet: a focus on intestinal health

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MOTS CLÉS

Microplastiques ;
Barrière intestinale ;
Digestion ;
Modèles *in vitro* ;
Toxicologie

Résumé Les plastiques jouent un rôle central dans notre vie quotidienne. Cependant, leur utilisation massive a mené à une forte augmentation de la pollution plastique à travers le monde, générant après dégradation d'importantes quantités de microplastiques. Leur omniprésence dans l'environnement, mais également dans les produits alimentaires ou encore les emballages, représente un risque potentiel pour la santé humaine. Dans cette revue, une attention particulière est portée au tractus gastro-intestinal, à la fois porte d'entrée et première barrière aux microplastiques. Les caractéristiques de ces derniers (origine, occurrence, taille, forme, type de polymère, propriétés de surface) ainsi que les premiers résultats sur l'exposition orale humaine sont présentés. Puis, nous mettons en lumière les transformations physico-chimiques des microplastiques au cours de la digestion. Par la suite, nous détaillons l'impact potentiel des microplastiques sur l'homéostasie intestinale, via le triptyque épithélium/mucus/microbiote, en considérant les études *in vitro* et *in vivo* chez le rongeur. Nous proposons enfin de futures orientations dans l'étude des microplastiques sur la santé intestinale humaine. Un accent particulier porte sur la nécessité de développer des modèles *in vitro* gastro-intestinaux robustes afin de simuler au mieux la physiologie digestive humaine, ce qui permettra une meilleure évaluation des risques sanitaires relatifs aux microplastiques.

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KEYWORDS

Microplastics;
 Intestinal barrier;
 Digestion;
In vitro models;
 Toxicology

Summary Because plastics exhibit various functional properties, they play a pivotal role in our daily life. However, their massive use has led to a sharp increase in plastic pollution worldwide, generating after degradation massive amounts of small-sized microplastics. Their presence in the environment but also in food products and packaging has revealed a potential threat to human health. In this review, a special emphasis is given on the human gastrointestinal tract, as a portal of entry but also first barrier for microplastics. First, we summarize the characteristics of ingested forms (origin, occurrence, size, shape, polymer type, surface properties) and give the current state of knowledge on human oral exposure to microplastics. Then, we highlight the physicochemical transformations of microplastics during digestion. Afterwards, we detail their potential impact on gut homeostasis disruption, *via* the three protagonists, epithelium/mucus/gut microbiota, considering *in vitro* and *in vivo* studies in rodents. Finally, this review underlines future directions about microplastics in the field of human intestinal health. Particular emphasis is given to the unmet need of developing robust *in vitro* gut models to adequately simulate human digestive physiology for better health risk assessment related to microplastics.

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Introduction

Au cours des dernières décennies, la production plastique a explosé, passant de 1,7 million de tonnes dans les années 1950 à plus de 367 millions de tonnes en 2020 [1]. Les plastiques jouent un rôle central dans notre vie quotidienne grâce à leurs nombreuses qualités (matériaux multifonctionnels, résistants, faciles à fabriquer et peu coûteux) et leur éloge fut fait par le français Roland Barthes dans son livre *Mythologies* « La hiérarchie des substances est abolie, une seule les remplace toutes : le monde entier peut être plastifié, et la vie elle-même, puisque, paraît-il, on commence à fabriquer des aortes en plastique ». Les plastiques sont des polymères de carbone facilement modelables à haute température et haute pression. Ils sont principalement produits à partir de carburants fossiles (pétrole, charbon, gaz naturels) mais peuvent également être fabriqués à partir de composés biosourcés tels que la cellulose et la lignine. A l'heure actuelle, les cinq plastiques les plus produits sont le polyéthylène haute ou basse densité (PEHD et PEBD), le polypropylène (PP), qui représentent à eux seuls 62 % de la demande mondiale [2], mais aussi le polystyrène (PS), le polychlorure de vinyle (PVC) et le polyéthylène téréphtalate (PET) pour une utilisation principalement dans les emballages (soit 40 % de la production globale) [1]. Cependant, l'utilisation massive des plastiques a conduit à une augmentation continue de la pollution environnementale à l'échelle mondiale. Les déchets plastiques sont transportés par les rivières, les ruisseaux, les marées, le vent et suite aux catastrophes naturelles mais aussi par les activités de pêche et d'aquaculture. Leur accumulation excessive a engendré la formation de plusieurs gyres piègeurs de déchets dont le plus connu reste le grand gyre du Pacifique nord, dénommé le septième continent. Ce sont 79 000 tonnes de déchets plastiques flottants et s'étalant sur 1,6 millions de kilomètres carrés (soit plus de

trois fois la taille de l'Espagne). Dans l'environnement, ces débris plastiques subissent une lente dégradation photochimique, physique et/ou biologique formant des petites particules plastiques, les microplastiques secondaires, par opposition aux microplastiques primaires produits intentionnellement par l'Homme pour des usages variés (produits cosmétiques—gommages, peintures...). Actuellement, il n'existe pas de consensus autour d'une définition précise des microplastiques [3] mais ils sont souvent caractérisés par des particules dont la taille varie entre 0,1 (ou 1) et 5000 µm [4]. Les microplastiques sont omniprésents dans l'environnement, aussi bien dans les eaux marines que souterraines, mais également dans les lacs, les sédiments, les sols et l'atmosphère [2]. Le devenir des plastiques constitue aujourd'hui un fort enjeu socio-économique, nécessitant des transformations majeures de l'économie au niveau mondial, comme l'ont récemment souligné Borrelle et ses collègues [5]. Face à cet enjeu, de nombreuses mesures et lois sont mises en place par les pouvoirs publics. La Commission Européenne dans sa stratégie de 2018 « the European Strategy for Plastics in a Circular Economy » [6] a souligné la nécessité d'une transition d'un schéma linéaire de « prendre-fabriquer-utiliser-jeter » à un circuit circulaire dans lequel les plastiques seraient continuellement réutilisés, recyclés et non plus rejetés directement dans l'environnement. Dans ce sens, la Commission a planifié l'interdiction de la production et de l'utilisation de dix types de plastiques à usage unique à partir de 2021 représentant 70 % des déchets plastiques maritimes [6]. De nombreuses autres actions ont été développées dans le monde pour lutter contre la pollution plastique, comme l'interdiction complète de l'utilisation, de la production et de la vente de sacs plastiques au Kenya depuis 2017, initiative suivie par de nombreux pays. Néanmoins, la crise sanitaire de la COVID-19 a confirmé la place centrale des plastiques dans nos vies. Klemeš et ses collègues ont mis en évidence la

demande accrue en plastique à destination des produits médicaux et des emballages au cours de la pandémie [7]. Ainsi, la ville du Wuhan, l'épicentre de la COVID-19 en Chine, a connu en mars 2020 une augmentation massive des déchets médicaux passant de 40-50 tonnes par jour à plus de 247 tonnes par jour [8]. Une situation similaire pourrait se produire dans l'industrie agroalimentaire, ou encore celle de l'emballage, du textile et de toutes autres activités qui ont différé leur stratégie d'avancement dans la lutte contre la pollution plastique [9]. L'utilisation des masques de protection à usage unique a également été ajoutée à la liste des sources potentielles de microplastiques [10].

L'impact de la pollution plastique sur l'environnement et sur les organismes est désormais bien établi [11]. Les particules plastiques entrent inévitablement dans la chaîne alimentaire et se retrouvent ingérées ou inhalées avec des effets probables sur la santé humaine, non établis à ce jour [12,13]. A peine abordée cinq ans auparavant, la question des microplastiques et de leurs risques sur la santé humaine fait aujourd'hui l'objet d'une attention grandissante de la part de la communauté scientifique mondiale. En termes d'exposition orale, peu d'études se focalisent sur le tractus gastro-intestinal, pourtant première barrière physique et biologique rencontrée [12,14–17]. Dans ce contexte, nous présentons dans un premier temps les données disponibles sur l'exposition orale humaine ainsi que les caractéristiques des microplastiques ingérés (origine, occurrence, taille, forme, type de polymère, propriétés de surface). Puis, nous exposons les résultats relatifs aux transformations physico-chimiques des microplastiques au cours de la digestion. Nous poursuivons sur l'impact potentiel des microplastiques sur la barrière intestinale à la fois sur des modèles *in vivo* (rongeurs) et *in vitro*, avec un accent particulier sur le triptyque épithélium/mucus/microbiote. Nous discutons enfin des futures directions de recherche et, plus particulièrement, sur le potentiel des modèles *in vitro* gastro-intestinaux humains afin de mieux évaluer les risques sanitaires liés aux microplastiques.

L'exposition humaine aux microplastiques

L'Homme peut être amené à ingérer chaque jour des microplastiques à travers l'alimentation, les boissons ou encore l'air *via* le mécanisme de clairance mucociliaire au niveau des poumons après inhalation [13,18]. À l'heure actuelle, de par le manque de méthodes standardisées, notamment sur les procédures d'échantillonnage et d'analyse, il est difficile d'évaluer l'exposition orale humaine aux microplastiques [18]. Dans un tel contexte, Schwabl et ses collègues ont été les premiers à rapporter la présence de microplastiques dans les selles de huit volontaires sains de différents pays (Japon, Russie, Pays-Bas, Royaume Uni, Italie, Pologne, Finlande et Autriche), avec en moyenne 20 particules pour 10 g de selle, composées de différents types de polymère (PP et PET étant les plus abondants) et de taille variable (entre 50 et 500 μm) [19]. La présence de microplastiques dans les selles humaines a ensuite été confirmée par d'autres études [20,21]. Des microplastiques ont également été retrouvés dans des échantillons de côlon humain, prélevés par colectomie chez 11 adultes, avec une moyenne de 331 particules en nombre par individu dont 50 % de polyamide (PA) et 40 %

de PP [22]. En 2019, une étude s'est intéressée à la quantification des microplastiques « consommés » par l'Homme *via* l'eau et l'alimentation et ce, en fonction du sexe et de l'âge (adultes et enfants) en considérant un régime américain standard. Ainsi, en moyenne, les enfants consomment 106 et 113 microplastiques en nombre par jour pour les filles et les garçons, respectivement alors que les femmes et les hommes en consomment 126 et 142, respectivement. Dans cette étude, les produits de la mer et les eaux en bouteille ont été identifiés parmi les contributeurs majeurs à l'exposition aux microplastiques, même si cette exposition peut varier grandement selon la localisation géographique et les habitudes alimentaires [23]. Plus récemment, Senathirajah et ses collègues ont été les premiers à donner une estimation en masse de la quantité de microplastiques ingérés, entre 0,1 et 5 g par semaine en fonction des scénarios d'exposition, ouvrant ainsi la voie à une meilleure évaluation des risques associés [24].

Des microplastiques dans les boissons

Les eaux en bouteille mais également du robinet contiennent des microplastiques provenant dans le premier cas des processus d'embouteillage et de dégradation des bouteilles plastiques alors que, dans le second cas, la pollution environnementale et la dégradation des tuyauteries en plastique seraient à l'origine de la contamination [25,26]. Les données quantitatives reportées dans les différentes études varient énormément selon les protocoles d'analyse appliqués. Les bouteilles en plastique contiennent néanmoins la plus grande quantité de microplastiques, pouvant par exemple s'élever à 325 particules par litre en moyenne alors qu'elle représente 5,45 particules par litre en moyenne pour l'eau du robinet [25,27]. Dans d'autres échantillons, un maximum de 4 889 et de 628 microplastiques par litre a été rapporté pour les eaux en bouteille et du robinet, respectivement. En considérant une consommation d'eau standard, cette voie d'exposition peut représenter pour un adulte une ingestion de 458 000 microplastiques *via* l'eau du robinet et 3 569 000 microplastiques avec des eaux en bouteille par an [28]. En termes de composition physico-chimique, de nombreux polymères différents ont été retrouvés dans les eaux potables mais les plus abondants restent le PP, le PET et le PE [28,29]. Les bouteilles d'eau contiennent principalement des fragments et des fibres de plastique de taille entre 6,5 et 100 μm [25] tandis que l'eau du robinet contient presque exclusivement des fibres de plastique dont la longueur peut varier entre 0,1 et 5 mm [27].

L'eau n'est pas la seule boisson contenant des microplastiques (Tableau 1). Ainsi, l'analyse de nombreux sodas, boissons énergisantes, thés glacés et bières, a révélé la présence de microplastiques pour 48 échantillons parmi 57 produits testés. Les quantités en présence peuvent atteindre $28 \pm 5,29$ particules par litre, incluant des particules de différents types de polymère (PE, PET...), formes (fibres et fragments), couleurs et tailles (0,1–3 mm) [30].

Des microplastiques dans les aliments

Outre les produits de la mer, incluant poissons et fruits de mer, de nombreux aliments comprenant des fruits et légumes, mais aussi des produits d'origine animale,

Tableau 1 Contamination en microplastiques de produits de consommation humaine : exemples de quelques aliments et boissons (en fonction des études, les chiffres sont rapportés de manière différente; ND : non détecté).

Produit	Contamination en microplastiques	Référence
Boissons		
Eau du robinet	5,45 particules/L en moyenne	[27]
	628 particules/L au maximum	[28]
Eau en bouteille	325 particules/L en moyenne	[25]
	4889 particules/L au maximum	[28]
Thés glacés	1 ± 0,57–6 ± 2 particules/L	[30]
Sodas	ND–7 ± 3,21 particules/L	[30]
Boissons énergisantes	ND–6 ± 1,53 particules/L	[30]
Bières	ND–28 ± 5,29 particules/L	[30]
	2–79 fibres, 12–109 fragments et 2–66 granules/L	[36]
Aliments		
Pommes	223 000 (médiane, 52 600–307 750) particules/g	[31]
Poires	176 500 (médiane, 98 325–302 250) particules/g	[31]
Brocolis	94 900 (médiane, 65 025–201 750) particules/g	[31]
Laitues	52 050 (médiane, 26 375–75 425) particules/g	[31]
Carottes	97 800 (médiane, 72 175–130 500) particules/g	[31]
		[31]
Bivalves	0,2–4 particules/g	[4]
Crevettes	0,75 particules/g	[4]
Poissons	1–7 particules/individu	[4]
Poulet	10,2 ± 13,8 particules/g gésier	[38]
Miel	40–660 fibres et 0–38 fragments/kg	[36]
Sucre	217 ± 123 fibres et 32 ± 7 fragments/kg	[36]
Sel	ND–13 500–19 800 particules/kg au maximum	[35]
Sardines en conserve et sprats	ND–de 1 à 3 particules selon la marque	[34]
Produits laitiers	6,5 ± 2,3 particules/L en moyenne	[37]

contiennent des microplastiques (Tableau 1). Une étude a montré une contamination s'élevant de 52 050 à 233 000 particules par gramme de produit, dépendant du type de légume ou de fruit considéré [31]. Cette contamination pourrait provenir de la pollution plastique des sols, menant à une absorption par les racines et à une translocation des particules dans les autres tissus. Les fruits sont en général plus contaminés que les légumes, la pomme étant le fruit le plus contaminé et la laitue le légume contenant le moins de microplastiques [31]. Par ailleurs, des microplastiques et/ou une fraction plutôt nano-dimensionnée (nanoplastiques) ont été détectés chez 201 espèces propres à la consommation humaine, dont 200 espèces marines et une espèce terrestre, le poulet [18]. Comme évoqué précédemment, les produits de mer sont particulièrement affectés avec en moyenne 0,2–4 particules/g chez les bivalves, 0,75 particules/g chez les crevettes et 1–7 particules/individu chez les poissons [4]. Même si les niveaux d'ingestion sont variables selon les travaux de la littérature, en fonction notamment de l'habitat, du comportement alimentaire et/ou du lieu géographique d'échantillonnage, il a été montré, par exemple, dans une étude réalisée au Portugal sur une zone estuarienne, que les poissons benthopélagiques (ici *Diplodus vulgaris*) peuvent avoir une occurrence de microplastiques plus importante que celle relevée chez les espèces démersales (ici *Dicentrarchus labrax* et *Platichthys flesus*), principalement sous forme de fibres en polyester, PP ou rayonne, de taille et couleur

variées [32]. De plus, parmi les espèces benthopélagiques, une étude menée en Chine a révélé que les poissons de mer (e.g. *Larimichthys crocea*) sont davantage contaminés par les microplastiques (en nombre de particules par individu) que les poissons d'eau douce (e.g. *Megalobrama amblycephala*) [33]. Cette contamination peut être généralisée puisque le miel (40–660 fibres ; 0–38 fragments/kg de miel), le sucre (217 ± 123 fibres et 32 ± 7 fragments/kg de sucre), le sel (0–19 800 particules/kg de sel) mais aussi les sardines en conserve et les sprats contiennent des microplastiques [34–36]. Pour d'autres aliments de consommation courante, les travaux sont plus limités. Toutefois, des microplastiques de différentes tailles (0,1–5 mm), formes (fibres et fragments) et couleurs ont été détectés (6,5 ± 2,3 particules par litre en moyenne) dans les produits laitiers [37]. Bien que les enjeux autour de la contamination des produits carnés aient été soulevés par certains auteurs [18], une seule étude à ce jour s'est intéressée à la contamination du poulet par des microplastiques (129,8 ± 82,3 et 10,2 ± 13,8 particules/g de selle et de gésier de poulet, respectivement) [38].

Des microplastiques provenant des emballages alimentaires

Une autre voie de contamination possible concerne les emballages alimentaires. Les microplastiques proviendraient des étapes de fabrication et d'emballage des différents aliments mais aussi directement des matériaux

utilisés. La simple action d'ouvrir un emballage plastique (récipients, sacs, bouchons, capsules...) en coupant avec un ciseau, un couteau ou encore *via* les systèmes d'ouverture facile peut générer entre 0,46–250 particules par cm en moyenne, selon les propriétés du plastique en question comme sa taille, sa densité, sa rigidité ou son épaisseur [39]. Comme énoncé ci-dessus, des microplastiques sont retrouvés dans des sodas, des boissons énergisantes, des thés glacés et des bières, qui semblent provenir des emballages et des textiles synthétiques utilisés [30]. De manière intéressante, il a été montré que les bouteilles en plastique libèrent des microplastiques (90 % des particules < 5 µm) à la surface des capsules en PEHD et des bouchons en PET à la suite d'une série d'ouvertures et de fermetures, l'abrasion différant très largement selon les marques [40]. Cependant, les bouteilles elles-mêmes ne sont pas source d'émission de microplastiques après l'application d'un stress mécanique par compression. Une autre étude révèle le relargage important de microplastiques (et également de nanoplastiques) dans l'eau par les sachets de thé plastifiés [41]. La composition physico-chimique de ces microplastiques correspond à celle des sachets de thé (nylon et PET), corroborant la source d'émission à l'emballage lui-même. De plus, les contenants en papier à usage unique, couramment utilisés pour le café et le thé, sont imperméabilisés à l'aide d'une fine couche de film plastique à leur surface, susceptible de se détériorer au contact d'un liquide chaud (85–90 °C) et de relarguer des microplastiques. Ainsi, il a été montré la libération de 25 000 microplastiques dans 100 mL de liquide en 15 minutes [42]. Concernant les populations vulnérables que représentent les enfants, les biberons en PP peuvent relarguer en moyenne 16 200 000 particules par litre au cours du processus de stérilisation et du contact avec un liquide chaud [43]. De la même manière, les tétines en caoutchouc libèrent des microplastiques (ainsi que des nanoplastiques) au cours de l'étape de stérilisation [44]. La présence de microplastiques a été montrée également pour plusieurs marques de lait. En termes de composition physico-chimique, le polyéthersulfone et le polysulfone ont été identifiés, ces deux polymères constituant les membranes de filtration utilisées au cours des transformations laitières [45]. De par leurs propriétés hydrophobes, les microplastiques sont capables de migrer des emballages alimentaires vers les aliments et notamment les aliments gras. Par ailleurs, une contamination aux microplastiques a été montrée dans du riz vendu dans des emballages plastiques avec un taux quatre fois plus important dans les riz instantanés que dans les riz crus [46]. Une étude récente a mis en évidence la présence de microplastiques (de 3 à 29 particules/contenant, majoritairement des fibres) dans des emballages alimentaires utilisés pour la vente à emporter et étant composés de PP, PS, PE ou PET. Les contenants en PS rugueux présentaient la plus forte contamination aux microplastiques, qui proviendrait principalement de la desquamation de la surface interne de ces contenants et/ou d'un dépôt par l'atmosphère ambiante dont l'importance varie selon le type de polymère [47] mais aussi du contenu (produits gras) et de la température [48]. Enfin, les sardines en conserve contiennent également des microplastiques (PET et fibres principalement) issus probablement des procédés de nettoyage et de mise en conserve [49]. En définitive, la pollution plastique

des emballages alimentaires peut représenter une ingestion additionnelle allant jusqu'à 203 microplastiques pour une personne consommant de 4 à 7 repas à emporter par semaine [47].

Le devenir et les transformations physico-chimiques des microplastiques au cours de la digestion

L'impact des microplastiques sur les fonctions digestives dépend à la fois de leurs propriétés physico-chimiques initiales (taille, distribution, forme, surface, fonctionnalisation, adsorption de contaminants chimiques et microbiens) et de l'évolution de ces propriétés suite à des changements de pH, de temps de transit, de potentiel d'oxydo-réduction tout au long de leur passage dans le tractus gastro-intestinal (TGI). La digestion est un processus en plusieurs étapes, dont des dégradations mécaniques et chimiques, au cours desquelles les aliments ingérés sont convertis en nutriments directement absorbés et assimilés par le corps humain pour assurer sa croissance, sa maintenance et le stockage d'énergie [50]. Après ingestion, les aliments sont fragmentés mécaniquement et chimiquement dans la bouche par l'action des dents et des enzymes salivaires (principalement la lipase et l'amylase). Par la suite, le bol alimentaire atteint l'estomac pour être soumis à une digestion acide par la pepsine et la lipase gastrique ainsi qu'à une dégradation mécanique par contractions et brassage. Le contenu gastrique (ou chyme) ainsi formé rejoint ensuite l'intestin afin de subir une digestion complémentaire par l'action combinée des acides biliaires, des enzymes intestinales sécrétées par les entérocytes et du jus pancréatique (contenant un cocktail d'amylases, de lipases, de protéases). L'intestin grêle est également le site principal d'absorption des nutriments. Les nutriments non absorbés restants atteignent le côlon, notamment les fibres alimentaires qui sont hydrolysées et fermentées par les communautés microbiennes résidentes, encore appelées le microbiote intestinal (voir la section dédiée ci-dessous).

Dans un tel contexte, connaître les transformations physico-chimiques des microplastiques au cours de la digestion est un élément clé pour déterminer leur impact sur la physiologie intestinale. À l'heure actuelle, les études sont rares et principalement centrées sur des approches *in vitro* exposant les microplastiques à des fluides digestifs simulés. Stock et ses collègues ont démontré, par l'utilisation d'un modèle *in vitro* en trois compartiments reproduisant les phases salivaire, gastrique et intestinale de la digestion humaine, que les fluides digestifs n'étaient pas capables de modifier la forme ou la taille de cinq types de microplastiques (PE, PP, PVC, PET et PS) [51]. De même, dans un modèle *in vitro* de digestion dynamique humaine mimant la bouche, l'estomac et l'intestin grêle, Liu et ses collègues n'ont montré aucune altération de la composition physico-chimique de microplastiques de PS mais décrivent plutôt la formation d'une couronne biomoléculaire à la surface des particules [52]. Composée de molécules organiques adsorbées en surface au cours de la digestion (protéines, mucines et lipides), cette couronne biomoléculaire reste encore peu

étudiée, mais du fait de son influence sur les propriétés physico-chimiques telles que l'hydrophobie et la charge de surface [52], son rôle semble essentiel pour une meilleure compréhension du comportement des microplastiques dans le TGI. Dans leur étude sur des microplastiques de PET, Tamargo et ses collègues ont récemment confirmé la formation d'un dépôt de matière organique et cristalline après l'application d'un protocole de digestion *in vitro* standardisé (selon la procédure INFOGEST) reproduisant les phases salivaire, gastrique et intestinale [78].

De manière très intéressante, une étude réalisée dans un système de digestion *in vitro* reproduisant les principaux processus de la digestion humaine (bouche, estomac et intestin grêle) a révélé la capacité de cinq types de microplastiques (PS, PET, PE, PVC et acide polylactique-co-glycolique) de diminuer l'efficacité de digestion des lipides [53]. Il a ainsi été montré que l'exposition aux particules de PS induisait le taux d'inhibition le plus élevé en réduisant la biodisponibilité des gouttelettes lipidiques *via* la formation de larges hétéro-agrégats lipidiques/microplastiques ainsi qu'en induisant des altérations de la structure secondaire et de l'activité des lipases suite à leur adsorption sur les particules plastiques [53].

L'impact des microplastiques sur la barrière intestinale

La barrière intestinale représente un rempart à la fois physique, chimique et biologique pour la protection de l'hôte incluant, sur son versant luminal, l'épithélium, le mucus, le microbiote, considérés isolément ou en interaction. Les résultats traitant de l'impact des microplastiques sur ce triptyque sont résumés dans les paragraphes suivants.

Les microplastiques et l'épithélium intestinal

Les caractéristiques et fonctions de l'épithélium intestinal

La fonction principale du TGI est la digestion et l'absorption de l'eau et des nutriments *via* l'épithélium intestinal agissant ici comme un filtre sélectif contre les pathogènes et les antigènes alimentaires [54]. La relation entre les microplastiques (mais également les nanoplastiques), la muqueuse intestinale et le système immunitaire ayant été récemment illustrée [15], ces aspects ne seront pas détaillés dans la présente revue.

L'épithélium intestinal est composé d'une monocouche de cellules organisées en cryptes et en villosités, permettant une augmentation considérable de la surface d'absorption de l'intestin grêle. Bien que les structures de l'intestin grêle et du côlon soient très similaires au sens histologique, ce dernier est dépourvu de villosités. De nombreux types cellulaires ont été décrits au niveau intestinal, comme les cellules absorbatives (entérocytes/colonocytes) et différenciées (cellules calciformes, cellules de Paneth, cellules entéro-endocrines et cellules tuft) mais aussi les cellules M spécialisées dans la capture d'antigènes et d'agents pathogènes pour présentation aux cellules immunitaires surmontant les plaques de Peyer. Les entérocytes/colonocytes restent cependant le type cellulaire le plus représenté

permettant l'absorption des nutriments et de l'eau dans l'intestin grêle et le côlon. En termes de cellules sécrétrices, ce sont les cellules calciformes les plus abondantes assurant la sécrétion de mucus pour une meilleure fonction de protection (voir section ci-dessous). Les cellules de Paneth sont, elles, spécifiquement localisées dans l'intestin grêle et protègent l'hôte de l'invasion de pathogènes tout en modelant la composition du microbiote commensal en sécrétant de nombreux peptides antimicrobiens [55]. L'intégrité épithéliale et la perméabilité intestinale sont régulées par un ensemble de protéines adhésives, telles que les jonctions serrées, les desmosomes et les jonctions adhérentes. Les jonctions serrées entourent les extrémités apicales des membranes latérales des cellules épithéliales et régulent la perméabilité paracellulaire sélective vis-à-vis des solutés [56]. Ce sont des protéines complexes, composées de quatre types de protéines transmembranaires dont les occludines, les claudines (famille regroupant 24 membres), les molécules d'adhésion jonctionnelle (JAM pour « junctional adhesion molecule »), les tricellulines et de nombreuses protéines cytosoliques. Le domaine intracellulaire de ces protéines transmembranaires interagit avec des protéines cytosoliques, telles que les protéines *zonula occludens*, assurant leur ancrage à l'anneau péri-jonctionnel d'actomyosine [57].

L'impact des microplastiques sur la barrière épithéliale intestinale

De nombreuses études s'intéressant à l'absorption, au devenir et aux effets des microplastiques sur la barrière épithéliale intestinale ont été réalisées sur des modèles cellulaires *in vitro* basés sur les cellules épithéliales Caco-2. Cependant, les évolutions actuelles tendent à promouvoir des modèles cellulaires plus sophistiqués, combinant plusieurs types cellulaires comme les cellules M [51,58,59] et/ou des cellules sécrétrices de mucus, avec la lignée HT29-MTX le plus fréquemment. Les questions relatives à l'influence des fluides digestifs simulés (transformation physico-chimique des microplastiques, formation d'une couronne biomoléculaire, voir section précédente) sont de plus en plus abordées, notamment pour évaluer les conséquences sur l'absorption et la toxicité des microplastiques [52]. De manière intéressante, certaines études proposent d'évaluer l'effet toxicologique des microplastiques sur un système « sensibilisé » en comparant des conditions d'exposition physiologiques et pro-inflammatoires [60].

La lignée cellulaire Caco-2, initialement isolée d'un adénocarcinome humain, a la particularité, une fois à confluence, de se différencier spontanément en une monocouche d'entérocytes polarisés comprenant des microvillosités, des transporteurs membranaires ainsi que les enzymes de la bordure en brosse. Il a été montré que les microplastiques peuvent fragiliser la barrière épithéliale intestinale, comme illustré par la diminution de la résistance électrique transépithéliale (i.e. résistance ohmique de la couche cellulaire permettant de caractériser la perméabilité intestinale) après exposition [59]. Sur ce même modèle, une concentration plus élevée de microbilles de PS (de 10^{-8} à 10^{-1} mg/mL) pendant 24 à 48 heures a induit une augmentation de la cytotoxicité, restant cependant

très modérée et non significative (10 % de perte de viabilité au maximum) [61]. Stock et ses collègues ont, quant à eux, étudié l'absorption de microplastiques de PS de trois tailles différentes (1, 4 et 10 μm), toujours sur une monoculture de cellules Caco-2 lors d'une exposition aiguë de 24 heures [62]. La plus forte absorption (d'environ 3 %) a été observée pour les particules de 4 μm . Fait intéressant, l'éthanol, qui agit comme un agent « dilatateur » des jonctions serrées, facilite le passage des particules à travers l'épithélium [59]. La réduction à la fois de la cytotoxicité et de l'altération de la fonction de transport, observée chez les cellules Caco-2 après exposition aux microplastiques de PS préalablement digérés *in vitro* (plus particulièrement pour les petites particules de 0,1 μm par rapport à leurs homologues plus larges de 5 μm), pourrait être expliquée par la formation d'une couronne biomoléculaire à la surface des particules [52], comme détaillé précédemment dans la section « Le devenir et les transformations physico-chimiques des microplastiques au cours de la digestion ».

Les microplastiques et le mucus intestinal

Les caractéristiques et fonctions du mucus intestinal

Le mucus est un gel viscoélastique composé principalement de glycoprotéines (les mucines, principalement MUC2 dans l'intestin grêle et le côlon chez l'Homme), qui tapisse et protège l'épithélium intestinal. Il fonctionne comme une barrière dynamique, perméable aux gaz, à l'eau et aux nutriments mais imperméable à la plupart des microorganismes et assure une lubrification de l'intestin, indispensable au transit intestinal [63,64]. L'intestin grêle se compose d'une seule couche de mucus discontinue tandis que le mucus colique s'organise, lui, en deux couches dont une couche interne, longtemps décrite comme fermement attachée à l'épithélium et dépourvue de bactéries et une couche externe, plus lâche, abritant les bactéries [65]. Une étude de Kamphuis et ses collègues chez le rongeur a proposé de revisiter ce modèle de structure bi-couche du mucus en montrant que ce dernier, au niveau du côlon distal, sert à recouvrir les fèces plutôt que l'épithélium [66]. Cette couche de mucus fécale permet alors de confiner le microbiote intestinal. Plus récemment, Bergstrom et ses collègues ont enrichi ce nouveau modèle au niveau du côlon proximal en établissant que, dans cette région, le mucus joue à la fois le rôle de niche pour certaines bactéries et de barrière primaire encapsulant le matériel fécal d'une première couche, renforcée ensuite par une seconde produite au niveau du côlon distal [67]. Il faut enfin souligner que le mucus constitue une barrière mais également une cible face aux polluants environnementaux (métaux lourds, pesticides, polluants organiques persistants), aux additifs alimentaires (émulsifiants, nanomatériaux) et aux pathogènes entériques, avec des relations bidirectionnelles [64,68]. Dans le cas des xénobiotiques, du fait de ses propriétés d'exclusion selon la taille et la charge, le mucus peut piéger ces xénobiotiques et ainsi diminuer leur toxicité. Inversement, ces derniers peuvent modifier les caractéristiques du mucus (épaisseur, expression et composition des mucines) tout au long du TGI, menant à des effets potentiellement néfastes pour l'homéostasie intestinale.

Les relations bidirectionnelles entre les microplastiques et le mucus intestinal

À l'heure actuelle, peu d'études ont évalué les interactions entre les microplastiques et le mucus intestinal. Au niveau des approches *in vitro*, afin de s'affranchir des limites de la lignée cellulaire Caco-2 ne produisant pas de mucus, des cocultures de cellules Caco-2/HT29-MTX ont été développées afin de se doter d'un modèle physiologiquement plus représentatif de la barrière intestinale [69]. En comparant l'exposition à des microplastiques de PS (1, 4 et 10 μm) d'une monoculture de cellules Caco-2 à celle d'une coculture de cellules Caco-2/HT29-MTX, Stock et ses collègues ont trouvé, de manière inattendue, une plus forte absorption des microplastiques de 1 et 4 μm dans la coculture par rapport à la monoculture [62]. Cependant, il est à noter que de nombreuses études ont mis en évidence la présence d'îlots de mucus isolés, donc potentiellement moins « protecteurs », à la surface des cocultures de cellules Caco-2/HT29-MTX plutôt qu'une couche uniforme [70] pourtant retrouvée dans d'autres études [71]. Ces disparités soulignent la nécessité de s'intéresser aux caractéristiques du mucus lorsque les cellules HT29-MTX sont employées pour les études toxicologiques *in vitro* sur les microplastiques. D'autres développements sont décrits dans la littérature, comme les modèles *in vitro* tridimensionnels de cellules intestinales Caco-2 et HT29-MTX en combinaison avec des cellules immunitaires (macrophages dérivés de monocytes de sang humain et de cellules dendritiques), afin d'évaluer l'impact de différents microplastiques (PP, PA et polyuréthanes, 50-500 μm) après 6, 24 et 48 heures en administration par voie aérosol afin d'éviter le flottement des particules dans le milieu de culture cellulaire [72]. Aucun effet délétère pour l'ensemble des microplastiques et des conditions d'exposition testés n'a été observé en termes de cytotoxicité, d'intégrité de la barrière intestinale et de réponse inflammatoire. Des études récentes chez la souris ont toutefois mis en évidence un impact négatif des microplastiques sur le mucus intestinal. En effet, après une exposition à des microplastiques de PS (0,5, 5 et 50 μm) à 100 et 1000 $\mu\text{g}/\text{L}$ dans l'eau de boisson pendant plusieurs semaines, la sécrétion de mucus au niveau du côlon était significativement diminuée, de manière corrélée à l'inhibition de l'expression de certains gènes impliqués dans les voies de biosynthèse et de sécrétion de mucus (*Muc2*, *Muc1* et *Klf4*) [73,74].

Les microplastiques et le microbiote intestinal

Les caractéristiques et fonctions du microbiote intestinal

Au cours de leur transit dans le TGI, les microplastiques sont au contact de la vaste communauté de microorganismes, composée de 100 milliards de bactéries, mycètes, archées et virus et appelée le microbiote intestinal. Ces populations se distribuent tout le long de l'intestin à la fois transversalement et longitudinalement selon le microenvironnement immédiat (pH, gradient d'oxygène, présence de peptides antimicrobiens, temps de transit...). Chaque individu abrite un microbiote intestinal unique dont la

densité la plus importante se trouve dans le côlon. Il est principalement composé de bactéries appartenant aux phyla Bacteroidetes, Firmicutes, Actinobacteria et, dans une moindre mesure, Proteobacteria et Verrucomicrobia [75]. L'écosystème microbien joue un rôle majeur dans l'homéostasie intestinale et pour la santé de l'hôte en assurant la digestion et l'assimilation de certains nutriments alimentaires non digestibles comme les fibres, menant à la production de produits fermentaires (gaz et acides gras à chaîne courte), véritables médiateurs métaboliques et immunitaires. Le microbiote intestinal est également impliqué dans l'immunité et la protection contre les pathogènes [76]. Depuis un certain nombre d'années, une attention grandissante est portée aux interactions entre le microbiote intestinal et le mucus. Ce dernier, au niveau du côlon, est un habitat naturel pour les bactéries commensales, leur apportant à la fois une source énergétique et des sites de fixation [77] via les glycanes des mucines [78]. De nombreuses bactéries mucolytiques ont ainsi été décrites, telles que *Akkermansia muciniphila*, *Bacteroides thetaiotaomicron*, *Bacteroides fragilis*, *Ruminococcus gnavus* et *Ruminococcus torques* [79]. Réciproquement, les bactéries commensales peuvent façonner le mucus et ses propriétés de barrière, comme démontré chez la souris [80]. De plus, les interactions entre le microbiote intestinal et les xénobiotiques sont de plus en plus reconnues comme un facteur clé dans la santé de l'hôte. Le microbiote intestinal a la capacité de métaboliser certains xénobiotiques apportés par l'alimentation et ainsi moduler leur mode d'action et leur toxicité. De manière concomitante, ces composés peuvent altérer la composition et/ou les fonctions métaboliques du microbiote intestinal [81].

Les relations bidirectionnelles entre les microplastiques et le microbiote intestinal

À l'heure actuelle, une seule étude s'est intéressée à l'impact des microplastiques sur le microbiote intestinal humain par une approche *in vitro* [82]. Des microplastiques de PET, digérés au préalable, ont été introduits à une dose unique de 0,166 g dans le modèle *in vitro* colique Simgi®, reproduisant les trois compartiments du côlon (ascendant, transverse et descendant), et inoculé à l'aide des selles d'un volontaire sain (expérience répétée avec un second donneur). La composition du microbiote intestinal a été analysée par séquençage de l'ARNr 16S sur 72 h à différents temps de prélèvement: 0, 24, 48 et 72 h. Une diminution (non significative) de la diversité α a été rapportée dans le côlon transverse et descendant chez le premier donneur et dans le côlon ascendant pour le second, comparativement à la condition contrôle. Selon le compartiment et le donneur, des modifications de la composition du microbiote intestinal ont été mises en évidence. Chez le premier donneur, tandis que l'abondance relative des Desulfobacterota augmente et celle des Bacteroidetes diminue dans les trois compartiments coliques, l'abondance des Firmicutes augmente dans le côlon ascendant. La population des Synergistetes augmente à la fois dans le côlon transverse et descendant tandis que celle des Proteobacteria augmente uniquement dans

le côlon descendant. Pour le second donneur, la proportion des Firmicutes augmente tandis que celle des Bacteroidetes diminue également dans l'ensemble des compartiments. La population des Desulfobacterota augmente cependant uniquement dans le côlon ascendant. La proportion des Proteobacteria est pour ce donneur diminuée au niveau du côlon ascendant et transverse. Chez le rongeur, les résultats décrits à ce jour portent exclusivement sur le modèle murin et montrent une altération de la composition du microbiote intestinal après exposition aux microplastiques. Des modifications ont ainsi été rapportées chez la souris dans le cas de l'administration de microplastiques de PS (0,5, 5 et 50 μm) via l'eau de boisson (100 et 1000 $\mu\text{g/L}$) pendant plusieurs semaines. Une réduction des indices de diversités α et β ainsi qu'une diminution significative de l'abondance caecale des Firmicutes, Actinobacteria et α -, β - et γ -Proteobacteria, évaluée par PCR quantitative, ont été mises en évidence. À cela s'ajoute, après analyse par séquençage de l'ARNr 16S, une diminution de l'abondance relative des Firmicutes, Actinobacteria, Bacteroidetes et Verrucomicrobia, en parallèle d'une augmentation de la proportion des Proteobacteria [73,74]. Au niveau du genre, toujours dans le contenu caecal, une diminution de *Bifidobacterium*, *Prevotella*, et *Veillonella*, accompagnée d'une augmentation de *Coproccoccus* et *Anaeroplasma*, a été décrite. Une autre étude, par analyse de séquençage de l'ARNr 16S, a révélé une augmentation de l'abondance et de la diversité du microbiote intestinal après exposition à des microplastiques de PE (10–150 μm) à 6, 60 et 600 $\mu\text{g/jour}$ pendant 5 semaines [83].

Un autre point majeur concerne la capacité du microbiote intestinal à dégrader les microplastiques, qui chez les mammifères reste largement méconnue. Cependant, chez le ver de terre, certains microorganismes du microbiote intestinal aptes à dégrader des particules de plastique ont été identifiés. À ceci s'ajoutent deux souches bactériennes, *Enterobacter asburiae* Y1 et *Bacillus* spp. YP1, présentes dans l'intestin des larves *Plodia interpunctella* ayant la capacité d'altérer le PE, comme le montrent la formation de trous et de cavités à la surface du polymère ainsi que l'addition de groupements carbonyle [84]. A l'heure actuelle, seule l'étude de Tamargo et ses collègues a abordé ce point, mais de manière encore très parcellaire. Une amorphisation progressive de la surface des microplastiques de PET (i.e. perte de cristallinité), essentiellement lors de la phase de fermentation colique, a été démontrée par spectroscopie Raman. Par ailleurs, des observations par microscopie électronique à balayage ont révélé la présence de bactéries adhérentes à la surface des particules, sans établir le lien (peut-être via des activités enzymatiques bactériennes de dégradation du PET) avec les modifications structurales mises en évidence au niveau du polymère [82]. La comparaison entre les bactéries capables de dégrader les microplastiques [85] et celles résidant dans le TGI humain, selon la base de données de Plaza Oñate et de son équipe [86], montre toutefois certains recouvrements de bactéries appartenant aux phyla Proteobacteria, Actinobacteria, Bacteroidetes et Firmicutes, ce qui méritera de futures investigations.

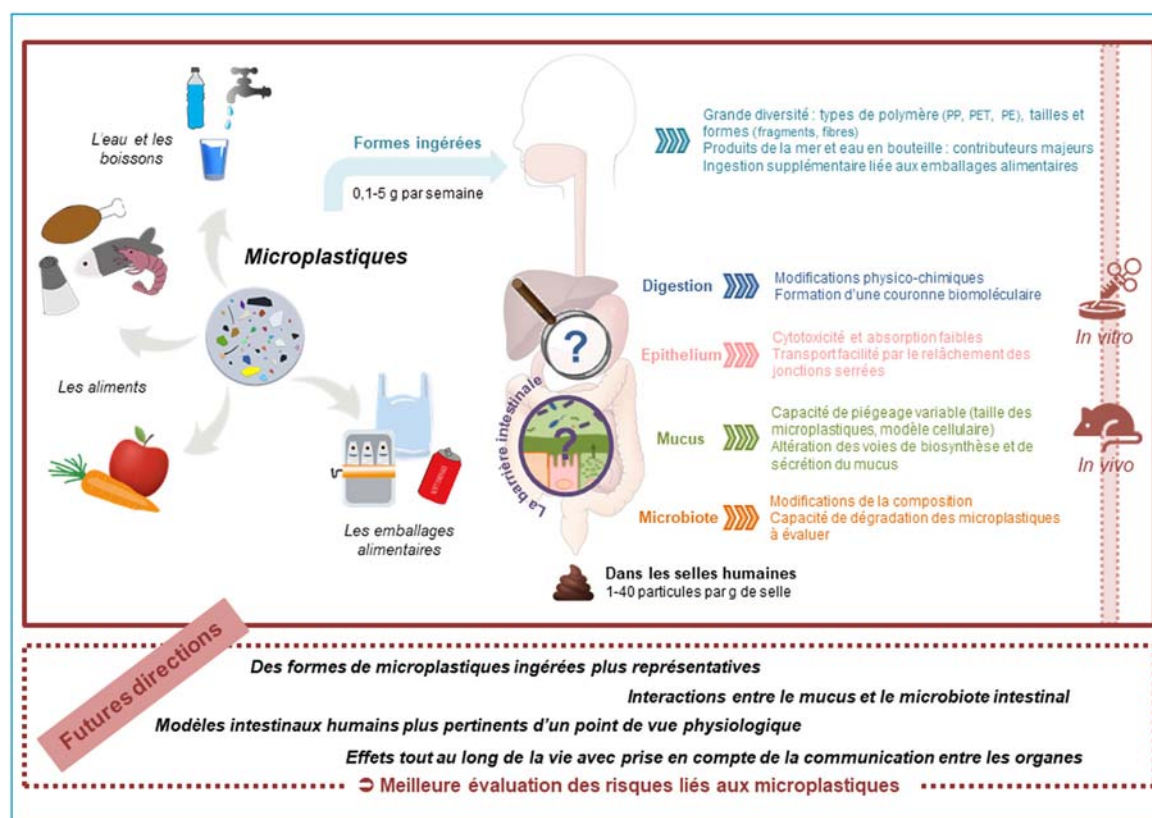


Figure 1. Représentation schématique de l'impact des microplastiques sur la santé intestinale : de l'occurrence (produits alimentaires, eau potable, boissons, emballages) à leur devenir et leur impact dans le tractus gastro-intestinal après exposition orale, notamment sur la digestion et les interactions avec les protagonistes de la barrière intestinale (épithélium, mucus et microbiote). Les principaux résultats de la littérature concernant les caractéristiques et les effets des microplastiques sont ici résumés. Certaines orientations pour les investigations futures sont également proposées.

Conclusions et futures directions pour la recherche sur les microplastiques en santé intestinale

Malgré un intérêt majeur en santé humaine, les effets potentiellement délétères des microplastiques sur la structure et les fonctions du TGI restent largement méconnus [16,17]. La Fig. 1 schématise les enjeux autour des microplastiques et résume les principales avancées décrites à ce jour, de leur occurrence dans les aliments, l'eau, les boissons et les emballages jusqu'à leur devenir dans le TGI (digestion et interactions avec la barrière intestinale, avec une attention particulière accordée à l'épithélium, au mucus et au microbiote intestinal). Certaines pistes pour des investigations futures sont également proposées ci-dessous.

Des formes de microplastiques plus représentatives de celles ingérées sont nécessaires, présentant probablement des différences de caractéristiques physico-chimiques, comportements et toxicité dans le TGI

Du fait de leur disponibilité et de leur facilité d'utilisation, la plupart des études jusqu'ici ont été réalisées sur des

microbilles de PS vierges, bien que ce polymère ne représente que 6 % de la production totale de plastique [1]. La forme sphérique n'est également pas complètement représentative des formes ingérées (fibres, fragments...). Dans ce cadre, de nouvelles études sont nécessaires pour évaluer les effets de microplastiques rencontrés dans la vie « réelle » et dotés de propriétés physico-chimiques spécifiques (type de polymère, forme, distribution de taille, etc.). À ceci devrait s'ajouter le développement de méthodes standardisées (dont l'échantillonnage et le traitement des échantillons) afin de détecter et de quantifier les microplastiques dans des milieux aussi complexes que les matrices et tissus biologiques, ce qui représente toujours à l'heure actuelle un verrou technologique majeur [18,87]. En outre, si les microplastiques vierges peuvent mimer une source directe d'exposition humaine, les formes « vieilles » sont également nécessaires à la simulation plus réaliste d'une exposition environnementale. Le processus de vieillissement des microplastiques mène, en effet, à de profondes modifications physico-chimiques dues à l'oxydation, à la fragmentation et/ou à la dégradation (photo-oxxydation, hydrolyse, abrasion mécanique, possible biodégradation par les microorganismes) mais aussi à la formation d'une couronne biomoléculaire en surface. Il a été décrit que la capacité d'absorption des microplastiques vieillis est significativement supérieure à celle des matériaux vierges [88]. Enfin, la capacité des microplastiques à agir comme des

vecteurs de contaminants chimiques et microbiens, comme les pathogènes (hypothèse du « cheval de Troie »), doit également être prise en compte [89].

Des modèles intestinaux plus représentatifs doivent être explorés pour étudier les conséquences d'une exposition orale humaine

Puisque les études directes chez l'Homme restent limitées pour des raisons évidentes en termes d'éthique, de réglementation et de coût, la grande majorité des recherches actuelles sur l'impact des microplastiques sur la santé intestinale chez les mammifères se focalisent sur des modèles *in vivo*, principalement chez la souris. Cependant ces études ne peuvent pas répondre à l'ensemble des questions soulevées, en raison de différences inter-espèces majeures au niveau de l'intestin (en termes de physiologie digestive et de microbiote intestinal, notamment). Certains modèles intestinaux humains ont ainsi été développés en guise d'alternative, comme les systèmes de digestion artificielle [16]. Ces dispositifs, souvent très simples, fonctionnent généralement en conditions statiques et sont uniquement composés de différents réservoirs reproduisant les principaux compartiments du TGI (sucs digestifs avec ajustement du pH, caractéristiques physico-chimiques du compartiment intestinal...). Des modèles physiologiquement plus représentatifs pourraient être pertinents pour une meilleure compréhension du devenir et des effets des microplastiques dans l'environnement digestif humain. Ainsi, le système gastro-intestinal TNO 1 (TIM-1) représente le simulateur de la partie haute du TGI le plus complexe à l'heure actuelle, reproduisant les principaux paramètres physico-chimiques de l'estomac et de l'intestin grêle (température, cinétique du pH gastrique et intestinal, temps de transit, sécrétions digestives de l'estomac, du pancréas et sécrétions biliaires, absorption passive des nutriments et de l'eau) [90,91]. Le TIM-1 pourrait être utilisé pour évaluer l'influence de la matrice alimentaire (comme par exemple les matrices riches en lipides) sur les transformations physico-chimiques spatio-temporelles des microplastiques, sur la dégradation et/ou la bioaccessibilité des contaminants chimiques associés, mais aussi sur la survie et la virulence des pathogènes potentiellement adhérents à la surface des particules de plastique. De plus, les modèles de la partie basse du TGI pourraient être mis à profit pour évaluer les interactions bidirectionnelles entre les microplastiques et le microbiote intestinal d'origine humaine [92]. Les effets d'une telle exposition seraient suivis au niveau de la composition et de l'activité métabolique du microbiote intestinal (production de gaz et d'acides gras à chaîne courte). Inversement, il serait possible d'étudier les capacités de dégradation et de métabolisation des microplastiques par le microbiote intestinal *via* notamment l'activation de voies métaboliques microbiennes spécifiques. Les variabilités interindividuelles pourraient également être appréhendées en inoculant les bioréacteurs avec des échantillons fécaux de différents donneurs (sexe, âge, régime alimentaire).

Les microplastiques pourraient compromettre l'homéostasie de la barrière intestinale en influençant le dialogue entre le mucus et le microbiote intestinal

Un autre challenge dans la compréhension de la toxicité intestinale des microplastiques est d'aller au-delà des interactions bidirectionnelles microplastiques/mucus d'un côté et microplastiques/microbiote de l'autre, en prenant en compte également le dialogue entre ces protagonistes 'mucus' et 'microbiote', intimement liés pour assurer le maintien de l'homéostasie intestinale. Pour répondre à cette question, toujours à l'échelle *in vitro*, il serait pertinent d'employer des modèles de la partie basse du TGI incorporant l'environnement mucosal. Ces études pourraient être envisagées dans un modèle mono-compartimenté simulant les principaux paramètres physico-chimiques et microbiens du côlon humain, comme le modèle artificiel du côlon (M-ARCOL), incorporant le mucus et le microbiote associé dans un compartiment spécifique contenant des billes de mucine et d'alginate [93]. Afin d'augmenter la complexité et la pertinence physiologique, un système multi-compartimenté reproduisant la totalité du TGI (estomac, intestin grêle et les trois parties du côlon), le simulateur de l'écosystème intestinal microbien humain (M-SHIME), incorporant des microcosmes recouverts de mucine et d'agar, pourrait être mis en œuvre [92].

Les microplastiques en conditions réalistes d'exposition par voie orale peuvent impacter la santé intestinale tout au long de la vie et influencer la communication inter-organes

Les connaissances sur les risques potentiels des microplastiques sur la santé humaine devront être approfondies selon des scénarios d'exposition réalistes (exposition chronique, doses de microplastiques utilisées) [87]. Il serait aussi important d'évaluer les conséquences toxicologiques sur des populations à risque (enfants, personnes âgées) pour lesquelles, là-encore, l'utilisation de modèles *in vitro* permettrait d'éliminer certaines contraintes éthiques et techniques [94,95]. Certaines conditions physiopathologiques (par exemple, patients obèses ou patients souffrant du syndrome de l'intestin irritable) pourraient également être simulées. De manière intéressante, ces populations sensibles se caractérisent déjà par une barrière muco-sale et épithéliale altérée ainsi que par une dysbiose du microbiote intestinal [96]. Enfin, l'interaction avec le système immunitaire mais également la communication inter-organes chez l'Homme (axe intestin-foie, axe intestin-poumons) pourraient être investiguées, en couplant les échantillons des modèles *in vitro* intestinaux avec des (co)cultures cellulaires de cellules intestinales, immunitaires et/ou extra-digestives.

Déclaration de liens d'intérêts

Les auteurs déclarent ne pas avoir de lien d'intérêt.

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Elora FOURNIER - Fate of microplastics in human digestive *in vitro* environment and study of the dialogue between epithelium, microbiota and mucus.

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Over the last half-century, plastic production has exploded resulting in environmental pollution. Accumulated plastics can be degraded into smaller pieces known as microplastics (MPs) found everywhere, and especially in the food chain. MPs have been detected in human feces and colon biopsies attesting of their transit through the gastro-intestinal tract (GIT). However, to date, little is known about the fate and potential effects of MPs in the human digestive sphere, particularly in the infant at-risk population. During their GIT travel, MPs can interact with physico-chemical digestive parameters, as well as gut microbiota, intestinal epithelium and its covering mucus layer. This joint PhD between MEDIS (Clermont-Ferrand) and Toxalim (Toulouse) aimed to investigate the fate of virgin MPs of polyethylene (PE), the most manufactured plastic polymer worldwide, in the adult and infant gut. This was studied using an original *in vitro* approach combining a human colon model and intestinal cells in culture. This doctoral research started with evaluating the impact of various stool conservative methods (48-h freezing -80°C, 48-h freezing -80°C with glycerol or lyophilization with maltodextrin/trehalose) on gut microbiota composition and activity in the Mucosal Artificial Colon (M-ARCOL), set-up under adult conditions. We showed that inoculating with raw frozen stool (48-h at -80°C) was the best option among those tested compared to fresh stools. Next, the M-ARCOL model was adapted to the specific toddler conditions in a new configuration termed Tm-ARCOL. This adaptation allowed to reproduce *in vitro* the main particularities associated to infant microbiota, such as lower bacterial diversity and higher abundance of *Bifidobacteriaceae*, together with higher concentrations of acetate and lower amounts of propionate and butyrate, as compared to adults. Then, we assessed the effects of a chronic ingestion of PE MPs on adult and infant microbiota using M-ARCOL and Tm-ARCOL, respectively, as well as the indirect impact of gut microbe metabolites after PE MP exposure on the intestinal barrier simulated by a co-culture of Caco-2 and mucus-secreting HT29-MTX cells. Interestingly, the effect of PE MP exposure on the gut microbiota was donor-dependent and resulted in an increase in potential pathobionts, like *Enterobacteriaceae*, regardless of age conditions. Regarding the activity of the gut microbiota, we showed for the first time that PE MP exposure was associated with a significant decrease in butyrate production in infant conditions, while skatole production increased significantly in adults. In contrast, no significant impact of PE MPs on the intestinal barrier, as mediated by changes in gut microbial metabolites, was evidenced. This PhD work provided pioneering and significant insights into the interactions of PE MPs with the adult and infant human gut microbiota and the intestinal barrier, filling some of the gaps in knowledge on MP behavior in the human GIT. This work contributes to a better understanding of MP health risk assessment for public policies. In the near future, our *in vitro* approach could be used for further investigations on more representative MP types (forms, sizes, aged and/or contaminated particles), in healthy but also disturbed situations including an altered gut barrier and dysbiotic microbiota (e.g. obesity, inflammatory bowel syndrome or inflammatory bowel diseases).

Keywords: Microplastics, polyethylene, *in vitro* gut models, human gut microbiota, intestinal cell model, fecal sample, preservation method, infant, mucus, toxicology

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