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Impact of polyphenols and feeding rhythms on the immunomodulation properties of the probiotic bacteria in the gastro-intestinal tract

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**PHD THESIS FROM THE UNIVERSITY OF BURGUNDY FRANCHE-COMTE PREPARED AT
THE UNIVERSITY OF BURGUNDY, DIJON**

Doctoral School n°554 Doctoral School E2S – Environment – Health

PhD in Microbiology

By

Ms. AL TARRAF Alissar

Impact of polyphenols and feeding rhythms on the immunomodulation properties of the
probiotic bacteria in the gastro-intestinal tract

Thesis presented and defended in Dijon, the 24th of June 2021.

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**THESE DE DOCTORAT DE L'ETABLISSEMENT UNIVERSITE DE BOURGOGNE
FRANCHE-COMTE PREPAREE A L'UNIVERSITE DE BOURGOGNE, DIJON**

Ecole doctorale n°554 Ecole doctorale E2S - Environnements – Santé

Doctorat de Microbiologie

Par

M^{lle} AL TARRAF Alissar

Impact des polyphénols et des rythmes d'alimentation sur les propriétés d'Immunomodulation
des bactéries probiotiques dans le tractus gastro-intestinal

Thèse présentée et soutenue à Dijon, le 24 juin 2021.

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DEDICATION

To my mom, dad, brothers, auntie & my future husband.

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What I have done is worthy of nothing but silence and forgetfulness, but what God has done for me is worthy of everlasting and thankful memory. I have always sensed your presence around me through my whole life journey and I have no way to express my real feelings but through my forever silent prayers and late-night talks to you ... thank you for the magical life coincidences you continuously offer me... Al Hamdulillah.

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LIST OF ABBREVIATIONS

A

AJ: Adherens junctions

AS: Atherosclerosis

AMP: Antimicrobial peptide

AMPK: adenosine monophosphate-activated protein kinase

AKT: RAC-alpha serine/threonine-protein kinase

AAD: Antibiotic-Associated Diarrhea

AA: Amino Acid

ANSM: Agence Nationale de Sécurité du Médicament

Ad-lib: Ad Libitum

A.M: After midday

B

BCR: B-cell receptor

BCAA: branched-chain amino acid.

BCFA: Branched-chain fatty acids

C

CMV: Cucumber mosaic virus

CO₂: Carbon Dioxide

°C: degree Celsius

CFU: Colony Forming Unit

CD: Crohn's disease

CR: Caloric Restriction

CRM: Caloric Restriction Mimetics

CFS: Chronic Fatigue Syndrome

Cm: centimeter

CD4+: Cluster of differentiation 4

ClpB: Caseinolytic peptidase B protein

CDI: *Clostridium difficile* infection

CRP: C-reactive protein

D

DC: Dendritic cells

DR: Dietary restriction

DSS: Dextran Sodium Sulfate

D: days

DPPH: Diphenyl-1-Picrylhydrazyl

DGGE: Denaturing gradient gel electrophoresis.

dhaT: 1,3-propanediol dehydrogenase T gene

E

EODF: Every other day fasting.

EGF: Epidermal Growth Factor

EGCG: Epigallocatechin gallate

ESCMID: European Society of Clinical Microbiology and Infectious Diseases

EFSA: European food Safety Authority

E.g: *exempli gratia*

F

FMT: Fecal Microbiota Transplantation

FMD: Fasting mimicking diets

FOS: fructo-oligosaccharides

FAMHP: Federal Agency for Medicines and Health Products

G

GRAS: Generally recognized as safe

GF: Germ-free

GM: Gut Microbiota

GOS: galacto-oligosaccharide.

GALT: Gut-Associated Lymphoid Tissue

GIT: Gastrointestinal tract

GI: Gastro-Intestinal

GTP: Green Tea Polyphenols

G: gram

GLP-1: Glucagon-like peptide-1

GPRR: G-protein-coupled receptor

H

Hsp: Heat shock proteins

HMO: Human milk oligosaccharides

H: hours

HDAC3: Histone deacetylase 3

HGD: High glucose diet

HFrD: High Fructose diet

HFD: High Fat Diet

HCF: High Cacao-Flavanol

H₂S: Hydrogen Sulfide

HIV: Human Immunodeficiency Virus

HP: High Protein

H₂O₂: Hydrogen Peroxide

HSV: Herpes simplex virus

I

IBS: Irritable Bowel Syndrome

IU: International Unit

IFL: Isolated-Lymphoid Follicles

IF: Intermittent Fasting

ITAD: Isocaloric-Twice-A-Day

IGFBP-1: Insulin-like growth factor binding protein 1

IBD: Inflammatory Bowel Disease

IL-10: Interleukin-10

IFN-gamma: Interferon- gamma

I.E: Id est

IgA: Immunoglobulin A

K

Kg: Kilogram

Kj: Kilojoule

L

LP: Lamina Propria

LPS: Lipopolysaccharides

LT: Lymphocyte T

LRGG: *Lactobacillus Rhamnosus* GG

LCF: Low-Cacao flavanol

LTA: Lipoteichoic acid

LAB: Lactic Acid bacteria

LcS: *Lactobacillus casei* Shirota

LFD: Low-Fat diet

M

MAP: mitogen-activated protein

MAM: Microbial Anti-inflammatory Molecule

Mg: Magnesium

mTOR: The mammalian target of Rapamycin

MD : Mediterranean Diet

MACs : Microbiota Accessible Carbohydrates

M²: Square Meter

m: Meter

M cells: Micro-fold cells

MALT: Mucosa-associated lymphoid tissue

MLN: Mesenteric Lymphoid nodes

mL: Milliliter

MPO: Myeloperoxidase

N

nm: Nanometer

NHANES: National Health and Nutrition Examination Survey

NF- κ B: Nuclear Factor Kappa B

NOD2 Nucleotide-binding oligomerization domain-containing protein 2

NCD : Non-communicable diseases

NSP : Non-Starch Oligosaccharides

NK: Natural Killer

NO: Nitric oxide

NGP: Next-Generation Probiotics

O

Oz: Ounce

Olf77: Olfactory receptor 77

P

PPI: Proton Pump Inhibitors

PYY: Hormone peptide YY

PRR: Pathogen Recognition Receptors

PAMPs: Pathogen-Associated Molecular Patterns

PP: Peyer's Patches

Ph: Potential of hydrogen

PK: Pyruvate Kinase

PI3K: Phosphatidylinositol 3-kinase

PCR: Polymerase Chain Reaction

PC: Phenolic Compounds

POM: Pomegranate

PGN: Peptidoglycan

PUFA: Polyunsaturated Fat

P.M: Post midday

?: Percentage

Q

QTL: Quantitative Trait Loci

QPCR: Quantative Polymerase chain reaction

QPS: Qualified Presumption of Safety

QS: Quorum Sensing

R

RCH: Reproductive and Child Health

ROS: Reactive Oxygen Species

RES: Resveratol

RS: Resistant Starch

RO: Resistant Oligosaccharides

S

SCFA: Short Chain Fatty Acids

Sirt: Sirtuin

SFA: Saturated-Fatty Acids

S6K: Ribosomal protein S6 kinase

T

TRF: Time-Restricted Feeding

TMA: Trimethylamine

TMAO: Trimethylamine N-oxide

TLR: Toll-Like receptors

TCR: T-cell receptor

TJ: Tight Junctions

Trp: Tryptophan

TOS: Total Oligosaccharides

U

UV: Ultraviolet

UC: Ulcerative colitis

US: United States

UNESCO: United Nations Educational, Scientific and Cultural Organization

V

VDR: Vitamin D receptor

VNR: Nutritional Value Daily

W

WHO: World Health Organization

X

XOS: Xylooligosaccharides

Z

Zn: Zinc

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ABSTRACTS

Title: Impact of polyphenols and feeding rhythms on the immunomodulation properties of the probiotic bacteria in the gastro-intestinal tract

Keywords: Probiotics, Gastro-intestinal Tract, Polyphenols, Biofilm, Bacteria, Immunomodulation

Abstract: The human intestinal microbiota is composed of several types of microorganisms, including archaea, fungi, unicellular eukaryotes, viruses, and bacteria. These bacteria actively participate in the maintenance of intestinal homeostasis. Dysbiosis of the gut microbiota, however, could be observed at course of many human pathologies, particularly inflammatory diseases intestinal chronic diseases (IBD), such as Crohn's disease (CD) or Ulcerative colitis (UC). Different intervention strategies, including fecal transplantation, administration of probiotics or even special nutritional diets have been developed to act on the microbial communities of the digestive tract and to restore homeostasis of host tissues by serving as substrates for microorganisms and indirectly by modulating intestinal homeostasis and components of the immune system associated, themselves contributing to regulate the composition microbiota. The discrepancies in results between pre-clinical models and clinical trials has encouraged a better characterization of the molecular mechanisms used by probiotics to exert their beneficial effects and especially better understand the relationship of these probiotic microorganisms with the resident microbiota and diet. In this work, we were interested in dietary interventions based on functional foods supplements, represented by *Lactobacillus* probiotics strains

and the plant-derived polyphenol like resveratrol in modulating the inflammatory response of immune and intestinal epithelial cell lines. Together, our *in vitro* data demonstrate that Resveratrol might be used to modulate the behavior of Lactobacilli with probiotic properties. Combination of probiotics and polyphenols could be considered to enhance the probiotic functionalities in further *in vivo* studies. Also, we investigated the combined effects of two interventions described separately to display immunoregulatory effects: fasting and lactobacilli administration. We demonstrated *in vitro* that fasting potentiates the ability of various lactobacilli strains to dampen LPS- or *Salmonella*-induced pro-inflammatory response in macrophages and intestinal epithelial cell lines.

Finally, we applied a fasting protocol *in vivo* to evaluate the beneficial combined effects of fasting and lactobacilli administration in a mice model of colitis, mimicking human IBDs. Altogether, the results presented in this study shed on light the positive potential of combining lactobacilli administration to host fasting.

Titre : Impact des polyphénols et des rythmes d'alimentation sur les propriétés d'immunomodulation des bactéries probiotiques dans le tractus gastro-intestinal

Mots-clés : Probiotiques, Tractus Gastro-Intestinale, Polyphénols, Biofilm, Bactérie, Immunomodulation

Résumé: Le microbiote intestinal humain est composé de plusieurs types de micro-organismes, dont les archées, les champignons, les eucaryotes unicellulaires, les virus et les bactéries. Ces bactéries participent activement au maintien de l'homéostasie intestinale. Cependant, une dysbiose du microbiote intestinal a pu être observée au cours de nombreuses pathologies humaines, en particulier les maladies inflammatoires des maladies chroniques intestinales (MII), telles que la maladie de Crohn (MC) ou la colite ulcéreuse (CU). Différentes stratégies d'intervention, y compris la transplantation fécale, l'administration de probiotiques ou encore des régimes nutritionnels spéciaux ont été développées pour agir sur les communautés microbiennes du tube digestif et restaurer l'homéostasie des tissus de l'hôte en servant de substrats pour les microorganismes et indirectement en modulant l'homéostasie intestinale et ses composants. du système immunitaire associé, contribuant eux-mêmes à réguler la composition du microbiote. Les écarts de résultats entre les modèles précliniques et les essais cliniques ont favorisé une meilleure caractérisation des mécanismes moléculaires utilisés par les probiotiques pour exercer leurs effets bénéfiques et surtout mieux comprendre la relation de ces microorganismes probiotiques avec le microbiote et l'alimentation des résidents. Dans ce travail, nous nous sommes intéressés aux interventions diététiques basées sur des compléments alimentaires fonctionnels,

représentés par les souches probiotiques de Lactobacillus et le polyphénol d'origine végétale comme le resvératrol pour moduler la réponse inflammatoire des lignées cellulaires épithéliales immunitaires et intestinales. Ensemble, nos données *in vitro* démontrent que le resvératrol pourrait être utilisé pour moduler le comportement des lactobacilles aux propriétés probiotiques. La combinaison de probiotiques et de polyphénols pourrait être envisagée pour améliorer les fonctionnalités probiotiques dans d'autres études *in vivo*. En outre, nous avons étudié les effets combinés de deux interventions décrites séparément pour afficher des effets immunorégulateurs: le jeûne et l'administration de lactobacilles. Nous avons démontré *in vitro* que le jeûne potentialise la capacité de diverses souches de lactobacilles à amortir la réponse pro-inflammatoire induite par LPS ou Salmonella dans les macrophages et les lignées de cellules épithéliales intestinales. Enfin, nous avons appliqué un protocole de jeûne *in vivo* pour évaluer les effets combinés bénéfiques du jeûne et de l'administration de lactobacilles dans un modèle de souris de colite, imitant les MICI humaines. Dans l'ensemble, les résultats présentés dans cette étude mettent en lumière le potentiel positif de l'association de l'administration de lactobacilles à l'hôte à jeun.

General Introduction – Main of the thesis

Chapter 1. The Intestinal microbiota

1. Definition

The human gastrointestinal (GI) tract constitutes astonishingly one of the sizeable interfaces (almost 250–400 m²) between the host, environmental factors and antigens in the human body (Iacob *et al.*, 2019), this human body hosts trillions of microorganisms at the level of the GI, skin, vagina and other mucous membranes. Together they build complex microbial communities, with multiple metabolic and immune functions that are essential for the survival of the human organism, leading to the holobiont concept (Lin *et al.*, 2017; Iacob *et al.*, 2019). The most important representation of this community, quantitatively and qualitatively, can be found in the gut as the intestinal microbiota. The term “microbiota” is used to describe the microbial taxa associated with a particular environment (e.g. the gut), whereas the term “microbiome” referred the catalog of genomes from all the microorganisms found in this particular environment, however both terms are often used interchangeably (Ursell *et al.*, 2012). The number of microorganisms inhabiting this GI tract has been estimated to exceed 10¹⁴, however yet most cannot be cultivated *in vitro* (Iacob *et al.*, 2019).

This gut-associated microbiota includes mainly bacteria but also archaea, fungi, protozoa, and viruses. It is primarily composed of bacteria from either *Bacteroidetes* phylum (mostly *Bacteroides* or *Prevotella* species), that are gram negative bacteria, or *Firmicutes* (mostly *Clostridium* and *Lactobacillus* species), that are gram positive bacteria (Lozupone *et al.*, 2013). The majority are strict anaerobes (more than 97 %), mostly belonging to the phyla *Firmicutes* (64 %), *Bacteroidetes* (23 %), *Proteobacteria* (8 %), and *Actinobacteria* (3 %); low numbers of the phyla *Fusobacteria*, *Verrucomicrobia*, and TM7 (2 %) are additionally present. The *Bacteroidetes* use a very wide range of substrates and are major producers of propionate, which represents, along with butyrate, the most essential short chain fatty acids (SCFA) for maintaining intestinal homeostasis and exerting multiple beneficial effects on mammalian energy metabolism (Den Besten *et al.*, 2013; Parada Venegas *et al.*, 2019). Among the *Firmicutes* are species that produce butyrate and that are specialist degraders of indigestible polysaccharides. *Actinobacteria* (that include *Bifidobacterium* spp.), *Proteobacteria* (including *Escherichia coli*), and *Verrucomicrobia* (including *Akkermansia muciniphila*) are typically present in smaller numbers in the healthy gut microbiota. Fungi and Archaea comprise less than 1 % of the total gut microbiota (Cardinelli *et al.*, 2015). Archaeae are

diverse microorganisms morphologically and physiologically and are remarkable for their ability to thrive in the most unlikely environments. In the intestinal microbiota, cultured archaea comprise anaerobic methanogens which specifically metabolize hydrogen produced by anaerobic fermentation of carbohydrates into methane; further transforming heavy metals and metalloids into methylated derivatives, along with the non-methanogen halophilic Archaea (Nkamga *et al.*, 2017). Besides, the gut virome is also defined as the viral component of the gut microbiome, defined itself as the microbial communities of the gut. It is composed of eukaryotic viruses that are effectively capable of replicating in human cells, as well as bacteriophages that replicate in gut bacteria, which are generally the most abundant. Regarding viruses of bacteria (bacteriophages), their presence is indeed modulated by the presence of their host bacteria and they might also regulate the bacterial populations and gut contents (Lecuit *et al.*, 2017).

Conversely to the extensive number of studies on the different communities of bacteria of the whole microbiota assessed in the last few decades, the fungal constituents of the microbiota, the mycobiome, received much slighter notice. Still, recent research acknowledged human mycobiome as a dynamic community, responsive to environmental and pathophysiological changes, and playing a vital role in host metabolism, as well as maintenance of host immune homeostasis (Moyes *et al.*, 2012; Seed *et al.*, 2015; Witherden *et al.*, 2017).

2. The intestinal environment

The dynamic balance between the bacterial and cellular populations of the intestine starts to be buildas soon as the birth. This construction of the intestinal ecosystem is progressive and sequential and create its proper environment.

2.1. Intestinal Architecture

The Intestinal architecture and its developmental features of different segments have been well defined. In the sections below, we provide a brief overview on this intestinal structure and its developmental aspects that are relevant to our understanding of GI growth and regulation.

Gastrointestinal tract,

The gastrointestinal (GI) tract is the largest part of the digestive tract. Besides the tract consisting of the stomach and intestines, other digestive organs are the liver along with the bile ducts and the

pancreas. Together, all the digestive organs are called the digestive system. It consists of a vacant muscular tube starting from the oral cavity, where food effectively enters the mouth, ongoing through the pharynx, esophagus, stomach and intestines to the rectum and anus, where food is ultimately expelled (Ogobuiro *et al.*, 2021).

Its primary purpose is to break food down into nutrients, which can be soaked up into the body to supply it with energy. Earliest, food must be ingested and inhaled into the mouth to be mechanically processed and moistened. Secondly, digestion occurs mainly in the stomach and small intestine where proteins, fats and carbohydrates are chemically broken down into their basic building blocks. Minor molecules are then absorbed across the epithelium of the small intestine and subsequently enter the circulation. The large intestine plays here a vital role in reabsorbing water excess. Various peripheral organs, such as liver, assist digestion by secreting enzymes and metabolites to help food break down and nutrients solubilization. Finally, undigested material and secreted waste products are excreted from the body via defecation (passing of faeces). Endocrine pancreas on the other side maintains the body's blood glucose (sugar) balance and produces insulin and glucagon which effectively regulates fuel homeostasis (Röder *et al.*, 2016). Apart from enzymes, various bacteria known as the human microbiota are also involved in digestion (Jandhyala *et al.*, 2015).

The intestinal epithelium,

The intestinal epithelial cells are at the heart of the host-microbiota interface. These cells form a very effective physico-chemical barrier separating the luminal intestinal microbiota from the underlying tissues. This barrier is highly dynamic and easily repaired in the event of aggression thanks to its rapid renewal from the stem cells present at the bottom of the crypts (Stedman *et al.*, 2016).

The epithelium found along the small intestine and colon is a simple prismatic epithelium made up of several cell types (Muniz *et al.*, 2012). The unique structure, in the form of invaginations and crypts called Lieberkühn crypts, as well as the presence of glove finger expansions called microvilli allows this tissue to have a very large absorption surface increasing the exchange surface between the organism and the content of the intestinal lumen, estimated for a human intestine, to approximately 200 m² (the equivalent of a tennis court) which optimizes the absorption of nutrients. The intestinal stem cells are located at the base of the crypts and allow throughout the

life the generation of all cell types of the intestinal lineage. The cells mainly found are the absorptive cells called enterocytes (or colonocytes in the colon). These are cells with microvilli with different functions, notably they allow the absorption of nutrients, thanks to the production of specific enzymes and also play a protective role through a barrier effect. Enterocytes represent 80% of all epithelial cells in the small intestine (Boonekamp *et al.*, 2020). They are characterized by their cylindrical shape and the presence at their apical pole of microvilli. Through the expression of the polymeric immunoglobulin receptors (pIgR), enterocytes allow the translocation of immunoglobulin A (IgA), produced by plasma cells, from the lamina propria to the gut lumen.

Four other cell types derive from intestinal stem cells and are secretory cells:

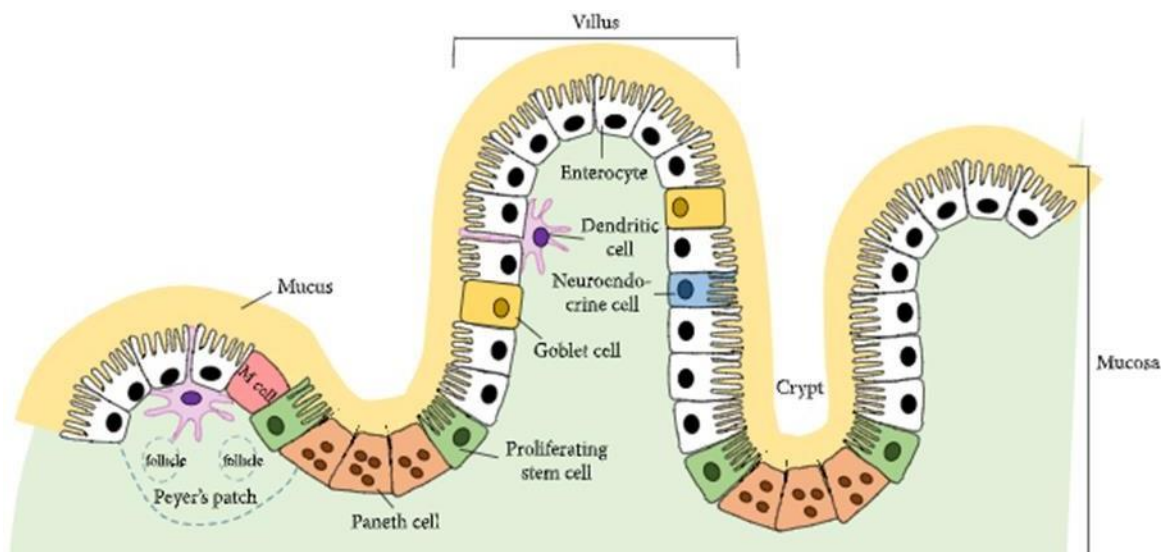


Figure 1: The cross-sectional structure of small intestine and the major cell constituents of epithelium

(Kong *et al.*, 2018)

- Goblet cells (4-12% of all intestinal epithelial cells) also called caliciform or mucus cells. These cells secrete mucus which contributes to the protection of the intestinal mucosa. It is interesting to note that they are distributed unevenly along the intestine.

Goblet cells are characterized by a large apical region and a thin basal part. The apical pole has a mass of mucin globules that move the nucleus toward the base. This mucous gel, composed of glycoproteins (mainly the mucin 2 protein), is secreted by the cell in order to protect the surface

of the epithelium against the intestinal contents and in particular the infectious microorganisms or commensals. This mucus also plays the important role of lubricator of the digestive tract (Deplancke *et al.*, 2001).

- Paneth cells (3-8% of all intestinal epithelial cells) that are exclusively located at the bottom of the crypts of the small intestine in direct contact with stem cells (Ouellette *et al.*, 2012) and participate in the innate immune system by secreting antimicrobial peptides (lysozyme, cathelicidins and defensins). They are characterized by large granules of eosinophilic secretion, located in the apical part of the cell. These granules contain antimicrobial molecules, such as lysozymes, phospholipase A2 and defensins. The release of these substances into the intestinal lumen represents one of the first element of innate immunity, provided by the intestinal barrier, since this event helps to restrain the development of the gut microbiota and to fight against pathogenic microorganisms. It is interesting to note that this is the only type of differentiated cell with a long lifespan, since it resides for three to six weeks in the crypt (Barker *et al.*, 2008). Studies have shown that these cells are likely to participate in the microenvironment, also called a niche, of intestinal stem cells.

- Enterodocrine cells (<1% of all intestinal epithelial cells) that produce a range of hormones involved in food intake, digestion regulation by promoting pancreatic and gallbladder secretions, and absorption such as serotonin, ghrelin or cholecystokinin, but also by controlling intestinal motility (Martin *et al.*, 2019). They divided into fifteen different subtypes depending on the hormone secreted (cholecystikinin, glucagon, secretin, etc.).

- Tuft cells (0,5-2% of all intestinal epithelial cells) that are chemosensory cells playing role in intestinal epithelial damage response and immunity (Banerjee *et al.*, 2018).

A last cell type in the intestinal epithelium is represented by micro-fold(M) cells (<1% of all intestinal epithelial cells). These environment sensing cells cover the surface of some lymphoid structures in the gut and are mainly found at the level of the Peyer's patches in the small intestine where they recognize and capture the antigens and microorganisms present in the intestinal lumen. Their function is to act as an interface between the contents of the intestinal lumen and the body's immune system (Neutra *et al.*, 1996).

Beneath the lining epithelium is a supporting connective tissue called the lamina propria or chorion. This tissue has a very dense vascular and lymphatic network which allows absorption of digested nutrients. It also contains many cellular elements involved in the immune system, which serve as a defense line against microbes that have crossed the intestinal epithelium.

The Intestine,

The intestine is the system in charge of digesting food, absorbing nutrients, and finally expelling organic waste. In humans, as in most mammals, it is divided into two main parts: the small intestine and the colon. The human gut is the home of more than 100,000 billion bacteria, mainly residing in the distal part of the small intestine (ileum) and have to cope throughout life with this strong exposure to microbial stimuli.

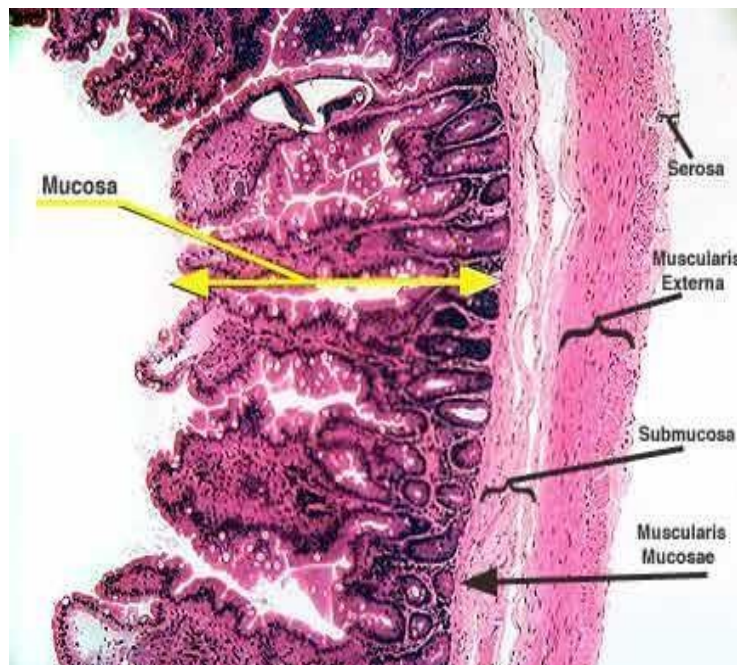


Figure 2: Histological layers of the small intestine

(Kelsey E *et al.*, 2020)

The intestinal wall is made up of 5 concentric layers, also called tunics (Moawad *et al.*, 2017).

From the outside towards the intestinal lumen, follow one another:

- the outer coat is a weed or a serosa, depending on its location in the digestive tract. It consists in particular of a loose connective tissue with a protective role at the ends of the tube.
- the muscularis (or the longitudinal muscle) is a thick tunic made up of smooth muscle cells ensuring peristalsis and intestinal segmentation, allowing the mixing as well as the advancement of nutrients.
- the submucosa consists of loose connective tissue, containing Meissner's submucosal plexus, as well as blood and lymphatic vessels.
- the muscle-mucosa is a thin layer made of smooth muscle cells.
- the lining is the inner lining, comprising a connective tissue called chorion (lamina propria or mesenchyme), rich in cells of the immune system, and an intestinal epithelium which corresponds to the layer of cells covering the interior of the intestine. This ensures the exchange and separation functions between the outside world (the intestinal lumen) and the inside of the organism.

The small intestine is located between the stomach and the colon and is in average 2.5 cm in diameter and 291cm long (Helander *et al.*, 2014). This part of the digestive tract has three successive sections: the duodenum (0.25 m), the jejunum (2.5 m) (Collins *et al.*, 2021), and the ileum (3.5 m) (Zhan *et al.*, 2004). The small intestine plays a major role in the absorption function. In order to optimize this function, it presents several surface amplification devices (the intestinal loops, intestinal villi and enterocytic microvilli), leading to an absorptive surface area of about 250 square meters.

The duodenum, coming from the Greek dodekadaktulon, meaning "12 fingers", the duodenum is so named because of its length, comparable to the width of twelve fingers, and follows the pylorus. In anatomy, it is described as forming a frame surrounding the pancreas. It is the only fixed segment of the small intestine, which however does not prevent it from having permanent peristaltic activity, like the rest of the small intestine.

The jejunum begins just after the duodenojejunal flexure (or Treitz angle) and is directly followed by the ileum which ends at the ileo-caecal valve. These two parts are suspended from the

mesentery, which gives them great mobility within the abdominal cavity. Finally, the jejunum-ileal segment is surrounded by the colonic frame.

Finally, the colon, also called the large intestine, is located between the cecum and the rectum. In adults, it measures almost 1.50 m in length and is broken down into 4 main elements: the ascending colon, the transverse colon, the descending colon and the sigmoid. The colon contains crypts but has a flat surface epithelium rather than villi observed in the small intestine. The colon is responsible for digesting nutrients that have not been absorbed by the small intestine. It is mainly dietary fiber which then undergoes the action of the bacterial flora present in the colon (Kay *et al.*, 1982). These fibers mainly come from flour, milk and vegetable products. The role of the colon is also to recover water, maintain water balance and absorb certain vitamins, such as vitamin K. Finally, the latter completes the transformation of waste from the small intestine into materials feces, which will then be expelled through the rectum located at its end.

2.1.2. The gut-associated immune system

Faced with the mass of food- or microbial-derived antigens that pass through the intestinal lumen, the intestinal epithelium must act as a barrier to maintain the whole organism homeostasis. In mammals, a complex immune system supports this first line of physical defense. However, the primary role of the gut-associated immune system is to tolerate the presence of the gut microbiota into the luminal compartment in order to sustain the symbiosis between the host and the resident microbes. This microbiota can be considered as an organ in its own, having coevolved with its host to achieve a symbiotic relationship leading to physiological homeostasis. The host provides an environment rich in nutrients that commensal bacteria use to perform their functions.

The intestinal immune system combines innate immune responses, that are quickly mobilized into adaptive immune responses taking a little bit longer to establish and whose particularity is the constitution of an immune memory. The bacteria of the intestinal flora contribute to the establishment of these innate and adaptive immune defenses during the development. As mentioned above, the intestinal immune system must constantly maintain a state of tolerance towards the intestinal flora, while being able to induce protective pro-inflammatory immune responses against gastrointestinal pathogens. Maintaining such a balance rests on the existence of regulatory mechanisms guaranteeing a reduced reactivity of the intestinal immune system in front of harmless commensal bacteria. Basically, the formation of the intestinal immune system is

initiated *in utero*, under the control of the host's genetic program. The maturation of this intestinal immune system occurs in the postnatal period (Georgountzou *et al.*, 2017) under the influence of bacterial colonization (Houghteling *et al.*, 2015).

Innate immunity involves unspecific receptors, PRRs (Pathogen Recognition Receptors), expressed on the different cell types present in the mucosa (enterocytes, polynuclear, mast cells, macrophages and dendritic cells (DC) (Suresh *et al.*, 2013).

Each cell type displays a particular repertoire of PRRs that might be modified depending on environmental stimuli. As a matter of fact, the innate immune response relies bigly on recognition of evolutionarily conserved structures on pathogens, termed pathogen-associated molecular patterns (PAMPs), alongside a restricted number of germ line-encoded pattern recognition receptors (PRRs), of which the family of Toll-like receptors (TLRs) has been studied most extensively Pathogen recognition and innate immunity (Akira *et al.*, 2006; Medzhitov *et al.*, 2009).

The family of TLRs is the major and most extensively studied class of PRRs. it appears that these TLRs can successfully recognize PAMPs either through direct interaction or via an intermediate PAMP-binding molecule. Thus, TLR4 recognizes lipopolysaccharide (LPS) through the accessory molecule MD2 (Kim *et al.*, 2007) whereas flagellin, the major constituent of the motility apparatus of flagellated bacteria, is recognized by TLR5 (Kau *et al.*, 2011). Upon PAMP recognition, PRRs present at the cell surface or intracellularly signal to the host the presence of microorganisms and in the case of infection by pathogenic microbes it can trigger set off proinflammatory and antimicrobial responses by switching on a multitude of intracellular signaling pathways, including adaptor molecules, kinases, and transcription factors (Akira *et al.*, 2004). PRR-induced signal transduction pathways ultimately contribute to the activation of gene expression and synthesis of a broad range of molecules, including cytokines, chemokines, cell adhesion molecules, and immunoreceptors (Akira *et al.*, 2006). Pathogens of quite different biochemical composition and with entirely different life cycles, including viruses, bacteria, fungi, and protozoa, are recognized by slightly different yet surprisingly similar and overlapping mechanisms by these host PRRs network (Akira *et al.*, 2006).

The adaptive immune system is mainly represented by the mucosal immune system (commonly described by the acronym Mucosa-associated lymphoid tissue (MALT)) and is located near the surfaces where most pathogens invade. The components of MALT are sometimes subdivided into many different associated tissues like GALT (gut-associated lymphoid tissue). The lymphoid elements of the gut-associated tissue comprise organized lymphoid tissues such as the Peyer's patches (PP), and the mesenteric lymph nodes (MLN). The effector sites of the intestine are the mucosal epithelium and underlying lamina propria (LP). There are many different immune cells including activated T cells, plasma cells, mast cells, dendritic cells and macrophages even under normal conditions. That this does not result in overt tissue pathology reflects the fact that the effector cells present are actively held in check by potent regulatory mechanisms (Kau *et al.*, 2011). Adaptive immunity mobilizes specific receptors expressed by B lymphocytes (BCR) and T lymphocytes (TCR) (Bonilla *et al.*, 2010). The intestinal immune system can be thus schematically divided into two compartments. A first compartment called the inducer, where the intestinal immune responses are initiated after antigenic stimulation, and a second effector compartment, where the cells activated by the antigen are established after recirculation via the hemolymphatic cycle. Peyer's patches, isolated lymphoid follicles (ILF) and mesenteric nodes are the main inducing sites; the intestinal epithelium and the underlying chorion represent the effector sites (Brandtzaeg *et al.*, 2008). In the reality, these two compartments are strongly intertwined and cooperate permanently to elaborate a proper immune response.

Physiologically, we find a phenomenon of tolerance of microbiota bacteria and food proteins by the intestinal immune system. PAMPs present on commensal bacteria are detected by PRRs present in particular on epithelial cells, which triggers the production of many cytokines by epithelial cells such as TGF- β , as well as by macrophages (Winkler *et al.*, 2007). Under the influence of this cytokine, the dendritic cells of Peyer's patches or of the lamina propria which have also recognized these non-pathogenic antigens will have a partial maturation and migrate towards the lymph nodes to synthesize a high-rate IL-10 (an anti-inflammatory cytokine). IL-10 will then orient the differentiation of naïve CD4 + lymphocytes T (LT) into regulatory LT which will synthesize IL-10 and IFN- γ to, on the one hand, inhibit the activation of LT effector LTh1, LTh2 and LTh17 responsible for increasing the rate of secretion of proinflammatory cytokines and on the other hand, inhibiting both the macrophages which allow the elimination of pathogens and the recruitment of neutrophils responsible for intestinal damage. Thus, this balance between

effector and regulatory mechanisms allow intestinal homeostasis and functional tolerance to be maintained (Ai *et al.*, 2014; Okumura *et al.*, 2017). When a pathogen enters the mucus layer and meets cells, the pathogen associated PAMPs are recognized by the PRRs and induce intracellular signals leading to the production and release of antimicrobial peptides and cytokines. These elements will lead to an appropriate inflammatory response limiting the spread of the pathogen and allowing the recruitment of immune cells to favor pathogen clearance (Mogensen *et al.*, 2009).

2.2. Intestinal Functions

The intestine, the reservoir of many microbes that make up the "microflora", a term borrowed from ecology in its nascent form, is the seat of digestion, absorption and protection in which their role became clearer at the end of the 19th century.

2.2.1. Digestion and Absorption

Digestion is a chemical and mechanical process, the purpose of which is to cut the ingested food in order to make it more easily absorbable. In the small intestine, digestion takes place in preferential regions, both intraluminal and in the mucous wall (Hornbuckle *et al.*, 2008) depending on the type of food ingested (Kong *et al.*, 2008), thanks to the enzymes of the biliopancreatic digestive secretions or of the brush border of the enterocytes (Hooton *et al.*, 2015). Carbohydrates, proteins and lipids are thus reduced respectively to oligosaccharides, oligopeptides and amino acids, and fatty acids and cholesterol, which are then absorbed to meet the needs of the body. Intestinal absorption takes place by transcellular route, crossing the apical membrane, by diffusion or active or facilitated transport (Snoeck *et al.*, 2005). In the enterocyte, the absorbed substances cross the cytoplasm and the basolateral membrane before being drained by blood or lymphatic route and being distributed in the body.

2.2.2. Physico-chemical barrier

The intestinal epithelium forms a selective physicochemical barrier that impedes enteric pathogens from invading the epithelium and causing disease but make it possible to absorb the nutrients necessary for the host (Cario *et al.*, 2008).

To colonize the intestinal mucosa, a particular pathogen needs to break and cross this barrier. The barrier consists of the intestinal microbiota, a low pH area (in the upper part of the GI tract), an

epithelial mechanical barrier maintained by intercellular tight junctions (TJ) and adherents' junctions (AJ), an apical actin cytoskeleton, a mucus layer along the epithelial surface, and antimicrobial peptides such defensins or immunoglobulin A (Ismail *et al.*, 2005; Cash *et al.*, 2006; Johansson *et al.*, 2011; Miki *et al.*, 2012; Diehl *et al.*, 2013; Ostaff *et al.*, 2013). In addition, this barrier produces cytokines and heat shock proteins (Hsps) playing a role in intestinal immunity (Malago *et al.*, 2010).

This epithelium also includes goblet cells as explained above, capable of secreting a layer of mucus whose chemical component allows the destruction or inhibition of bacterial or mycological growth. This mucus layer located in the colon consists of two parts, an outer loose one allowing the growth of specific bacterial species and an inner one that restrains the presence of microorganisms. These mucus-secreting cells are in fact most abundant in the distal part of the small intestine and colon, producing thus a more important layer of mucus in these areas of the GI tract and ensuring primarily lubrication after water resorption from the stool (Johansson *et al.*, 2013).

Most of the cells of the digestive epithelium are capable of directly producing antimicrobial peptides (defensins in particular) or induced (by microbial compounds that stimulate epithelial receptors for innate immunity). Some cells, however, specialize in the synthesis and excretion of antimicrobial molecules AMPs. This is for example the case of the cells of Paneth (Beaugerie L *et al.*, 2014). Controlled by only one single gene, these AMPs can be produced rather quickly upon infection with narrow energy consumption such as β -defensins (Sechet *et al.*, 2018); nonetheless, some are constitutively expressed (Rahnamaeian *et al.*, 2011) such as the antibacterial chemokine BRAK/CXCL14 (Frick *et al.*, 2011).

In some disease states, such as obesity or type 2 diabetes, the existence of a low-grade inflammation leads to an increase in intestinal permeability and the translocation of bacterial products, such as the Caseinolytic Protease B. protein ClpB and lipopolysaccharides (LPS), compromising the barrier function of the intestinal epithelium (Cani *et al.*, 2018). When this barrier is altered, as observed in various physio pathological situations, the antigens present in the intestinal lumen are found directly in contact with the villi of the enterocytes, which can shrink and cause hyperpermeability (Wells *et al.*, 2017). The intestinal lining is no longer sufficiently tight and can pass macronutrients which may turn out to be allergens, toxins, viruses, or bacteria.

There is enough evidence for the crosstalk between the stable microbiota present in the intestinal lumen and these components in favor of intestinal immunity (Shimada *et al.*, 2013). Thus, the intestinal microbiota crosstalks with this physicochemical barrier to maintain the functions of the barrier and institute gut immunity. Mounting evidence shows that it influences the development and regulation of the host's immune and non-immune defenses, regulates mucin gene expression by goblet cells, modifies glycosylation of mucus to interfere with bacterial adhesion, colonization and invasion (Caballero-Franco *et al.*, 2007), induces secretion of antimicrobial peptides notably by intestinal Paneth cells (Frantz *et al.*, 2012), regulates alterations of intestinal permeability caused by infection, stress, and inflammation (Lutgendorff *et al.*, 2008), and influences development of mucosal and systemic immunity (Tlaskalová-Hogenová *et al.*, 2004).

The host's microbiota and intestinal epithelial cells have a symbiotic relationship, the consequence of which is an effective protective barrier effect. By occupying the ecological niches, the resident flora prevents colonization of the intestine by potentially pathogenic bacteria and can protect in some extent the host from environmental substances which could be harmful when they are present in the digestive tract (Kenneth *et al.*, 2020).

3. Composition and distribution of the Intestinal Microbiota

Each individual shelter in its digestive tract a unique niche of microbes that may be as unique as a fingerprint and that make up its intestinal microbiota (Turner *et al.*, 2018).

The microbiota is localized between the lumen of the digestive tract and the mucus present on the surface of the intestinal epithelium, it is present throughout the digestive tract, but its concentration is maximum in the distal portion of the small intestine and the colon (Pei *et al.*, 2004). The esophagus contains a mainly transient microbiota, but a study reveals the existence of a resident microbiota in its distal part with most bacteria belonging to the phyla *Firmicutes* (*Streptococcus* and *Veillonella*) and *Bacteroidetes* (*Prevotella*). In the stomach, duodenum, jejunum, and proximal ileum: the flora is rather sparse, and although variable depending on food intake it does not normally exceed concentrations of 10^5 germs / ml.

The distal ileum marks a transition zone with the presence of an aero-anaerobic flora (dominant and sub-dominant flora) at concentrations of 10^6 - 10^7 germs / g. On crossing into the colon, the bacterial concentration and variety of the enteric flora change dramatically. Concentrations of 10^{12}

CFU/mL or greater may be thus found and are comprised mainly of anaerobes such as *Bacteroides*, *Porphyromonas*, *Bifidobacterium*, *Lactobacillus*, and *Clostridium* (Quigley *et al.*, 2013).

3.1. Composition

Certain dominant species, which are present in the majority of individuals, remain stable and allow the essential functions of the microbiota to be carried out; they are associated with minority populations which are specific to each of us.

An estimation of 500–1000 species of bacteria exist in the human body at any one time (Gilbert *et al.*, 2018), however, the number of unique genotypes per individual (called sub-species) could be orders of magnitude greater than this (Locey *et al.*, 2016) and wherein each bacterial strain owns a specific genome containing a library of thousands of genes, presenting considerably more genetic richness and diversity, and hence a certain kind of flexibility than the human genome. Yet, different people lodge entirely different collections of microbes with significantly varying densities even among conserved taxa, and it is until today that we still understand too little about what leads to and what regulates this vast variation (Gilbert *et al.*, 2018).

However, on the metagenomics level, the same proportion of gene categories are found suggesting that functions are a more relevant readout than the composition itself (Vidulin *et al.*, 2018). This composition is highly variable and could easily change, but it finally fits with host requirements. The dominant bacteria of the human gut microbiota belong to 3 major bacterial phyla: *Firmicutes*, *Bacteroidetes* and *Actinobacteria* (Barbut *et al.*, 2010). This feature is shared with the microbiota observed in the gut of other vertebrates (Ley *et al.*, 2008).

Bacteria belonging to the *Firmicutes* phylum are gram positive. They usually represent more than half of the microorganisms in the flora. This phylum has 3 classes of bacteria: class I of Clostridia which contains the genera *Clostridium*, *Ruminococcus* and *Faecalibacterium*, Class II Mollicutes containing bacteria of the genus *Mycoplasma*, Class III of *Bacilli* containing the genera *Listeria*, *Staphylococcus*, *Lactobacillus*, *Enterococcus* and *Streptococcus*.

The *Bacteroides* phylum represents up to 30% of the bacterial population. It includes bacteria of the genus *Bacteroides* which are bacteria in the form of an anaerobic gram-negative *bacillus* and the genus *Prevotella*.

The phylum of *Actinobacteria* generally represent less than 10% of the microbiota population. They are gram positive bacteria, in particular of the genera *Actinomyces*, *Mycobacterium* or *Bifidobacterium*.

There are also bacteria from the *Proteobacteria* phylum, containing the order *Enterobacteriales* which are facultative anaerobic bacteria which are found in weak amount. In a minority, we find bacteria phyla *Fusobacteria*, *Verrucomicrobia* and *Spirochaetes*. The fungal component consists of fungi and yeasts. Fungi capable of growing in and colonizing the gut are limited to a small number of species, mostly *Candida* yeasts and yeasts in the family Dipodascaceae (*Galactomyces*, *Geotrichum*, *Saprochaete*). *Malassezia* and the filamentous fungus *Cladosporium* are also potential colonizers; but more work is needed to clarify their role. Other commonly detected fungi come eventually from the diet or the environment but either cannot or do not colonize (like *Penicillium* and *Debaryomyces* species, which are common in fermented foods but cannot technically grow at human body temperature), while still others have dietary or environmental sources (*Saccharomyces cerevisiae*, a fermentation agent and sometimes considered as probiotic; *Aspergillus* species, ubiquitous molds) yet are likely to impact gut ecology (Hallen-Adams *et al.*, 2016).

Archaea, accounting for a major portion of the microbial population in a variety of “normal” biological niches, are also found these are prokaryotic unicellular microorganisms. They have long been considered bacteria, but genetic analysis and phylogenetic classification methods have helped to justify the creation of a full-fledged group. Thus, they are single-celled prokaryotes with cellular characteristics distinct from bacteria and eukaryotes (Coker *et al.*, 2020). Many archaea are found in ecosystems with extreme environments, and may be acidophilic, alkaliphilic, halophilic or thermophilic (Eme *et al.*, 2017). Some archaeal species are however mesophilic⁸ and have been isolated from human skin, nose, lungs, oral cavity and vagina (Lurie-Weinberger *et al.*, 2015). They have been reported to be stable commensals of the gastrointestinal tract where they participate in functions such as methanogenesis, where most of these archaea are methanogenic,

transformation of heavy metals, trimethylamine metabolism and immune modulation (Blais Lecours *et al.*, 2014; Brugère *et al.*, 2014).

In the human digestive tract, most of these archaea are methanogenic (Gaci *et al.*, 2014). Finally, large population of viruses are also found in the GI tract. There is a large amount of bacteriophage viruses, archaeophages or prophages, inserted in certain bacterial genomes (Gaci *et al.*, 2014). Phages, by infecting and lysing certain bacteria are suggested to maintain the diversity of microbial species and to shape in some extent the bacterial composition of the gut microbiota.

All these microbial species participate in the biodiversity of the intestinal flora which increases all along the GI tract. If we find similarities in terms of biotope composition at the level of large phylogenetic groups, we find many subject-specific species. An individual's microbiota is therefore positively balanced and there does not appear to be an exemplary microbiota as long as the microbiota host interaction supports the health of the host. Schematically, we can consider three populations characterizing the flora in the digestive tract (Rodríguez *et al.*, 2015).

The dominant flora, which is most numerous, is localized essentially at the level of the colon where the colonization rate of each of the bacterial groups which compose it reaches 10^9 to 10^{11} germs / g or ml of intraluminal content with very little interindividual variations, it is essentially composed of anaerobic germs. Mainly, the predominant phyla that inhabit the large intestine include *Firmicutes* and *Bacteroidetes* while *Bacteroides*, *Bifidobacterium*, *Streptococcus*, *Enterobacteriaceae*, *Enterococcus*, *Clostridium*, *Lactobacillus* and *Ruminococcus* are counted as the predominant luminal microbial genera (and can be identified in stool), only *Clostridium*, *Lactobacillus*, *Enterococcus* and *Akkermansia* are the predominant mucosa and mucus associated genera (detected in the mucus layer and epithelial crypts of the small intestine) (Swidsinski *et al.*, 2005).

The sub-dominant flora is localized at the level of the colon at rates lower than those of the germs of the dominant flora, i.e., 10^6 to 10^8 germs / g or ml of intraluminal content, it is composed of optional aero-anaerobic germs (*Enterobacteriaceae*, *Streptococci*).

The flora of passage, variable, transient, is normally in low concentration ($<10^4$ - 10^6 germs / g or ml of intraluminal content). It is polymorphic made up of all that can be ingested (bacteria,

viruses, yeasts), and except in pathological circumstances it is unable to establish itself in the digestive tract and to express its pathogenic potential.

3.2. Gut microbiota establishment and its evolution through life

The microbial ecosystem is established during the first decade of life then tends to stabilize before deteriorating with senescence (Derrien *et al.*, 2019). In fact, the gut microbiota is generally believed to begin from birth, although this dogma is challenged by a limited number of studies, sometimes controversial, in which microbes were detected in womb tissues, such as the placenta (Zhu *et al.*, 2018).

In early stages of development, the microbiota is generally low in diversity and is dominated by two main phyla, *Actinobacteria* and *Proteobacteria* (Ottman *et al.*, 2012).

During the first year of life, the microbial diversity increases, and the microbiota composition converges towards a distinct adult-like microbial profile with temporal patterns that are unique to each infant (Palmer *et al.*, 2007). By around 2.5 years of age, the composition, diversity, and functional capabilities of the infant microbiota resemble those of adult microbiota (Koenig *et al.*, 2011), however recent studies suggest that mature development of the gut may take longer, occurring in the first decade of life (Derrien *et al.*, 2019). Although, in adulthood, the composition of the gut microbiota is relatively stable, it is still subject to perturbation by life events as mentioned above (L. Dethlefsen *et al.*, 2011). Extreme-age people (centenarians) have a microbiota that differs from those of older adults, consistent with general age-related microbiota trends. Lifestyle, and particularly diet, play a large role, since aging is often accompanied by a reduction in the amount and variety of fiber-containing foods, and there is often a risk of malnutrition (Amarya *et al.*, 2015; O'Toole *et al.*, 2015).

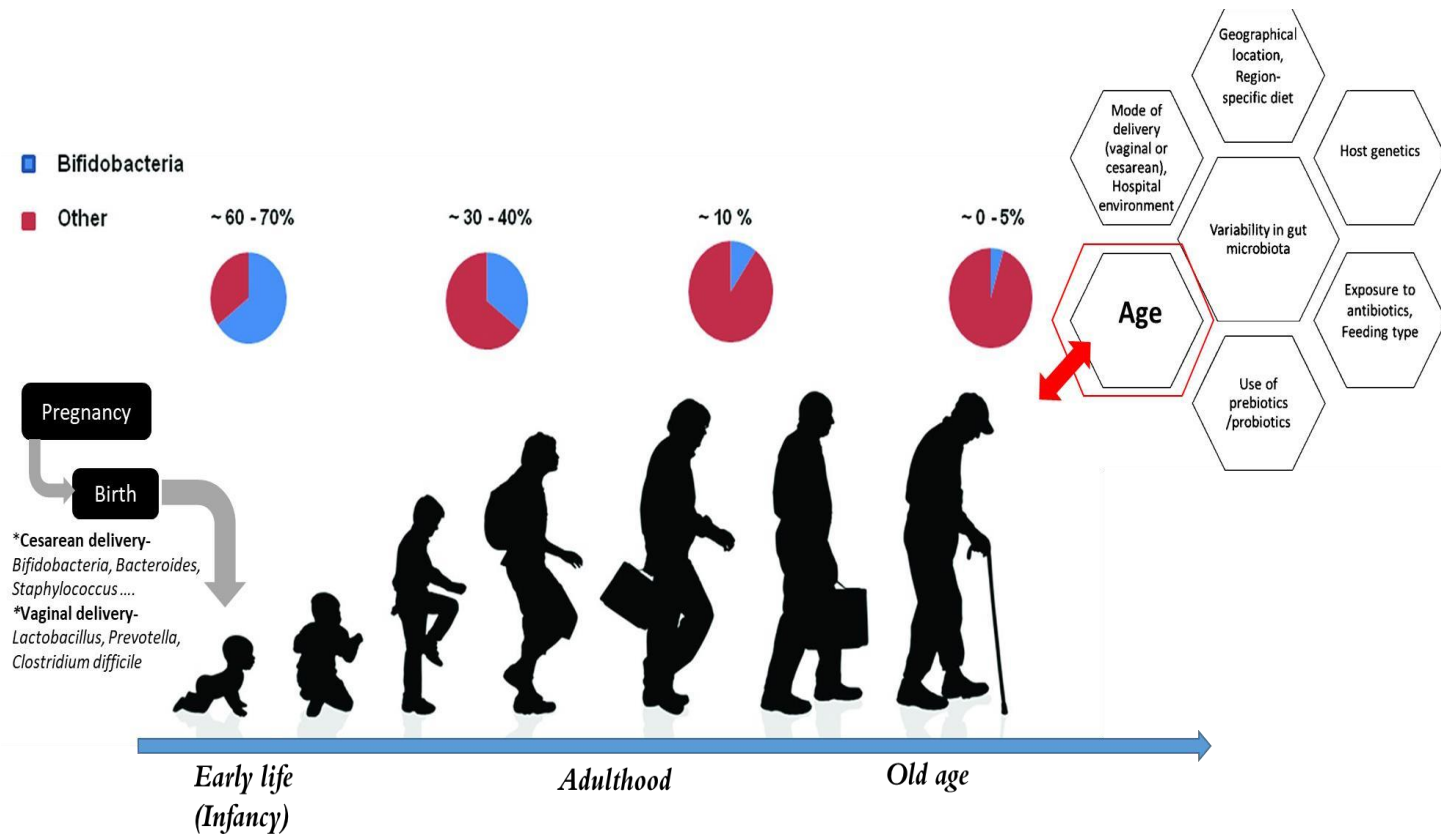


Figure 3: Evolution of gut microbiota through life

Adapted from (Kumar et al.,2016)

3.3. Intrinsic and extrinsic factors modulating gut microbiota

Several environmental factors have been implicated in shaping the gut microbiota (Tyakht *et al.*, 2013). In fact, hosts use specific and nonspecific factors to select their own gut microbiota.

Generally, the species in bacterial communities are important for normal tissue and immune development (Kamada *et al.*, 2014; Kubinak *et al.*, 2015; Smith *et al.*, 2015). They provide metabolic functions (Stanley *et al.*, 2013), and help prevent pathogen colonization (Kaltenpoth *et al.*, 2009; Koch *et al.*, 2011). However, the beneficial properties of the microbiota are highly dependent upon its composition (Mendes *et al.*, 2011; Willing *et al.*, 2011; Stanley *et al.*, 2013). Moreover, evolutionary and ecological dynamics repeatedly threaten to disarrange a given community whenever nonbeneficial species can establish themselves (Lozupone *et al.*, 2013; Jarry

et al., 2015). This suggests that there is strong natural selection on hosts to control and manage the composition of their microbiota (Schluter *et al.*, 2012).

There is extensive evidence that hosts exert some control over their microbiota in humans and other systems (Chu *et al.*, 2013). While correctional host mechanisms have the potential to influence the microbiota, as an alternative way for a host to influence its microbiota positively. Accordingly, a host acts in a way that promotes beneficial microbes rather than inhibits harmful ones. Theoretical work suggests that positive control can be more effective than negative control because the former encourages growth of beneficial species near the epithelium and thereby pushes harmful species away (Hasan *et al.*, 2019).

Intrinsic factors influencing gut microbiota

Host genetics,

Despite the growing volume of data explaining how the gut microbiota affects host physiology and health, explanations of how host genetics shapes the structure of the gut microbiome are very scarce. In general, the authors usually propose immune functions, metabolism, energy regulation, gut motility, and adhesion interactions as the most expected genetics-dependent physiological phenomena that may impact the composition of the gut microbiota (Benson *et al.*, 2010; Leamy *et al.*, 2014; Davenport *et al.*, 2015).

(Benson *et al.*, 2010) pointed out that quantitative trait loci (QTL) for *Coriobacteriaceae* and *Lactococcus* (located on MMU10) identified in their study were closely positioned with several genes engaged in immune responses and regulation. These comprised genes involved in the TLR2 pathway, IFN-gamma, and IL-22, all of them important in the immune response at mucosal surfaces. The authors also discussed a microbiome-related QTL on MMU1 that overlaps the conserved gene ATG16L1, and the region is syntenic with a region of human chromosome 2 already shown to be associated with Crohn's disease (Parkes *et al.*, 2007; Benson *et al.*, 2010). The pathogenesis of Crohn's disease has been so far recognized as a result of the gut microbiome and environmental factors leading to an abnormal immune response in a genetically predisposed patient. Possible factors promoting and mitigating Crohn's disease have been recently discussed in an extensive review by (Manuc *et al.*, 2016). Interestingly, some Crohn's associated gene polymorphisms have been demonstrated as affecting both the immune response and the gut

microbiota composition. For instance, the innate immune response is affected by the polymorphism of nucleotide-binding oligomerization domain-containing protein 2 (NOD2)/caspase recruitment domain-containing protein 15 (CARD15). NOD2 is an intracellular receptor involved in the sensing of the muramyl dipeptide, a component of the bacterial cell wall, and able to signal through the NF- κ B pathway, important for the elaboration of immune response.

Other worth noting observations propose that gene-encoded metabolic characteristics influence the microbiome structure. As an example, a correlation has been identified between a bacterial taxon associated with obesity (genus *Akkermansia*) and a variant near PLD1, a gene related to body mass index in Everard's study (Everard *et al.*, 2013; Davenport *et al.*, 2015). Also, evolutionary studies of vertebrates and typical composition of their gut microflora claim that the basal microbiota composition is shaped by stomach acidity of a species; this was suggested by the analysis of microbiome modifications correlating with evolutionary changes of animals (Beasley *et al.*, 2015).

Thus, the relative contribution of the host genetics in shaping the gut microbial structure and function is not yet clearly defined and remains a subject of ongoing debate. Like in the following recent study where the consumption of the same high-fat, high-sugar diet by different genotyped mice, reproducibly altered the gut microbiota despite differences in host genotype. Repeated dietary shifts demonstrated that most changes to the gut microbiota are reversible, while also uncovering bacteria whose abundance depends on prior consumption. These results emphasize the dominant role that diet plays in shaping interindividual variations in host-associated microbial communities (Carmody *et al.*, 2015). This concept will be further discussed in detail in the second part of the introduction.

Exogenous factors influencing emerging biodiversity

Delivery method,

Initial postnatal microbial exposure occurs during and shortly after birth therefore, the mode of delivery is a determining factor in the initial colonization of the newborn's digestive tract (Gagliardi *et al.*, 2018). It is suggested that the composition of this microbiota in infants may remain disturbed for months or even years. A number of studies have indicated that the mode of delivery affects the development of the gut microbiota in early life. Interestingly, the GM of a

newborn will closely resemble the microbiota that it encountered during birth. In vaginally delivered infants, gut microbiota resembles most likely to their mothers' vaginal microbiota, which are dominated by *Lactobacillus*, *Prevotella*, or *Sneathia*, whereas the microbiota of infants born by caesarean section are most similar to skin microbiota, which is dominated by *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* (Dominguez-Bello *et al.*, 2010). In addition, some studies have shown that colonization by *Bacteroides* and *Bifidobacterium* is delayed for one month after birth, whereas *Clostridium difficile* was abundant at one month (Penders *et al.*, 2006; Biasucci *et al.*, 2008). Underrepresentation of *Bacteroides* was also observed in the microbiota of infants born by caesarean section for three to four months after birth. In addition, the same studies have shown that infants born by elective caesarean section had particularly low bacterial diversity (Azad *et al.*, 2013). These modifications in the composition of the gut microbiota during the early life might have long term effects since Caesarean birth has been associated with an increased risk for immune disorders such as allergic rhinitis, asthma, and celiac disease (Renz-Polster *et al.*, 2005; Marcobal *et al.*, 2010; Decker *et al.*, 2011).

Diet of the newborn or infant,

- Breast milk: and baby formula

The breastfed microbiota was traditionally characterized by a predominance of *Bifidobacterium*, which has long been associated with health (Harmsen *et al.*, 2000), although some reports found no differences between breastfed and formula-fed infants (Adlerberth *et al.*, 2009). The predominance of *Bifidobacteria* in breastfed infants has been partly attributed to the prebiotic effect of HMOs (Garrido *et al.*, 2012). The supplementation of formula with prebiotics, i.e., galactooligosaccharides and fructooligosaccharides, has greatly contributed to reducing these differences and increasing *Bifidobacterium* and *Lactobacillus* counts (Veereman-Wauters *et al.*, 2011; Sierra *et al.*, 2015) and HMOs can thus act as decoys, preventing pathogenic bacteria from binding to intestinal cells (Wang *et al.*, 2012).

Several studies point to the existence of an enteromammary pathway in which dendritic cells and maternal macrophages transport components of the bacteria or the bacteria themselves from the maternal intestine to the mammary glands. It is estimated that bacterial content of milk can reach 10^5 to 10^7 bacteria per 800 mL of milk (corresponding to the daily intake of the newborn)

(Fernández *et al.*, 2013). A recent study also suggests the existence of a very diverse fungi microbiota in the breast milk (Boix-Amorós *et al.*, 2019).

The maternal biotope thus seems to influence the biotope of the child. The health of mothers before or during pregnancy could influence the baby's gut microbiota and therefore to some extent their health. But this statement should be put into perspective because the quality and composition of breast milk is very variable. Overall, the nutritional quality of human milk is highly conserved. However, macronutrient composition differs between preterm and term milk specifically during the lactation period (Léké *et al.*, 2019), with preterm milk is higher in true protein than term milk (Gidrewicz *et al.*, 2014). Fat is however the most highly variable macronutrient of milk (Ballard *et al.*, 2013).

This certainly depend on the immunological and dysbiotic status of the mother but also on her nutritional habits, her lifestyle, and the duration of lactation. More studies need to be done before making a causal link between the composition of the maternal microbiota and the composition of the infant's microbiota (Castanys-Muñoz *et al.*, 2016).

Not only but also, Human milk oligosaccharides (HMOs) are sugar molecules, that are part of the oligosaccharides group and which can be found in high concentrations exclusively in human breast milk. Though non-nutritive to the infant, HMOs constitute a remarkable quantity of human milk, similar to the quantity of total protein. These structures are synthesized by glycosyltransferases, enzymes that also synthesize similar structures in other human secretions and on mucosal surfaces. HMOs are therefore considered as prebiotic agents that selectively encourage the growth of beneficial micro-organisms such as bacteria belonging to the *Bifidobacterium* genus (Walsh *et al.*, 2020). In addition, HMOs and their protein conjugates are recognized as pathogen-binding inhibitors that function as soluble decoy receptors for pathogens that have an affinity for binding to oligosaccharide receptors expressed on the infant's intestinal surface (Ballard *et al.*, 2013).

Infants receiving formula seem to have a more varied biotope. By compared to breast-fed infants, the microbiota of formula-fed infants has more abundant populations of *Bacteroides*, *Clostridium* and *Enterobacteriaceae* (Harmsen *et al.*, 2000), breast milk being a richer source of *Bifidobacterium* and *Lactobacillus* (Penders *et al.*, 2006).

Food diversification,

Food is a primordial need for our survival and well-being. However, diet is not only essential to maintain human growth, reproduction, and health, but it also modulates and supports the symbiotic microbial communities that are responsible of colonizing the digestive tract—the GM (Makki *et al.*, 2018).

Diet has also a major impact on gut microbiota composition, diversity, and richness. Additionally, different components of the diet will shape the gut bacterial communities in a time-dependent manner whereas long-term dietary patterns, particularly the intake of protein and animal fat (*Bacteroides*) versus carbohydrates or plant-based foods (*Prevotella*), are associated with so-called enterotypes (Wu *et al.*, 2011).

Ecologically, dietary administration of fiber alters the niche environment in the gut by providing substrates for microbial growth, allowing microbial species that are able to utilize these substrates to expand their populations (Deehan *et al.*, 2017). For example, a diet rich in fiber contributes to the maintenance of a healthy gut microbiota associated with increased diversity and functions such as the production of short-chain fatty acids (SCFAs).

With food diversification, the microbiota of infants is gradually approaching that of adults. These changes are more significant in the breastfed infant. There is then a decrease in the number of *Bifidobacteria*, *enterobacteria* and *Clostridium* while the proportions of *Bacteroides* remain relatively stable (Castanys-Muñoz *et al.*, 2016). This section will be further discussed in detail in the second part of the manuscript.

Diet of children and adults,

The diversified diet contributes to the establishment of a specific intestinal ecology. Among the dietary factors, the only food additives, the amount of non-fiber digestible, the presence of disruptive drug treatments such as antibiotics or Proton Pump Inhibitors (PPI), the consumption of prebiotics, probiotics and symbiotics (the definition of which will be explained later) modify the composition of the microbiota (Gagliardi *et al.*, 2018). This section will be further discussed in more detail in (**Section 2.**) of the second chapter of the manuscript.

Antibiotics,

Generally, perturbation and disruption of optimal microbiota development, arising from preterm birth or antibiotics has likely long-term implications for microbial diversity and consequent health. Short-term antibiotic treatment can significantly affect the evolution of the infant gut microbiota; in fact, the colonization pattern of *Bifidobacterium* seems to be particularly disturbed up to 8 weeks after treatment while *Proteobacteria* are increased (Rodríguez *et al.*, 2015).

Antibiotics are, therefore, the main risk factor associated with damage to the gut microbiota and decreased phylogenetic diversity. The administration of these treatments during the first months of life favors colonization by opportunistic pathogens resistant to antibiotics, such as *Clostridium difficile* (Bonnemaison *et al.*, 2003; Langdon *et al.*, 2016).

Not only but also, antibiotic treatment in adult perturbs the intestinal microbiota, leading to an immediate reduction in microbial abundance and species diversity (Antonopoulos *et al.*, 2009) and suppression of the innate immune system (Bartlett, 2006; Rupnik *et al.*, 2009). Several studies indicate that the gut microbiota of healthy young adults is resilient after four days of broad-spectrum antibiotic treatment with recovery of most bacterial communities in approximately 6 months, with an individualized response of the human distal gut microbiota due to this repeated antibiotic perturbation (Les Dethlefsen *et al.*, 2011) whereas repeated perturbations may be particularly likely to cause such shifts, even when the community seems to have recovered from the initial perturbation (Paine *et al.*, 1998). One potential ramification of the altered community is an enhanced carriage of antibiotic-resistance genes in the human population (Salyers *et al.*, 2004). Therefore, antibiotic exposure reduces colonization resistance by freeing niches and nutrients and creating an immunosuppressed host state for invading pathogens to exploit. For example, *C. difficile* is the leading cause of antibiotic-associated diarrhea in the healthcare setting, colonizes patients (mainly by environmental spores) and rapidly overgrows in the intestine (Ghose *et al.*, 2013). *Clostridium difficile* produces two potent enterotoxins, harboring glucosyltransferase activity, that are translocated into enterocytes, resulting in irreversible disassembly of the actin cytoskeleton.

Environment and lifestyle,

Interestingly, the members of the same family and the close relatives (siblings) have been described as a relevant environmental factor influencing infant GM colonization. Thus, relatives have bigger proportion of *Bifidobacterium* spp. than single infants (Rodríguez *et al.*, 2015). for example, in a recent study conducted on baboons, it was found that social group membership and social network relationships predicted both the taxonomic structure of the gut microbiome and the structure of genes encoded by gut microbial species. Hence, Jenny and colleagues strongly implicate direct physical contact among social partners in the transmission of gut microbial species (Tung *et al.*, 2015).

Geographical location also has an impact on the microbiota, as microbiota differences are related to dietary patterns and lifestyle in a specific area (city, town, country, religion, etc.). It has been reported earlier that a ‘geographical gradient’ exists in the European infant microbiota whereas infants from Northern areas have higher levels of *Bifidobacterium* spp. and specific *Clostridium* spp. and *Atopobium* spp., while Southern infants had higher levels of *Eubacteria*, *Lactobacillus*, and *Bacteroides* (Rodríguez *et al.*, 2015). Significant differences between the microbiota of Finnish and German infants (Ruokolainen *et al.*, 2020) or between that of Estonian and Swedish ones (Rodríguez *et al.*, 2015) have been also reported. In the first study, the proportion of *Bifidobacterium* spp. was higher in the Finnish than in the German infants, who showed a higher abundance of the *Bacteroides*, *Prevotella*, and *Akkermansia muciniphila*. Another study reported higher levels of *Bifidobacterium* spp., *Bacteroides*, *Prevotella*, and *Clostridium histolyticum* in Malawi infants than in Finnish infants at the age of 6 months (Fouhy *et al.*, 2012). Furthermore, the gut microbiota of infants from Burkina Faso (BF) (rural village) was characterized by an enrichment of *Bacteroides*, while *Enterobacteriaceae* was predominant in Italian children, which is mainly due to dietary habits, whereas Burkina Faso children eat exclusively raw vegetables (De Filippo *et al.*, 2010) therefore having a gut microbiota that is capable of harvesting energy from plant-derived food, while metagenomic results showed that the gut microbiota harbors many genes encoding enzymes made to degrade cellulose and other plant-wall compounds. This could indicate how that this microbial simplification harbors the risk of depriving our microbial gene pool of potentially useful environmental gene reservoirs that allow adaptation to peculiar diets, as we observed in BF population.

In a recent study, the healthy infant microbiota from the Amazonas of Venezuela, rural Malawi and US metropolitan was compared (Yatsunenکو *et al.*, 2012). Shared patterns of gut microbiome development were identified during the first year of life in all populations, and *Bifidobacteria* was the most prevalent group, dominating the infant microbiota of all three groups during this period.

3.4. Microbiota functions

It is paradoxical that finding that the intestinal microbial community of each individual is highly subject-specific at the bacterial species level, while the microbiota functions are highly conserved between individuals and vital for their health. The intestinal microbiota plays multiple and complex roles in digestive, metabolic, immune, and neurological functions (Bull *et al.*, 2014). It ensures its own metabolism by drawing its energy sources from carbohydrates and proteins of food origin not digested in the upper part of the digestive tract and from endogenous secretions (mucopolysaccharides, debris cellular, enzymes, sterols,).

3.4.1. Gastrointestinal tract maturation

The microbiota intestinal content plays an important role in the participation of the maturation and the evolution of the digestive tract and helps to define the structure of the GIT via direct interaction with mucosal cell and immune cells resulting thus in the thickness of the intestinal mucosa, the size of the villi, mucus production, epithelial vascularization, and enzymatic activity of the mucosa (Di Mauro *et al.*, 2013). It also has a role similar to that of a barrier whereas the commensal microbiota is proven to be involved in maintenance of barrier function prompting an increased epithelial cell proliferation and enhancing intestinal epithelial integrity, through tight junction proteins translocation and gene up-regulation of those involved in desmosome maintenance (Ashida *et al.*, 2012).

To support this statement, some studies have observed structural aberration affecting germ-free mice wherein greatly enlarged cecum, lessened intestinal surface area, decreased epithelial cell turnover, smaller Peyer's Patches and disordered gut-associated lymphoid tissue and smaller villous thickness were noted (Shanahan *et al.*, 2002).

3.4.2. Metabolic "functions"

Microbiota bacteria play a direct role in digestion by taking part in all stages of the metabolism of food, by ensuring the fermentation of non-digestible substrates and food residues, by facilitating the assimilation of nutrients thanks to its enzymes and by ensuring the hydrolysis of complex polysaccharides such as starch or cellulose (Rowland *et al.*, 2018; Vernocchi *et al.*, 2020).

Some GI microbes could also participate effectively in the synthesis of certain types of vitamins (like vitamins K, B12, B8 and others) and the regulation of few metabolic pathways like the degradation of cholesterol, the absorption of fatty acids, calcium or magnesium (Weaver, 2015; Rowland *et al.*, 2018).

a) Carbohydrate metabolism:

Carbohydrates are metabolized on the one hand by digestive enzymes and on the other hand by microorganisms. Carbohydrate fermentation in the gut is an important source of energy. Carbohydrates, not digestible by intestinal enzymes, include large polysaccharides (cellulose, hemicellulose, lignin, pectin, and gums), some oligosaccharides or so-called prebiotics (fructooligosaccharides, inulin, galactosaccharides and lactulose), sugars unabsorbable and alcohols. The main final product of this metabolism is the generation of short chain fatty acids (den Besten *et al.*, 2013).

SCFAs, particularly acetate, propionate, and butyrate, are mainly generated by the fermentation of digestible dietary fibers (soluble fiber) by the intestinal microbiota (G. Huang *et al.*, 2018). They are the end products of this anaerobic bacterial fermentation in the GI tract. They are a major source of energy for the host, in general, and the colon, in particular. While there is broad agreement that SCFAs confer beneficial effects on metabolism, mechanisms that mediate interactions remain under debate. Some of the benefits can be presumed to result directly from their provision of key sources of energy. For example, SCFAs used by various tissues would seem to reduce the need for glucose production from the liver. However, it is also now clear that a significant portion of the impact of SCFAs is mediated by one or more SCFAs. SCFAs are then reported to activate at least 4 distinct G-protein-coupled receptors including GPR41, GPR43, GPR109A, and Olfactory receptor 78 (Olf78) whereas GPR41 and GPR43 are the best studied of these, at least in terms as their role as receptors for SCFAs and are widely expressed and reported

to confer an array of metabolic effects. Briefly, these SCFA receptors are expressed on pancreatic β -cells, adipocytes, and enteroendocrine cells wherein they can activate a series of events including production of GLP-1, insulin, and leptin that would generally favor energy storage and reduce energy intake. GPR43, in particular, is also reported to drive enteroendocrine cell production of appetite-suppressing hormone peptide YY (PYY) (Zou, Chassaing, Singh, Pellizzon, Ricci, M. D. Fythe, *et al.*, 2018). SCFAs play a significant role as well in regulating glucose, lipid and energy metabolism, and may effectively reduce the risk of developing gastrointestinal disorders, cancer, and cardiovascular disease, among others (Tungland *et al.*, 2018).

All these fatty acids have important functions in the host physiology. Butyrate is almost entirely consumed by the colon's epithelium and is a major source of energy for the colonocytes. Acetate and propionate pass into the bloodstream from the portal circulation and are eventually metabolized by the liver (propionate) or by the peripheral tissues, more specifically, the muscle (acetate). Acetate and propionate may act as modulators of glucose metabolism by improving insulin sensitivity (Guarner *et al.*, 2003; Franks *et al.*, 2013).

b) Lipid metabolism

The intestinal flora exerts an indirect action by modifying the metabolism of cholesterol and bile salts. Ninety-five percent of bile acids follow the enterohepatic cycle before being re-secreted into the bile. Only 5% of the bile acids secreted in the bile therefore reach in the colon where they are metabolized (deconjugation, oxidation, epimerization, 7- α -dehydroxylation, desulfation, etc.) by bacteria of the microbiota into so-called secondary bile acids (Ridlon *et al.*, 2006). Deconjugation (species of the genera *Bacteroides*, *Bifidobacterium*, *Clostridium*, etc.) makes bile acids more hydrophobic and promotes their passive absorption (Landman *et al.*, 2016).

Lipids serve as a substrate for *Lactobacilli* and *Bifidobacteria*, hence the production of SCFAs, and more specifically, propionate. Propionate would then act in the same way as bile salt chelating resins, fixing bile acids in the form of an insoluble complex. The enterohepatic cycle of bile acids is then inhibited, and their faecal elimination increased. In addition, the absorbed cholesterol is then used for the synthesis of new bile acids and the blood cholesterol level decreases (Eckburg *et al.*, 2005).

c) Protein metabolism

The anaerobic metabolism of peptides and proteins by the microbial microflora produces SCFAs, but also at the same time a series of potentially toxic substances including ammonia, amines, phenols, thiols, and indoles. Branched-chain fatty acids (BCFAs), such as isovalerate and isobutyrate, can also be produced as end-products. Additionally, some gut microbial species, mainly from the class Bacilli, also possess a specialized branched-chain keto acid dehydrogenase complex to yield energy from the oxidized forms of the branched-chain amino acids directly, which also leads to BCFA production (Portune *et al.*, 2016). Proteins available include food-borne elastin and collagen, pancreatic enzymes, degenerate epithelial cells, and lysed bacteria (McConnell *et al.*, 2013).

Nevertheless, amino acids can also implicate bacterial flora. It can be either utilized for the synthesis of bacterial cell components or catabolized through different pathways. This diversity of amino acid metabolism in gut bacteria may have either positive or negative effects on the host. In consequence, modulating dietary protein or amino acid intake may lay out a strategy for shaping the amino acid fermenting bacteria and their metabolic pathways, thereby potentially affecting host metabolism (Neis *et al.*, 2015).

In fact, microbiota can curiously use some amino acids such as Tryptophan (Trp), functional in regulating host physiology and metabolism (Liang *et al.*, 2018; Miklavcic *et al.*, 2018) by the direct transformation of Trp by intestinal microorganisms into several molecules, such as indole and its derivatives such as AhR ligands that are finally able to tune local and distant host functions, including immune homeostasis and barrier physiology (Agus *et al.*, 2018). Also, a recent study conducted by Liang demonstrated that dietary Tryptophan supplementation for 4 weeks altered the intestinal microbiota and enhanced the abundance of intestinal tight-junction proteins. The beneficial effect of Trp was hence associated with an increased abundance of microflora (Liang *et al.*, 2018).

d) Mineral and vitamin metabolism

Colon microorganisms also play a role in the synthesis of vitamins and in the absorption of calcium, magnesium, and iron. Some bacteria have the ability to synthesize vitamins as mentioned above, including vitamin K, vitamin B 12, folic acid (B9), biotin (B8), riboflavin (B2) and

pantothenic acid (B5) (Morowitz *et al.*, 2011; Yoshii *et al.*, 2019). However, some intestinal bacteria are unfortunately unable to synthesize B vitamins and must earn them from the host diet or from other intestinal bacteria for their growth and survival. This claims that the composition and function of the intestinal microbiota may affect host B vitamin usage and, by extension, host immunity (Yoshii *et al.*, 2019). Also, numerous studies have shown that minerals functions are also affected by the microbiota wherein some *in vitro* studies have confirmed that *Bifidobacteriaceae* are capable of binding iron in the large intestine, thereby limiting the formation of free radicals synthesized in the presence of iron, and thus reducing the risk of colorectal cancer (Skrypnik *et al.*, 2018). While other *in vivo* studies have revealed that probiotics and/or prebiotics and synbiotics supplementation has a significant effect on bone calcium, phosphate and bone metabolism. The dynamic interaction between microbiota and other minerals like zinc was also shown.

3.4.3. Immune functions

The gut microbiota stimulates the development of the innate and adaptive immune system of the host. It plays an essential role in the maturation of the intestinal immune system, the regulation of the immune response and the protection against external pathogens by preventing their implantation and their multiplication. In addition, it has been shown that in animals without microbiota (called axenic or germ free), the immune system is far less developed (Kennedy *et al.*, 2018). It has also been proven that certain bacteria have the capacity to prevent the development of inflammatory diseases (*Saccharomyces boulardii* and *Lactobacillus* spp.), while on the flip side, other bacteria are capable of causing inflammation and that depending on the bacterial response (*Staphylococcus aureus*), the microbiota therefore order pro- and anti-inflammatory responses. This underlines the importance of the diversity of bacteria in the microbiota. This diversity prevents colonization of the digestive tract by a single bacterial species, colonization which would then be detrimental for the host (Iacob *et al.*, 2019) for that lower diversity is considered as a marker of dysbiosis. The strong connection between reduced diversity and disease indicates perhaps that a species-rich gut ecosystem is more robust against environmental influences, as functionally related microbes in an intact ecosystem can compensate and pay back for the function of other missing species. Consequently, diversity seems to be a generally good marker of a healthy gut (Valdes *et al.*, 2018).

Once detected, bacteria in the intestinal flora can stimulate the synthesis of antimicrobial peptides by host cells such as intestinal epithelial cells and in particular Paneth cells. These peptides have an antibiotic type of activity by bactericidal or bacteriostatic effect.

The microbiota can also stimulate the immune system by inducing the production of secretory immunoglobulin A (sIgA) by plasma cells (Strugnell and Wijburg, 2010) (which favors both maintenance of non-invasive commensal bacteria and neutralization of invasive pathogens through multiple mechanisms (Johansen *et al.*, 1999; Strugnell *et al.*, 2010).

A study on the murine transcriptome shows that it is the genes involved in the immune system that are most regulated in the presence of the microbiota (Mutch *et al.*, 2005). In addition, *Bacteroides fragilis* has been shown to be able, through its capsular polysaccharides, to stimulate a wide variety of T cells, illustrating the ability of commensal bacteria to modulate the adaptative immune system (Mazmanian *et al.*, 2006).

3.4.4. Protective ‘functions’

The intestinal microbiota has been shown to protect against a large number of enteropathogenic agents, such as *Clostridium*, *Escherichia coli*, *Salmonella*, *Pseudomonas*, and limit the multiplication of saprophytic yeasts such as *Candida albicans*. This protection is exerted by bacterial interference.

The slightly acidic pH, resulting from the fermentation process of lactic acid bacteria and the production of SCFAs, inhibits the development of potentially pathogenic germs in the GI tract. In contrast, many species of aerobic and anaerobic bacteria are capable of producing harmful substances for the body such as ammonia, amines, thiols, phenols and indoles. These products can cause intestinal damage (Salminen *et al.*, 1995). This explains the importance of maintaining the commensal intestinal flora by promoting its growth to ensure an effective protective role to limit these effects. Apart of the acidic pH, bacteria can produce bacteriocins that are bacterially produced peptides that are active against other bacteria and against which the producer has a specific immunity mechanism (Cotter *et al.*, 2005). They may function as colonizing peptides, facilitating the introduction and/or dominance of a producer into an already occupied niche. Alternatively, bacteriocins may as well act as antimicrobial or killing peptides, directly inhibiting competing strains or pathogens. Lastly, bacteriocins may function as signaling peptides, either

signaling other bacteria through quorum sensing (QS) and bacterial cross talk within microbial communities or signaling cells of the host immune system (Dobson *et al.*, 2012).

The microbiota also promotes direct colonization resistance through killing and competition for resources. Bacteria must compete for limited nutrient sources in the gut (Pickard *et al.*, 2017). At the same time, they have developed an array of armaments to directly kill competitors. The pair mechanisms tend to act between more closely related species whereas similar bacteria tend to utilize similar nutrients or niches and have evolved targeted killing mechanisms to compete with their own kind.

4. Dysbiosis state

4.1. Dysbiosis definition

The co-existence of the gut microbiota and the host reveals the determining role of the gut microbial flora in host health, and maintenance of the gut microbiota equilibrium is highly principal to the host gut and overall systemic physiology.

The equilibrium of the microbial ecosystem is reached when there is a mutualistic relationship between the members of the intestinal flora, the metabolic products, and the immune system of the host. This mutualistic relationship is called symbiosis and could lead to the emergence of the holobiont concept. In a basal state, the intestinal microbiota lives in symbiosis with its host and their interactions are necessary to support and maintain host homeostasis. It is now considered an organ on its own, relatively stable over time and resilient to external events in a physiological situation.

In certain pathological situations, this symbiosis microbiota host can be disrupted and then create an imbalance associated with harmful consequences for the host; creating what we call dysbiosis (Marteau P *et al.*, 2012). It can be characterized by an excess of potentially harmful microorganisms called pathobionts, a lack of beneficial microorganisms, a restriction of biodiversity, a reduction in the number of microbial genes or even microbiota interactions, a loss of richness or diversity, an imbalance between phyla and / or genera and specific depletions of certain species. Although the microbiota is highly implicated in inflammatory diseases, no single species has been found to be fully responsible. In fact, several observations demonstrate that the

immune system, the first line of dialogue with our microbiota, responds to a wide range of bacterial markers, making the overall scene a little complex to decode.

The factors of variation can be endogenous: an immunodeficiency, various infections, or diseases, or exogenous such as a sudden change in environment or diet (food substrates, deficiency in digestive enzymes more or less associated with food intolerance (intolerance to milk or meat for example) (Gagliardi *et al.*, 2018) and the administration of certain drugs, more particularly, antibiotics or proton pump inhibitors, laxatives, transit retarders (Belkaid *et al.*, 2014; Syer *et al.*, 2014).

At the level of the intestinal ecosystem, this dysbiosis can be explained by several points. First, the need for "keystone" species to keep the system stable and explain this functional redundancy shared by all individuals. When one is swept away due to one of the exogenous and endogenous factors cited above, the ecosystem will start being permanently disturbed, leading to illness. Although all these host factors can induce dysbiosis in gut microbiome, the main debate remains whether these elements are rather risk factors inducing the occurrence of dysbiosis or causes leading to the dysbiosis state and thus developing other dysbiosis- associated diseases afterwards.

In summary, dysbiosis can be defined as an unfavorable disruption of the balance of the gut microbiota (Moré *et al.*, 2015). Even if there is a functional microbial core (40% of microbial genes are shared by half of the world's population) (Gagliardi *et al.*, 2018). the presence of significant intra- and inter-individual variability in the composition of the digestive flora complicates the definition of a healthy microbiota. Dysbiosis cannot therefore be considered as a single pathological profile. It is described as a microbiota profile where the species beneficial bacteria (*lactobacilli* and / or *bifidobacteria* for example...) see their overall concentrations reduced. Likewise, the concept of "beneficial" or "harmful" microbes is not universally acceptable. Germs can be harmful or beneficial in one individual but not in another. In addition, among one species some bacterial can be commensal whereas some other are highly pathogenic, as illustrated by the *E. coli* species. The habitat and context in which a microbe is introduced or multiplies must also be considered. A microbe having a different behavior depending on the environment in which it is found, it is considered that the composition and functioning of the microbiota depends on multiple factors. The phylogenetics of the species constituting the digestive flora, their diversity and even the existence of central species (or dominant) and the functional dynamics of the species

present confronted with the host's genotype, its environment (taking treatment for example, etc.), its diet and the occurrence of pathology (s) modulate the functionality of the microbiota and can induce dysbiosis (Doré *et al.*, 2010).

Hence, dysbiosis is likely to impair the normal functioning of gut microbiota in maintaining host wellness, and potentially induce selective-enumeration of certain microbiota member including pathobionts, leading to dysregulated production of microbial-derived products or metabolites which might be harmful to the host, causing diverse range of diseases on local, systemic or remote organ (Kho *et al.*, 2018).

4.2. Functional consequences of gut microbiota dysbiosis

Firstly, the non-trivial question is whether dysbiosis is the cause or consequence of inflammation as already discussed above. It is important to understand whether changes in microbial ecosystems are causally linked to the pathology and to what extent disease risk is predictable based on characteristic changes in community structure and/or function.

As for the functional consequences that a dysbiotic GM could generate, a rich literature proposed various forms of disfunctions associated with the gut dysbiosis. As the GM plays an important role in the development of the normal mucosal immune system (whether humoral or/and cellular), including the development of gut-associated lymphoid tissues (Round *et al.*, 2009), a negatively altered microbiome could logically lead to a weakened immunity characterized by a disturbed immune homeostasis (Toor *et al.*, 2019), leading to various forms of inflammation contributing thus to diverse inflammatory diseases that will be furthermore discussed in part 3.3 of this section, as well as a disturbed intestinal mucosa (Toor *et al.*, 2019).

Other functional consequences could also be translated through various food imbalance features. In fact, studies in some human cohorts have shown that there is a consequential association between dysbiosis and pathogenesis of food allergy, while studies from animal models have demonstrated the capacity of specific species in the GM to alter immune response, which may lead to the desensitization of food allergy (Lee *et al.*, 2020). Although all these studies were not able to identify the specific bacterial taxa that are somehow consistently associated or responsible of food

allergy, due to heterogeneity in study design, they still show that microbiota diversity and composition are considerably associated with the onset of food allergy, perhaps by affecting host metabolism (Sicherer *et al.*, 2014; Iweala *et al.*, 2016) and the alteration of adaptive immunity (Brandt *et al.*, 2003; McCoy *et al.*, 2006), by creating an aberrant immune response (Plunkett *et al.*, 2017). Several sequencing-studies have identified a lower relative abundance of bacterial class Clostridia (bacterial phylum *Firmicutes*) in children with food allergy compared to healthy children (Dominguez-Bello *et al.*, 2010; Cao, Feehley *et al.*, 2014; Bäckhed *et al.*, 2015) while other epidemiologic studies noted an association between *Bacteroidetes* phyla in the gut and food allergy (Cho *et al.*, 2012; David *et al.*, 2014). The reason behind this is that the intestinal microbiota owns a great importance in the development of oral tolerance which prevents the immune system from reacting to harmless commensal bacterial and food antigens (Wambre *et al.*, 2018). Therefore, a healthy immune response to food antigens is seemingly a state of non-responsiveness, referred to usually as oral tolerance. Mechanistically, when oral intolerance is rather induced, food antigens can instead evoke a response characterized by the release of mast cell mediators, such as histamine, leading to the symptoms associated with food allergy like in the case of anaphylaxis (Beghdadi *et al.*, 2011).

4.3. Associated diseases

As a result of these many disfunctions, the intestinal microbiota could cause any imbalance and can therefore be at the origin of the development of pathologies (Mitsuoka *et al.*, 2009). Technically, a heterogeneity of pathologies is associated, in a way or another, with changes in both the community structure and function of the GM, suggesting some sort of connection between dysbiosis and disease etiology. It is important to understand whether changes in microbial ecosystems are causally linked to the pathology and to what extent disease risk is predicable based on characteristic changes in community structure and/or function.

Once dysbiosis occurs, it manifests itself in several ways such as digestive disorders, including transit problems (diarrhea, constipation), bloating or sometimes irritable bowel syndrome (disorder of the functioning of the colon), immune disorders, including infections of the respiratory tract, urinary tract, vaginal tract and allergies such as atopic dermatitis (or eczema), or by metabolic problems, namely an increase in cholesterol, obesity, or diabetes (Festi *et al.*, 2014).

By antonymy to eubiosis, a dysbiosis of the intestinal ecosystem is not only associated with intestinal disorders but also with other diseases such as obesity (Ley *et al.*, 2005; Turnbaugh *et al.*, 2006). A recent work conducted *in vivo* on mice models and that aimed to determine the association between non-high-fat diet-induced obesity and the associated gut microbiome dysbiosis with certain gut abnormalities (Nagpal *et al.*, 2018) found that obesity eventually impacted cellular turnover of the intestine with by its turn increased cell death and cell survival/proliferation gene expression with significantly enhanced stemness, which are linked with increased intestinal permeability, changes in villi/crypt length, and decreased expression of tight junctions and mucus synthesis genes along with dysbiotic gut microbiome signature (Nagpal *et al.*, 2018).

Also, in patients with Crohn's disease, a study has shown that the "*Clostridium leptum*" group is greatly reduced, both in diversity and in abundance (Manichanh *et al.*, 2006). In another context, the increase in *Bacteroides* and the fall in *Firmicutes* would be accompanied by a faculty of the microbiota to more easily store the energy brought by food, which would constitute a risk factor for obesity (Backhed *et al.*, 2004; Ley *et al.*, 2006).

Abundant literature has suggested that a reduced bacterial diversity and an imbalance between harmful and protective bacteria in the intestine are largely responsible for the rising incidence of IBD and other infectious and chronic inflammatory pathologies (Gevers *et al.*, 2014; Petersen *et al.*, 2014).

Current theories suggest that the rising incidence of IBD is a result of one, if not more, of various causative factors (Kho *et al.*, 2018) including (i) innate genetic defects in the intestinal epithelial and mucosal barrier, which may thus lead to bacterial translocation; (ii) microbial imbalance or dysbiosis, in the intestine and (iii) A sort of dysfunction in the intestinal inflammatory cascade, contributing to an eventual pathologic proliferation of inflammatory cytokines (Colombel *et al.*, 2007).

What is worth mentioning here is the fact that for some diseases, dysbiosis can actually contribute to the disease's onset. This circle sticks to the fact that for some cases, these diseases are the main cause for dysbiosis while for some other cases it is only a consequence. A third case also exists whereas the role of the dysbiosis is absolutely ambiguous with no definitive elements completing the idea that these diseases are the outcomes of the dysbiosis state.

Chapter 2. Strategies to manipulate the gut microbiota and Intestinal Homeostasis.

Homeostasis is defined as the ability of an organism to maintain a state of relative stability between the various components of its internal environment, despite constant variations in the external environment. As seen in the below section, many diseases are associated to gut microbiota dysbiosis but in most cases, it remains unclear whether dysbiosis is a cause or a consequence of the disease state. According to the characteristics of gut microbiota dysbiosis and its suggested impact on the whole-body homeostasis, several nutritional and therapeutic approaches can be considered and developed in order to modulate the gut microbiota and thereby restoring locally the homeostasis in the GIT and in the whole body.

1. Fecal transplantation

The fecal microbiota transplantation (FMT) is a promising strategy for the treatment of a wide range of diseases suspected to be caused, at least in part, by a dysbiosis of the gut microbiota (Ianiro *et al.*, 2020).

1.1. Principle:

The modulation of the gut microbiota by FMT primarily follows the probiotic principle, but instead of treating the patient with specific strains, a complex community of microorganisms coming from a healthy donor is used. In 1958, the first study using feces as a therapy was documented in humans for the treatment of pseudomembranous colitis (Rao *et al.*, 2015). Since then, FMT has gained increasing attention. A further flashback on its history, fecal transplantation has been used in Chinese medicine since the 4th century. Human fecal suspensions were administered as “golden soups” or ‘yellow juice’ to treat severe diarrhea (Shi *et al.*, 2018). Since then, this technique has been used in Western countries in veterinary medicine on farm animals (Ma *et al.*, 2017). Recently, this technique is recognized in France by the national agency for the safety of medicines and health products (ANSM) which has issued recommendations to supervise clinical trials since 2014. Moreover, today in France, samples are taken in hospitals, but in some countries stool banks have been opened, as in the United States since 2012 or in the Netherlands more recently (Terveer *et al.*, 2017). Today, FMT is widely known as an emerging technique to treat patients with dysbiosis by the restoration of their abnormal gut microbiota composition *via* transplantation of normal fecal microbiota obtained from healthy donors. Interestingly, this transplantation can be remarkably stable

over time, with modifications in the composition of the patients gut microbiota lasting for months or years after the transplantation (Goloshchapov *et al.*, 2019).

1.2. Impact on human diseases:

Today, FMT has been highlighted as a new therapeutic approach treating many diseases (Antushevich, 2020). Among them, FMT is successfully used as a therapeutic tool in patients suffering from severe relapsing *Clostridium difficile* infection (CDI) (Berg *et al.*, 2015). Interestingly, FMT has been shown to be one of the moderately effective available treatments for such recurrent CDI in many studies (Wortelboer *et al.*, 2019): A systematic review evaluated the efficacy and safety profile of FMT in recurrent CDI patients and found that 87% of 536 subjects experienced resolution of diarrhea (Ma *et al.*, 2017). In fact, the composition of the donor microbiota is a relevant key factor in determining treatment efficacy. Microbiota replacement via FMT in CDI patients was shown to produce a microbiota like that of the donor 2 weeks post-transplantation, as determined by 16S rRNA profiling (Ott *et al.*, 2017). Indeed, microbiota modulation strategies for the donor before FMT may further profit the recipient. However, little is known about the complete mechanisms underlying FMT therapy in such diseases. As a matter of fact, FMT has been shown to be more efficient than treatment with a 'synthetic microbial mixture', which was characterized as transplantation with a microorganism community that could be controlled and tested for the presence of viruses or pathogens (Vos *et al.*, 2013). Possible explanations for this difference include bacterial quantity and quality, as well as their postbiotic ability (Vieira *et al.*, 2016). Another recent randomized controlled trial evaluated the efficacy of FMT administered *via* duodenal infusion and found significantly higher eradication rates (81%) of vancomycin-induced diarrhea for FMT administered *via* duodenal infusion than for the control treatment. Based on this study, the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) approved the use of FMT for multiple recurrent CDI (Debast *et al.*, 2014).

Apart from treating CDI, FMT was notably tested in the context of **Inflammatory Bowel Diseases (IBDs)**, a group of intestinal disorders comprising Crohn's diseases (CD) and Ulcerative colitis (UC) (Lopez *et al.*, 2016). After all the studies, IBD etiology is still not completely understood, but a key feature of IBD we know is an inappropriate inflammatory response to the intestinal microbiota in genetically susceptible individuals (Kaser *et al.*, 2010). It is known that IBD patients have greater microbiota instability and lower abundance of Firmicutes than healthy subjects (Gong

et al., 2016; Clooney *et al.*, 2021). An increased abundance of Actinobacteria and Enterobacteriaceae has also been reported, and these findings suggest that dysbiosis is associated with IBDs development (Lane *et al.*, 2017). Therefore, microbiota modulation strategies have been introduced as promising alternative IBDs therapies. Several approaches (antibiotics, probiotics...) have been tested in IBDs as strategies to restore the unbalanced gut microbiota, however, there are no conclusive data concerning their efficacy, explaining the necessity to develop new strategies such as FMT.

FMT has productively gained interest as a novel treatment option for IBDs. A rigorous systematic review of numerous studies that used FMT as the primary therapeutic agent in IBDs described some benefits of this strategy (Lopez *et al.*, 2016). The first effort of FMT to treat UC was made in 1989 (Bennet *et al.*, 1989). The largest study conducted on this topic, which covered a wide range of CD patients, reported that FMT might be one of the moderately safe and effective treatment for refractory CD (Cui *et al.*, 2015). Similarly, a randomized trial including approximately 70 UC patients, conducted by Moayyedi and colleagues, demonstrated that FMT was safe and induced disease remission in more patients compared to placebo (Moayyedi *et al.*, 2015). Currently, the number of controlled clinical trials testing FMT in IBDs with large cohorts of patients are very limited, thus further clinical studies are required to evaluate the short- and long-term beneficial effects of FMT for the treatment of IBD.

Beyond the treatment of CDI and IBD, a growing number of clinical trials assess the efficiency of FMT in a wide range of human diseases including the irritable bowel syndrome, obesity, cirrhosis, multidrug resistant infections, neuropsychiatric illnesses (autism, schizophrenia) (Gupta *et al.*, 2020; Bajaj *et al.*, 2019).

1.3. Current limits of FMT and perspectives:

There are many issues and important points that remain confusing and uncertain and are worth mentioning when evaluating FMT, such as the likelihood of side effects, including the following phenomenon's bacterial stability and translocation, genetic factors of individual recipients that may influence the success of FMT and bacterial colonization, or transplant rejection similar to organ transplantation, although generally well tolerated, there have been some serious adverse events, notably with the transfer of various pathogens for that it is complicated to check all pathobionts and slightly hard to characterize and well define a healthy gut microbiota (Merrick *et*

al., 2020). As an illustration of this issue, a study recently assessed the transmission of potential pro-carcinogenic bacteria (e.g., enterotoxigenic *Bacteroides fragilis*, *Fusobacterium nucleatum*, and *Escherichia coli* Pks+) from donors to patients during FMT and concluded that durable transmission of these pro-carcinogenic bacteria can occur during FMT, suggesting potential long-term side effects.

Hopefully, the efforts will probably lead to the refinement of synthetic stool that may have a beneficial effect in IBD and other diseases. As the age of personalized medicine occurs, the future of FMT may involve microbiome profiling of patients with individualized microbial treatments as opposed to a one-microbiome-fits-all approach. However, while the future holds promise, there exists not enough data at this time to support the routine use of FMT for IBD. Further studies are thus necessary to optimize and improve this technique and expand the knowledge of FMT in treating a range of microbiota-associated inflammatory diseases.

2. Dietary interventions

Hippocrates' said: "Let food be thy medicine and medicine be thy food" remains highly relevant millennia later but requires consideration of how diet can be used for modulation of gut microbial ecology to promote health (Makki *et al.*, 2018).

Diet has a major impact on gut microbiota composition, in term of diversity and richness, mostly by bringing substrates necessary to the survival and growth of gut-associated microorganisms. In order to understand how diet shapes the composition of the microbiota, we will further discuss the influence of certain diets on the equilibrium, the quality and the balance of this gut microbiota. The contribution of diet to modulating the microbiota and its crucial role in orienting the host–microbiota crosstalk is evident from the beginning of life, when human milk oligosaccharides (HMOs) participate in the maturation of the microbiota in early infancy (Charbonneau *et al.*, 2016) followed by increased bacterial richness associated with the introduction of solid food and diversification (Laursen *et al.*, 2017) and concludes with decreased richness observed in frail ageing populations in long-stay care, probably due to reduced food diversity (Claesson *et al.*, 2012). Mainly, the major aspect by which diet influences the microbiota is its contents — namely, the macronutrients and micronutrients that make up consumed meals. This aspect of nutrition has been broadly investigated as it is largely believed that the striking stream in metabolic diseases and other sequelae in modernized societies can be assigned to changing dietary trends in the past

century (Sonnenburg *et al.*, 2016). In fact, gut microbiota has co-evolved with the host over thousands of years and generations to form an intricate and mutually beneficial relationship. The evolution of the human diet over the past 10,000 years from a Paleolithic diet to our present modern pattern of intake has resulted in intense changes in feeding behavior, especially during the last century. Past cultural deviations and changes in habitat and ecology, there have been quite a good number of major dietary shifts in human evolution, including meat eating, cooking, and those linked with plant and animal domestication (Luca *et al.*, 2010). These shifts have occurred from diets high in fruits, vegetables, lean meats, and seafood to processed foods high in sodium and hydrogenated fats and low in fiber. These dietary changes have adversely affected dietary parameters known to be related to health (Jew *et al.*, 2009) and has led towards a whole different shaped diet, that might have affected the symbiotic relationship of the human body with the resident gut microbiota. Different components of the diet will shape the gut bacterial communities. The influence of such specific foods on the composition of this microbiota has been studied mainly for specific compounds such as macronutrients such as polysaccharides (fibers and sugars), fats and proteins consumed by the host are partially degraded by the gut microbiota and represent a continuous source of substrate for these microorganisms. As a consequence, certain dietary fibers, soluble fibers such as inulin (present in artichoke or endive in particular), are considered as prebiotics, stimulating the growth of bacterial species associated with health benefits among the intestinal flora. Beyond macronutrients, the micronutrients, such as vitamins, polyphenols or minerals can also modulate the gut microbiota composition (Dostál *et al.*, 2013). For example, the consumption of iron-fortified cookies has been shown to be ineffective in reducing anemia in children (their initial purpose) but leads to a change in their microbial profile (Zimmermann *et al.*, 2010) and microbiota (Greenhalgh *et al.*, 2019).

First clues of the relevance of long-term dietary intervention to modulate the gut microbiota came from the comparison of gut microbiota composition between populations following a certain extreme diet like a vegetarian or gluten-free one compared to the rest of the population (Tomova *et al.*, 2019) whereas vegans and the vegetarian population have significantly higher counts of certain *Bacteroidetes*-related operational taxonomic units compared to omnivores. Changes in this microbiota composition might be due in majority to differences in bacteria directly consumed through food, differences in some substrates consumed, variations in transit time through the gastrointestinal system, pH, host secretion influenced by dietary patterns, and regulation of gene

expression of the host himself and/or his/her microbiota (Salonen *et al.*, 2014). In addition to that, there seem to be striking differences linked to the country, even within countries with the same standard of living, which suggests that different eating habits and lifestyles strongly impact the intestinal microbiota in the long term. A better understanding of the long-term dynamic of the gut microbiota upon diet changes will be helpful to design interventions aiming to bring amicrobiota back to a pre-disease state in patients (Costea *et al.*, 2018).

Changes in the composition and nature of food can have a very rapid effect on the composition of the microbiota as exemplified by this study in human demonstrating that shift from a conventional diet to an animal-based or a plant-based diet for 4 days is sufficient to induce deep changes in the gut microbiota composition (Lawrence A. David *et al.*, 2014). Diet has been also demonstrated to be capable of reconstructing and restructuring the microbiome within quite a couple of days yet is typically reversible on a similar timescale (Zhang *et al.*, 2012; Lawrence A David *et al.*, 2014; Carmody *et al.*, 2015). It is therefore theoretically possible to modify the biodiversity of the bacteria present by dietary interventions, to modify the energy production or more broadly the metabolism of the microbiota and consequently that of the host, however diet-induced disturbances on the gut microbiota composition and functions cannot be easily and robustly predicted. This is, for example, what certain so-called functional foods (such as oatmeal) claim with most often a low level of scientific evidence (Morand *et al.*, 2017). In fact, the study of diet on the intestinal microbial ecosystem still shows a certain resilience of communities and the significant changes in the composition of the gut microbiota in individuals following a specific dietary intervention for few days are only transient and the gut microbiota returns to its initial state rapidly after the intervention (Qin *et al.*, 2010; Lawrence A. David *et al.*, 2014). Once fixed, a digestive ecosystem therefore seems relatively stable and resilient to short-term interventions. Long-term dietary interventions seem more promising, but individuals can have more difficulties to adhere to important dietary changes over the long term (Sacks *et al.*, 2009). A significant change in eating habits (by modifying fat, protein, and carbohydrate contents) over a period of one year was studied in humans, (Houghton *et al.*, 2016) and aimed to modulate the gut microbiota in both the composition and activity (Houghton *et al.*, 2016) wherein most GM alterations have been based around changes in the *Firmicutes:Bacteroidetes* ratio that is by far considered to be a quite relevant marker of gut changes (Magne *et al.*, 2020) and has been described in being the dominant groups in the distal gut microbiota (Eckburg *et al.*, 2005; Qin *et al.*, 2010). While short-term therapeutic

antibiotic treatment was shown to induce substantial, partially recoverable shifts in the GM of humans (Les Dethlefsen *et al.*, 2011; Voigt *et al.*, 2015), it was also shown that it can however lead to a complete deterioration of the community and subsequent pathogen invasion (e.g., *C. difficile*) (van Nood *et al.*, 2013), effectively leading to a failure to recover the original community state (Costea *et al.*, 2018). Contrarily, dietary interventions, which cause considerably quite less perturbation to the microbial ecosystem of the human gut, may thus be a more convenient fit for investigating community resilience. Interestingly, such compositional changes interventions could be observed within only four days, and enterotypes appear to be quite steady and stable in a period of around ten days, suggesting a quite good tendency of recovering the original state. Stability was as well tracked in a six-month intervention, using the ratio of *Prevotella* to *Bacteroides* (obtained by qPCR method) as a proxy for entero-type assignments. The results above enable to suggest that there are several limitations on how much an individual's microbiome may be disturbingly perturbed by short-term dietary interventions, and support thus enterotype resilience. In contrast to that, long-term perturbations have been shown to possess a more profound effect with dietary modulation over the period of a year having a rough impact on the *Bacteroidetes/Firmicutes* ratio, potentially leading to enterotype switches. As enterotypes are generally considered to be stable over the time, no follow-up studies were claimed to be necessary nor exist for the long-term interventions, therefore, no available approximation of their resilience rate, either in terms of overall community resemblance or enterotype assignment, could be concluded from the already existing data library (Costea *et al.*, 2018). While long-term diets influence the structure and activity of the trillions of microorganisms residing in the human gut (Lawrence A. David *et al.*, 2014), it remains unclear how rapidly and reproducibly the human gut microbiome responds to short-term macronutrient change and what are the more sensitive species to these changes in the GIT. Some particular nutrients are suggested to favor the growth and colonization of the GIT by beneficial microbes and are called prebiotics. They are usually digested and fermented in the colon by resident microorganisms and could be used in synergy with probiotics, in formulation called synbiotics (de Vrese *et al.*, 2008; Markowiak *et al.*, 2017). There are also many indications in the literature that, due to the use of prebiotics, probiotic microorganisms acquire larger tolerance to environmental conditions and stresses, including: oxygenation, pH, and temperature in the intestine of a particular organism (Sekhon *et al.*, 2010).

The following section focuses on the effects of the most extensively studied diet modifications: fiber and sugar-rich diet, protein and lipid-rich diet, and high-fat diet (HFD) (Pickel *et al.*, 2020). Of all the macronutrients, carbohydrates and nitrogen sources have been demonstrated to be the most influential on this microbiome (Sonnenburg *et al.*, 2016; Holmes *et al.*, 2017; Reese *et al.*, 2018). Also, simple sugars can override host genetic effects on the microbiome (Kashyap *et al.*, 2013).

2.1. Global food intake

Food is a pivotal adjustable factor influencing the composition of the GM, indicating the potential for therapeutic dietary strategies to manipulate microbial diversity, composition, and stability. While different regimens can induce a shift in the gut microbiota, these changes appear to be temporary. Whether prolonged dietary changes can induce permanent alterations in the gut microbiota is relatively unknown, mainly due to a lack of long-term human dietary interventions, or long-term follow-ups of short-term dietary interventions (Leeming *et al.*, 2019). In the sections below, we describe how modulation of the global food intake in term of quantity and temporality could affect the GM.

2.1.1. Caloric Restriction

Calorie restriction (CR) is a nutritional intervention of reduced energy intake or caloric intake, typically by 20 - 40% of *ad libitum* consumption, without it causing malnutrition and while maintaining adequate nutrient intake (Cantó *et al.*, 2009; Jasper Most *et al.*, 2017). It appears that CR is the most effective and reproducible dietary intervention known to take part in the regulation of aging and the increase of the healthy lifespan in various model organisms, ranging from the unicellular yeast to worms, flies, rodents, and primates (Lee *et al.*, 2016). It also protects against age-associated diseases including chronic inflammatory disorders such as cardiovascular disease and diabetes (Ferrucci *et al.*, 2018). However, caloric restriction could be considered as a severe intervention that results in both beneficial and detrimental effects (like anemia, migraines, extreme fatigue, and vitamin-related deficiency issues such as muscle weakness, hair loss and scaly skin, brittle nails, reduced bone strength, increase risk of fractures and abnormal heart rhythms (Otten *et al.*, 2006), as this phenomenon has been scrutinized further, it appears that prevention of free radical formation and the downregulation of inflammatory responses contributes to the benefits of CR (Anderson *et al.*, 2010). The main mechanisms behind this observation gained much attention

in the recent research field and identification of these metabolic pathways influenced by CR has brought out interest in finding molecular targets that could be modulated by drugs mimicking CR. It has been shown that both short- and long-term CR may have an impact on immunity (Jolly *et al.*, 2004). Animal studies (i.e. rodents) suggest that while CR could have beneficial effects on the adaptive immune response, particularly T-cells, it may have a detrimental effect on the innate immune response with impaired ability to control infections by monocytes/macrophages (Jolly *et al.*, 2004; González *et al.*, 2012). Specifically, it has been proven that calorie restriction ameliorates IL-2 production and T cell proliferation that is usually reduced through aging (Goonewardene *et al.*, 1995; Pahlavani *et al.*, 1997) and reduces the incidence, duration and severity of experimental autoimmune uveoretinitis in rats correlating with lower levels of IFN γ and auto-antibody production (Abe *et al.*, 2001). Caloric restriction has been proved to increase autophagy through inhibition of the mammalian Target of Rapamycin (mTOR) pathway that is known to be related to autophagy signaling changes and is activated in the presence of nutrients or growth factors; by reducing insulin and IGF levels (Chung *et al.*, 2019). Caloric restriction achieves thus mTOR inhibition through two pathways: decreased PI3K activity and increased AMPK activity. Usually, cells in low energy states (calorically restricted) have low PI3K activity, lowering Akt activity, which then lowers mTORC1 via inhibition by Tsc1/2 (Arbor *et al.*, 2019). The molecular pathways increased by caloric restriction that increase lifespan are also targeted by rapamycin, resveratrol, and metformin (Blagosklonny *et al.*, 2021). Normally, the (mTOR) pathway is activated by the presence of nutrients (glucose and amino acids), growth factors and insulin, and regulates a wide range of cellular processes, including protein synthesis and pro-inflammatory gene expression modulation notably *via* the activation of the transcription factor nuclear factor kappa B (NF- κ B). On the other hand, the mTOR kinase is directly inhibited by rapamycin, and indirectly by resveratrol, which activate AMPK, a kinase inhibiting mTOR activation (Hindupur *et al.*, 2015). Upon mTOR inhibition, the histone deacetylase, Sirtuin-1, considered a master metabolic regulator, exhibits the ability to run and to modulate numerous transcription factors including de-acetylation (inactivation) of NF- κ B, resulting in reducing the transcription process of pro-inflammatory genes. Resveratrol also enhances activation of Sirtuin-1 (negative regulator of NF κ B) and inhibits PI3K and S6K (positive regulators of NF- κ B).

2.1.2. Differences between caloric restriction and dietary restriction

There are two possible ways of defining CR that have distinct and noninterchangeable meanings and the distinction is important to make, because the latter term can yield insights into the physiological mechanisms by which diet affects life span, whereas the former term cannot.

Whereas CR is a reduction of caloric intake, Dietary Restriction (DR) is a specific reduction/manipulation in nutrient intake. DR do not always necessarily result in caloric restriction, because the reduced intake of a particular nutrient (typically a macronutrient) may be offset by an increased intake of one or more other nutrient. As an example, if carbohydrate intake is reduced, then protein and/or lipid intake can perhaps be increased in the purpose to maintain a quite normal caloric intake. Given this, DR seems to be considerably more bearable than CR, especially when taking into consideration the fact that some researchers estimate that energy consumption must be reduced by a minimum of 20 - 25% in order to beget any life-extending benefits from a CR regimen (Djuric *et al.*, 2002). Instead, specific types of chronic, intermittent, or periodic dietary restrictions without chronic caloric restriction have therefore the potential to provide a significant health span increase while minimizing adverse effects. Hence, improved periodic or targeted dietary restriction regimens that uncouple the challenge of food deprivation from the beneficial effects will perhaps allow a safe intervention achievable for a major portion of the population (Lee *et al.*, 2016). The definition of DR has been therefore expanded from an alternative description of CR to also encompass a broader scope of interventions, including short-term starvation, periodic fasting, fasting-mimetic diets, intermittent fasting, normo-caloric diets with planned deficiencies (in particular, macronutrients: proteins, carbohydrates, etc.), and time-restricted feeding. Most of these relatively novel interventions are reported to have beneficial effects on overall health and in some cases longevity.

Simply reducing protein intake can deliver an equally potent impact on lifespan as dietary restriction in multiple model organisms (Mirzaei *et al.*, 2014). A recent analysis of the National Health and Nutrition Examination Survey (NHANES) showed that low protein intake was associated with reduced overall mortality for those under 65 years of age. Also, a high-carbohydrate, low-protein diet resulted in longer lifespan and improved cardiometabolic health, despite increased food intake and body fat (Solon-Biet *et al.*, 2014). Furthermore, the restriction of a single essential amino acid in a normal diet increased lifespan and stress resistance (Richie *et*

al., 1994; Zimmerman *et al.*, 2003a) whereas reducing the tryptophan content in the diet showed to extend maximum life span. Also, lowering the content of sulfhydryl-containing amino acids in the diet by removing cysteine and restricting the concentration of methionine has been shown to extend all parameters of survival (Zimmerman *et al.*, 2003b). In flies, adding back essential amino acids to the caloric restricted diet decreased lifespan to that of the normally fed group. Laboratory rodents fed a methionine-restricted diet displayed an extended lifespan with decreased age-dependent diseases and increased resistance to oxidative stress, in part due to increased antioxidant capacity (Orentreich *et al.*, 1993; Richie *et al.*, 1994). A tryptophan-restricted diet also provided longevity and reduced age-dependent deterioration (Lee *et al.*, 2016) but has mainly been explored for neurological benefits, due to its role in serotonin synthesis.

Finally, wherein all these diet types focus on food deprivation, a main important factor seems to influence largely on the response of the gut microbiota and hence, on the host's status: the feeding rhythms.

2.1.3. Fasting and feeding rhythm

Overview

Fasting is an extreme emerging dieting pattern consisting in abstinence from all solid food for a defined time period (usually practiced by restricting eating periods from 12 to 24 h) that typically range from 12 h to three weeks (Longo *et al.*, 2014). This phenomenon has been regularly practiced for millennia, for a variety of reasons that range from dieting to religious beliefs to medical testing (like for blood glucose and lipid markers) and—involuntarily—probably for much longer (millions of years) during human evolution, in this way shaping human metabolic flexibility (Freese *et al.*, 2018).

Fasting is distinct from CR in which the daily caloric intake is reduced chronically by 20–40%, but meal frequency is maintained. Starvation is instead a chronic nutritional insufficiency that is commonly used as a substitute for the word fasting, particularly in lower eukaryotes, but that is also used to define extreme forms of fasting, which can result in degeneration and death.

CR and fasting share similar but often distinct effects on a number of biomarkers (e.g., reduced glucose, and insulin levels) suggesting that partially overlapping mechanisms are involved (Lee *et al.*, 2011).

Fasting protocols,

Among alternative interventions for the prevention and treatment of chronic diseases and severe illnesses, different forms of fasting and fasting mimicking diets have the topmost potential of being integrated into the standard medical care and treatment schedule. These range from time restricted feeding (TRF), feeding every other day (alternate day fasting), adopting a reduced calorie regimen twice a week (5:2 fasting), or undergoing a simple periodic cycle of regimes that grant a relatively high caloric content but able to copy or adequately mimic many of the fasting effects (Fasting Mimicking Diets) (Longo *et al.*, 2016). A fasting-mimicking diet, consisting of very low calorie and protein that leads to similar physiological response to fasting, including reduced levels of glucose and IGF-1 and increased levels of ketone bodies IGFBP-1, enhanced health span and rejuvenated the hematopoietic system while promoting adult neurogenesis (Lee *et al.*, 2016). Intermittent fasting however refers to practicing this intervention every other day whereas periodic fasting refers to severe restriction for two or more days periodically (every two weeks, month, etc.). The strategies of intermittent fasting (IF) can differ adequately, for instance according to different daily hours of fasting. A well-known intermittent fasting pattern is Ramadan fasting, which entails abstinence from eating and drinking from sunrise to sunset over a period of approximately 30 days during the month of Ramadan (Lessan *et al.*, 2018) and is being widely studied for its impact on human health and disease in population-based studies (Rouhani *et al.*, 2014). Both intermittent and periodic fasting can increase lifespan, even when there is little or no overall decrease in calorie intake (Anson *et al.*, 2003; Brandhorst *et al.*, 2015). A great number of studies have provided evidence for health benefits of intermittent fasting to host (De Cabo *et al.*, 2019). Understanding the mechanistic link between nutrients and fasting benefits is leading more and more to the identification of newer fasting mimicking diets (FMDs) that achieve purposes comparable to those caused by fasting just like in the case of Martinez-Lopez's study where they suggested that consuming two meals a day in similar food amounts at two short windows early and late in the diurnal cycle called Isocaloric Twice a Day (ITAD) feeding, and without a CR regimen is sufficient to induce autophagy, alter nutrients metabolism and prevent the metabolic syndrome (Martinez-Lopez *et al.*, 2017) just like fasting is hypothesised to achieve in stricter conditions. Also, other studies indicated that when mice were given access to food for only 8–9 hours during the active phase of the day, metabolic diseases induced by a high-fat, high-fructose, and high-sucrose diet, were reduced without lowering caloric intake (Hatori *et al.*, 2012).

The benefits of time-restricted feeding against such obesogenic diets were proportional to the duration of the fasting each day (Chaix *et al.*, 2014). Ad libitum feeding during the weekend did not however interfere with the protective effects of TRF (Chaix *et al.*, 2014). Notably, the restricted feeding pattern reversed the progression of pre-existing obesity and type II diabetes, suggesting it has the potential to be a clinically relevant and feasible dietary intervention, useful to prevent and treat obesity and metabolic disorders (Chaix *et al.*, 2014).

In addition to all listed strategies above, the restriction of specific macronutrients (or macronutrient restriction) without the restriction of calories is among the most promising interventions that have emerged to promote healthy aging in humans. Among the different types of macronutrient restriction, reduced intake of proteins and amino acids is the most effective pro-longevity intervention (Grandison *et al.*, 2009; Mirzaei *et al.*, 2014).

Circadian clock,

Basically, life forms on our planet have evolved under the strong influence of a daily light/dark cycle. Daily rhythm in environmental factors and food availability has led to the evolution of a 24 h internal timing mechanism or circadian clock to enable organisms to predict or anticipate daily changes and to upgrade and optimize fitness. Fundamental to this 24 h rhythms are the ability to acquire food when it is available and to store a portion of these resources for utilization during the rest of the day (like the fasting period) without compromising fitness and vitality. The fasting period also serves as a break time for standby and repair so that the organism is fit, ready and competent to bring in and harvest energy when food or light becomes available enough once again (Longo *et al.*, 2016).

In brief, feeding in most creatures is confined to a defined and determined time periods, leaving short periods of fasting that might coincide with sleep. Fasting on the other hand, enables organisms to enter alternative and substitute metabolic phases, which rely less on glucose and more on ketone body-like carbon sources. Both intermittent and periodic fasting result in benefits ranging from prevention to the enhanced treatment of different diseases (Longo *et al.*, 2016). Similarly, TRF, in which feeding time is restricted to certain hours of the day, allows the daily fasting period to last up to >12 h, thus imparting pleiotropic and various benefits in multiple organisms (Longo *et al.*, 2016). Considering that key metabolic factors, such as AMPK, sirtuins,

and AKT, are regulated by a cooperation of circadian rhythm and feeding time as a teamwork (Vollmers *et al.*, 2009; Eckel-Mahan *et al.*, 2013), dietary schedules should therefore be more carefully studied in the context of dietary restriction. Not only but also, emerging evidence suggesting that rhythmic signals play a major role in immune (Lee *et al.*, 2008; Silver *et al.*, 2012) and metabolic (Feillet *et al.*, 2006) functions naturally leads to the possibility of exploring biological rhythms as targets of quite interventional strategies. A persisting question of interest, however, is whether the readout of intermittent fasting under longer daily duration is due to fasting duration, reduction of food intake or both together. The answer will also be in favor of the mechanistic distinction between intermittent fasting and the very classical caloric restriction diet.

Effects of fasting and feeding rhythm on the gut microbiota,

Fasting and different feeding rhythms can also trigger important GM changes which may in turn influence host health and immunity (Jabbar *et al.*, 2003). One recent study assessed the effects of different daily fasting hours on shaping the gut microbiota in mice where healthy male mice were subjected to 12, 16 or 20 h fasting per day for 1 month, and then fed ad libitum for an extended month and resulted into alterations in the composition of gut microbiota by all types of intermittent fasting. At genus level, 16 h fasting led to increased level of *Akkermansia* and decreased level of *Alistipes*, but these effects disappeared after the cessation of fasting. Thus, the obtained data indicated that intermittent fasting shapes gut microbiota in healthy mice, and the length of daily fasting interval may influence the outcome of intermittent fasting (Li *et al.*, 2020). Numerous additional scientific reports demonstrated the beneficial effects of fasting, short-term calorie restriction, or protein-restricted diets in mice models of certain types of cancer (Brandhorst *et al.*, 2013; Fontana *et al.*, 2013). One of the main mechanisms through which fasting induces metabolic improvements is certainly mediated by the gut microbiota (Li *et al.*, 2017). For instance, every-other-day fasting (EODF) treatment led to a remarkable shift in the gut microbiota composition, ascending in the levels of *Firmicutes* while descending in most other phyla and consequently increasing the production of SCFAs as compared to control animals fed ad libitum. In addition to the fasting effect, food withdrawal decreased the abundance of potentially pathogenic *Proteobacteria* while it increased in *Akkermansia muciniphila* levels (Zheng *et al.*, 2018).

While the fundamental bacterial taxa are resilient to most temporary external influences, the gut microbial community displays an eminent inter-individual day-to-day variability (Leeming *et al.*,

2019). Within 24 to 48h of a dietary intervention, swift changes are thought to be made to the microbial composition on diverse species and family level (but not phyla) (Sonnenburg *et al.*, 2016).

This striking variability is explained only partially by the diet composition itself, with a good number of intrinsic and extrinsic factors thought to contribute like feeding behaviors and practices, and a circadian rhythm (Liang *et al.*, 2015; Parkar *et al.*, 2019). Although the GM is not accessible nor exposed to the dark and light cycle associated with the circadian rhythm, its composition and function are thought to still be affected by this cyclical ups and downs (Thaiss *et al.*, 2014). Basically, in humans, at least 10% of Operational Taxonomic Units (OTUs) oscillate due to the circadian rhythm (Thaiss *et al.*, 2014). The microbiota fluctuates thereby based on nutrient availability and depending on the level of host-derived autoantibodies and peptides, both of which are associated with circadian rhythm oscillations (Thaiss *et al.*, 2014, 2016; Liang *et al.*, 2018). The microbiota is thought to program these synchronized diurnal oscillations by rhythmic histone acetylation through epithelial histone deacetylase 3 (HDAC3). HDAC3 integrates microbial and circadian signals, through which metabolic gene expression and nutrient intake are affected. Jetlag, an acknowledged disrupter of the body's internal clock and eating patterns, generates real changes in the microbial composition in an investigational study in two humans and mice (Broussard *et al.*, 2016). Likewise, disrupted sleep patterns, often common in shift workers, has been found to alter the gut microbiota, increase dietary intake (Poroyko *et al.*, 2016). The feeding regimen itself has a powerful strapping training effect on peripheral oscillators such as the liver and intestine (Oosterman *et al.*, 2015). It is therefore very possible that manipulation of feeding times and control of fasting periods, including time and duration of consumption and frequency, may affect the gut microbial composition and function and potentially the overall host health (Kaczmarek *et al.*, 2017). In humans, Kaczmarek *et al.* observed that a couple of bacteria were connected to the eating times, like the *Barnesiellaceae* genus which decreased with higher eating frequency in the behavioral model. Also, new relations emerged with overnight fast duration, whereby the relative abundance of *Coprococcus* increased with longer overnight-fast duration and whereas *Holdemania* decreased with longer overnight-fast duration (Kaczmarek *et al.*, 2017). Likewise, in mouse models Thaiss *et al.* noted that a rhythmic food intake not only leads to 15% of commensal bacterial taxonomic units swinging throughout the course of a day, but also increased microbial abundance (Thaiss *et al.*, 2014). The impact of this specific meal timing in humans on

The gut and oral microbiotas were successfully discovered in a 2018 randomized crossover experimental study by Collado et al. where the timing of a meal was found to strike the diurnal rhythms of the salivary microbial profile with eating the main meal late shown to increase salivary taxa (Collado *et al.*, 2018). With the capacity of the gut microbiota to swing in as short a time span as an hour (Sonnenburg *et al.*, 2016), this poses the question of whether hunger related to delayed feeding could potentially affect the composition of the GM or not. Despite this, no significant effect of the exact eating time as in eating early or late was observed on the overall faecal microbial composition in this study (Collado *et al.*, 2018). In fact, in a mouse model, several bacteria and bacterial metabolites have been shown to be involved in regulation of hunger and satiety, with their production dependent on bacterial growth cycles (Fetissov *et al.*, 2017). Anyhow, basic characteristics of the effect of fasting or time-restricted feeding on the gut microbiota are still unknown until today with a limited number of observational studies of religious fasting and some modest experimental studies, most with fewer than 50 participants (Zarrinpar *et al.*, 2016). Due to the co-evolution that exists between us and our gut ecology (Garud *et al.*, 2019) the GM's ability to rapidly respond to dietary changes and contributions may be the reflective mirror of our dietary intake collection that was based on necessity for dietary flexibility with periods of feast and famine (Lawrence A. David *et al.*, 2014).

These relatively rapid changes to the gut microbiota could be a 'shock reaction' to an invasion of incoming nutrients, possibly causing a transient disruption of microbial composition (Klimenko *et al.*, 2018). However, coping with this kind of stress or collapse is part of the inherent plastic nature of the normal microbiota. In this way, the gut microbiota can adapt and adopt a new beneficial or detrimental state when faced with a continuous perturbation or disruption (Leeming *et al.*, 2019).

Vegan Diet,

Some long or permanent diet restrictions are based on the intake of specific kind of nutrients, whether depending on an ethical point of view or a health matter such as the Vegan or the Vegetarian kind of diets. Vegetarian diets are defined as a way of living that attempt to exclude all forms of animal exploitation and cruelty, whether for food, clothing, or any other purpose. A vegan diet is, however, another form of vegetarianism where only plant foods are eaten and all foods from animal sources are avoided (meat, seafood, eggs and dairy). Both diets are generally enriched in fermentable plant-based foods and are known to lower rates of illness and death from

some degenerative diseases (Steyn *et al.*, 2000). One study compared vegan and vegetarian diets to an unrestricted control diet and found that both vegans and vegetarians had significantly lower counts of *Bifidobacterium* and *Bacteroides* species (G. D. Wu *et al.*, 2016). Interestingly, another different study found a very modest difference in the gut microbiome's profiles of vegan versus omnivorous subjects (Zimmer *et al.*, 2012). The discrepancy between the two studies may be due to different methodologies for microbiome profiling including culture- vs sequencing-based approaches, different control group diets, and/or individual host genetics. Moreover, microbial counts of *Bacteroides* spp., *Bifidobacterium* spp., *E. coli*, *Enterobacter* spp., *Enterococcus* spp., *Clostridium* spp., *Klebsiella* spp. and *Lactobacillus* spp. are not significantly different between vegans and vegetarians according to J. Zimmer *et al.* (Zimmer *et al.*, 2012). It has also been shown that a vegetarian diet affects intestinal microbiota, specifically by decreasing and modifying the diversity of the *Clostridium* cluster IV (Liszt *et al.*, 2009). Future research with precise experimental designs will be required in the future in order to decode the differences between vegan and vegetarian diets and hence provide more insight into the differential effects of the two diets on the gut microbiome. Plant-based diets high in fiber intake also encourages the growth of species that ferment fiber into metabolites as short-chain fatty acids (SCFAs), including acetate, propionate, and butyrate (Tomova *et al.*, 2019).

The results of different studies showed that GM composition differs widely between different areas and between different ethnic groups within the same area. The conclusions highlight that gut microbiota composition differs according to diet and eating habits which are closely correlated to geographical location suggesting therefore, the need for more in-depth research, looking at ethnic diversity and eating habits.

2.2. Sugars and fibers

On the basis of the number of sugar units and how the sugar units are chemically bonded to each other, carbohydrates are subdivided into several categories. These categories include sugars, and fibers, and starches, and will be respectively described each apart in the text below.

Simple sugars,

Sugar seems to have developed a reputation as the big bad wolf in relation to health as the excess sugar in diets has been hypothesized to be causative of numerous modern diseases prevalent in westernized cultures. These diseases include metabolic syndrome and its component diseases obesity, type 2 diabetes, cardiovascular disease (Ginsberg *et al.*, 2009), liver disease, tooth decay, and neurodegenerative disorders, including Alzheimer disease (Di Rienzi *et al.*, 2020). The link between sugar and these diseases has been interestingly postulated to be at least partially through the gut microbiome (Di Rienzi *et al.*, 2020). The implication is that increased consumption of existing sugars and novel sweeteners has altered the carbohydrate pools available to the microbiome, creating distinct environments in the gut that are filled by exogenous microbes or endogenous microbes that have undergone adaptation, some of which are potentially pathogenic and called pathobiont. Scientific evidence suggests that consumption of diets enriched in simple sugar condition the microbiota, resulting in the acquisition of a westernized microbiome as hypothesized above, which is characterized by a substantial reduction in the gut microbial diversity (Klement *et al.*, 2019). According to Moon Ho Do, high-sugar consumption has been noted to induce changes in the gut microbiota composition characterized by a lower proportion of Bacteroidetes and a markedly increased proportion of Proteobacteria, as well as induction in obesity and metabolic disorder through recent studies (Do *et al.*, 2018), wherein few of these studies have reported that high-dose fructose or glucose intake correlates with detrimental health outcomes (Do *et al.*, 2018). Accordingly, Crescenzo *et al.* reported that obesity and insulin resistance are elicited by a high-fructose diet (HFrd) in adult rats (Crescenzo *et al.*, 2017). Moreover, a diet high in fructose is suggested to effectively induce inflammation and metabolic dysregulation in the gut and liver due to alterations in gut microbial communities characterized by a significant decrease of lactobacilli in HFD group (Jena *et al.*, 2014).

Fibers,

Some prebiotics, including FOS, inulin-type fructans, and galacto-oligosaccharides (GOS) were observed to exert beneficial effects by increasing the proportion of *Bifidobacteria* in the gut, especially in the context of a detrimental high fat diet that usually reduced the prevalence of these bacteria (Davani-Davari *et al.*, 2019). Other carbohydrates, with prebiotic properties, such as arabinoxylans can also have a similar potential (Reis *et al.*, 2014).

Dietary fibers can be defined as plant polysaccharides and lignins resistant to human digestive enzymes. By contrast to simple sugars, they are mostly not digested in the small intestine and therefore enter the colon largely undegraded, representing substrates of interest for the gut microbiota. The classification of fibers in figure 4 is based in the first instance on whether the site of action is in the upper or lower gut and then further according to the nature of action (degraded microbially or not), the physical origin of the fiber (plant cell wall material or not); and the chemical identity of each fiber (Ha *et al.*, 2000).

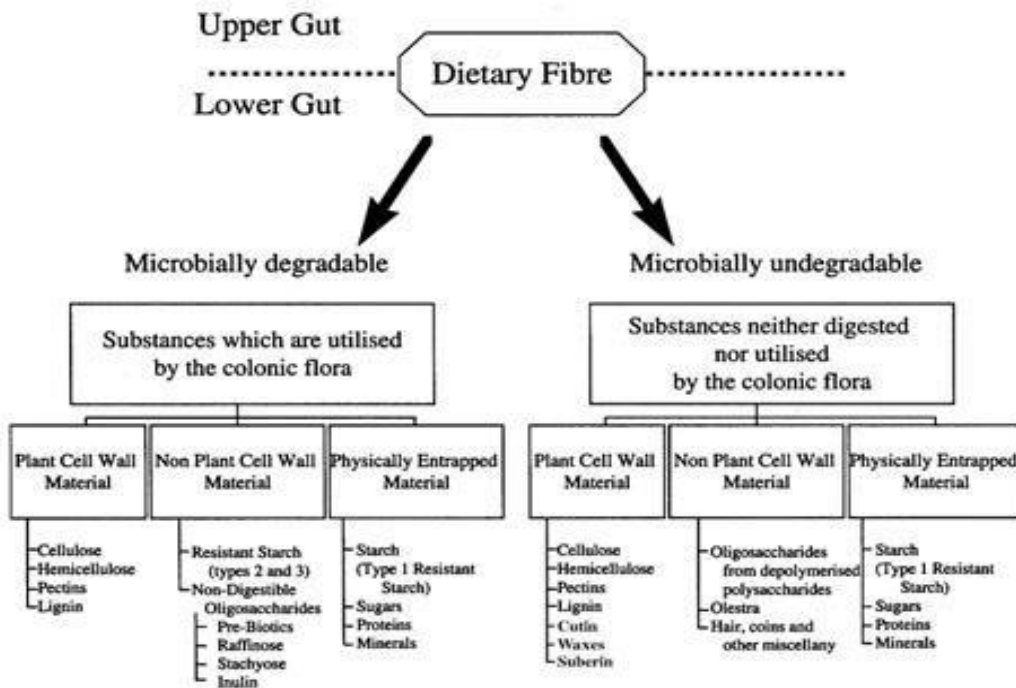


Figure 4: Proposed classification of dietary fibers

(Ha *et al.*, 2000)

Dietary fibers are also classified according to further several parameters, including their water solubility, and viscosity, and their fermentability and are subdivided either into polysaccharides (non-starch polysaccharides (NSPs), resistant starch (RS), and resistant oligosaccharides (ROs) or into insoluble and soluble forms (Deehan *et al.*, 2021). While soluble fibers absorb water to become a gelatinous substance during intestinal transit, insoluble fibers pass through the intestinal tract while remaining largely unchanged. As an example, a study has shown that the degradation rate of cellulose is lower (15 to 25%) than that of non-cellulosic polysaccharides (70 to 95%)

(Cummings *et al.*, 1997). The only plant polysaccharide known to be partially hydrolysable by human enzymes is exclusively starch that is made up of many glucose units linked together. Chemically, dietary fiber consists of non-starchy polysaccharides, such as cellulose, and many other non-cellulosic components such as dextrans, inulin, waxes, chitins, pectins, beta-glucans, gums, substances obtained from marine algae, as well as lactulose, soy oligosaccharides, fructooligosaccharides, galactooligosaccharides, xylooligosaccharides, and isomaltooligosaccharides (Eastwood *et al.*, 2005). Fibers are therefore a heterogeneous mixture both chemically and physically and it is quite difficult to generalize in terms of their effects on the human intestine or gut microbiota composition (Motulsky *et al.*, 1990).

Impact of dietary fibers on the gut microbiota,

As mentioned above, during their passage through the digestive tract, a large part of fibers is fermented by the gut microbiota affecting therefore the gut resident microorganisms by changing their metabolic activities as well as their quantities and relative abundance in the GI tract. These activities largely determine the physicochemical environment of the GI system, and that is what make the impact of dietary fiber intake on the microbiota intensely important. After fermentation, the bacteria fermentation product becomes absorbable, mainly in the form of SCFA (including butyrate, propionate, and acetate) which is nutritionally, essential, and indispensable for the host. Interestingly, studies in mice have shown that the beneficial gut microbes of mice fed with a high-fiber diet promotes the production of the SCFA propionate (Parada Venegas *et al.*, 2019). In contrast, diets high in animal protein and simple sugars tend to reduce the levels of SCFA and bacteria belonging to the genus *Bifidobacterium* present in the gut (Gagliardi *et al.*, 2018).

Mechanistically, *Bacteroides* groups have a high proportion of *cazyme* and enzymes specific in carbohydrate metabolism. Several studies link western-type diets and *Bacteroides* enterotype while diets rich in plant polysaccharides are associated with a major proportion of *Prevotella* (Firmicutes enterotype) (Gagliardi *et al.*, 2018), which are able to degrade plant fibers thanks to the expression of hydrolases enzymes (Arumugam *et al.*, 2011). Individuals bringing many fibers in their diet seem to constitute therefore a *Firmicutes* enterotype with many bacteria of the genus *Prevotella* (Arumugam *et al.*, 2011) as species belonging to *Firmicutes* (and *Actinobacteria*) are the main responders to dietary fiber (Deehan *et al.*, 2021), although they contain relatively few fiber-metabolizing enzymes per organism. However, they generally have more specialized roles

such as the initiation of complex substrate degradation. It is not exclusively the enzymatic capacity (as a primary fiber degrader) that determines the ability of a microbe to benefit from a dietary fiber, but also its ability to successfully ‘adhere to a certain substrate, tolerate the physico-chemical conditions generated by the fibers degradation (e.g., increased acidity through fermentation), and benefit from carbohydrate breakdown products (secondary fiber degraders) and metabolites (through cross-feeding) (Deehan *et al.*, 2021). Primary fiber degraders can hereby function as keystone species that initiate the utilization of a complex fiber (Zhao *et al.*, 2018). For example, *Ruminococcus bromii* is considered as a keystone species for the degradation of RS and contributes significantly to butyrate production in the colon, although the species itself does not produce butyrate. Altogether, it is estimated that the gut microbiome harbors approximately 130 glycoside hydrolase, 22 polysaccharide lyase, and 16 carbohydrate esterase families, which allow the microbiome flexibility to switch between different energy sources of fibers, depending on their availability (Makki *et al.*, 2018).

This dichotomy in the *Prevotella/Bacteroides* ratio was also observed between industrialized and non-industrialized human populations (Makki *et al.*, 2018).

Similar observations were made in animal models, in fact, Sonnenburg *et al.* investigated the consequences of the lack of fiber (referred to as Microbiota accessible carbohydrates MACs) intake in mice colonized with a human microbiota and showed that a low-MACs diet led to dramatically reduced microbial diversity in just three generations, which could not be restored when mice were moved to a normal-MAC diet (Sonnenburg *et al.*, 2016). In point of fact, creating the food web to bacteria, dietary administration of fiber alters the niche environment in the gut by supplying substrates for microbial growth, allowing the expansion in the population of microbial species that are able to utilize these substrates (Deehan *et al.*, 2021). Additionally, taxon-based analysis of a recent study showed marked changes in the gut microbial compositions of the HFD, HGD, and HFrD groups. At the phylum level, these groups had a significantly lower relative abundance of *Bacteroidetes* and significantly increased abundance of *Proteobacteria* compared to the normal diet group (Do *et al.*, 2018). In an early study, it was suggested that diets rich in complex carbohydrates increased the levels of beneficial *Bifidobacteria* such as the subspecies *Bifidobacterium longum*, *Bifidobacterium breve*, and *Bifidobacterium thetaiotaomicron* (Pokusaeva *et al.*, 2011) while, on the other hand, reducing the growth of opportunistic species such as *Mycobacterium avium* subspecies paratuberculosis and *Enterobacteriaceae* (Brown *et al.*,

2012). In brief, dietary fiber, arabinoxylan, GOS, Inulin type fructan, resistant starch, and polydextran have major bifidogenic effects and can positively modulate health-beneficial microbes in the gut and the utmost potentially health-beneficial microbes modulated by these main carbohydrates are *Bifidobacterium* sp., *Lactobacillus* sp, *Akkermansia* sp, *Fecalibacterium* sp., *Roseburia* sp., *Bacteroides* sp. and *Prevotella*, *Roseburia*, *Clostridium lepum* and *Ruminococcus intestinalis* (Yang *et al.*, 2020). Thus, some dietary fibers are usually regarded as prebiotics and could represent a promising dietary intervention to sustain diversity in the gut microbiota. Basically, vegetables, fruits, whole grains, milk, milk products and other edible plants are major sources of carbohydrates constituting potential prebiotics (Markowiak *et al.*, 2017). Also, some artificially produced prebiotics are, among others: lactulose, galactooligosaccharides, fructooligosaccharides, maltooligosaccharides, cyclodextrins, and lactosaccharose whereas lactulose constitutes a significant part of produced oligosaccharides (as much as 40%). Fructans, such as inulin and oligofructose, are believed to be the most used and effective in relation to many species of probiotics (Zou, Chassaing, Singh, Pellizzon, Ricci, M. Fythe, *et al.*, 2018).

Consequences of dietary fibers intake on host intestinal homeostasis and disease prevention,

What is also worth noting is that dietary fibers can mechanically stimulate the intestinal epithelium to secrete mucus (McRorie *et al.*, 2017) for that insoluble particles have a mechanically irritating effect on the mucosa of the large bowel, stimulating secretion of water and mucous as a defense mechanism to protect from abrasion (McRorie *et al.*, 2017). Subsequently, prolonged lack of dietary fibers reduces the mucus barrier and is associated with increased abundance of the mucin-degrading bacteria such as *Akkermansia muciniphila* (Desai *et al.*, 2016).

On the other hand, there are also several links between fiber intake, the immune system, and associated diseases for that microbial metabolism of fibers by a fermentation of fibers to SCFAs are known to promote a generation of colonic regulatory T cells (Tregs) in a GPR43-dependent manner as well as by inducing histone H3 acetylation (Smith *et al.*, 2013), emphasizing therefore the important role of SCFAs in regulating and maintaining a normal function of the innate and adaptive immune system.

Based on the positive impact of dietary fibers on the host homeostasis, especially in the GI compartment, it can be expected that dietary fibers might have beneficial effects in human diseases related to gastrointestinal disorders such as IBD. Inulin, for example, a soluble fiber molecule,

and a commonly available prebiotic, was suggested in previous studies that it can reduce symptoms in dextran sodium sulfate (DSS)-induced colitis in mice (Nolan L.W. Nadolski *et al.*, 2020). In a study conducted in 2012, results indicated that colitis was significantly reduced in all fructo-oligosaccharides (FOS)-fed rats compared to the control diet, whereas inulin decreased chronic intestinal inflammation in only half the number of animals (Koleva *et al.*, 2012). Nevertheless, a fiber-rich diet composed of the soluble and fermentable fiber inulin (20% of food) was proven to prevent high-fat diet (HFD)-induced metabolic syndrome in mice, notably by reducing weight gain and adipose tissues development, by lowering levels of cholesterol and by preventing dysglycemia in a 2017 study (Zou, Chassaing, Singh, Pellizzon, Ricci, M. Fythe, *et al.*, 2018).

2.3. Lipids

Overview,

As important elements of the diet, found predominately in butter, oils, meats, dairy products, nuts, seeds, and in many processed foods, dietary lipids influence host physiology directly but also indirectly through interaction with the gut microbiota (Schoeler *et al.*, 2019). In addition, the GM can influence the host lipid metabolism through metabolites produced by the gut microbiota such as SCFAs, secondary bile acids, trimethylamine and by pro-inflammatory bacterially derived factors such as lipopolysaccharide as described in many mouse models (Schoeler *et al.*, 2019).

First of all, speaking metabolism, although most of the fatty acids consumed can be digested and absorbed in the small intestine and it is estimated that less than 5% reach the colon (Mu *et al.*, 2004; Morales *et al.*, 2016), Thus there is still a minority of fat components that enter the colon (Liu *et al.*, 2016) where they can potentially affect the colonic microbial composition. Some microorganisms in the gut are known to possess lipases and can degrade triglycerides and phospholipids into their polar head groups and free lipids (Jaeger *et al.*, 1994; Morales *et al.*, 2016). Triglycerides represent 95% of total dietary fat, whereas phospholipids, mostly in the form of phosphatidylcholine, constitute a minor portion, but are also derived endogenously from bile acids (Wang *et al.*, 2013).

Lipids affect the gut microbiota both as substrates for bacterial metabolic processes, and by inhibiting bacterial growth by toxic influence (Schoeler *et al.*, 2019).

Negative effects of lipids on the gut microbiota,

Fatty acids have a broad spectrum of antibacterial activity including lysis and solubilization of bacterial cell membranes (Jackman *et al.*, 2016) and inhibition of Adenosine Triphosphate (ATP) production (Yoon *et al.*, 2018). The antibacterial action of fatty acids is affected by carbon chain length, saturation, and double bond position (De Carvalho *et al.*, 2018) such as linoleic acid where Zheng *et al.* demonstrated that these unsaturated fatty acids show antibacterial activity against *S. aureus* (Zheng *et al.*, 2005).

Modulation of the gut microbiota by lipids,

However, the impact of fatty acids on the gut microbiota is not only limited to antimicrobial action. Dietary supplementation with saturated long-chain fatty acids, which were metabolized by and promoted growth of *Lactobacillus*, reversed alcohol-induced dysbiosis, stabilized the intestinal gut barrier, and reduced liver injury (Chen *et al.*, 2015). In the following section, we will be discussing the impact of these diets including a high rich one and a low rich one, on the ecology of the GM.

Speaking type of diet, the latest version of dietary guidelines issued in 2015 by the US Departments of Agriculture and Health no longer call for a reduction in total fat intake but rather for optimization of fat types in the diet, and specifically reduced intake of saturated and trans-fat (Mozaffarian *et al.*, 2015). This recommendation is supported by mechanistic studies demonstrating that the quantity and the source of fat can have differential effects on the host (Hirt-Burri *et al.*, 2011) and that some of these fat-mediated effects are transmitted through changes induced in the gut microbiome whereas a gut microbiota modified by a diet rich in fat is characterized by over-representation of LPS-expressing bacteria of both mice (Choi *et al.*, 2015) and humans (Rial *et al.*, 2016).

Through the literature, fat-rich diets associated effects are thought to be driven, at least in part, by high-fat-diet (HFD)- induced alterations in gut bacterial composition in the GI tract of adult mice, especially a decrease in bacterial diversity (Clarke *et al.*, 2012; Zhang *et al.*, 2013; Daniel *et al.*, 2014; Murphy *et al.*, 2015). Interestingly, blooms of *Bacteroides*, *Turicibacter* and *Bilophila* spp. seems to be specific to saturated fat. By contrast, mice fed unsaturated fat were characterized by expansion of *Bifidobacterium*, *Akkermansia* and *Lactobacillus* spp. and did not demonstrate metabolic impairments (Zmora *et al.*, 2019). Curiously, replication of the metabolic phenotype in

germ-free (GF) mice transplanted with these distinct microbial compositions suggested a role for the gut microbiota in mediating the differential effects of fat type on the host health (Tremaroli *et al.*, 2012). Specifically, it was reported earlier that body fat percentage growth was somehow negatively associated with the abundance of the genus *Akkermansia* (phylum *Verrucomicrobia*) yet positively associated with the relative abundances of *Lactococcus* from phylum *Firmicutes* and with the genus *Allobaculum* (phylum *Bacteroidetes*) (Parks *et al.*, 2013). Moreover, GF mice were inoculated with fresh faecal samples from human, and results showed that high fat diets significantly reduced numbers of *Lactobacillus-Enterococcus* compared to control diet. Additionally, a dietary pattern high in saturated and/or total fat have consistently shown to have adverse effects on intestinal microbiome in a recent work: fifteen clinical reports (including six randomized controlled interventional studies and nine observational studies) have shown that diets high in total fat and saturated fat have a negative effect on the richness and diversity of gut microbiota (Wolters *et al.*, 2019).

In parallel with HFD, and in order to specifically investigate the effects of different kinds of dietary fat on the human gut microbiota, Fava *et al.* conducted a study that had subjects consume diets of varying fat content after which the authors noted that consumption of a low-fat diet (LFD) led to increased fecal abundance of *Bifidobacterium* with concomitant reductions in fasting glucose and total cholesterol, compared to baseline (Singh *et al.*, 2017). Similarly, it was found that *Blautia* and *Faecalibacterium* genera, known to contain butyrate-producing bacteria, were increased in the LFD group. In contrast, the abundance of *Faecalibacterium* was decreased while *Bacteroides* and *Alistipes* were increased after the HFD intervention. These variations at genus level indicated that dietary fat content had a selective effect on the human GM, which might have clinical indications among healthy young adults. As proven from an earlier study, within the *Bacteroidetes* phylum, the genus, *Bacteroides* has been shown to decrease in most murine studies following a HFD (Cani *et al.*, 2007; Patrone *et al.*, 2012). However, within the phylum *Firmicutes*, specific bacterial changes are more varied. For example, *Lactobacillus* spp. (Patrone *et al.*, 2012; Druart *et al.*, 2013) decreased following HFD. Alterations observed in bifidobacterial rates following a HFD vary effectively. In contrast to that, in a few murine studies, numbers of *Bifidobacteria* in caecal samples have been seen to decrease following a HFD (Cani *et al.*, 2007). While some other studies showed different results in terms of *Bifidobacteria*. For example, in the human study of Fava *et al.* (Fava *et al.*, 2013; Respondek *et al.*, 2013) HFD had no significant effect on Bifidobacterial numbers in

collected faecal samples. However, in two murine studies, *Bifidobacteria* in caecal samples were shown to be significantly higher following a HFD compared to the control group (Neyrinck *et al.*, 2011; Patrone *et al.*, 2012). As the Bifidobacterial change following a HFD is indecisive in murine studies, the impact needs to be further investigated in human trials.

On the other hand, Morales *et al.* observed that a HFD including fiber supplementation induces inflammation without altering the composition of the gut microbiota (Morales *et al.*, 2016). Changes in other bacteria induced by a HFD were also interestingly modulated and normalized by prebiotic supplementation (Delbès *et al.*, 2018) whereas in a 2020 study, the ratio of Firmicutes: Bacteroidetes was significantly lower following supplementation of GOS compared to that of a HFD treated group (Stojanov *et al.*, 2020). Although, there was no low-fat group in this study, which was a limitation of this experiment design.

Though the influence of dietary lipids on intestinal permeability is well acknowledged, an unusual relationship between the degradation of epithelial barrier integrity and alterations in the gut microbiota in the context of diet-induced obesity remains to be defined. According to Cani *et al.*, a HFD-induced changes in the gut microbial community enhance intestinal permeability and promote the leakage of LPS into circulation by decreasing the expression of intestinal tight junction proteins (Cani *et al.*, 2009).

However, the effect of lipid-rich diet on the human gut remains quite complex given the considerable individual variation in the response of the gut microbiota to dietary intervention (Salonen *et al.*, 2014).

2.4. Proteins

Major food sources of proteins include meats, dairy products, seafood, and a variety of different plant-based foods (e.g., soy). Proteins are macromolecules composed of chains of subunits called amino acids. Also, factors associated with dietary protein, such as protein source, concentration, and amino acid composition or balance (Millward *et al.*, 2008), are important elements for host health and can influence the intestinal microecosystem: If dietary protein is in excess of requirements, the homeostasis of gut microbiota can be disrupted resulting in intestinal disorders, waste of nitrogen resources, and environment pollution (Fan, P. *et al.*, 2015). Therefore, it is important to understand the interactions between protein metabolism (Davila *et al.*, 2013) and the gut microbiota as these microbes play important roles in the relationship between nutrient

utilization and host response (Bishu *et al.*, 2016). Additionally, understanding the effects of different components of dietary protein on intestinal microbiota and function is also important.

Protein source and quantity have a large effect on gut bacterial community composition (Neis *et al.*, 2015; Egerton *et al.*, 2020).

Protein quantity,

In one study (McAllan *et al.*, 2014), like in the following one where mice were fed with a LFD or a HFD for 21 weeks, with either casein (20% kJ) or whey protein isolate (WPI) at 20%, 30%, or 40% kJ. The study outcomes showed an increase in abundance of the phylum *Proteobacteria* and *Actinobacteria* in the gut microbiota for the experimental animal groups that received 20% WPI. Yet, when the protein was increased from 20% to 40% the results were opposite for the phylum *Actinobacteria* compared to that of the HFD group (Du *et al.*, 2020). In addition, high concentrations of protein supplementation can result in increases in numbers of potential pathogens due to disruption of the homeostasis of the gut micro-ecosystem with reductions of beneficial microbes. Possibly, this GM may directly determine to what extent dietary proteins are converted into other metabolically active compounds such as SCFAs, branched chain fatty acids, or different nitrogen containing compounds (Lin *et al.*, 2017).

In contrast, low concentrations of dietary protein reduce the amount of substrate for pathogenic bacteria proliferation in a pig model (Chen *et al.*, 2018). For instance, *E. coli* communities were reduced on the mucosal surface by the low concentrations of protein in the diet in humans (Kau *et al.*, 2011), which is in agreement with other studies that reported that lower dietary protein decreased the substrate is available for *E. coli* proliferation (Gensollen *et al.*, 2016; Hancock, Haney *et al.*, 2016). Thus, the change of gut microbes under lower protein conditions contributes to less toxic nitrogenous bacterial metabolites, e.g., polyamine, which can impair the intestinal integrity and immune defense (Zitvogel *et al.*, 2016). However, when the concentration of protein in the diet is too low to meet the basic requirement for host, it can increase the abundance of potentially pathogens such as some proteobacterial strains and decrease the population of potentially beneficial microorganisms like *Lactobacillus reuteri* (H. Wang *et al.*, 2019).

A study showed that the number of aerobes and anaerobes of feces increased when weaned animals were fed with dietary protein from 100 to 200 g/kg, with these protein levels resulting in an

increase of *Lactobacilli* and reduction of *Coliforms* and *Staphylococci* in the gut. However, when the level of dietary protein was greater than 200 g/kg, there were interestingly increases in the populations of pathogens, such as *Coliforms*, *Streptococcus* and *Bacillus* (Windey *et al.*, 2012).

At the same time, lower concentrations of dietary protein decreased butyrate-producing bacteria including *Lactobacilli*, *Bifidobacteria* and *Saccharolytic*, which serve as anti-inflammatory agents in intestinal disorders (Louis *et al.*, 2014). In older animals, populations of intestinal microbiota are relatively robust to small changes in protein levels. For instance, there was little difference in the number of bacteria in fecal samples when adult animals were fed with 190 g/kg and 150 g/kg protein, respectively (Sandrini *et al.*, 2015) which suggests that the gut microbiota composition is well established and stable under regular diet patterns in adults (Jalanka *et al.*, 2015). There is also a strong evidence to support the assertion that dietary protein consumption elicits both compositional and functional changes to the gut microbiota (Sheflin *et al.*, 2016) wherein David *et al.* (Lawrence A David *et al.*, 2014) showed a rapid shift in gut microbial community composition and increased populations of *Alistipes*, *Bilophila*, and *Bacteroides* after consuming a high-protein diet for 5 days.

The effect of 10 weeks of daily consumed protein supplementation in cross-country runners (estimated to 10 g whey isolate and 10 g beef hydrolysate per day) successfully decreased SCFA-producing bacteria while increased bacteria with proteolytic activity in the microbiota without affecting SCFAs, ammonia, or fecal pH of endurance athletes (Moreno-Pérez *et al.*, 2018). The amount of additional dietary protein was small but yielded a significant 17% increase in dietary protein for these athletes. Specifically, protein supplementation increased the abundance of the *Bacteroidetes* phylum and decreased the presence of health-related taxa including *Roseburia*, *Blautia*, and *B. longum*. In contrast to Clarke *et al.* (Clarke *et al.*, 2014), no changes in compositional microbiota diversity were detected after the ten-week intervention, which may relate to the low percentage of protein intake. On the flip side, a 70-day protein supplementation in a healthy athlete's diet had a negative impact on gut microbiota in a 2020 study, which resulted in a decreased level of the health-beneficial microbiota *Roseburia*, *Blautia*, and *Bifidobacterium longum*, and an increase in the microbiota of the phylum *Bacteroidetes* (Mohr *et al.*, 2020).

Protein Quality (Plant versus Animal Protein),

Also, in other animal models, Zhu et al. recently conducted a study on young rats where rats were fed a diet with proteins from chicken (17.7%) for 14 days. This diet has led to an increased relative abundance in the genus *Lactobacillus*. However, the opposite pattern was demonstrated in middle-aged rats (Zhu et al., 2016). It has also been demonstrated in another study that in comparison to casein, whey protein isolate (WPI) intake increased levels of *Lactobacilli* and *Bifidobacteria* in a rat model of colitis (Sprong et al., 2010) which suggests inter differences regarding protein families. Meanwhile, in what concerns the quantity of protein administered in a certain diet, one study has justified that low-protein diets alters the intestinal bacterial community in finishing pigs (Fan et al., 2017). In a preclinical early study, the cheese whey proteins were shown to have the ability to act as growth factors for fecal counts of *Lactobacilli* and *Bifidobacteria* compared with Caesin (Sprong et al., 2010). In contrast to the evidence from plant-based protein interventions, diets containing casein increased fecal *Enterobacteriaceae* and decreased fecal *Lactobacilli* in piglets (Rist et al., 2014). Changes to the gut microbiota have also been documented when dietary protein is increased: *Bacteroides* spp. are highly associated with animal proteins, whereas *Prevotella* spp. are highly associated with increased intakes of plant proteins (Wu et al., 2011). Interventional studies have demonstrated as well that high-protein diets (mainly animal protein) reduced fecal butyrate concentrations and butyrate-producing bacteria to a certain point, such as *Bifidobacteria* spp., *Roseburia* spp., and *E. rectale* (Brinkworth et al., 2009; Russell et al., 2011). All these evidence from animal models suggest that the protein quality and quantity affect the composition of gut microbiota and that these changes are thought to be driven in part by an increased bile secretion.

2.5. Micronutrients

Despite the fact that micronutrients are the nutrients your body needs in smaller amounts in comparison with macronutrients, their importance in modulating the gut microbiome in favor of host health is not minimal nor neglectable.

Aside of macronutrients discussed above, micronutrients represent one of the most studied molecules in the recent science through the nutritious, molecular, and medical field. They are

called micronutrients because they are needed only in minuscule amounts, these substances are required to enable the body to produce enzymes, hormones, and other substances essential for proper growth and development.

In the following paragraphs we will be discussing the effects of some micronutrients on the composition of the gut microbiome as an effective strategy manipulating the gut microbiota and rebuilding the intestinal homeostasis.

2.5.1. Vitamins

Vitamins are organic compounds that are essential in very small amounts for supporting normal physiological functions in the organism. They often serve a variety of roles in the body—one of the most important roles is their action as cofactors for enzymes. Basically, the diet is the primary source of these vitamins, as our bodies cannot synthesize them to meet our daily needs, but certain vitamins, notably vitamin K, and B group vitamins, are synthesized by gut microbiota (LeBlanc *et al.*, 2013). Chronic health conditions can be created or exacerbated when one or more of these vitamin groups are deficient, and it is common for people to consume individual or multiple vitamin supplements simultaneously, which can provide very high doses of specific vitamins. Subsequently, minimal absorption of these vitamins in the upper gut can therefore be in favor in modulating the abundance and diversity of the gut microbiota.

Vitamin A, a fat-soluble vitamin, has been indicated as an adjuvant therapy for infectious diseases (Mawson *et al.*, 2013; Huang *et al.*, 2018) and has a potential adjunct therapeutic effect on children with autism spectrum of disorders (ASD) (Bjørklund *et al.*, 2019), possibly by altering gut microbiota. A recent study demonstrated that vitamin A supplementation in the form of retinoic acid (that is a physiologically active metabolite of vitamin A) in a murine model could inhibit murine norovirus replication (Lee *et al.*, 2017). In this study, the researchers also demonstrated that the administration of retinoic acid significantly increased the abundance of *Lactobacillus* sp. during a norovirus infection. At the same time, in an *in vitro* model, *Lactobacillus* showed antiviral activity against norovirus, and based on the obtained results, the authors hypothesized that the abundance of *Lactobacillus* in the gut was partially responsible for norovirus inhibition (Lee *et al.*, 2017; Gombart *et al.*, 2020). It was also shown in H Lee study that retinoic acid administration increased the abundance of *Allobaculum*, *Aggregatibacter*, *Bifidobacterium*, *Dialister*, and *Enhydrobacter*. In addition to all previous results, vitamin A supplementation was reported to

reduce both mortality and morbidity associated with infectious gastrointestinal diseases (Thornton *et al.*, 2014). Which may be somehow linked to the floral diversity alterations happening in the GIT, as a consequence of Vitamin A intake. This alteration that is more likely to be defined as an enhancement of beneficial bacteria and/or the suppression and unfortunate development of the pathogenic ones.

Moreover, better vitamin A status in infancy may influence health, both in infancy and later in life, by promoting the establishment of a healthy microbiota (Huda *et al.*, 2019). What's worth mentioning is that the supplementation of infants in early (6–15 week) or late (2 year) infancy with 50,000 IU vitamin A was reported to have the ability to increase the abundance of *Bifidobacterium* and *Akkermansia* in their feces but did not affect the abundance of *Proteobacteria*.

Besides Vitamin A supplementations, the administration of vitamin D also suggests a positive effect on Crohn's disease (CD) patients by modulating the intestinal bacterial composition and also by increasing the abundance of potential beneficial bacterial strains where the microbiota of the members of the genera *Alistipes*, *Barnesiella*, unclassified *Porphyromonadaceae* (both Actinobacteria), *Roseburia*, *Anaerotruncus*, *Subdoligranulum* and an unclassified *Ruminococcaceae* (all *Firmicutes*) were increased significantly after administration of vitamin D for one week in CD patients (Schäffler *et al.*, 2018). Furthermore, maternal administration of vitamin D during pregnancy had a negative linear association with *Bifidobacterium* sp. and a positive association with the *Bacteroides fragilis* group in infants, suggesting therefore that the prenatal vitamin D administration influenced bacterial diversity in the infants (Yang *et al.*, 2020). Reduced abundance of *Clostridium difficile* was associated with vitamin D supplementation of breast-fed infants whose mothers were more likely to adhere to a lifestyle with regards to dietary habits as vegetarians, or organic/macrobiotic diets. These data suggest that pre/postnatal vitamin D exposure affects the wealth of several key bacterial taxa within the infant microbiota, leading thus to the maturation of health beneficial/detrimental microbiota in infant gut (Luthold *et al.*, 2017; Talsness *et al.*, 2017).

Moreover, vitamin D receptor (VDR) function has been positioned to be a critical aspect of immune response and gut homeostasis wherein literature shows an interesting, correlated hypothesis that lays between vitamin D, VDR function, and gut microbiome and autoimmune diseases (Clark *et al.*, 2016).

The effect of vitamin D on immune cells is complex. However, the first hint of the significant role of vitamin D on the immune system was made out of the discovery of the presence of the VDR on almost all cells of the immune system which's expression is differently controlled according to their corresponding activation status and the vitamin D activating enzyme 1- α -hydroxylase (CYP27B1) are as well expressed in many cell types such as intestine (Prietl *et al.*, 2013).

2.5.2. Minerals and Trace Elements

Minerals and trace elements are essential micronutrients for human metabolism and perform active interaction with the gut microbiome (Yang *et al.*, 2020).

Both nutritional deficiency and an excess of minerals and trace elements are responsible for various diseases in humans. The role of trace element excess or deficiency in modulating gut microbiota is an emerging field, and some of the major findings of published articles are discussed below.

Epidemiological data suggests that magnesium deficiency is associated with an increased incidence of chronic disease (DiNicolantonio *et al.*, 2018), but the real proof for the role of the microbiome in this association is not quite clear yet. It was reported previously that four days of magnesium (Mg) deficiency could reduce the bifidobacterial content in mouse cecum, but with prolonged magnesium deficiency (three weeks) there was an increase in the intestinal content of *bifidobacteria* and *lactobacilli* (Pachikian *et al.*, 2010). Magnesium, the second most abundant cation in bacterial cells, is also important for bacterial heat-shock survival. It has been reported that magnesium, more than sodium and zinc, ameliorates the thermotolerance of probiotic *L. rhamnosus* GG, *Lactobacillus casei* Zhang and *Lactobacillus plantarum* P-8 (Yang *et al.*, 2017). Likewise, recent studies have demonstrated that Iron significantly affects the intestinal microbiota, as it is in charge for the acquisition of energy by intestinal bacteria from some nutrients ingested by the host (Dostal *et al.*, 2015). In *in vitro* cultures of the human intestinal microbiota, an increase in the iron content of the culture medium in amounts simulating oral Iron supplementation causes a decrease in the number of commensal bacteria (Kortman *et al.*, 2016). However, according to Ng *et al.*, Bifidobacteriaceae can bind to Iron present in the large intestine, thereby limiting the formation of free radicals synthesized in the presence of Iron and thus reducing the risk of colorectal cancer (Ng *et al.*, 2016).

As for Calcium, a nutritional intervention of 54 days, high calcium supplementation (12 g/kg) modulated gut microbiota in a prebiotic manner by increasing the number of *Bifidobacterium* sp., and increasing *Bacteroides/Prevotella* ratio in the cecal sample of an HFD mouse model. The number of *Bifidobacterium* sp. in this study was negatively correlated with the plasma LPS level, indicating the reduction in LPS producers in the gut microbial pool post-calcium intake (Yang *et al.*, 2020).

Zinc is an essential micronutrient for the host that operates in order to maintain the epithelial integrity, possibly by modulating the beneficial gut microbiota (Usama *et al.*, 2018). In fact, animal experimentation has suggested that zinc supplementation (120 mg/kg) (Shao *et al.*, 2014) in a “*Salmonella typhimurium*-challenged broiler” model increased the number of beneficial bacteria, such as *Lactobacillus* sp., while lowering the rates of harmful and detrimental bacteria, including *Salmonella* sp. However, the clinical data on shaping the gut microbiota by dietary zinc in humans are until today lacking. In contrast, iron supplementation is a common strategy to correct iron-deficiency in clinical settings. However, still no consistent conclusion has been achieved for the effect of iron supplementation on gut microbiota (Kortman *et al.*, 2014; Yang *et al.*, 2020).

Some recent research concerning the role of dietary vitamins in the modulation of gut microbiota is conducted using both animal models and clinical trials and could be summarized in the following table (**Table 1**).

Table 1: Modulations of dietary vitamins, minerals, and trace elements on potentially beneficial gut microbiota.

| Vitamin/Mineral | Dose and treatment duration | Test model | Modulations of dietary vitamins, minerals, and trace elements on potentially beneficial and detrimental gut microbiota. | References |
|-----------------|---|---|---|------------------------------------|
| Vitamin A | 50,000 IU vitamin A within 48 h | Infants | <i>Bifidobacterium sp.</i> ↑ <i>Akkermansia sp.</i> ↑ | (Huda <i>et al.</i> , 2019) |
| Vitamin A | 200,000 IU vitamin A once orally for a 6-month | children with autism spectrum disorder | <i>Bifidobacterium sp.</i> ↓ <i>Bacteroides sp.</i> ↑ | (Liu <i>et al.</i> , 2017) |
| Vitamin D | 0µg/day, <10 µg/day, or ≥ 10 µg/day | Maternal supplementation during pregnancy and the fecal samples from their one-month-old babies | <i>Bifidobacterium sp.</i> ↓ <i>Bacteroides sp.</i> ↑ <i>Clostridium sp.</i> ↓ | (Talsness <i>et al.</i> , 2017) |
| Vitamin D | (50,000 IU) supplementation for 12 months | on stable normal glucose tolerance for 12 months | <i>Fecalibacterium sp.</i> ↓ <i>Ruminococcus sp.</i> ↓ <i>Dialister sp.</i> ↓ <i>Bifidobacterium sp.</i> ↓ | (Ciubotaru <i>et al.</i> , 2015) |
| Magnesium | Magnesium-deficient diet (70 mg/kg) for 21 days | control diet (500 mg/kg) group as reference/mice model | <i>Bifidobacterium sp.</i> ↑ <i>Lactobacillus sp.</i> ↑ | (Pachikian <i>et al.</i> , 2010) |
| | Magnesium-deficient diet (70 mg/kg) for 4 days | control diet (500 mg/kg) group as reference/mice model | <i>Bifidobacterium sp.</i> ↓ | |
| Calcium | HFD enriched with calcium supplementation (12 g/kg) for 54 days and | HFD group (4 g/kg) as reference/mice model | <i>Bifidobacterium sp.</i> ↑ <i>Lactobacillus sp.</i> ↑ | (Chaplin <i>et al.</i> , 2016) |
| Calcium | Supplementation of 1000 mg calcium | Healthy men | <i>Bifidobacterium sp.</i> ↓ <i>Clostridium sp.</i> ↑ | (Trautvetter <i>et al.</i> , 2018) |

| | | | | |
|------------|--|--|--|--|
| | +1000 mg phosphorus/day for 8 weeks | | | |
| Zinc | Supplemental Zn (120 mg/kg) diet for 42 days | diet without Zn as reference/ <i>Salmonella Typhimurium</i> -challenged Broiler chicken model | <i>Lactobacillus sp.</i> ↑ <i>Salmonella sp.</i> ↓ | (Shao <i>et al.</i> , 2014) |
| Phosphorus | Diet mixed with the supplementation of Phosphorus (3 g/kg) for 10 days | diet without calcium group as reference/broiler chickens' model | <i>Fecalibacterium sp.</i> ↑ | (Borda-Molina <i>et al.</i> , 2016) |
| Iron | High-iron-fortified formula (6.4 mg Fe/day) for 45 days | 6-month-old healthy Swedish infants | <i>Bifidobacterium sp.</i> ↓ <i>Lactobacillus sp.</i> ↑ | (Simonyté Sjödin <i>et al.</i> , 2019) |
| Iron | Multiple micronutrient powder containing 12.5 mg iron daily for 3 months | multiple micronutrient powder without the iron as reference/clinical trials (6-month-old Kenyan infants) | <i>Bifidobacterium sp.</i> ↓ | (Tang <i>et al.</i> , 2017) |
| Iodine | 18µg/kg/d iodine for 8 weeks | control group as reference/mice model | <i>Bifidobacterium sp.</i> ↑ <i>Lactobacillus sp.</i> ↑ <i>Fecalibacterium sp.</i> ↑ <i>Allobaculum sp.</i> ↑ <i>Clostridium sp.</i> ↑ <i>Blautia sp.</i> ↓ | (Shen <i>et al.</i> , 2019) |

In summary, there is limited evidence supporting specific mechanisms whereby minerals and trace elements modulate the gut microbiome particularly probiotic-related gut bacteria and hence a thorough study in this area is mandated. However, we cannot deny the positive effects of some minerals upon some probiotic-related bacteria resident in the GIT.

2.6. Polyphenols

2.6.1. Definition of polyphenols

Phenolic compounds or polyphenols are widely distributed secondary metabolites in the plant kingdom being found in all fruits and vegetables. These compounds are present in all parts of the

plants but with a distribution which vary between species and the different parts of the plant (e.g., leaves, stems, roots, fruits). More than 8000 structures have been identified (Tsao *et al.*, 2010) ranging from simple molecules like phenolic acids to highly polymerized substances like tannins (Dai *et al.*, 2010). They are synthesized by all plants and they participate in defense reactions to different biotic stresses (pathogens, injuries, symbiosis) or abiotics (light, UV radiation, low temperature, deficiencies), allowing plants to cope with environmental changes. Growing evidence supports the ability of these molecules to modulate GM composition and function, interfering with bacterial quorum sensing, membrane permeability, as well as sensitizing bacteria to xenobiotics. Their effects on microorganisms are highly variables from one species to another, and sometimes from one strain to another. Regarding the biological activities of polyphenols on microbial communities, these compounds have a wide range of anti-microbial properties, inhibiting bacterial growth and biofilm formation (Besednova *et al.*, 2020). These compounds include prenylated isoflavonoids, stilbenes, coumarins, flavonols or even auronnes. Although further research is still required, particularly translational and clinical studies, the biotechnological progresses achieved during the last years open up good outlook and a greater perspective to, in a near future, be able to improve the use of dietary polyphenols modulating GM in a broad range of disorders characterized by a dysbiotic phenotype to another eubiotic one (Kumar Singh *et al.*, 2019) and shaping the intestinal flora towards a healthy one.

2.6.2. Structures and families

Polyphenols are characterized by the presence of at least one benzene nucleus to which is directly linked at least one hydroxyl group, free or engaged in another function: ether, ester, heteroside. (BRUNETON *et al.*, 1999). Based on their chemical features, polyphenols may be classified into different groups as a function of the number of phenol rings contained and the structural elements that bind these rings to one another. Presently, it is widely accepted that polyphenols can be grouped into two main categories: flavonoids and nonflavonoid compounds. Figure 4 illustrates the different classes phenols based on their chemical structures whereas numerous quantities and combinations can arise affecting their physiochemical characteristics and thus their bioavailability and biological targets (Hollman *et al.*, 2004; Popkin *et al.*, 2012). Phenolic acids are a subclass of the phenolic compounds that contain at least one aromatic ring and one hydroxyl group. Flavonoids are the largest subclass of polyphenols in the human diet and are characterized by two or more

aromatic rings containing at least one hydroxyl group in each and connected with a heterocyclic pyran. Flavonoids can be sub-divided further into two main groups, anthocyanins (glycosylated derivative of anthocyanidin) and anthoxanthins. Anthoxanthins are composed of several categories, such as flavones, flavanones, flavonols, flavanols, isoflavones and their glycosides (Beecher *et al.*, 2003).

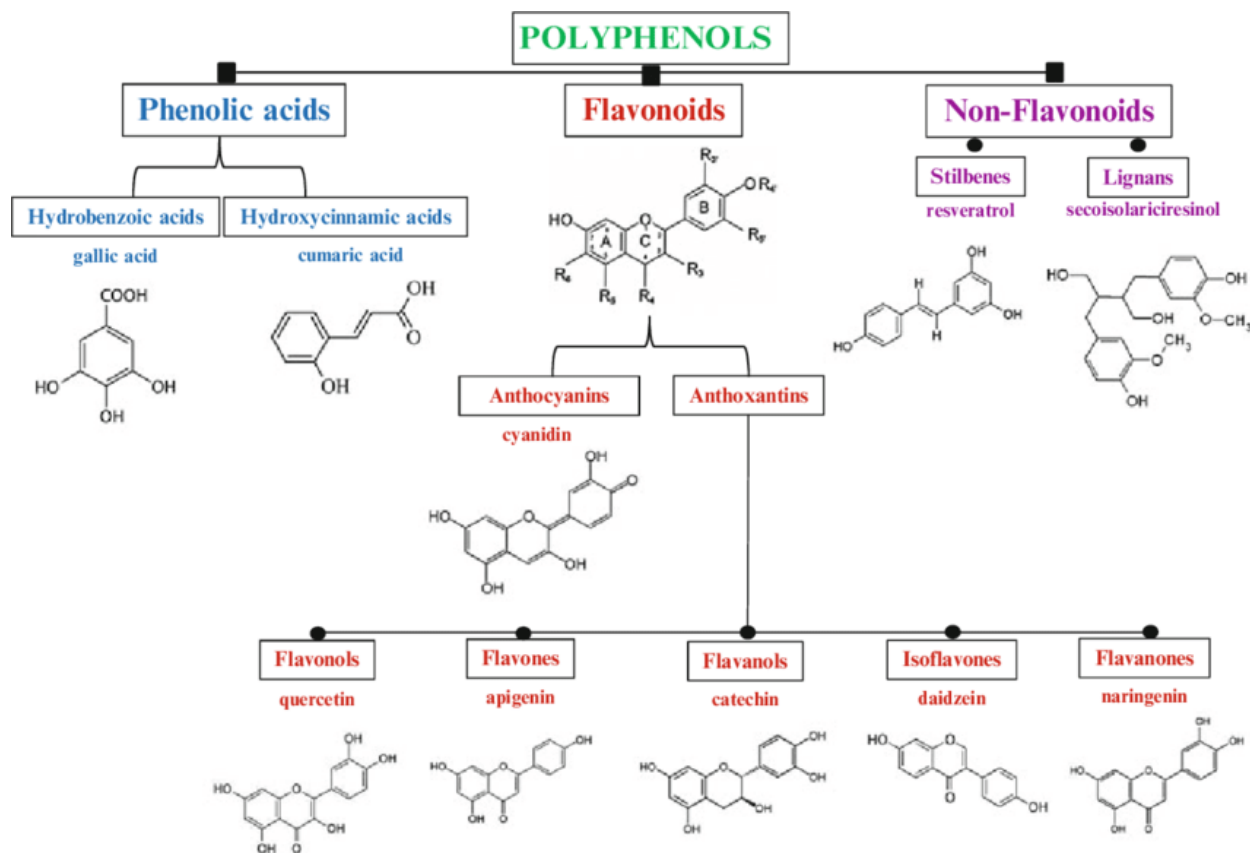


Figure 5: Schematic classification and structure of the main Polyphenol classes

It was generally believed that the phenols exerted their health benefit by directly scavenging free radicals or reactive oxygen species (ROS) or chelating of redox metals, but these properties are now largely attributed to the phenols' ability to act as signaling agents of cellular endogenous responses (Pham-Huy *et al.*, 2008; Del Rio *et al.*, 2013).

From an applied point of view, polyphenols act as signaling molecules interacting with a wide range of intracellular signaling pathways in mammalian cells, thereby affecting many cellular functions. For example, Resveratrol, a stilbene, is a potent stimulator of the autophagy pathway, a

degradative process crucial for cell homeostasis. Autophagy inducer activities of resveratrol rely on its ability to modulate PI3K/AKT and mTOR signaling pathways, but also by affecting host cell transcriptional program through the modulation of the Sirtuins histone deacetylases (Ng and Tang, 2013; N. Wang *et al.*, 2019). Despite their extensive biological activities, many difficulties are encountered during their production to ensure their stability and functionality *in vivo*. In addition, once consumed, dietary polyphenols are perceived as xenobiotics in humans and their biological availability is reasonably poor as compared with others micro- and macro-nutrients, due to their extensive conjugation and clearance.

2.6.3. Effects on the gut microbiota

The influence of dietary polyphenols on gut ecology and the mechanism underlying the assumed beneficial effects on GI and extra-intestinal diseases have been outlined during the last decade (Kumar Singh *et al.*, 2019). Structural complexity and polymerization limit the absorption polyphenols in the small intestine (about 5–10%) (Cardona *et al.*, 2013). The leftovers of polyphenols (90–95%) may accumulate up to the millimolar range in the colon along with the bile conjugates released into the lumen and are exposed to the gut microbial enzymatic activities (Grootaert *et al.*, 2015). Recent studies support the idea that dietary phenolic substances reaching the gut microbes, as well as the aromatic metabolites generated, may modify, and produce variations in the GM by exhibiting dual effects, with prebiotic effects on some species and antimicrobial action against some others (Kumar Singh *et al.*, 2019), highlighting the strain-dependent effects of polyphenols.

Mechanisms of action of dietary polyphenols differ in Gram positive and Gram-negative bacteria due to alterations in cell membrane structure. Polyphenols have ability to tie up bacterial cell membranes in a concentration-dependent manner, therefore modifying functional aspects of the membrane and/or cell wall. These modifications of the physico-chemical properties of the bacterial surface can lead to various phenotype, including growth inhibition or modulation of the interactions with other microorganisms (aggregation, adhesion, or biofilm formation) (Al Azzaz *et al.*, 2020). During human intervention study designed as a randomized, double-blind, crossover, controlled intervention trial in which healthy volunteers were asked to consume either an HCF (high-cocoa flavanol) or LCF (low-cocoa flavanol) drink namely, catechin and epicatechin, were reported to stimulate growth and proliferation of *Bifidobacterium* spp. and *Lactobacillus* spp.

(Tzounis *et al.*, 2011; Kumar Singh *et al.*, 2019) which might have been partially accountable for the perceived decline in the concentration of plasma C-reactive protein (CRP), an inflammatory blood biomarker and a hallmark of the acute phase inflammatory response (Sproston *et al.*, 2018).

Conversely, colonic microflora may effectively transform the phenolic compounds into actual bioactive compounds, which have the ability to positively influence the intestinal ecology and affect human health. Studies in animals and humans have shown that prescribed amounts of particular polyphenolic compounds may edit the gut microflora composition resulting in inhibition of certain bacterial groups, while others can flourish in the available niche of the ecosystem (Kumar Singh *et al.*, 2019). An *in vitro* study in 2008 reported that flavan-3-ol monomers, namely, catechin and epicatechin, may have ability of impelling the bacterial population in large intestine (Tzounis *et al.*, 2008) where catechin subdued the growth of *Clostridium histolyticum* and boosted the growth and development of members of the *Clostridium coccooides-Eubacterium rectale* group and *E. coli*. Also, Proanthocyanidin-rich red wine extract has been shown to swing the preponderance of *Bacteroides*, *Propionibacterium* and *Clostridium* spp. towards the predominance of *Bacteroides*, *Bifidobacterium* and *Lactobacillus* spp. in a colon cancer animal model (Tombola *et al.*, 2003).

Thus, polyphenols might influence the resident GM, and thereby contribute to correct a dysbiotic state and restore intestinal eubiosis. Overall, dietary polyphenols have shown, both in preclinical and in clinical studies, several benefits on distinct disorders due to effects on gut microbiota, although further experimental evidence are still warranted to elucidate the precise molecular mechanisms involved.

These findings suggested that Phenolic Compounds, particularly quercetin, may enhance adhesion to intestinal epithelial cells by the tested probiotic strains, Altogether, these studies showed that PC or PC-rich extracts are able to modulate, positively or negatively, the adhesion, growth, and diversity of probiotic strains, in a strain-, PC- and very dose-dependent manner (**Table 2**).

Table 2: Modulations of dietary polyphenols on potentially beneficial gut microbiota.

| Polyphenols | Dose and treatment duration | Test model | Potentially affected probiotic Microbiota | Diversity | References |
|---|---|---|--|-----------|------------------------------------|
| Catechin | 21 mg/day for 4 weeks | Human models | <i>Bifidobacterium sp.</i> ↑ <i>Lactobacillus sp.</i> ↑ <i>Clostridium sp.</i> ↓ | | (Tzounis <i>et al.</i> , 2011) |
| Caffeic Acid | 10 ug/ml for 48h | <i>In vitro</i> , and a control without polyphenol as reference | <i>Bifidobacterium sp.</i> ↑ <i>Lactobacillus sp.</i> ↑ <i>Bacteroides sp.</i> ↓ | | (Parkar <i>et al.</i> , 2013) |
| Chlorogenic Acid | 30 and 100 ug/ml for 48h | <i>In vitro</i> and a control without polyphenol as reference | <i>Bifidobacterium sp.</i> ↑ <i>Lactobacillus sp.</i> ↑ <i>Bacteroides sp.</i> ↓ | | (Parkar <i>et al.</i> , 2013) |
| Genistein | 0.25 g/kg for 4 weeks | Mice model and a control without polyphenol as reference | <i>Lactobacillus sp.</i> ↑ <i>Bifidobacterium sp.</i> ↑ <i>Bacterioides sp.</i> ↑ <i>Ruminococcus sp.</i> ↑ <i>Clostridium sp.</i> ↑ | | (Paul <i>et al.</i> , 2017) |
| Epigallocatechin-3-gallate and Resveratrol (EGCG + RES) | 282 mg/day, 80 mg/day for 12 weeks | Human models | <i>Fecalibacterium sp.</i> ↓ <i>Eubacterium sp.</i> ↓ <i>Bacteroides sp.</i> ↓ | | (J Most <i>et al.</i> , 2017) |
| Fruit-derived polyphenols (Tart cherry juice consumption) | 8 oz/day) for 5 days | Human models | <i>Lactobacillus sp.</i> ↑ <i>Bacteroides sp.</i> ↑ <i>Prevotella sp.</i> ↑ <i>Ruminococcus sp.</i> ↓ | | (Mayta-Apaza <i>et al.</i> , 2018) |
| Pomegranate (POM) extract | (1000 m/d, total phenolic content expressed as gallic acid equivalents of 680 µg/g) for 4 weeks | Human models | <i>Lactobacillus sp.</i> ↑ <i>Akkermansia sp.</i> ↑ <i>Prevotella sp.</i> ↑ | | (Li <i>et al.</i> , 2015) |

| | | | | | |
|------------------------------|--|--------------------------------------|---|-----------------------|--------------------------------------|
| grape phenolic compounds | (2.5 and 5 mg/kg/d diluted in 0.1% Dimethyl Sulfoxide) for 14 months | Rat model | <i>Bifidobacterium sp.</i> ↑ | ↑ | (Chacar <i>et al.</i> , 2018) |
| Tea-derived polyphenols | Green tea (1000 mL/day) for 10 days | Clinical trials | <i>Bifidobacterium sp.</i> ↑ | | (Jin <i>et al.</i> , 2012) |
| Red wine-derived polyphenols | Red wine (50 mg/kg) for 16 weeks | Rat model and HFD group as reference | <i>Bifidobacterium sp.</i> ↑ <i>Lactobacillus sp.</i> ↑ <i>Clostridium sp.</i> ↓ | No significant effect | (Dolara <i>et al.</i> , 2005) |
| Red wine-derived polyphenols | red wine (272 mL/d) for 30 days | Human models | <i>Bifidobacterium sp.</i> ↓ <i>Lactobacillus sp.</i> ↑ <i>Fecalibacterium sp.</i> ↑ <i>Akkermansia sp.</i> ↑ <i>Roseburia sp.</i> ↑ <i>Enterobacter sp.</i> ↓ | ↑ | (Moreno-Indias <i>et al.</i> , 2016) |

3. Antibiotics

The term antibiotic was coined from the word „antibiosis“ which literally means „against life“. In the past, antibiotics were considered to be organic compounds produced by one microorganism which are toxic to some other microorganisms (Russell *et al.*, 2004). As a result of this notion, an antibiotic was originally, broadly defined as a substance, produced by one microorganism (Denyer *et al.*, 2008), which at low concentrations can inhibit the growth of, or are lethal to other microorganisms (Russell *et al.*, 2004).

3.1 Classification and mechanisms of action of antibiotics

There are a few ways of classifying antibiotics, but the most common classification schemes are based on their molecular structures, mode of action and spectrum of activity (Schwalbe *et al.*, 2007), while some other ways include the route of administration (injectable, oral and topical). Antibiotics within the same structural class will generally show similar pattern of effectiveness, toxicity and allergic-potential side effects. Some very common classes of antibiotics based on

chemical or molecular structures include Beta-lactams, Macrolides, Tetracyclines, Quinolones, Aminoglycosides, Sulphonamides, Glycopeptides and Oxazolidinones (van Hoek *et al.*, 2011; Frederick Adzitey *et al.*, 2015).

The antimicrobial potency of most classes of antibiotics are directed at some unique feature of the bacterial structure or their metabolic processes. The mechanism of antibiotic actions can be summarized hence as follows: inhibition of cell wall synthesis, breakdown of cell membrane structure or function, inhibition of the structure and function of nucleic acids, inhibition of protein synthesis and blockage of key metabolic pathways such as folate synthesis inhibition (Wright *et al.*, 2010).

3.2 Positive modulation of the gut microbiota by antibiotics

Antibiotics are able to affect the gut microbiota from a therapeutic point of view. Although antibiotics are the basis of the treatment of infectious disease, they are largely known to impair the GM in association with a consequent increase in susceptibility to microbiota-associated diseases such as IBD (Xu *et al.*, 2020). Indeed, antibiotics display usually a broad spectrum of action, altering numerous bacterial communities in the GM, including beneficial microbes. Additionally, antibiotic treatment has been recognized as a trigger for *Clostridium difficile* infection (CDI) (Giau *et al.*, 2019). On the other hand, an interesting recent study using different regimens of antibiotic therapy demonstrated that different types of antibiotic administration in an experimental model of CDI induced distinct changes in microbiota structure by recovering an intestinal microbiota that was more resistant to CDI (Lewis *et al.*, 2015). In addition, a single bacterial species, *Clostridium scindens*, was able to confer resistance to infection by synthesizing *C. difficile*-inhibiting metabolites from host-derived bile salts in the intestinal content from antibiotic-exposed animals. This effect was neutralized when intestinal content was pre-incubated with cholestyramine, proving that *C. scindens*-mediated *C. difficile* inhibition is dependent upon accessing and modifying endogenous bile salts and recapitulates a natural mechanism of microbiota-mediated infection resistance (Buffie *et al.*, 2015).

Antibiotics can also be used to treat intestinal dysbiosis by depleting pathobionts from the GM. However, their effects are generally not very specific for a particular bacterial species, due to their broad spectrum of action, which can expose in the long term to other anomalies of the composition of the intestinal microbiota. The intensive use of antibiotics to promote the growth of farm animals

(Poultry for example) is currently being questioned because of the ecological risks induced in the short term (resistance) but also in the medium term, because the antibiotic residues in meat for human consumption exposes consumers to dysbiosis.

Interest in antibiotics use should be emphasized in certain groups of patients at risk: in severely malnourished children in Malawi, the administration of oral antibiotics for 1 week has improved nutritional status and reduced mortality (Trehan *et al.*, 2016). This suggests that children with uncomplicated severe acute malnutrition who qualify for outpatient therapy remain at risk for severe bacterial infection and that the routine inclusion of antibiotics as part of their nutritional therapy is warranted. This prospective, randomized, double-blind, placebo-controlled study showed no benefit of routine amoxicillin therapy. Antibiotic can also cause a change in the composition of the flora responsible for the loss of the mucosal barrier effect (Kim *et al.*, 2017). The bacterial metabolism is also found to be modified, causing a drop in hydrolytic activities and fermentation of the flora.

3.3 Side effects of antibiotics on the gut resident microbiota

Yet, given that antibiotic therapy may lead to the development of antibiotic resistance, the use of antibiotics as a new strategy for GM modulation, despite their effectiveness in the treatment of IBD, should be intensely and more carefully investigated. Unfortunately, diarrhea remains a common side effect of antibiotic therapy until today and it is called antibiotic-associated diarrhea (AAD). It generally occurs during antibiotic treatment and sometimes leads patients to interrupt their treatment but can also take place several days after stopping antibiotics (PICHE *et al.*, 2004). In fact, two main factors contribute to the variability in the impact of antibiotics on the gut microbiota: the level of exposure of the microbiota to the antibiotic, and the intrinsic antibacterial activity of the antibiotic on the bacteria that make up the microbiota. The intrinsic antibacterial activity of antibiotics is the basis for their use in infected patients. The choice of empirical antibiotic therapy is based on epidemiological evidence of bacterial resistance, considering the organ supposedly infected. Most often, broad-spectrum antibiotic therapy is administered initially, after taking microbiological samples. Antibiotic sensitivity of the identified bacteria (s) is usually achieved after 2 to 3 days. The antibiotic therapy is then modified for a molecule with a narrower spectrum of activity, centered on the sensitivities identified in the isolated strain. However, if the effect of antibiotics on cultivable bacteria in the gut microbiota is fairly well described (Rs *et al.*,

2017) this should be reassessed using new culture-independent analysis methods, in order to have a better knowledge of the overall effect of antibiotics on the gut microbiota (Fouhy *et al.*, 2012). But in general, the microbiota becomes balanced again soon after stopping the antibiotic therapy, which suggests that the microorganisms responsible for the barrier effect are only temporarily eradicated, or rather that their multiplication is only inhibited during treatment antibiotic (Haddad *et al.*, 2005).

From another perspective, concerning their effect on the composition of the intestinal microbiota, dramatic advances over the past decade in culture-independent techniques, such as next-generation sequencing, have demonstrated that the vast majority of commensal flora is composed of bacterial species that are unknown because they cannot be cultivated using the techniques used until then (Tlaskalová-Hogenová *et al.*, 2004). A better visualization of the modifications of the intestinal microbiota induced by antibiotics has thus been made possible (Whangbo *et al.*, 2017). In their study of healthy adult subjects treated with ciprofloxacin for 5 days, Dethlefsen *et al.* observed that the administration of antibiotics had a rapid and transient effect in terms of reducing the bacterial diversity and changes in the composition of the gut microbiota (Kim *et al.*, 2017). Another study based on elderly subjects treated with antibiotics showed a significant reduction in the abundance of some commensal strains such as *Alistipes* but without reduction in species richness (Milani *et al.*, 2016). Despite a certain degree of resilience, characterized by a recovery in terms of diversity within one week after the treatment, the return to the pre-therapeutic state does not seem complete (Les Dethlefsen *et al.*, 2011). Long-term reduction in bacterial diversity has been observed, as has reduced resistance to colonization following repeated administration of antibiotics. Some results suggest that anaerobic bacteria may be linked to resistance to colonization to outsider pathogens (Pickard *et al.*, 2019) where they are reduced following antibiotic treatment (Reeves *et al.*, 2012; Buffie *et al.*, 2013; Petrof *et al.*, 2013). Similar results have been observed following treatment with clarithromycin and metronidazole (Löfmark *et al.*, 2010) or 3rd generation cephalosporins (Burdet *et al.*, 2019). The long-term consequences of this shift to an alternate state of equilibrium are not yet known. Moreover, adverse intestinal effects following antibiotics have been naturally linked to disturbance of the gut microbiota secondary to treatment. Thus, patients have reduced gut bacterial diversity, and their microbiota has a different typology from that of patients free from infection (Bien *et al.*, 2013). Non-targeted metagenomic sequencing analysis revealed that the feces of patients with CDI were depleted of commensal organisms with a protective role, such as

Bacteroides, *Alistipes*, *Lachnospira*, and *Barnesiella*, and were enriched in opportunistic pathogens. The pathophysiology of CDI, and, in particular, the relationship between disturbance of the intestinal microbiota and the development of infection, has been extensively studied using animal models, especially murine ones. Among them, the golden hamster infection model is particularly used. Its high susceptibility to infection makes it particularly suitable for studying the pathophysiological mechanisms during acute infection. The disturbances of the gut microbiota induced by the administration of antibiotics and their links with the pathophysiology of CDI have been recently synthesized (Theriot *et al.*, 2015). Mechanistically, the development of infection has thus been linked to cholesterol metabolism, and more particularly, to metabolization of primary bile acids to secondary bile acids mediated by the gut microbiota. In the absence of antibiotic disturbance, secondary bile acids generated by the resident gut microbiota inhibit the sporulation and growth of *C. difficile*. Antibiotics, by disrupting the structure of the microbiota, prevent this metabolism, thereby removing the inhibition exerted by secondary bile acids and allowing infection to develop.

One direct adverse effect of antibiotic's administration upon microbial flora is that it can drive the generation and spread of antibiotic-resistance genes in gut microbiota. Technically, under selective pressure by antibiotics, sensitive strains will be eliminated, giving the antibiotic-resistant strains a growth advantage (Willing *et al.*, 2011). What is worse is the readily transmitted resistance across bacterial species, which may lead to a rapid dissemination of antibiotic resistance in other members of the gut microbiota (Zhang *et al.*, 2019). Antibiotics also influence host immunity by changing the bacterial metabolites and the signals transmitted from GM to the host, especially the recognized signals by intestinal epithelial cells and immune cells (Zhang *et al.*, 2019). Meanwhile, a small number of studies suggest that it is possible to reduce the impact of antibiotic therapy on the gut microbiota by considering the route of elimination of the antibiotics administered (Pilmis *et al.*, 2020). With the rapid development of microbial omics technologies, our understanding of the deleterious effects of antibiotics on host intestinal flora and immune system will become more comprehensive. In consideration the broad impact of antibiotics on GM, succeeding studies need to evaluate the effect of antibiotics on composition and functionality of GM and host immunity. Luckily, probiotics and FMT (discussed in the section above) are two promising therapeutic methods in the management of antibiotic-induced gut microbial dysbiosis and restoring intestinal homeostasis. Yet, long-term follow-up concerning safety issues, the impacts of probiotics and

FMT on intestinal microbiota and host immunity, and the impacts on nutrients metabolism remain to be assessed in future investigations. In the following sections, we will be discussing the probiotic concept along with its diverse functionalities and interactions with the host.

4. Phage-therapy

Among the perspectives on therapeutic modulation, a fourth promising alternative to manipulate bacterial population of the microbiota, could be the use of bacteriophages (McCarville *et al.*, 2016; Lee *et al.*, 2018), also defined as natural born killers during the 5th World Congress on Targeting Phage and Antibiotic Resistance (WCTAR) (Paule *et al.*, 2018).

The application of phages as a therapeutic strategy has been broadly discussed in Europe by the European Medicines Agency for ethical policy reasons since this therapy includes a live medicine. In contrast, in Eastern European countries and Switzerland, phage therapy has been widely used for trials and therapies for long time (Lee *et al.*, 2018; McCallin *et al.*, 2018) and has been also used to reduce pathogenic bacteria and has lastly arisen as a new method to modulate microbiota diversity. Phages from different families like families of *Myoviridae*, *Siphoviridae* and *Podoviridae* (Ackermann *et al.*, 2007) have been therefore selected and “trained” to infect a wide spectrum of bacteria or tailored to infect specific antibiotic resistant bacteria present in patients since that time.

The viral component of the human GM is interestingly dominated by bacteriophage, which are known to play main roles in shaping microbial composition and function of the human gut microbiome in both health and disease, driving bacterial diversity of bacterial communities in many environments, and facilitating horizontal gene transfer (Sutton *et al.*, 2019).

The new development of genetically modified phages may be therefore an efficient tool, whether used alone or in combination with probiotics as vectors for nutrient biosynthesis or degradation that could favor the host, to positively remodulate the microbiota quality and mitochondrial dynamic, to treat the gut microbiota dysbiosis associated with different pathologies and increased production of bacterial metabolites and subsequently decrease systemic low-grade chronic inflammation associated with chronic diseases in the purpose to restore the equilibrium and homeostasis of the whole system. Manipulation of GM by lytic phage can be effectively used to selectively reduce pathogenic bacteria according to recent studies (Saint-Georges-Chaumet *et al.*, 2016; Durand *et al.*, 2018). In addition, prophages that carry biosynthesis genes of metabolites that

positively regulate mucosal inflammation can be engineered to genetically modify the functions of commensal bacteria. As an example, we can imagine increasing the capacity of these commensal bacteria to synthesize SCFA and Hydrogen Sulfide H₂S using genetically modified phages, which could be furthermore used as a treatment for obesity and affected mitochondrial metabolism, respectively knowing that the positive effect of SCFA produced by commensal bacteria has been previously shown for the benefit of obese patients (Yadav *et al.*, 2013). A clear evidence confirming the potential of the virome as a tool to shape the microbiome and its role in the efficacy of FMT treatment was reported by Ott *et al.* in 2017 (Ott *et al.*, 2017) where patients with relapsing CDI received fecal filtrates from healthy donors in the study and that resulted in CDI symptoms being eliminated for up to a period of 6 complete months and wherein recipient phage populations were substantially modified, resembling those of the donor for a minimum of a period of 6 weeks. Surprisingly, *Lactococcus* phage were noted to dominate both donor and recipient virome, despite the fact that *Lactococcus* spp. represents only a minor proportion of the GM. This result could reflect a dominance of lactococcal phage in the donor and recipient, implying that lactococcal phage plays an important role in homeostasis in CDI (Moineau *et al.*, 2004).

On the flip side, just like every therapy having adverse effects or limitations, an example of a restriction on the use of phage therapy could be a hazardous use of engineered constructs where a possible insertion could be translocated by a recombination to bacterial genomes that would acquire functions noncompatible with the environment, such as promoters or resistance genes. For that reason, additional studies should include and discuss the validity of those aspects (McCarville *et al.*, 2016).

To sum up, new data concerning the role of the phages on the modulation of gut bacterial community are required to better understand the potential effect of phages on microbiota in order to further clarify the possible detrimental effects of their application on the human immune system and the long-term effects of such human-bacteriophage interaction.

Chapter 3. Probiotics and their modulation by diet

1. Overview on probiotics

1.1. Overview

The principle describing that particular bacteria, and notably lactic acid bacteria, offer health benefits was defined notably by the Russian Elie Metchnikoff, Nobel Prize in medicine in 1908 (Guarner *et al.*, 2011). He said at the time that "the dependence of gut microbes on food makes it possible to adopt measures to modify the flora in our bodies and replace dangerous microbes with useful microbes ". He developed a diet based on milk fermented by a bacterium called the *Bulgarian bacillus* (now called *Lactobacillus delbrueckii subsp. Bulgaricus*) aiming at replacing the putrefactive bacteria responsible, according to him, for auto-intoxication. The first *Bifidobacterium* was isolated by a member of the Pasteur Institute: Henry Tissier in a healthy breastfed child. He named it *Bacillus bifidus communis* (now called *Bifidobacterium bifidum*) and recommended that it be administered to infants with diarrhea. In 1917, a non-pathogenic strain of *Escherichia coli* was isolated by the German professor Alfred Nissle from the stool of a soldier who did not develop enterocolitis during a severe outbreak of shigellosis. The term "probiotic" was first introduced in 1965 as opposed to antibiotics. It has been defined by Lilly and Stillwell as factors derived from microorganisms and stimulating the growth of other microorganisms. The prefix "pro" means for and the suffix "-biotic" means life. Probiotics, as defined today by the WHO are living microorganisms which, when ingested in sufficient quantities, have positive health effects beyond traditional nutritional effects (Kechagia *et al.*, 2013). Conferring a wide range of effects on the host, they are proposed to prevent gut microbiota dysbiosis in the event of exposure to antibiotics, in the event of intense physical and / or mental stress, in the event of digestive infectious diseases recurrent or any other predisposing conditions.

Probiotics are usually made up of bacteria or yeasts declared as non-pathogenic microorganisms. A probiotic strain (Guarner *et al.*, 2011) is identified by its genus, its species, and by alphanumeric characters designing the strain name. There is no exact requirement for a microorganism to be recognized as a probiotic, but there are minimum criteria considered including proper strain characterization, strain have to be safe for the intended use and have to display beneficial effects in at least one positive relevant human study, and strain viability should be maintained until end of shelf life of the products (Binda *et al.*, 2020). Indeed, in-depth strain characterization is crucial

in probiotic development since effects of probiotic are highly strain-dependent and therefore results for one given strain cannot be generalized to any commercial product containing a different strain of the microorganisms belonging to the same genus. This concept is well-illustrated by a study from Meijerink and collaborators comparing immunomodulatory properties of 20 *Lactobacillus plantarum* strains and presenting huge differences in their abilities to modulate the secretion of the anti-inflammatory cytokine IL-10 and two pro-inflammatory cytokines, TNF- α and IL-12p70 by primary human dendritic cells (Meijerink *et al.*, 2010). Some *L. plantarum* strains displayed a strong anti-inflammatory profile (IL-10 high and TNF- α + IL-12p70 low) whereas some others are thought to be pro-inflammatory (IL-10 low and TNF- α + IL-12p70 high).

Strain viability is another key point of probiotics since some of their beneficial effects can rely on *in situ* metabolic activities of the probiotic in the GI tract whereas others beneficial effects can be recapitulated by the dead microorganisms (Adams *et al.*, 2010). Even if dead microorganisms are not considered as probiotics in the current definition, the development of products based on inactivated microorganisms can be of interest since ingestion of live microorganisms might represent safety issues (e.g., sepsis) in some consumers such as neonates or vulnerable patients (Piqué *et al.*, 2019). A number of case reports describe episodes of infection caused by microorganisms consistent with probiotic strains in patients who consumed probiotics prior to symptom onset. Nine cases of overt sepsis have been reported back then (Burkhardt *et al.*, 2005; E F Zein *et al.*, 2008), associated with *S. boulardii cerevisiae*, *Lactobacillus rhamnosus* GG, *Bacillus subtilis*, *Bifidobacterium breve*, or probiotic combinations.

Regarding posology, the dosages of probiotics are usually expressed in Colony forming unit (CFU) / dose. The microorganisms studied for their probiotic properties being very different from each other and their precise molecular mechanisms of action not established, it is not possible to compare the doses of one product to another nor to define doses universally necessary to hope for an effect. Depending on the strain, the amounts needed to prove effective in controlled human studies can vary from 10^8 CFU for one strain to 1.10^{11} CFU for another (Ouwehand *et al.*, 2017).

1.2. Selection criteria

In 2005, the European Food Safety Authority (EFSA) defined the first quality criteria for a probiotic strain in France, with a revision of these criteria in 2010 (EFSA (2007). Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA - Opinion of the Scientific Committee. EFSA J. 5:587. 10.2903/j.efsa.2007.587) (Binda *et al.*, 2020). These criteria allow us to understand the importance of the selected strain, as well as the means of administration of the strain, that is to say the influence of the vector food and of the pharmaceutical form for the production of products containing probiotics.

A probiotic had to meet the following quality criteria:

- To be classified taxonomically (genus, species, strain), its strain must belong to an internationally recognized strain bank and be deposited in the national collection of cultures of microorganisms at the Pasteur Institute,
- Belong to the group of microorganisms considered safe, including in immunocompromised patients. It therefore presents no risk of toxicity and must be perfectly harmless. Lists of these microorganisms have been drawn up and possess a Qualified Presumption of Safety (QPS) status in Europe (attributed by the EFSA) or Generally Recognized As Safe (GRAS) status in the United States (attributed by the FDA),
- Reach then be active within the intestinal environment and survive it (including during exposures to gastric juices, pancreatic and bile acids).
- Be associated with a clinical or functional benefit for the patient in a documented way by clinical studies with scientific methodology (since January 2010),
- Be cultivated, handled and stored without being destabilized until the date of optimum use indicated (the probiotic bacteria should not be killed if the resistance of the product is tested from 12 to 24 months, at 4 and 20°C),
- The dose of probiotic must be between 10^9 and 10^{11} CFU / day.

Similar criteria have also been described by FAO/WHO (Hill *et al.*, 2014).

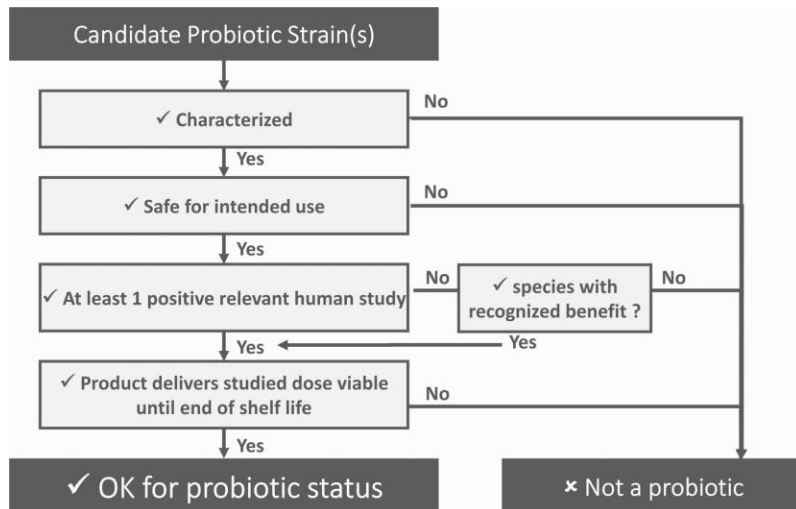


Figure 6: Summarized chart of criteria for probiotic selection

(Binda *et al.*, 2020)

1.3. Main species used as probiotic

As for the main species,

There are many different microorganisms currently used as probiotics (Heyman *et al.*, 2002). Based on the empirical use of probiotics during the last century, the most common microorganisms used as probiotics come from the *Lactobacillus* genus followed by the *Bifidobacterium* genus. Other bacterial strains such as *Enterococcus*, *Streptococcus* and *Escherichia* are also used (Mercenier *et al.*, 2003). Among the fungi kingdom, the yeasts from the genus *Saccharomyces* are the most employed as probiotics.

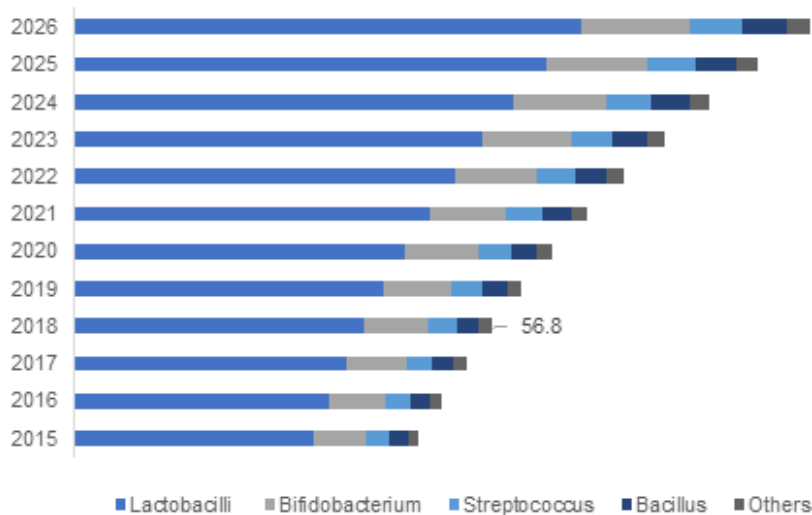


Figure 7: Probiotics Market, by ingredients, 2018 to 2026 (in USD million): Increasing use of Lactobacilli strain in milk & dairy products to fuel the industry outlook in 21 countries including U.S., Canada, Mexico, Germany, UK, France, Italy, Spain, Russia, Netherlands, Belgium, China, India, Japan, South Korea, Indonesia, Malaysia, Brazil, Saudi Arabia, UAE, South Africa.

(Pratap *et al.*, 2020)

Lactobacillus species

Lactobacillus is a heterogeneous group of bacteria, including includes more than 170 species, widespread in the plant, animal, and human environment (Goldstein *et al.*, 2015). Their ability to produce lactic acid refers to a group of lactic acid-producing Gram-positive rods, belonging to the Firmicutes phylum. They are obligate and facultative anaerobes non-sporulating and non-motile bacteria presented as long and thin rods, or very short, or curved or even ovoid, mostly present in the human gastrointestinal and genito-urinary tracts (Rossi *et al.*, 2019). Recently, in consequence to this heterogeneity, a reclassification of the *Lactobacillus* genus into 25 genera has been proposed, based on genetic, physiological, and ecological criteria (Zheng *et al.*, 2020). To illustrate this, the bacteria belonging to the former *Lactobacillus casei* group (comprising the former *Lactobacillus casei*, *Lactobacillus paracasei*, and *Lactobacillus rhamnosus*) are now reclassified as the genus *Lacticaseibacillus*. The formation of chains is common in the bacteria of the Lactobacillaceae family. Most *lactobacilli* multiply in a temperature range of 15°C to 42°C. Their metabolism is exclusively saccharolytic and lactate represents at least half of the final metabolites produced from assimilated carbon sources. There are two types of metabolism: strict homofermentary by producing only lactate and heterofermentary by synthesizing another end

products such as acetate, ethanol, succinate. Depending on species, they are normal inhabitants of various mucosa of the human body, including the oral cavity (*L. gasseri* and *L. fermentum*), the stomach (*L. gastricus* and *L. reuteri*), the intestine and the colon (*L. fermentum*, *L. plantarum*, *L. casei* and *L. rhamnosus*) and the vaginal mucosa (*L. crispatus*, *L. gasseri*, *L. vaginalis*, *L. iners* and *L. jenssonii*) (Lamont *et al.*, 2011; Caufield *et al.*, 2015).

First, *Lactobacilli* and other lactic acid producing bacteria are important organisms used in industrial food production (Hammes *et al.*, 1994). They are used to ferment common foods, such as yogurt, cheese, pickles, sauerkraut, and sourdough bread (Bernardeau *et al.*, 2006). Fermentation produces lactic acid that causes a decline in pH. This drop in pH then inhibits the growth of putrefactive and pathogenic bacteria. In addition, these organisms also increase the nutritional value of fermented foods. This occurs because lactic acid-producing bacteria cause an increase in the production of essential amino acids and vitamins, along with an increased bioavailability of minerals. Over the last century, the food microbiology industry has extensively studied *lactobacilli* and judged the bacteria safe for human consumption.

Beyond these nutritional aspects, *Lactobacillus* strains might constitute a promising new source for the development of innovative anti-infectious agents that act both lumenally and intracellularly in the GI tract (Liévin-Le Moal *et al.*, 2014). For instance, hydrogen peroxide-producing *lactobacilli* are bactericidal to the vaginal pathogen *Gardnerella vaginalis*, and their presence in the vagina has been associated with decreased frequencies of bacterial vaginosis and trichomoniasis (Turovskiy *et al.*, 2011). In the vagina, lactic acid from *lactobacilli* lowers vaginal pH, which can avert pathogen maturation and growth. Similarly, *Lactobacillus* strains isolated from human intestinal microbiota (i.e., *L. rhamnosus* GG, *L. casei* Shirota YIT9029, *L. casei* DN-114 001, *L. johnsonii* NCC 533, *L. acidophilus* LB, and *L. reuteri* DSM 17938) have been well characterized with regard to their potential antimicrobial effects against the major gastric and enteric bacterial pathogens and rotavirus (Liévin-Le Moal *et al.*, 2014).

Most researchers agree that the effectiveness of *lactobacilli* and other probiotics for all indications depends on their ability to colonize an area of tissue (Khalighi *et al.*, 2016). To do this, *lactobacillus* preparations must contain live and viable organisms. Products stored for long periods of time or stored improperly may contain few live and active organisms. For oral preparations, bacteria must also remain viable after passing through the gut, and then they should be able to clamp on to the intestinal

epithelium. Thus, natural resistance of *lactobacilli* strains to industrial process-related stress (drying, osmotic stress or freezing) and to GI tract conditions are important selection criteria in the development of new probiotic strains. *Lactobacilli* strains might vary in their effectiveness due to differences in their ability to adhere to the epithelial cells by host factors such as hormone levels (Dizzell *et al.*, 2019). Likewise, in a study where colonization of *Lactobacillus* in the gut depended on several factors like the amino acid metabolism, the genetic environment and such factors needed for gut persistence (Licandro-Seraut *et al.*, 2014). *Lactobacilli* are the bacteria mainly used as probiotics, in particular *Lactobacillus acidophilus*, *Lactobacillus casei* and *Lactobacillus rhamnosus*, because these three species offer good resistance to gastric acidity and have a strong capacity for adhesion to intestinal cells (Seppo Salminen *et al.*, 1998).

They are considered as beneficial bacteria providing nutritional benefits, notably by producing a range of bioactive compounds during fermentation processes and impacting host metabolism, but also by increasing the bioavailability of some vitamins and minerals (Khalighi *et al.*, 2016). *Lactobacilli* also might contribute to strengthen the mucosal barrier and to decrease intestinal permeability, allowing to restrict potential colonization by pathogenic organisms (Rao *et al.*, 2013; Pickard *et al.*, 2017). The theory is that taking *lactobacillus* probiotics during antibiotic treatment can fend off or minimize normal flora depletion and pathogenic bacteria colonization. There is some evidence to support this theory: there are perhaps three areas in which probiotics may act as adjuncts to antibiotics such as in secreting antibacterial substances that lower pathogenic bacterial populations locally and at distant mucosal sites, and disrupt biofilms, making it easier for antibiotics to function; or by enhance generalized mucosal immunity, which in turn aids in the eradication of the organisms at the mucosal site (Yoon *et al.*, 2018).

Lactobacillus strains have been proposed to modulate the digestive immune responses, their intake might be beneficial in the case of prevention or management of some digestive disorder's pathologies including necrotizing enterocolitis of premature newborns, digestive infections, diarrhea associated with antibiotics or the irritable bowel syndrome (Mu *et al.*, 2018). *Lactobacilli* can also be used in the prevention or treatment of colic in infants, in particular, with some strains belonging to the *Lactobacillus reuteri* species that are recognized for their good tolerance in children and for their multiple supposed benefits (reduction of infections and bacterial translocations, modulation of the

host immune response, regulation of food tolerance and participation in the absorption of nutrients, vitamins and minerals) (Mu *et al.*, 2018). Taking *L. acidophilus* in very low birthweight infants would reduce moreover the risk of morbidity, the time of hospitalization and would also increase the weight gain (Härtel *et al.*, 2014). Concerning the effect of their supplementation on the composition of the gut microbiota, a recent study investigated the use of the probiotic strain *Lactobacillus rhamnosus* GG (LGG) on gut microbiota composition in Human Immunodeficiency Virus (HIV)-infected individuals whereas a reduction of Enterobacteriaceae and Erysipelotrichaceae in the gut microbiome was noted, with reduced Enterobacteriaceae among individuals (Arnbjerg *et al.*, 2018).

***Bifidobacterium* species**

Bifidobacterium is a strict anaerobic, Gram-positive, pleomorphic rod that are part of the phylum Actinobacteria, non-sporulated, irregularly shaped, often with branches. In the genus *Bifidobacterium*, there are currently 42 species and nine subspecies. They are saccharolytic organisms that produce acetic acid and lactic acid as by-products of glucose utilization. They are found in the mouth, intestine, colon and vagina. This bacterial genus is very well adapted to the GI tract despite physiological stress. In humans, whereas *lactobacilli* are present in the intestinal ecosystem in small amounts that vary greatly from one individual to another, *Bifidobacteria* are part of the dominant flora with differences in terms of species depending on the age of the individual (Turroni *et al.*, 2012). This genus establishes itself at high levels in the first days of life in a full-term newborn (Milani *et al.*, 2017). When newborns born vaginally and breastfed, colonization with *bifidobacteria* is rapid and dominant due to the ingestion of HMOs during breastfeeding, that promote their implantation. In addition, it has recently been shown that breast milk may contain indigenous *bifidobacteria*, supporting a possible direct colonization from the mother's microbiota (Milani *et al.*, 2017). A delay in implantation can be seen in children born by cesarean section and those born prematurely (Dolié *et al.*, 2018). The many nutritional requirements of the genus *Bifidobacterium* (like their need for amino acids and carbon sources as well as their mineral salt and vitamin requirements) (Bezkorovainy *et al.*, 1981) and its sensitivity to oxygen and antibiotics (especially those of gram-positive spectrum (macrolides) and those with broad spectrum (rifampicin) (Masco *et al.*, 2006) make it slightly more complicated to select strains as probiotics, suitable for industrial processes. The most used species as probiotics include *B. breve*, *B.*

adolescentis, *B. animalis*, *B. bifidum*, *B. infantis*, *B. lactis* and *B. longum* (Mercenier *et al.*, 2003). The genus *Bifidobacterium* is classified in the category of organisms devoid of pathogenicity, it is therefore considered non-pathogenic and rarely involved in infections. Rare cases of infections linked to probiotic strains have been reported by the EFSA but only in immunocompromised people. Since this report dating from 2012, only one case of bacteremia has been reported in a very low birth weight neonate receiving a mixture of two probiotic strains (Underwood *et al.*, 2017). Also concerning its effect on microbiota's composition in the gut, a recent study investigated the effects of *Bifidobacterium longum* strain BB536 supplementation on intestinal microbiota composition and the immune response in term infants and suggested that this strain has some positive effects on establishing a healthy intestinal microbiota whereas the amount of *bifidobacteria* and the *bifidobacteria/Enterobacteriaceae* ratio (B/E) were significantly higher in the BB536 supplementation group at months 2 and 4 than those in control group (B.-B. Wu *et al.*, 2016).

Concerning their application, some strains show less importance than others like in dairy products production for example, where the growth of *bifidobacteria* in milk is often slow or limited compared with lactic acid bacteria used in fermented dairy products, and this appears partially due to low proteolytic activities (Gomes *et al.*, 1999). However, *Bifidobacteria* probiotic strain has been used as a major ingredient to produce nutraceutical products and as a dairy starter since 2000 (Ku *et al.*, 2016) making it one of the most used and studied probiotic bacteria (Song *et al.*, 2012). The varied bio-functional out-turns and potential for industrial application of *Bifidobacteria* has been characterized and demonstrated by *in vitro* (i.e., phytochemical bio-catalysis, cell adhesion and anti-carcinogenic effects on cell lines, and immunomodulatory effects on immune cells), *in vivo* (i.e., suppressed allergic responses and colitis in mouse model), and clinical studies (eczema in infants and adults with irritable bowel syndrome) (Ku *et al.*, 2016).

***Streptococcus* species,**

The genus *Streptococcus* consists of Gram-positive, spherical ovoid, or coccobacillary cells, with a diameter less than 2 µm, that form chains or pairs. Cells in older cultures may appear Gram variable, and some strains are pleomorphic. *Streptococcus spp.* are nonsporing and nonmotile. They ferment carbohydrates to produce mainly lactic acid, but no gas, and have complex nutritional requirements. Under glucose limiting conditions, formate, acetate, and ethanol are also

produced. Most are facultatively anaerobic or aerotolerant anaerobes; some are capnophilic (CO₂-requiring) (Issa *et al.*, 2020). The *streptococci* are characteristically found on the mucous membranes of the mouth, upper respiratory tract, alimentary tract and genitourinary tract, as well as on the skin, of man and other animals (Köhler *et al.*, 1979). Some *Streptococcus* species benefits from probiotic properties like the *Streptococcus thermophilus* strain. However, in contrast with other lactic acid bacteria, the probiotic status of this strain remains still questioned and considered as a promising probiotic candidate (Uriot *et al.*, 2017).

Concerning their effect on microbiota's composition, a recent study observed that the administration of *S. thermophilus* as probiotics could alter the gut microbiota composition of untreated mice or mice with LPS-induced sepsis (Han *et al.*, 2020).

Streptococcus species are widely used in food fermentations, especially for yogurt manufacturing whereas several of *Streptococcus* strains showed characteristics adequate for their use as starter culture for yoghurt production, with valuable biotechnological properties for industrial application, such as high antibacterial activity and exopolysaccharides (EPS)-producing capacity (Cartasev *et al.*, 2018).

***Bacillus* species**

Bacillus species are Gram-positive rod, belonging to the Firmicutes phylum, which produces lactic acid, and therefore is often misclassified as lactic acid bacteria, such as *Lactobacillus*. As a matter of fact, some commercial products containing *Bacillus* are marketed as *Lactobacillus* sporogenes or "spore-forming lactic acid bacterium." It forms spores, which is an important factor in differentiating these species. *Bacillus* is used therapeutically in a similar manner as other probiotics such as *Lactobacillus* and *Bifidobacterium*; however, *Bacillus* is not a component of the normal human flora. In order to be effectual for restoring normal flora and avert pathogenic colonization, probiotics must have the ability to persist and colonize in the intestinal mucosa. Three *B. cereus* strains were shown to persist in the mouse GI tract for up to 18 days post administration, confirming that these organisms have some ability to colonize this environment. Products containing endospores of members of the genus *Bacillus* (in single doses of up to 10⁹ spores/g or 10⁹ spores/ml) are used commercially as probiotics, and they offer some superiority over the more common *Lactobacillus* products in that they can be stored indefinitely in a desiccated form (P. Mazza *et al.*, 1994). Originally, many commercial products were sold as products that carry

Bacillus subtilis spores, but recent studies have shown that most products are mislabeled and carry other *Bacillus* species, including *Bacillus clausii*, *Bacillus pumilus*, and a variety of *Bacillus cereus* strains (Green *et al.*, 1999). When the *Bacillus* spore is ingested by humans, it is unknown what happens to the spore. It is also unknown if the *Bacillus* spore is capable of germinating in the GI tract or if colonization occurs (Swick, Koehler and Driks, 2016). However, strains of *Bacillus* are very good potential candidates to be used as probiotics. Metabolically, *Bacillus* species are very active and previous research has identified several useful enzymes (amylases and proteases) (Priest *et al.*, 1977) and numerous antibiotics they produce like in the case of some forms of *B. brevis* producing gramicidin or *B. subtilis* producing Bacilysin (Mannanov *et al.*, 2001). In addition to these secreted products, most *Bacillus* survive the rigors of food processing, including those designed to deplete microorganisms such as pasteurization (Ehling-Schulz *et al.*, 2019). Proponents of *Bacillus* strain suggest that this species of probiotics offers interesting biotechnological properties over others such as *Lactobacillus* (Duc *et al.*, 2004). A study concerning the effect of these strains on microbiota composition was also the interest of a recent study conducted in 2017. This study demonstrated that *B. subtilis* CSL2 supplementation in the diet along with *Salmonella Gallinarum* infection in broiler chickens influenced the diversity, composition, and functional diversity of the faecal microbiota resulting in higher abundance of *Firmicutes* and lower abundance of *Bacteroidetes* and *Proteobacteria* in probiotic supplemented groups (Jin *et al.*, 2017).

Bacillus species have been known to be implicated in various industrial applications. These incorporate industrial production of enzymes with considerable interest in detergent and food sectors; the production of primary metabolites such as vitamins and ribonucleosides; of secondary metabolites including bacteriocins and biosurfactants and of plant growth promoting formulations. Besides, recent studies have shown that the aerobic spore formers can produce fine chemicals with compelling biotechnological applications like for example carotenoid pigments and a variety of biopolymers including poly- γ -glutamic and lactic acids. These findings open perspectives for new biotechnological applications of *Bacillus* and related species (Raddadi *et al.*, 2012).

2. Probiotics functionalities

Probiotics have been known to have numerous advantageous functionalities in human organisms (Markowiak *et al.*, 2017). However, the functions of these probiotics vary significantly within the same species, mostly up to and dependent on some specific strain. Thus, in evaluating the functions of the probiotics, it is essential to characterize the functions of each probiotic to the specific strain (Tsai *et al.*, 2019).

2.1. Competition of nutrients

Probiotic bacteria may be able to restrain pathogens growth in the GI tract, exerting an important effect on the host's health condition (Hemarajata *et al.*, 2013). This inhibition of the growth of pathogens can sometimes take place through a process of restriction of nutrients. In fact, there are competitions between the bacteria present at the digestive level for the nutrients. Probiotics usually compete with pathogens using the same substrates found in the intestinal lumen such as vitamins, amino acids, or dietary fiber that are consumed by the host (Hemarajata *et al.*, 2013) such as arginine, asparagine, and tryptophan, that are considered to be central points of competition between the host and pathogen (Ren *et al.*, 2018). The decrease in available substrates makes the environment unfavorable for the growth of pathogens (Delcenserie *et al.*, 2008).

2.2. Amelioration of barrier function

The presence of the epithelium acts as an essential physical barrier in order to separate the microorganisms present in the intestinal lumen from the intestinal mucosa. The barrier effect also consists of mechanical actions induced by the peristaltic movements of intestine and colon and chemical actions (acid pH, enzymes, and anti-microbial peptides). Probiotics are suggested to play a beneficial role by inducing the production of mucus and anti-microbial peptides (Resta-Lenert *et al.*, 2006) and by strengthening the integrity of the intestinal barrier.

The increase production of mucins by probiotics can limit the adhesion of pathogens to the intestinal epithelium. For example, in the presence of *Lactobacillus plantarum* or *Lactobacillus rhamnosus* GG, Mack *et al.* observed an increase in the secretion of MUC3 mucins (Mack *et al.*, 2003). In another *in vitro* study, Collado's team found that twelve different strains of probiotics were able to inhibit the adhesion to human intestinal mucus of *Bacteroides vulgatus*, *Clostridium histolyticum*, *Clostridium difficile*, *Staphylococcus aureus* and *Enterobacter aerogenes*. These same probiotics also appear to be able to displace the cited pathogens from their binding sites (Singh *et al.*, 2018).

As mentioned below, these beneficial effects are strain-dependent, as exemplified by the improvement in the intestinal barrier integrity achieved by the administration of *Bifidobacterium lactis* or by the association of *Lactobacillus rhamnosus* 19070-2 and *L. reuteri* DSM 12246 in a model of preterm infants whereas administration of the well-known probiotic strain *L. rhamnosus* GG or *L. plantarum* 299v did not modulate the intestinal integrity in the same model (Stratiki *et al.*, 2007). These results illustrate how the mechanism of each strain must be elucidated to effectively use probiotics (Sacks *et al.*, 2018). Moreover, some studies have demonstrated that probiotics could enhance the barrier function through the reinforcement of intercellular tight junctions (TJ) by inducing synthesis and assembly of tight junction proteins (such as occluding and claudin), but also preventing disruption of tight junctions by injurious factors and releasing bioactive factors triggering activation of various cell signaling pathways that can therefore lead to the strengthening of tight junctions and the barrier function (Rao *et al.*, 2013). Other studies evoked the importance of some *Bifidobacterium* and *Lactobacillus* species in preventing epithelial barrier disruption induced by TNF- α , wherein live *Bifidobacterium* species (*B. bifidum* strain WU12, WU20, WU57, and *B. longum* strain WU16) were shown to significantly promote wound repair in Caco-2 cell monolayers treated with TNF- α for 48 h (Hsieh *et al.*, 2015) and hence, restore of intestinal epithelial TJ function.

2.3. Immunomodulation

Probiotic organisms produce several compounds that can influence the host's immune system as components of the wall, DNA and various metabolites (Kalliomäki *et al.*, 2008). Just like those produced by pathogenic bacteria, these products, named as Pathogen Associated Molecular Patterns (PAMPS) are recognized by molecular sensors (Pathogen Recognition Receptor PRRs) of the immune system which generates an appropriate immune response following integration of various signaling cascades. As an example, the Lipoteichoic acid (LTA) exposed at bacterial surfaces is recognized by the host receptor Toll-like receptor 2 (TLR2). Various receptors from the TLR family are exposed at cell surface or on endosomes to detect bacterial- or fungal-derived molecules, that in most cases are exposed on microorganism surface (Figure 8).

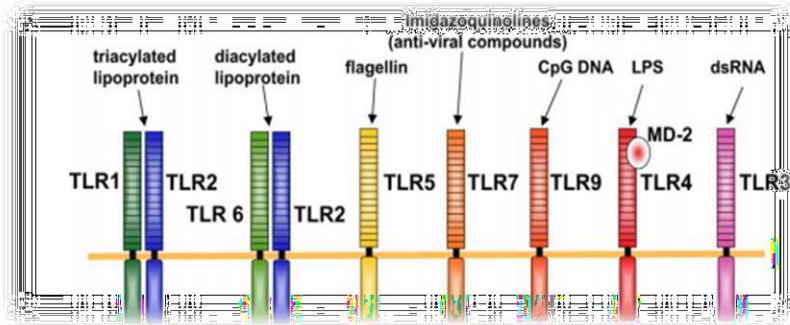


Figure 8: TLRs and their ligands. TLR1–TLR7 and TLR9 have been characterized to recognize microbial components. TLR2 is essential for the recognition of microbial lipopeptides. TLR1 and TLR6 associate with TLR2 and discriminate subtle differences between triacyl- and diacyl lipopeptides, respectively. TLR4 recognizes LPS. TLR9 is the CpG DNA receptor, whereas TLR3 is implicated in the recognition of viral dsRNA. TLR5 is a receptor for flagellin. Thus, the TLR family discriminates between specific patterns of microbial components (Takeda *et al.*, 2004)

The composition of LTA, in particular their D-alanine content could be responsible for the modulation of cytokines release. For example, bacteria exposing at their surface LTA with a low D-alanine content stimulate the secretion of IL-10 by lymphocytes (Coleski *et al.*, 2009). Beyond membrane- or cell wall-bound compounds, other active compounds with immunomodulatory properties are found in the bacterial supernatants such as the Microbial Anti-inflammatory Molecule (MAM) protein produced by *Faecalibacterium prausnitzii* (Valcheva *et al.*, 2016) and as the GroEL protein produced by some *Lactobacillus* strains (Le Loir *et al.*, 2005; Rieu *et al.*, 2014). Certain bacterial metabolites such as nitrogen monoxide (NO) and butyrate are also capable of exerting an effect on the secretion of pro-inflammatory cytokines in experimental models of colitis (Lenoir *et al.*, 2006). This innate immune response following probiotic treatment may also take the form of increased secretion of IgA just like in the case of *Lactobacillus casei* when expressing of the receptors involved in the innate immune response in BALB/c mice (Galdeano *et al.*, 2006; Hawrelak *et al.*, 1995), elevated numbers of natural killer cells, or enhanced phagocytic activity of macrophages (Schiffrin *et al.*, 1995). Increased secretion of IgA may decrease numbers of pathogenic organisms in the gut, thus improving the composition of the microflora (Fuller *et al.*, 1997). Due to these immunomodulating effects, some researchers think probiotics might not only restrain the growth and colonization of intestinal and urogenital pathogens, but might also be helpful for conditions, such as IBDs, pouchitis, food allergy, and for use as an adjuvant to vaccination (Schultz *et al.*, 2000).

2.4. Production of antimicrobial compounds

Many microorganisms produce antimicrobial compounds, providing them a competitive advantage over other microorganisms in a given ecological niche. Probiotics can display antimicrobial effects by at least two ways: directly by producing anti-microbial substances or indirectly by inducing the production of these substances by host cells (Dobson *et al.*, 2012). Probiotic strains are described to produce antimicrobial compounds contributing to their ability to maintain in various ecological niche including food products as well as the GI tract. The probiotic bacteria usually produce several useful compounds such as bacteriocins, exopolysaccharides, SCFAs, free amino acids, bioactive peptides, vitamins, digestive enzymes, immunomodulatory compounds, and oligosaccharides (Vidya Prabhakar *et al.*, 2013).

The efficacy and spectrum of antimicrobial products of lactic acid bacteria in particular are broad and include organic acid (lactic and acetic acid, formic acid), oxygen catabolites (hydrogen peroxide), sugar catabolites (carbon dioxide, diacetyl, ethanol) as well as bacteriocins or bacteriocin-like substances (Mishra *et al.*, 1996) and antibiotic-like substances (reuterin and reutericyclin), and others (Vuyst *et al.*, 2004). The major bacterial growth inhibitory principles produced by *Lactobacilli* are organic acids that are most potent at low pH, but other antimicrobial substances, different from organic acids, may contribute to this killing. Among all these compounds bacteriocins have attracted most attention in recent years and it has been shown that many *Lactobacillus* strains produce different kinds of bacteriocins. Bacteriocins are antibacterial polypeptides that target their activity towards killing or inhibiting bacterial strains often non-related or closely related to produced bacteria or produced species, in order to reduce environmental competitors for acquiring nutrients and living space like Colicins for example, originally produced by *E. coli* as the primary defense systems in response to various triggers: DNA-damaging agents, stringent response, oxidative stress, growth phase, osmolarity, cold shock, or nutrient deprivation (Gillor *et al.*, 2004; Ghazaryan *et al.*, 2014; Yang *et al.*, 2014). Nisin is also a bacteriocin presenting antimicrobial activities and are produced by some *Lactococcus* species. In addition, nisin has been widely accepted as a safe and natural preservative in food industry (Ibarra-Sánchez *et al.*, 2020). They are produced by all major lineages of bacteria and archaea and constitute a heterogeneous group of peptides with respect to size, structure, mode of action, antimicrobial potency, immunity mechanisms and target cell receptors. The production of antimicrobials is often regarded a priori as an important trait in the context of bacterial fitness but

also in terms of probiotic efficacy for that some antimicrobial compounds like bacteriocins could contribute to probiotic functionality in several ways including functioning as colonizing peptides, facilitating the introduction and/or dominance of a producer into an already occupied niche (Riley *et al.*, 2002). Alternatively, they may also act as killing peptides, directly inhibiting competing strains or pathogens (Majeed *et al.*, 2011). Lastly, through functioning as signaling peptides, either signaling other bacteria through quorum sensing (QS) and bacterial cross talk within microbial communities or signaling cells of the host immune system (Czárán *et al.*, 2002; Di Cagno *et al.*, 2007; Meijerink *et al.*, 2010; Dobson *et al.*, 2012). Traditionally, bacteriocin production has been an important criterion in the selection of a probiotic strain, albeit that few studies have definitively demonstrated the impact of bacteriocin production on the ability of a strain to compete within the GI tract and/or positively influence the health of the host. Lastly, bacteriocins may function as signaling colonizing peptides facilitating the introduction and/or dominance of a producer into an already occupied niche (Dobson *et al.*, 2012), either by signaling other bacteria through QS and bacterial cross talk within microbial communities or by signaling cells of the host immune system (Dobson *et al.*, 2012; Miller *et al.*, 2018).

2.5. Metabolic interactions

Probiotic microorganisms may also exert beneficial effects on host by modifying its metabolic processes, particularly those occurring in the gut environment. Such beneficial effects could be achieved in theory by a variety of mechanisms: by cutting off reactions that result in the formation of toxic or carcinogenic metabolites, by stimulating enzymatic reactions involved in detoxification of potentially noxious substances, by enhancing mammalian enzymes involved in the digestion of complex nutrients, or where such enzymes are absent (due to genetics or disease) providing a bacterial source of these enzymes, by synthesizing vitamins and other indispensable nutrients not provided in enough amounts in the diet. Although probiotics induce positive metabolic reactions in the digestive tract, they can promote on the other hand deleterious metabolic reactions in the host. During bacterial colonization of the small intestine, excess microorganisms can induce diarrhea (Monreal *et al.*, 2005). Other studies revealing probiotic's functionalities through metabolic interactions showed that patients with ileostomy using probiotics have been proved to increase the transformation of conjugated primary bile acids into free secondary bile acids. However, in order to avoid these exaggerated reactions of deconjugation, and dihydroxylation, it is essential to carry out further tests *in vitro* to assess metabolic reactions of probiotics (Marteau *et al.*, 2003).

Nevertheless, in recent years, probiotics, prebiotics, and synbiotics are considered to be the best documented substances with the potential to produce SCFAs (Usta-Gorgun *et al.*, 2020). In a recent study, the production of SCFA by a panel of *Bifidobacterium* probiotic species was assessed *in vitro* and it turned out that the largest increase in total SCFAs was observed for *Bifidobacterium infantis* and *Bifidobacterium lactis*, respectively, due to their higher metabolic activity comparing to the others (Usta-Gorgun *et al.*, 2020). Due to such beneficial health effects, some probiotics like *Bifidobacterium/Lactobacilli* species are used as food supplements or as SCFA producers and therefore added into some solutions such as yogurt. On the flip side, some other probiotic strains could contrarily participate in the production of some molecules that could act negatively on the health host. A recent study proved the role of probiotics in the amelioration of lipid metabolic disorders through the reduction of intestinal Trimethylamine (TMA), a biologically active molecule metabolized and generated by the gut microbiota from choline and L-carnitine (Liu and Dai, 2020), which when passed through the blood, could form the Trimethylamine N-oxide (TMAO) which by its turn, enters in a great association with the risk of developing atherosclerosis (AS), which is considered to be a chronic inflammatory disease initiated by vascular endothelial inflammatory injury. In Liang's study, five of 10 lipid-lowering strains (*Bifidobacterium animalis* subsp. *Lactis* F1-3-2) effectively degraded TMA *in vitro*, and the TMA level in the cecum of mice were reduced after probiotic intervention, alleviating thus AS (Liang *et al.*, 2020).

3. Molecular mechanisms of interactions with host cells

The mechanisms of action of probiotics are complex, often multiple and, as mentioned above, highly depend on the bacterial strain considered. The molecular details of these mechanisms have been extensively studied during the last decades, allowing on one side to identify many active principles of probiotics and on the other side the signaling networks involved in host cells. (Boirivant and Strober, 2007; O'Toole and Cooney, 2008; Bai and Ciacci, 2017). In the section below, we will describe the main bacterial components involved in such interactions with host cells.

3.1. Peptidoglycans:

Peptidoglycans (PGN) are the largest component of the bacterial cell wall in gram positive bacteria accounting for up to 90% of their weight (Warshakoon *et al.*, 2009) and is an essential polymer in *lactobacilli* that determines the shape and preserves their integrity (Sengupta *et al.*, 2013). PGN is an indispensable molecule of LAB and is suggested to be important for the functionalities of a probiotic bacterium (K, Jamila *et al.*, 2012). Studies have also found that *Lactobacillus* strain-specific anti-inflammatory capacities are somehow correlated with the PGN structure (Fernandez *et al.*, 2011). PGN can be therefore recognized beyond mucosal surfaces, and their receptors, such as NOD1/2, NLRP3 or TLR2, can be expressed in tissues and cells that are far from the niches where bacteria reside (Wheeler *et al.*, 2014).

PGNs can inhibit LPS-induced pro-inflammatory response in RAW264.7 cells. This anti-inflammatory effect relies on the modulation of the signaling downstream the LPS receptor TLR4 and depends on the diversity of PGNs derived from various *Lactobacillus* strains (Wu *et al.*, 2015). Thus, illustrating the fact that the immune modulation mediated by the host receptors to PGN, notably TLR2, following the binding of the PGN is highly strain specific due to modifications in its composition (Turroni *et al.*, 2014). In fact, considerable variations occur in the basic compositions of the glycan strands and pentapeptides which impart strain-specific characteristics to the bacteria (Sengupta *et al.*, 2013).

3.2. Exopolysaccharides:

Another example is the role of probiotics exopolysaccharides (EPS) in modulating the host's immune system. EPS are extracellular carbohydrates excreted as tightly bound capsule or loosely attached slime layer in microorganisms. As polysaccharides display an excessive diversity among genera, including *lactobacilli*, they are thought to be involved in determining strain-specific properties prime for probiotic action, such as adhesion, stress resistance, and interactions with specific receptors and effectors of the host defense system (Delcour *et al.*, 1999). The term EPS have sometimes a broader definition, encompassing extracellular polymeric substances other than carbohydrates such as proteins, DNA or lipids. They play the most prominent role against desiccation, antimicrobial peptides, phagocytosis, cell recognition, phage attack, antibiotics or toxic compounds and osmotic stress (Lebeer *et al.*, 2009; Angelin *et al.*, 2020).

In particular the EPS retrieved from probiotic bacteria with varied carbohydrate compositions possess immunomodulatory properties by stimulating the proliferation of T and B lymphocytes, by enhancing natural killer (NK) cell tumoricidal activity and by strengthening mononuclear cell phagocytic capacity (Angelin *et al.*, 2020). In macrophages, EPS increase their viability, enhance phagocytosis and stimulate macrophage activation, promoting the secretion of NO, TNF- α , IL-1 β , IL-6 and IL-10 suggesting that EPS may have significant effect on immune response (You *et al.*, 2020). The EPS from *L. casei* strain Shirota are known to be involved in suppressed pro-inflammatory cytokine responses by macrophage *in vitro*, suggesting that this polysaccharide is the relevant immune modulator which may function to lessen excessive immune reactions during the activation of macrophages by *L. casei* Shirota (Yasuda *et al.*, 2008).

Nevertheless, other properties were elucidated through multiple studies describing EPS, like being antimicrobial substances or antioxidant (Serafini *et al.*, 2013) just like in the case of a study that demonstrated that EPS from *B. bifidum* WBIN03 and *L. plantarum* R315 display strong antibacterial ability against a wide range of bacterial and fungal pathogens (*Candida albicans*, *Cronobacter sakazakii*, *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella typhimurium*, and *Shigella sonnei*). Similarly, they showed strong scavenging activity against Diphenyl 1-picrylhydrazyle (DPPH), hydroxyl, and superoxide radicals, and inhibitory effects on lipid peroxidation and erythrocyte hemolysis (Li *et al.*, 2014). The EPS from *L. rhamnosus* strains isolated from the human breast milk show strong anti-bacterial activity against pathogenic *E. coli* and *Salmonella typhimurium* under *in vitro* condition (Riaz Rajoka *et al.*, 2018). For some bacteria, EPS seem to be essential components in their ability to restrain colonization by pathogenic bacteria, as exemplified by an EPS-deficient mutant of *B. subtilis* unable to prevent *C. rodentium*-associated intestinal disease compared to the corresponding wild-type strain (Jones *et al.*, 2014).

EPS can also display antiviral properties as shown against the Cucumber mosaic virus (CMV-Y) and herpes simplex virus type 2 (HSV-2) (Gugliandolo *et al.*, 2014).

Anti-tumor activities as illustrated by the fact that EPS from *Lactobacillus kefir* strain MSR101 can stimulate the expression of pro-apoptotic genes (BAX, BAD, caspase3, caspase8, and caspase9) in HT-29 colorectal cancer cells and thereby decrease their survival (Riaz Rajoka *et al.*,

2019), as well as some anti-viral (against, anti-diabetic, and cholesterol lowering activities (Hussain *et al.*, 2017).

Of note, *L. rhamnosus* GG limits the production of EPS to optimize its adhesion to intestinal epithelial cells (Bron *et al.*, 2012).

3.3. Surface proteins:

Proteins exposed at microorganisms' surfaces are described to play various roles in host-probiotics interactions. As previously described in the 'Production of antimicrobial compounds' some of them have anti-pathogens activities, whereas some others modulate the production of cytokines or inhibit apoptosis of colonic epithelial cells (Turroni *et al.*, 2014) such as the surface-layer protein (Slps), which may have an immunomodulatory effect mediated by C-type lectin and TLR receptors within GALT, however, the effective role of these proteins remains to be confirmed *in vivo* (Do Carmo *et al.*, 2018).

Two proteins (p40 and p75) secreted from *L. rhamnosus* hampered cytokine-induced apoptosis in epithelial cell lines by activating the EGF receptor and its downstream target Akt, as well as inhibiting p38 MAP activation, *in vitro* and *ex vivo* (YAN *et al.*, 2007). Akt promotes cell survival by inactivating proapoptotic proteins, including caspases 3 and 9 (Hanada *et al.*, 2004). Expression of p40 and p75 is strain specific because *L. casei*, but not *L. acidophilus*, also produces these proteins (YAN *et al.*, 2007). Additionally, pretreatment with either p40 or p75 protected several cell lines from H₂O₂-induced disruption of barrier function. This effect was *via* inhibition of H₂O₂-induced cytosolic localization of the TJ proteins occludin and ZO-1 and the AJ proteins E-cadherin and β -catenin. All these effects were all closely dependent on activation of PKC ϵ , PKC β I and the MAP kinases ERK1/2 (Seth *et al.*, 2008). Therefore, bacterial proteins isolated from *L. Rhamnosus* cultures effectively block the induction of apoptosis, helping to enhance epithelial barrier function suggesting that the improvement of the intestinal barrier function seems to be dependent on the action of some specific surface proteins in several probiotic strains. With the demonstration of the therapeutic effects of the membrane proteins of probiotics, the definition of post-biotics (metabolites and cell membranes of probiotics) was born and since then, the latest scientific literature has highlighted the widely accepted definition of paraprobiotics/post probiotics as "the inactivated/dead/non-viable microbial cells of probiotics (intact or ruptured containing probiotic cell components upon lysis) or crude cell extracts (i.e. with complex chemical composition)"

(Taverniti *et al.*, 2011). By contrast, postbiotics are the complex mixture of healthy metabolic products or secreted components of probiotics in cell-free supernatants such as enzymes, secreted proteins, short chain fatty acids, vitamins, amino acids, peptides, organic acids, etc. (Tsilingiri *et al.*, 2012). The therapeutic interest of these post biotics is thereby under study (Turroni *et al.*, 2014).

4. Biofilm formation and bacterial Adhesion to intestinal epithelium

In the second half of the 1980s, it was shown that lactic acid bacteria (LAB) of genitourinary origin were able to adhere to epithelial cells of the urinary tract and thereby preventing colonization by pathogenic microorganisms (Vieco-Saiz *et al.*, 2019). Back then, the first *in vitro* tests in the 1990s were performed on models of differentiated human intestinal cells exhibiting the same phenotypic characteristics as a portion of the intestinal epithelium in the body. For example, the Caco-2 cell line, widely used in the pharmaceutical industry to predict the intestinal absorption of active ingredients, has made it possible to study the epithelial adhesion capacities of different probiotic strains. By testing twenty-five strains of *Lactobacillus*, Chauvière *et al.* show that not all of them show the same adhesiveness to Caco-2 cells (Sacks *et al.*, 2018). Several subsequent studies aim to estimate this adhesion capacity of probiotics more precisely. Overall, they agree that human LAB readily adhere to Caco-2 cells *in vitro*. Strong adhesiveness is found for *Lactobacillus johnsonii* LaI, *Lactobacillus rhamnosus* GG, *Lactobacillus acidophilus* LB (Marcotte *et al.*, 2017), as well as for *Bifidobacterium breve* 4 or *Bifidobacterium infantis* I (Toscano *et al.*, 2015). The adhesiveness of probiotics to the intestinal mucosa has also been evaluated *in vivo* on athletes, confirming that this is a strain-dependent property (Jäger *et al.*, 2019). However, one worthy of notice point is the fact that *in vitro* tests on Caco-2 cells or other IEC lack the presence of many factors to mimic the GI tract environment such as the presence of the mucus layer. Thus, *in vivo* validation is required to confirm results. By adhering to the intestinal mucosa, probiotics compete with potential pathogens for the same binding receptors like in the case of inhibition of binding of *Helicobacter pylori* to the glycolipid receptors by probiotic *Lactobacillus reuteri* (Mukai *et al.*, 2002). The adhesion of bacteria to host surfaces is considered of major importance to the permanent, if not transient, establishment of probiotic species in any environmental niche. Adhesion properties might delay their clearance, thereby increasing the chance of probiotics to bring benefits to the host (Monteagudo-Mera *et al.*, 2019). By adhering to surfaces, probiotic bacteria are able to, eventually, initiate the formation of biofilms.

4.1. Definition

The definition of a biofilm has evolved a lot since its discovery attributed to Van Leeuwenhoek in the 17th century. Indeed, he would have observed, for the first time, microorganisms adhered to the surface of the teeth. In the early 20th century, the work of Claude E. Zobell, a pioneer in biofilm research, has greatly contributed to the development of the concept of bacterial attachment ([Zobell *et al.*, 1933](#)). Researchers still disagree on certain points but a commonly accepted definition is that of Donlan and Costerton ([Donlan *et al.*, 2002](#)): “a biofilm is a sessile microbial community characterized by cells irreversibly attached to a substrate, an interface or quite simply between them, coated with an extracellular matrix of polymeric substances (notably exopolysaccharides) which they themselves have produced and which exhibit a particular phenotype in terms of growth rate and of gene transcription. Basically, bacteria can be found in nature under two forms: either freely floating planktonic bacteria or as sessile colonies of microorganisms forming these biofilms. Some studies suggest that biofilm life mode is the dominant form of growth for bacteria and archaea in many habitats on earth ([Flemming *et al.*, 2019](#)).

4.2. Stages of biofilm formation

The biofilm formation model has four stages (figure 5): so-called reversible adhesion, irreversible adhesion, maturation, and detachment. To achieve the reversible adhesion step, the bacteria must first approach the support. Mechanisms in which the cell is not active intervene Brownian motion, sedimentation, and mass transfer by convection ([Ghalambaz *et al.*, 2014](#)), but also mechanisms where the cell is active such as chemotaxis and the establishment of motion-generating appendages such as flagella ([O’Toole *et al.*, 1998](#)). This approach leads to a transient attachment (between 20 and 50 nm from the support), the so-called reversible adhesion, relying on Van der Waals forces, Lewis’s acid-base forces and electrostatic forces. Secondly, a stable association with the surface is established thanks to adhesive structures such as filamentous adhesins (fimbriae, pili) or secreted compounds (EPS, capsule, etc.) and to the establishment of numerous low energy bonds such as hydrogen bonds, hydrophobic and ionic bonds ([Palmer *et al.*, 2007](#)). Once the irreversible attachment is established, micro-colonies will be able to form. The adhered bacteria will multiply and synthesize an extracellular matrix in which they will be included. The biofilm will then enter

the maturation phase. It is during this stage that bacteria are grouped into microcolonies separated by aqueous channels. This network of canals ensures the transport of oxygen and nutrients as well as the evacuation of waste. Thus, the constituent's soluble matter capable of diffusing through the matrix can be used by bacteria. A gradient of nutrients and dioxygen is observable from the top of the biofilm to its base where an anaerobic microenvironment prevails (De Beer *et al.*, 1999). Finally comes the detachment step during which cells will be released in planktonic form for the colonization of new surfaces.

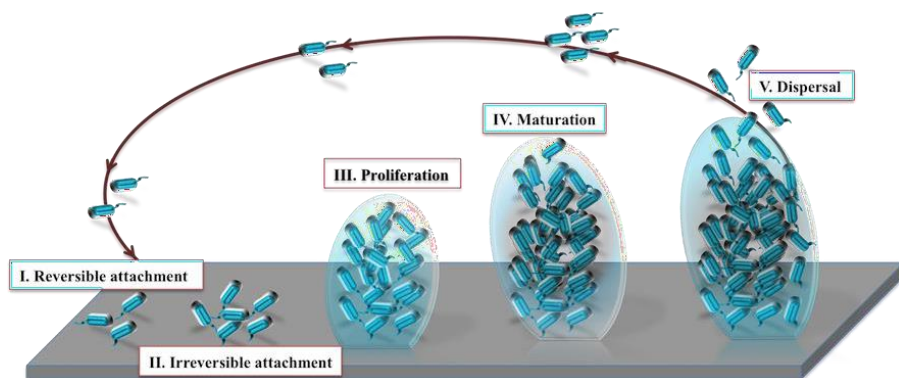


Figure 9: Stages of a biofilm formation model

(Rajput *et al.*, 2018)

The structure of the biofilm is reinforced by the extracellular matrix of exopolymers, consisting essentially of EPS, but also other molecules of organic and inorganic nature, such as extracellular DNA, proteins and rhamnolipids (Allesen-Holm *et al.*, 2006; Pamp *et al.*, 2007). This matrix represents 85% of the total volume, allowing the biofilm to retain a high plasticity and protecting the resident bacteria against a wide range of environmental challenges. Notably, it has been shown that the EPS matrix can physically prevent the entry of certain antimicrobial agents into the interior of the biofilm, by acting as an ion exchange for example, and thus reducing the diffusion of compounds from the external environment to inside the biofilm (Teitzel *et al.*, 2003).

While much is known about the composition, structure and metabolism of oral biofilms, their study has largely been neglected in the GI tract. However, based on what we know of biofilms in the oral cavity, it is likely that particle-associated and mucosal biofilm communities in the lower digestive tract, particularly the large bowel are highly evolved assemblages. Due to its unique anatomy and the mechanics of movement of particulate substances through the gut, bacteria that

are able to colonize food residues in the cecum and maintain significant populations in the proximal bowel serve as inocula for new digestive materials entering the colon. These organisms may therefore be of particular ecological importance in maintaining the stability of the colonic microbiota. However, little is known about how the colonization of particulate substances occurs in the large intestine, but it is likely that the organisms involved in the initial stages of this process form biofilms (Macfarlane *et al.*, 2006). It is proposed that biofilms formed in the GI tract may be involved at least in transmitting anaerobes to the newborn baby and in cooperative degradation of complex polymeric substrates (de Vos *et al.*, 2015)

4.3. Positive and negative features of biofilms

Biofilm communities often exhibit coordinated multicellular behavior, within and between species, and many biofilm properties are dependent on local cell population densities. A good example of this is provided by QS transcriptional activation in certain Gram-negative bacteria (Salmond *et al.*, 1995). There is also a mounting interest in mucosal biofilms in the colon, especially with respect to their role in IBD. Because bacteria growing in biofilms are more resistant to antibiotics than unattached organisms, it is often laborious to modify the structure and composition of these communities, or to eradicate them from the body. However, emerging technologies will improve our understanding of the temporal, metabolic and spatial organization of these biofilms. The increasing shift in emphasis away from culture-based studies, and further development of molecular techniques (Aminov *et al.*, 2006), together with the exposure and the great emergence of methodologies for investigating gene expression *in situ* (Hoshino *et al.*, 2001), will greatly facilitate future work on biofilm structures in the GI tract.

Besides all the described biofilm behaviors, some other kind of studies assessed the effect of probiotic bacteria under biofilm mode of life bringing stronger functionalities like in the following study where results demonstrated that the biofilm life mode promotes colonization and longer persistency of *Lactobacillus* in the mucosa of the host, avoiding then colonization by pathogenic bacteria (Armando *et al.*, 2012). In addition, biofilm formation enhances the immunomodulatory effects of *Lactobacillus sp.* (Rieu *et al.*, 2014). Indeed, in comparison with the planktonic one, such bacteria grown in biofilms expressed higher amount of the GroEL protein, a protein having immunomodulatory effects. It is also suggested that probiotic bacteria under biofilm mode of life increase stress resistance, gastro-intestinal colonization, and the anti-inflammatory properties (Rieu *et al.*, 2014; Heumann *et al.*, 2020).

5. Limitations of probiotics

Our understanding of mechanisms involved in beneficial effects of probiotics is not superficial at all. However, there exists a sort of a paradoxical theory between the fact that a large number of extremely promising *in vitro* and *in vivo* results were obtained in animal models such as mice and rats in the favor to treat multiple diseases like IBD, cancer and other with probiotics, and the fact that there is a major lack of efficacy regarding the same probiotics in human models concerning several aspects. Wherefore, insufficient information about probiotic dosages required for clinical effects adds to the need for molecular characterization of probiotics for initiation and establishment of health requests. Usually, organisms need certain characteristics to enable them to exert maximum therapeutic effects. Of these critical characteristics, there are some that are considered almost very essential for a probiotic to have, including gastric acid and bile salt stability, ability to adhere to the intestinal mucosa, and ability to colonize the GI tract (Dunne *et al.*, 2001). However, in most people, this colonization is rather transient. In contrast to that, in a recent study, humans promoted person-, region- and strain-specific mucosal colonization patterns, marked by anticipating baseline host and microbiome traits and characteristics, but identical in terms of probiotics present in stool (Zmora *et al.*, 2018). In consequence to that, probiotics might have finally brought on a temporary transient, individualized impact on mucosal community structure and gut transcriptome. Collectively, empiric probiotics supplementation may be limited in universally and persistently impacting the gut mucosa, meriting development of new personalized probiotic approaches (Zmora *et al.*, 2018). Another limitation might be the antibiotic-resistance of some probiotic strains such as *Lactobacillus* and which poses a threat to food safety. For the simple reason that LAB is not generally targeted by antibiotic treatments as they are considered to be non-pathogenic and non-opportunistic pathogens (Liu *et al.*, 2009). Some of these antibiotics are vancomycin, metronidazole, erythromycin, and aminoglycosides. Some resistances are innate, others have been acquired by genetic mutations (mutation of ribosomal DNA 235 in *L. rhamnosus* giving it a reduced affinity for erythromycin for example). There are multiple resistance mechanisms (enzymatic inactivation of the antibiotic, modification of antibiotic targets, etc.).

Some *lactobacilli* are also able to transfer resistance genes to other microorganisms (Boone DR *et al.*, 2009).

In the empire of manufacturing process and subsequent formulation there is quite very well published literature out there. However, lot needs to be done to improve the survival of strains during formulation and storage. In fact, several factors have been reported to affect the viability of probiotic cultures in industrial matrix such as fermented milks: acidity, pH, dissolved oxygen content, redox potential, hydrogen peroxide, starter microbes, potential presence of flavoring compounds and diverse additives (including preservatives). For those fermented vegetables can offer an alternative suitable media to deliver probiotics and has been known since ancient time. Yet, it shows that the low incubation temperature of vegetable fermentation is quite a problem for the introduction of the traditional *L. acidophilus* and *Bifidobacterium* probiotic bacteria (Champagne *et al.*, 2009). Thus, the development of new probiotic vegetable products requires further investigations.

As well, it should be surveyed if the probiotic can be prophylactically applied in prevention for an individual with a predisposition for the maturation of a specific condition, the maintenance of health status or for the treatment of an ongoing disease, adapted towards the restoration of the microbial ecological gut network (O'Toole *et al.*, 2017). It is however important to note that despite the improvement in animal models studies, there is a need for developing systems that will reflect the physiological complexity of host-gut microbiome interactions in human diseases (Sadaghian Sadabad *et al.*, 2015; Ulluwishewa *et al.*, 2015) to dim the contrasting results between reported benefits in cell lines models, experimental animal models and human clinical trials. Differences in the nature of this treatment in animal models versus human models is quite a critical limitation point in most studies, whereas a preventive treatment is used in animal models as a prophylactic one preventing the occurrence of some diseases before they take place while most of the human trials represent curative ones with very short administration in time.

Another important limitation is the design of clinical trials. In functional food research, nutritional and clinical sciences use similar methodology. Experimental scientists have already published on several aspects of probiotic trials (Shane *et al.*, 2010). The panel agreed that the best study design is a randomized, placebo-controlled, and double-blinded human trial, either of parallel or crossover design. In certain circumstances a crossover design may not be appropriate, as the functional effect

of a probiotic may not disappear during the intervening washout period (e.g., in children if maturation of the gut barrier has improved). The length of study and duration of intervention should be appropriate for the endpoint as well as reflecting consumption patterns recommended for the probiotic. An acute effect associated with intervention may be seen in short-term interventions, but if people are recommended to take a product for a longer time period, it might be better to test the efficacy of intervention over a more extended period (Gibson *et al.*, 2011). Thus, clinical trials should be harmonized in term of protocols, design, study length and intervention period.

Correspondingly, more studies aiming to develop successful formulations with nutraceutical or therapeutic applications are urgently needed. This kind of formulations should synchronously confer better bacterial viability, high efficacy of the probiotic action and should be indeed safe for humans representatively. Thus, the development and market implementation of novel probiotics demand a close interaction between research institutions, pharmaceutical industries, and regulatory agencies. Altogether, classical probiotics reveal limited effects on the human GM seeking the need for a better selection and formulation of bacterial strains (Neef *et al.*, 2013). These next generation probiotics (NGP) were evaluated previously in preclinical trials and yielded positive outcomes for inflammatory and metabolic disorders (Patel *et al.*, 2015). In addition to that, newer techniques stand in need of the development of new probiotic products containing strains from human origin. This is to say, these strains must come from the major groups of the intestinal microbiota, quite very close to the intestinal microbiota, they have to be defined to have a safe status and proven to have boosted potential beneficial effects in comparison with resident ones (Martín *et al.*, 2017). Numerous studies results suggest that *Faecalibacterium prausnitzii* and *Akkermansia muciniphila* can be considered as promising probiotic candidates for NGPs with exciting potential for the treatment of dysbiosis-associated diseases and pathologies characterized by chronic gut inflammation (Sokol *et al.*, 2008) or as a prognostic tool for the success of diet interventions (Dao *et al.*, 2016) respectively (El Hage *et al.*, 2017). The challenges of these non-conventional native gut bacteria lie mainly on their extreme sensitivity to O₂ traces. If these strains are to be used successfully in food, supplements, or drugs they need to be conditionally stable and active in human models.

6. Interaction of probiotic with diet

It is thought that the introduction of probiotics, prebiotics, or symbiotic into human diet might be favorable for the intestinal microbiota equilibrium and associated intestinal homeostasis. In this context, during the last years the concept of functional food has been enormously matured in order to characterize and define functional foods containing ingredients with positive effects on host health beyond their nutritive value and these foods included products that contain biologically active components that promote health, such as probiotics as we already mentioned above (Ziemer *et al.*, 1998; Kechagia *et al.*, 2013). Within this market, probiotics have been assimilated in a diversity of products, mainly fermented dairy foods. In light of this ongoing trend and nutritious orientation and despite the solid scientific evidence associating these microorganisms to multiple health favors, further investigation and analysis is required in the purpose to establish them and assess their safety as well as their nutritional aspects. Many questions remain: Is the beneficial relation between food and probiotics reciprocal? If so, then how does the assimilation of specific foods enhance the functionalities of these bacterial strains and on which levels? And how do we ensure the engraftment of a specific bacterial strain in this purpose? Perhaps the answer lies in the setting up of a metabolic niche that operates by upholding selected bacteria with targeted atypical dietary substrates or specific food products. Most probably, synergistic symbiotic products coupled with either prebiotics and/or probiotics to profitably affect the host and are then matured to beat possible survival difficulties and deadlocks for probiotics (Markowiak *et al.*, 2017). In this way, the prebiotic element is specifically designed and constructed to maintain the growth of the cognate probiotic. With the response to a dietary intervention being highly individual, synergistic symbiotics have the position of favorably providing both the strain and its growth substrate *in situ* (Leeming *et al.*, 2019). In the following section, we will be presenting the activity of probiotics on various ingested food and nutrients and then discussing the effects of some diet modifications upon the functionalities of probiotics, with a major emphasis on polyphenols, since the vast majority of the current available literature is focused on this.

6.1. Probiotics activities on ingested food/nutrients/micronutrients

Some probiotics have interestingly shown to have an activity on the digestion and absorption of particular ingested nutrients which could be linked in a way or another with the weight of the host.

While increased proportions of *Lactobacillus* have been observed in HFD fed mice (Clarke *et al.*, 2013), certain species of *Lactobacillus*, such as *L. plantarum* (Isokpehi *et al.*, 2017) were recently linked to weight-loss in humans. Further, supplementation with *L. curvatus* HY7601 and *L. plantarum* KY1032 in diet-induced obese mice was associated with gut microbial changes and reduction in obesity (Parks *et al.*, 2013). The two probiotic strains *L. plantarum* KY1032 and *L. curvatus* HY7601 have also been confirmed to reduce adipose tissue mass in human and animal studies investigating the effect of soy on the microbiota, and recently, consumption of soy foods has appeared to increase the levels of *Bifidobacteria* and *Lactobacilli* genera and alter the ratio between *Firmicutes* and *Bacteroidetes* (Huang *et al.*, 2016) in diet-induced obese mice (Yoo *et al.*, 2013).

On the other hand, despite the fact that probiotics function predominately in the large intestine, there is currently no evidence that amino acids liberated from bacterial fermentation in the large intestine alter plasma amino acid concentrations of the host; however, certain probiotic strains have proteolytic properties and have been linked to an increased production of digestive enzymes and subsequently improved host protein utilization (Jäger *et al.*, 2020) just like in the case of lactic acid bacteria (LAB) such as *L. paracasei*, having proteases and peptidases providing the bacteria with free amino acids for optimal growth. They are not classified as strongly proteolytic bacteria. However, their role on protein digestion and absorption of amino acids by the host is currently unknown until today.

For example, in Jäger's recent study, probiotic supplementation results in favorable changes in the gut microbiota, aiding the absorption of amino acids from plant proteins by the host. As results, turned out that probiotic administration (*L. paracasei* LP-DG and *L. paracasei* LPC-S01) significantly increased methionine, histidine, valine, leucine, isoleucine, tyrosine, total BCAA (branched-chain amino acid) blood concentrations. Thus, probiotic strains supplementation can be an important nutritional strategy to improve post-prandial changes in blood amino acids and to overcome compositional shortcomings of plant proteins (Jäger *et al.*, 2020).

Apart of macronutrients, some micronutrients were also noted to be driven by the activities of some probiotic species, like phenolic compounds (PC). To exert systemic beneficial effects on host, PC must be absorbed in the intestinal tract and be bioavailable in the circulatory system. When they are not absorbed in the small intestine, phenolic compounds reach the colon, where they may undergo extensive biotransformation by the resident microbiota, which may improve their absorption and bioavailability. Interestingly, probiotics, including *Lactobacilli* and *Bifidobacteria*, have been shown capable of causing biotransformation of PC like of isoflavones and lignans through the action of different glycosylhydrolases *via* the release of aglycones from glycol-conjugated phenolic compounds (Rossi *et al.*, 2013) in the small intestine, whereas 90–95% reach the colon because of insufficient gastric residence time, low permeability or solubility in the intestine (Pereira-Caro *et al.*, 2015; Cueva *et al.*, 2017; de Llano *et al.*, 2017). It is also very known that intestinal bacteria are able to convert resveratrol to dihydroresveratrol, which, at least partially, is effectively absorbed and further metabolized to conjugated forms and that can be thus excreted in urine (Walle *et al.*, 2004). Multiple bacterial strains participate in this conversion such as *Slackia equolifaciens*, *Adlercreutzia equolifaciens*, *Slackia isoflavoniconvertens* and a consortium of many more microbes (Bode *et al.*, 2013).

6.2. Current knowledge on the effects of diet on probiotics functionalities

Effectiveness of probiotics is conditioned by several factors and very little of literature is available until today when it comes to various diet types conditioning these effects. Some evidence is however currently available about the impact of the food in which or with probiotics are delivered on their beneficial features, like the adhesion to human gut for example. This vehicle, intended as food, is crucial for the probiotic strain survival to ensure its effects according to several studies. Different food matrices can be used as probiotic delivery systems. Usually, dairy products are the most common ones due to their high fat contents which protect the strain during the digestion process (Mollakhalili *et al.*, 2017). Strikingly, with the introduction of new diet types and the rising knowledge on the disadvantages of meat-based diets, the market is trying to focus the attention on other types of food today, such as fruits and vegetables (PEM *et al.*, 2015). Delivery of probiotics through new functional vegetable-based food represents therefore a very interesting opportunity for producers and consumers. One very recent study investigated whether probiotic bacteria, when grown in weaning food matrices, change their *in vitro* gut adhesion in order to enhance their efficacy with improved colonization by beneficial microorganism (Bocchi *et al.*, 2020).

Food matrix,

Based on the hypothesis that probiotic strains applied to food fermentation can synergistically interact with the substrate, they selected newly isolated strains of *Lactobacillus spp.* and *Bifidobacterium spp.*, which are able to take advantage of the interaction with their substrate and to express this synergism with improved beneficial features for the host, such as adhesiveness to the gut mucosa. Hence, results indicated that food matrices affect adhesion for certain probiotic species depending on their origin. For example, the adhesion capacity of *L. rhamnosus* strains were highly affected by their origins; *L. rhamnosus* LMG S-29885 isolated from plants exhibited no loss of adhesiveness independently of the tested food, whereas *L. rhamnosus* strains of human origin exhibited reduced adhesion following the fermentation of food. These results highlight the fact that the adhesion of probiotic strains is influenced by both the strain and the matrix. Therefore, functional foods could be designed for specific requirements such as those characterizing the weaning period in the previous study, in order to minimize for example, the impact of the transition from milk diet to solid foods on the human microbiota (Castenmiller *et al.*, 2019).

Beginning with proteins-rich diets, a study was conducted by Sabran to elucidate the effect of High-protein (HP) diet on the ability of probiotic *Lactobacillus casei* Shirota LcS to reduce urinary AFM1 (Aflatoxin biomarker) and certain aflatoxicosis symptoms in AFB1-induced rats (metabolites of aflatoxin, aflatoxin B1 well known as hepatotoxicant and genotoxicant). A randomized, double-blind, cross-over, placebo-controlled study with two 4-week intervention phases was therefore conducted (Redzwan *et al.*, 2016). The authors found that probiotic supplementation with HP diet alleviated the adverse effects of AFB1 by preventing AFB1 absorption in the small intestine and reducing urinary AFM1 in certain subjects and thus to take part in the reduction of the overall genotoxicity (Nurul Adilah *et al.*, 2018). In addition, high intake of macronutrients can influence the metabolism of aflatoxin and subsequently affect the circular production of AFB1 metabolites (Redzwan *et al.*, 2016). This confirms therefore the effect of protein on the ability of LcS and other probiotics to reduce the negative effect of AFB1. Concerning lipid-rich diets effects on probiotic functionalities, and as polyunsaturated fatty acids (PUFA) possess antimicrobial activities, they may deter the action of probiotics. A study conducted in 2001 by Seppo J Salminen assessed whether free linoleic, gamma-linolenic, arachidonic, alpha-linolenic and docosahexaenoic acids at physiological concentrations in the

growth media would influence the growth and adhesion of *Lactobacillus GG*, *Lactobacillus casei* Shirota and *Lactobacillus bulgaricus* (Kankaanpää *et al.*, 2001). Results proved that higher concentrations of PUFA (10-40 microg PUFA ml (-1)) inhibited growth and mucus adhesion of all tested bacterial strains, whilst growth and mucus adhesion of *L. casei* Shirota was promoted by low concentrations of gamma-linolenic acid and arachidonic acid (at 5 microg ml (-1)), respectively. PUFA also altered bacterial adhesion sites on Caco-2 cells. Caco-2 cells grown in the presence of arachidonic acid were less adhered to by all three bacterial strains. Yet, *L. casei* Shirota adhered better on Caco-2 cells grown in the presence of alpha-linolenic acid. As the adhesion to mucosal surfaces is pivotal in promoting effects of probiotics, the previous obtained results clearly suggest that the action of probiotics in the gut may be modulated by dietary PUFA.

Regarding Sugar-rich diets, a study examined the effect of 3 simple saccharides (glucose, galactose, and lactose) on the adherence of 5 probiotic type strains (2 lactococci starter cultures, and 5 potential dairy probiotic strains: *Lactobacillus rhamnosus* CCDM 598T, *Lactobacillus acidophilus* CCDM 79T, *Lactococcus lactis ssp. lactis* CCDM 617T, *Enterococcus durans* CCDM500T, and *Bifidobacterium animalis ssp. lactis* CCDM 374T) whereas adherence was measured on widely used models: on polystyrene alone and polystyrene coated with either mucin or cocultures of Caco2:HT29-MXT. The most commonly observed effect of prebiotics was decreased adherence of the tested strains observed on all types of substrates. The tested saccharides, which are part of the residual compounds of the used prebiotics, had a very similar effect—eliciting a decrease in adherence ability in most of the probiotic strains. Thus, although the charge of bacteria plays a very important role in adherence, it appears that in these combinations, additional factors exert a greater influence. However, this measurement was only tentative, and the exact mechanism of inhibition adherence should be the subject of future research. Meanwhile, Koh *et al.* (2013) observed a neutral effect of tagatose (found in some varieties of fruit and extracted from lactose (also called milk sugar)) on the adherence of both *Lactobacillus casei* 01 and *Lb. rhamnosus* GG (Koh *et al.*, 2013). It should be mentioned that those authors used HT29 cell lines that only remotely resemble intestinal cells. Contrarily, Kavanaugh *et al.* (2013) observed a positive effect of human milk oligosaccharides on the adhesion of *Bifidobacterium longum ssp. Infantis* (Kavanaugh *et al.*, 2013). However, oligofructose (which is a one of the best researched prebiotic fibre ingredients worldwide known as Orafti P95 formulation) had a neutral effect on adherence

in the same study and those authors also observed that this effect related to adherence was strongly strain specific.

6.3. Modulation of probiotics activities by polyphenols

For fifteen years, researchers and industrialists in the food industry have been increasingly interested in a category of antioxidants, polyphenols. Recognition of the antioxidant properties of these compounds, their abundance in food and their likely role in the prevention of diseases associated with oxidative stress are the main reasons for this craze. These substances arouse a lot of interest in several fields because of a wide range of biological activities, at least demonstrated *in vitro*, and including antibacterial, anti-carcinogenic and anti-inflammatory, properties as already developed in the second part of the thesis.

The polyphenols have gained lately a great interest in their interactions, positive or negative, with microorganisms on different levels (Ozidal *et al.*, 2016). A very interesting reciprocal relation exists between polyphenols and probiotics: several foods are rich sources of phenolic compounds, and their beneficial effects on human health may be combined to the positive functionalities of probiotics. Additionally, probiotics may use PC as substrates, increasing their survival and functionality. In fact, there are many available studies on the effects of PC on probiotics, including their physiological functionalities, interactions and capacity of surviving during industrial processes and during exposure to harsh gastrointestinal.

Effects of PC on the adhesion of probiotics,

Several studies have assessed the effects of polyphenols on the adhesion capacity of probiotics and many of them were able to prove that PC can actually improve this capacity even during exposure to conditions that mimic the GI tract. Adhesion of microorganisms to the intestinal mucosa is, actually, important for GI tract residence time and correlated to the ability of the strains to beneficially influence host health including immune modulation and competitive exclusion of pathogens (Duda-Chodak *et al.*, 2008; Tassell *et al.*, 2011; Gonza *et al.*, 2012; Marín *et al.*, 2015). Favorable effects of polyphenols were reported for the probiotic *Lactobacillus rhamnosus* 299 enhancing proliferation and adhesion simultaneously with inhibition of growth and adhesion of

pathogens (Baur *et al.*, 2006). The impact largely depended on the phenolic structure and its dosage (Bustos *et al.*, 2012) in conferring beneficial effects. Somehow, PC seem to influence bacterial adhesion because the presence of hydroxyl groups in the molecules of the former, which enable the occurrence of protein-protein interactions (Bustos *et al.*, 2012). In fact, protein-like components are considered to be key factors in the adhesion of bacteria to intestinal mucin and/or epithelial cells (Izquierdo *et al.*, 2008). Many current studies are assessing the *in vitro* effects of PC or PC-rich source extracts on the adhesion, growth or survival of probiotics. However, molecular mechanisms underlying these effects are often not fully characterized, especially in the case of microorganisms treated with PC rich extracts that represent highly complex mixtures of bioactive molecules. *Lactobacillus acidophilus* ATCC 1643 have shown to exhibit higher adhesion to abiotic polystyrene support in the presence of aqueous or ethanolic apple peel extract, or in the presence of purified phenolic compounds rutin, epicatechin, phlorizin, chlorogenic acid, quercetin and p-coumaric acid, in comparison to the adhesion observed for untreated bacteria. The stronger effect on adhesion is obtained with bacteria treated with the aqueous apple peel extract. The coprecipitation of polyphenols due to their interaction with apple cell wall components (e.g., proteins) could cause the solubility of apple polyphenols in polar (ethanolic) extract, causing decreased availability of these compounds to act on probiotic cells (Shinde *et al.*, 2015).

In contrast, different flavan-3-ols inhibited the adhesion of *L. acidophilus* LA-05 and *L. plantarum* IFPL379, except for epigallocatechin gallate, which increased the adhesion of *L. acidophilus* LA-05 to colonic Caco-2 cells (Bustos *et al.*, 2012). Procyanidins B1 and B2 markedly increased the adhesion of *L. casei* LC115 to colonic HT-29 cells, whereas epigallocatechin increased the adhesion of *L. casei* LC115 to Caco-2 cells (Bustos *et al.*, 2012). Selective enhancing effects of naringenin, phloridzin and rutin on bacterial adhesion were similarly observed. Naringenin and rutin increased the adherence of *L. rhamnosus* to Caco-2 cells, whereas naringenin and phloridzin decreased the adherence of the pathogen *Salmonella typhimurium* to these cells (Parkar *et al.*, 2008).

Apple peel and pulp ethanolic extracts were evaluated considering their influence on the adhesion of *L. gasseri* R and *L. casei* FMP to Caco-2 and HT29-MTX cell lines. Apple pulp extracts decreased the adhesion of *L. gasseri* R and *L. casei* FMP to epithelial cell lines. In contrast, apple peel extract (richer in polyphenols) increased the adhesion properties of these strains in the same

cellular models. This study found quercetin to be the most active polyphenol, increasing adhesion by 95% in *L. gasseri* R (Volstatova *et al.*, 2017).

In addition, the adaptation of *Streptococcus thermophilus* CHCC 3534 in media with catechin (0.3%) resulted in decreased bacterial adherence (Khalil *et al.*, 2010). What's also worth mentioning is that both cell surface hydrophobicity and aggregation are strongly related to the ability of probiotics to adhere to intestinal mucosa (Deepika *et al.*, 2012), which reinforces the importance of the overall positive modulatory effects exerted by Quercetin and Resveratrol on these physiological features in *Lactobacillus* strains used in this study.

Although previous studies have shown variable results concerning the effects of polyphenols on adhesion properties of probiotics (Bustos *et al.*, 2012; Volstatova *et al.*, 2017), it has been suggested that polyphenols could increase the adhesion capacity of specific probiotic strains by inducing biosynthesis or secretion of multifunction proteins (named moonlighting proteins; e.g., glycolytic enzyme pyruvate kinase) engaged in adhesion of bacteria to epithelial cells (Celebioglu *et al.*, 2018). However, we could not exclude that polyphenols are also able to act by other mechanisms, especially by modifying physico-chemical properties of bacteria cell surface, like in the study of de Souza where Quercetin increased cell surface hydrophobicity of most of the tested *Lactobacillus* strains and increases or decreases in cell surface hydrophobicity varied in the presence of different resveratrol concentrations among some strains (Dos Santos *et al.*, 2019). The effects of five plant phenolics on the probiotic *Lactobacillus acidophilus* NCFM were also monitored by determining adhesive capacity onto a mucin coating and an HT-29 cell layer as well as by differential whole cell and surface proteome analyses. The adhesive ability of NCFM varied with the different phenolics and their concentrations supplemented during growth. Resveratrol (100 µg/mL) significantly increased adhesion to mucin and HT-29 cells and increased significantly after growth in the presence of 250 µg/ml of resveratrol, while Tannic acid (100 µg/ml) increased the adhesion of HT-29 cells and so did Ferulic Acid (100 µg/ml). Early studies have reported that phenolic-rich matrices could protect probiotic *Lactobacillus* strains during the passage through the GI tract. Grape marc protected *L. plantarum* 12A, *L. plantarum* PU1, *L. paracasei* 14A and *Bifidobacterium* breve 15A when exposed to a stomach-mimicking condition (Campanella *et al.*, 2017) and mashed tomato protected *L. reuteri* ATCC 55730 when exposed to simulated GI tract conditions (Garcia-Hernandez *et al.*, 2018). These possible protective effects of phenolic

compounds have been primarily attributed to their antioxidant properties, which could protect probiotic cells from the damage caused by exposure to the harsh conditions found in the GI tract (Maukonen *et al.*, 2015).

Effects of PC on bacterial aggregation,

PC are also able to affect the aggregation properties of microorganisms, an important feature for biofilm formation by probiotic bacteria, assisting them in adhering to intestinal mucosa (Dos Santos *et al.*, 2019). No decrease in auto aggregation capacity of tested *Lactobacillus* strains was caused by either of the tested concentrations of Quercetin and Resveratrol. All tested concentrations of Quercetin increased the auto aggregation capacity of *L. plantarum* 49 and *L. fermentum* 296 while Resveratrol increased the auto aggregation capacity of *L. plantarum* 49 and *L. plantarum* 53. The maintenance or improvement of the capacity of probiotics to coaggregate with pathogens should be considered as a positive feature of some polyphenols like Quercetin and Resveratrol, since it has been cited as one of the initial steps to prevent colonization of the host GI tract by pathogens (Todorov *et al.*, 2008). Together aggregation and coaggregation enable probiotics to better compete with pathogens for host binding sites (Ferreira *et al.*, 2011). Nevertheless, polyphenols have been recently established to bind to the bacterial surface including *Lactobacillus* and *Bifidobacterium* species and significantly enhance their total oxidant- scavenging capacities (Koren *et al.*, 2009).

Effects of PC on bacterial survival and growth,

Not only on adhesion and antioxidant properties but also on the survival ones, researchers have consistently observed that phenolic-rich foods can commonly improve the survival and growth of some probiotic strains during GI digestion and in different food matrices (Possemiers *et al.*, 2010; Campanella *et al.*, 2017; Succi *et al.*, 2017; Silva *et al.*, 2018). In the same study, lactic acid fermentation increased the antioxidant properties of grape marc on intestinal cells. These findings suggested mutual beneficial interactions among dietary phenolic compounds and probiotic strains mentioned above. Interestingly, the content of phenolic compounds (e.g., gallic acid) was decreased in grape marc after fermentation, indicating that the tested probiotic strains were capable

of metabolizing some phenolic compounds present in grape marc (Campanella *et al.*, 2017). The growth of *L. acidophilus* CECT 903 was not affected by grape polyphenols or by caffeic acid, gallic acid, tannic acid, catechin, epicatechin and quercetin, however, Grape pomace phenolic extract and tannic acid remarkably stimulated the growth of this bacteria (Hervert-Hernández *et al.*, 2009).

Another study with red fruit (plum skin, Italian red grape skin and different parts of elderberry) ethanolic extracts characterized as polyphenol/anthocyanin-rich sources observed stimulatory effects of the tested extracts on the growth of *L. rhamnosus* IMC 501 and *L. paracasei* IMC 502 alone or in combination, as well as of *L. plantarum* IMC 509 in laboratory media. These combined formulations of plum skin extract or elderberry skin or seeds extracts and each of the four tested probiotics exhibited higher antioxidant activities than the extract alone, indicating the ability of the probiotics to enhance the antioxidant properties of the tested fruit extracts. Specifically, the highest stimulatory effects on the probiotic's growth were exerted by fruit extracts rich in anthocyanins, suggesting that anthocyanins and/or their metabolites may modulate positively the intestinal bacterial population (Hidalgo *et al.*, 2012). Further studies using flavan-3-ol-enriched grape seed extract and oligomeric and monomeric fractions containing different polyphenol concentrations found a higher maximum growth rate for *L. plantarum*, *L. casei* and *L. bulgaricus* in media supplemented with the tested extract or fractions. However, exposure to 1 mg/mL grape seed extract caused slight inhibitory effects on all tested *Lactobacillus* strains. These impairing effects were attributed to the high amounts of gallate-derived compounds (e.g., (±)-epicatechin-3-O-gallate) in the tested grape seed extract (Tabasco *et al.*, 2011). These compounds have been demonstrated to have higher antimicrobial activity than their homologues lacking this trait (Caturlaet *et al.*, 2003). Dark chocolate with high cocoa content (80%) and total phenolics has also showed protective effects on probiotic survival during exposure to simulated GI tract conditions. Dark chocolate presented protective effects on freeze-dried *L. paracasei* F19 and *L. rhamnosus* GG during passage through simulated GI tract conditions (Succi *et al.*, 2017). The viability of immobilized *L. casei* 01 and *L. acidophilus* LA5 in three different types of chocolate (white, milk and dark) rich in high levels of polyphenols and flavonoids, were evaluated during exposure to simulated GI tract conditions. All chocolate types exerted protective effects on the tested strains, but *L. casei* 01 presented higher survival rates than *L. acidophilus* LA5 during exposure to *in vitro* digestion. Intrinsically, dark chocolate presented higher protective effects on *L. casei* 01 and *L.*

acidophilus LA-05 (Kemsawasd *et al.*, 2016). More studies using dark chocolate suggested that adding lyophilized *L. rhamnosus* GG, *L. paracasei* F19, *L. casei* DG or *L. reuteri* DSM 17938 to dark chocolate with 80% cocoa stored at 18°C might have strain-dependent effects on probiotic survival. In this study, the phenolic contents in dark chocolate did not seem to influence probiotic survival over time. Dark chocolate containing the probiotics presented good acceptance during a 90-day storage period (Succi *et al.*, 2017).

In other studies, the growth and metabolism of *L. casei* LC2W, *L. casei* BD-II and *L. casei*-01 were enhanced by the incorporation of rich phenolic ingredients in cow milk, such as green tea infusion. Higher bacterial counts, reduced fermentation time, abundant production of flavor compounds and high release of free amino acids were achieved during the fermentation of milk supplemented with green tea using the tested *L. casei* strains, suggesting a better optimization of the properties of these bacterial strains when PC are incorporated (Souza *et al.*, 2019). Similarly, the presence of green tea extract and flavan-3-ols epigallocatechin-3-gallate, epigallocatechin, epicatechin-3-gallate, epicatechin and catechins stimulated the growth of different probiotics (*Enterococcus faecium* AdF1-GU396270, AdF2-GU396271, AdF3-GU396272 and AdF11-GU396279; *Bacillus coagulans* AdF4-GU396273, *L. plantarum* AdF5-GU396274, AdF6-GU396275 and AdF10-GU396278; and *L. fermentum* AdF7-HQ677597, AdF8-GU396276 and AdF9-GU3962770) (Sourabh *et al.*, 2014). Curiously, *B. animalis* LAFTI-B94 presented increased survival rates in different varieties of phenolic-rich green tea extracts during their exposure to gastrointestinal conditions and when incorporated into food matrices in comparison to *L. paracasei* LAFTI-L26 and *L. acidophilus* LAFTI-L10 (Souza *et al.*, 2019). Available literature also indicates that metabolites of PC formed by intestinal microorganisms, including probiotics, exert a variety of benefits on host health. These metabolites are typically more active than parental dietary PC. For example, the survival of *L. acidophilus* ATCC 1643 in a cow milk-based drink increased with supplementation with aqueous apple peel phenolic extract or purified polyphenols over a 50-day refrigeration storage period. However, apple skin phenolic extract promoted the growth of *L. acidophilus* more than either of the tested polyphenols, with the exception of rutin. These findings indicate the potential use of apple skin as a source of polyphenols to enhance probiotic bacteria stability in dairy foods in addition to adding value to this waste stream agro-industrial material (Shinde *et al.*, 2015). Between all previously tested polyphenols, resveratrol, in particular, has scored high rates of interest in the field of research when studying probiotic bacterial

functionalities. Like in a very recent study where data showed that a short exposure to resveratrol provides numerous protective mechanisms to *L. plantarum* and confer advantages to this microorganism to compete in the GI tract (Reverón *et al.*, 2018). The impact of resveratrol on the redox homeostasis of the gut is of particular relevance and authors have described the ability of this phytochemical to promote the growth and health of *Bifidobacterium* and *Lactobacillus* spp. (Cardona *et al.*, 2013).

Resveratrol (100 μ M) is also interestingly found to protect *L. reuteri* against protein carbonylation plausibly through various mechanisms including direct scavenging of ROS, upregulation of the *dhaT* gene involved the oxidoreductase activity, and promoting the synthesis of sulfur containing compounds with protective effect. Some researchers have hypothesized that the stimulatory effects of phenolic compounds on probiotics could be associated with their effective antioxidant and oxygen-scavenging properties, which could modulate the oxidative stress generated from microbial metabolic activities, providing a favorable environment for probiotic growth and survival (Chaikham *et al.*, 2015). Like in the following study with the polyphenols present in green tea where Lacey observed that glycosylated flavonoids stimulated the growth of *Bifidobacterium animalis* B94 whereas the antioxidant activity of the tested polyphenols increased following incubation with *B. animalis* B94, which could indicate the use of catechins by this bacterium. Additionally, there was a direct relationship between the reduction of catechin content in the medium during the monitored incubation period and the increase in its antioxidant activity (López de Lacey *et al.*, 2014). On the flip side, recent studies have shown that PC mostly modulate the composition of gut microbial communities through the inhibition of pathogenic bacteria and stimulation of beneficial bacteria. In the latter, PC may exert a prebiotic function and increase the population of beneficial bacteria, including lactic acid bacteria suggesting a mutual relationship between phenolic compounds and probiotics (Ozidal *et al.*, 2016; de Llano *et al.*, 2017; Souza *et al.*, 2019). Besides the multiple effects that PC can have on probiotic bacteria, some studies conducted research on the number of polyphenols used in the same experience suggesting a ‘Combination vs. Alone’ theory: In a study testing the effect of individual PC on probiotic growth, results indicated that *Lactobacilli* showed better adaptation to gallic, protocatechuic, vanillic acids and catechin than pathogenic bacteria while combined PC caused a lower growth, as compared to the individual compounds. Catechin, when combined with protocatechuic or vanillic acid, allowed the growth of both probiotics. When gallic acid was combined with either protocatechuic acid or

catechin, the combination only allowed the growth of *L. rhamnosus* and did not have an effect on the growth of the others.

On the other hand, Tabasco et al. (2011) observed that a grape seed extract supplemented with catechin and gallic acid at the same time, had an inhibitory effect on the growth of different species of *Lactobacillus* while a noticeable increase in the response of gallic acid was observed after incubation of the extract with only *L. plantarum* IFPL935 (Tabasco et al., 2011). The effect of five phenolic compounds (catechin and gallic, vanillic, ferulic and protocatechuic acids) identified in different fruits, particularly in mango, was evaluated on the growth of two probiotic (*L. rhamnosus* GG ATCC 53103 and *L. acidophilus* NRRLB 4495) in a recent study done by R. Pacheco-Ordaz in 2017 wherein the presence of catechin and gallic, protocatechuic and vanillic acids allowed the growth of *lactobacilli* while catechin combined with protocatechuic or vanillic acid mildly allowed the growth of both probiotics. In conclusion, phenolic compounds were selectively able to inhibit the growth of pathogenic bacteria without affecting the viability of probiotics. These effects were also evaluated in another *in vitro* study where PC selectively allowed the growth of probiotic *lactobacilli* (*L. rhamnosus* GG and *L. acidophilus*) in the presence of gallic acid, vanillic acid, ferulic acid, and protocatechuic acid and inhibited pathogenic bacteria (*Escherichia coli* and *Salmonella typhimurium*) (Pacheco-Ordaz et al., 2018). Nevertheless, on the molecular level, proteins changing in abundance remarkably differ for resveratrol and tannic acid, suggesting that these polyphenols have different roles in molecular reactions eliciting beneficial effects and thus act in a cooperative manner. Regarding the surface protein profiles, some interesting observations emerge (Bergonzelli et al., 2006). Surface proteomes after resveratrol treatment revealed increase in the glycolytic enzymes pyruvate kinase (PK) that may be responsible for adhesion, being identified as a moonlighting protein in adhesion to mucin, HT-29 cells, and yeast mannan (Katakura et al., 2010; Wang et al., 2016).

Perspectives on products formulation assembling probiotics and PC,

Overall, the data from available literature suggest synergetic effects between polyphenols influencing probiotic bacteria functionalities (growth, antimicrobial activities, prebiotic activities) and a potential interaction among phenolic compounds and probiotics for mutual boosting of the health-promoting properties and food functionalities. Thus, there exist plenty of possibilities to establish a combination of polyphenols and probiotic bacteria for utilizing the polyphenols to

selectively stimulate the proliferation and/or activity of probiotics. This synergetic combination of polyphenols and probiotics may be used and might also provide an opportunity to produce cells with enhanced attachment ability and tolerance to environmental stresses, including industrial processes and GI tract environment.

A variety of beneficial effects of phenolic compounds on human health (Li *et al.*, 2013; Igwe *et al.*, 2017; Theodotou *et al.*, 2017), however, there has been little clinical evidence on the beneficial effects caused by the interaction of phenolic compounds and probiotics on the host and a complete evidence has not yet reached a definitive consensus in clinical trials (Ganai *et al.*, 2015; Nuñez- Sánchez *et al.*, 2017). In part, this limitation may be due to the difficulty of attributing the observed beneficial effects to specific PC, since most of the available studies have used foods containing different PC or phenolic-rich extracts (Hossen *et al.*, 2017). Furthermore, most of these studies have not considered the use of a realistic ingestion dose of phenolic compounds and plausible metabolic forms (e.g., conjugated, glucuronidated and sulphated) that can circulate in the bloodstream and reach tissues (González-Sarrías *et al.*, 2015). The large inter-individual variations in the effects of phenolic compounds observed in clinical trials have been partially related to differences in the intestinal microbiota composition and consequently to how microorganisms forming these communities metabolize these compounds (Espín *et al.*, 2017).

Nevertheless, foods containing combinations of phenolic compounds and probiotics could be promising added-value products for the food industry, considering that they have already demonstrated good acceptability and that there are well-known health benefits associated with the consumption of these bioactive components.

6.4. Development of synbiotics

The synbiotic concept was first introduced in earlier in 1995, along with prebiotics, as "mixtures of probiotics and prebiotics that beneficially affect the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria, thus improving host welfare" (Gibson *et al.*, 1995). Later on, for practical reasons, the combination of probiotics and prebiotics has been described as conbiotics by certain authors and as symbiotics by others (Kechagia *et al.*, 2013) and since then, there have been many *in vitro* and *in vivo* studies concentrating on the application of prebiotics, firstly in health and

gradually in disease states. Only recently have studies on synbiotics started to emerge with the main focus being on applications against disease (Kolida *et al.*, 2011).

Among these studies, many conducted on the relationship of the microbiota of human gut and animal gut and immunology have highlighted the role of synbiotics in the health of mammals. There is proof to propose that synbiotics influence the microbial ecology of the intestines of humans and animals and play a role in alleviating and relieving various ailments. Upgrading in the count of beneficial bacterial groups and the shrinkage in the potentially pathogenic loads has been detected following administration of synbiotics in farm animals. Despite the fact that the understanding of the effects of synbiotics has effectively increased, important information relating to their impact on the host is so far incomplete. Though, it has been advocated those potential combinations of most suitable probiotics and prebiotics may act on reducing the risk associated with intestinal diseases and abolish specific microbial disorders. This might perhaps result in the modulation of the metabolic activity in the intestine with the conservation of the intestinal biostructure, the continual growth of beneficial microbiota, and the restraint of potential pathogens present in the gastrointestinal tract (de Vrese *et al.*, 2008).

Mechanistically, the promising effects of synbiotics are generally attributed to the benefit from prebiotics, the function of probiotics, and the combinational effectiveness of both. Yet, some *in vivo* studies suggest that trial outcomes of prebiotics vary significantly depending on the condition being treated, along with the choice of the probiotic strains in employment, and lastly, the types of prebiotics used. However, synbiotic products lay out the potential for development of new types of prebiotics that could target specific probiotic strains for the improvement of human health. Thus, an error-free mixture of prebiotics and probiotics could be an excellent strategy for the formulation of probiotic supplements and other food products.

A successful combination of *Bifidobacterium* or *Lactobacillus* genus bacteria with fructooligosaccharides in synbiotic products seems to be the most popular until today (Markowiak *et al.*, Śliżewska, 2017). **Table 3** presents the most commonly used combinations of probiotics and prebiotics.

Table 3: Examples of prebiotics and synbiotics used in human nutrition.

| Human Nutrition | | |
|----------------------|---|---|
| Prebiotics | Synbiotics | References |
| FOS | <i>Lactobacillus</i> genus bacteria + | (Oliveira and González-Molero, 2016; Sáez-Lara <i>et al.</i> , 2016;Crittenden R <i>et al.</i> ,2009) |
| GOS | inulin | |
| Inulin | <i>Lactobacillus</i> , <i>Streptococcus</i> and | |
| XOS | <i>Bifidobacterium</i> genus bacteria + | |
| Lactitol | FOS | |
| Lactosucrose | <i>Lactobacillus</i> , <i>Bifidobacterium</i> , | |
| Lactulose | <i>Enterococcus</i> genus bacteria + | |
| Soy oligosaccharides | FOS | |
| TOS | <i>Lactobacillus</i> and <i>Bifidobacterium</i> genus bacteria + oligofructose <i>Lactobacillus</i> and <i>Bifidobacterium</i> genus bacteria + inulin | |

Further research is needed to evaluate interaction between probiotics and prebiotics, and the effects of bacterial profiles of the host on the responsiveness of the treatment should be studied. The well-designed and carefully conducted randomized controlled trials of synbiotics with relevant inclusion/exclusion criteria and adequate sample sizes are needed. Furthermore, these studies should use validated clinical outcome measures to assess the effects of synbiotic supplementation and define their optimal doses and intake durations, as well as provide more information about the long-term safety of synbiotics (Braegger *et al.*, 2011). Obligatory and standardized reporting on adverse events has been suggested in studies with synbiotics (Van den Nieuwboer *et al.*, 2015).

Aim and objectives of this thesis.

The general aim of this thesis was to explore dietary interventions that could be applied as novel strategies to modulate innate immune responses and to restore intestinal dysbiosis states. In this context, we gave interest in dietary and food supplements, mainly probiotics and micronutrients, in the aim to investigate their impact on the modulation of the autophagic mechanism in the intestine. Additionally, we studied the effect of micronutrients (i.e., resveratrol polyphenolic compound) on probiotics implementation within the gut targeting their adhesive capacities to human intestinal epithelial cells as well as their immunomodulatory properties, involved in the maintenance of the intestinal homeostasis.

The specific aims of each individual paper were:

Paper I: To investigate the impact of polyphenols on modulating the ability of probiotic strains belonging to the *Lactobacillus* genus to form biofilm and thereby to possibly enhance their beneficial properties.

Paper II: To explore the impact of fasting on the ability of probiotic *Lactobacillus* bacteria to exercise their immunomodulatory activities and colonize the gastrointestinal tract.

Results

PAPER 1

Paper 1: Resveratrol Favors Adhesion and Biofilm Formation of *Lacticaseibacillus paracasei* subsp. *paracasei* Strain ATCC334

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Abstract: Bacterial strains of the Lactobacillaceae family are widely used as probiotics for their multifaceted potential beneficial properties. However, no official recommendations for their clinical use exist since, in many cases, oral administrations of these bacteria displayed limited beneficial effects in human. Additional research is thus needed to improve the efficiency of existing strains with strong potential. In this context, we assess in vitro the effects of nine polyphenols to stimulate biofilm formation by lactobacilli, a feature enhancing their functionalities. Among these polyphenols, we identify trans-Resveratrol (referred to hereafter as Resveratrol) as a potent inducer of biofilm formation by *Lacticaseibacillus paracasei* (formerly designated as *Lactobacillus paracasei*) ATCC334 strain. This effect is strain-dependent and relies on the enhancement of *L. paracasei* adhesion to abiotic and biotic surfaces, including intestinal epithelial cells. Mechanistically, Resveratrol modifies physico-chemical properties of the bacterial

surface and thereby enhances *L. paracasei* aggregation, subsequently facilitating adhesion and biofilm development. Together, our in vitro data demonstrate that Resveratrol might be used to modulate the behavior of Lactobacilli with probiotic properties. Combination of probiotics and polyphenols could be considered to enhance the probiotic functionalities in further in vivo studies.

Keywords: Probiotic; *Lacticaseibacillus*; *Lactobacillus*; Resveratrol; polyphenols; biofilm; adhesion

1. Introduction

The lactobacilli, and more generally lactic acid bacteria, have been used for centuries for the production of fermented foods [1]. Since bacteria belonging to the *Lactobacillus* genus are extremely diverse, it has been recently proposed a reclassification of this genus into 25 genera taking into account genetic, physiological and ecological criteria [2]. These bacteria are considered as normal beneficial residents of the mammalian gastrointestinal (GI) tract, but also in the oral cavity and vagina, representing in this latter environment the dominant flora [3]. Regarding their relative abundance in the human gut microbiota, bacteria from the Lactobacillaceae family are among the first colonizers and dominant bacteria after birth [4]. In adult, Lactobacillaceae are still present however their proportion is highly variable from one individual to another, and they become subdominant, representing about 0.04% of total bacteria in fecal samples [5]. More than 60 species belonging to the Lactobacillaceae family have been retrieved from the human GI tract, including *L. rhamnosus*, *L. casei*, *L. Int. J. Mol. Sci.* 2020, 21, 5423; doi:10.3390/ijms21155423 www.mdpi.com/journal/ijms *Int. J. Mol. Sci.* 2020, 21, 5423 2 of 20 *paracasei*, *L. plantarum*, *L. ruminis*, *L. gasseri*, *L. acidophilus*, *L. delbrueckii*, *L. namurensis*, *L. rogosae* and *L. murinus* as predominant species [3,5,6]. Thus, their status of normal GI tract residents and their wide use in traditional and functional foods make Lactobacillaceae strains as bona fide candidates for the development of probiotics. Particularly, the former *L. casei* group, reclassified as the genus *Lacticaseibacillus* [2], consists of three of the most studied Lactobacilli owing to their beneficial properties on the GI tract health. These Lactobacilli are namely *L. casei*, *L. paracasei* and *L. rhamnosus*. Nowadays, the most accepted definition of probiotics is “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host”, but new terms are also emerging to define new concepts in the field of probiotics, such as Live Biotherapeutic Product (LBP) or Next-Generation Probiotic (NGP) [7]. The latter term is often used to refer to non-traditional probiotics such as commensal strains with putative beneficial properties isolated from the gut microbiota (i.e., *Akkermansia muciniphila* or *Faecalibacterium prausnitzii*). An extensive literature exists describing in vitro and in vivo the multiple beneficial properties on host exhibited by Lactobacillaceae strains, including, but not limited to, the modulation of inflammatory responses, antimicrobial effects to limit pathogens, the modulation of host metabolism or positive effects on the gut/brain axis [8]. Despite there are clinical indications, supported by clinical trials, for the use of some Lactobacillaceae strains in the treatment of human

diseases such as antibiotic-associated diarrhea, necrotizing enterocolitis or irritable bowel syndrome (IBS) [9–11], no official recommendations for their clinical use exist. In addition, for some human diseases such as the inflammatory bowel diseases (IBDs), comprising Crohn's disease (CD) and ulcerative colitis (UC), even if some Lactobacillaceae strains are highly effective to cure colitis (mimicking human IBDs) in rodent models, these bacteria displayed very limited effects in human [12,13]. The most promising results to date for probiotics-based approaches have been obtained for induction and maintenance of remission in UC [14,15]. Thus, additional research is required to better understand the probiotic mechanisms of action and to develop next generation probiotics or improve the efficiency of existing strains.

In this context, our group and others have demonstrated that, compared to the traditional planktonic culture of Lactobacillaceae, the growth of Lactobacillaceae bacteria under biofilm condition can enhance their functionalities by, for instance, protecting them against GI tract stresses (low pH, bile salts), promoting their immunomodulatory properties or stimulating their anti-pathogenic activities [16–18]. Biofilms are defined as communities of microorganisms attached to an inert or living surface, enclosed in a self-produced extracellular polymeric substance and thus representing a higher level of organization than single planktonic cells do [19,20]. Since bacteria living under biofilm condition express totally different phenotype traits, compared to the planktonic condition, stimulating the biofilm growth of probiotics might represent an appealing way to improve their efficiency as mentioned above.

Polyphenols are one of the most diverse group of biologically active plant compounds and are mainly studied for their antioxidants and anti-inflammatory properties that they exert on host cells by acting on a wide range of signaling pathways [21–23]. These properties have strong implications for their potential use in the treatment of cancers, neurodegenerative disorders and during aging. Besides their roles in modulating eukaryotic cells, there is now a growing literature on the effects of polyphenols on bacteria activities and even on gut microbiota composition and functions [24]. For decades, many studies pointed out only their antibacterial properties [25], but at lower doses, in the nano- or micromolar range, polyphenols can regulate bacterial metabolisms and processes. The influence of polyphenols on bacterial growth and properties is highly dependent on the polyphenol considered, its dose, but also on the bacterial strain considered. Regarding the Lactobacillaceae family, berries-derived polyphenols have been shown to increase their proportions in the gut microbiota of human healthy subjects [26]. Similar results were

obtained using polyphenols derived from cocoa, green tea, grape or red wine extract in various model organisms, suggesting that some polyphenols might act as prebiotics for beneficial bacteria, including those belonging to the Lactobacillaceae family [27–30]. However, the precise molecular mechanisms by which polyphenols might favor, among others, Lactobacilli in the GI tract remain unclear.

In the present study, we compared the effects of nine polyphenols on the ability of two *Lacticaseibacillus* strains, *L. paracasei* subsp. *paracasei* ATCC334 (referred as *L. paracasei* hereafter) and *L. rhamnosus* GG to form biofilm. Among the polyphenols tested, trans-Resveratrol (referred to hereafter as Resveratrol) was the most potent to promote biofilm formation by *L. paracasei* ATCC334, in a strain-dependent manner. Mechanistically, Resveratrol enhanced biofilm formation by modifying the physico-chemical surface properties of *L. paracasei*, hence promoting its adhesion capacities. This work provided new mechanistic insights into how polyphenol, and especially Resveratrol, can change the behaviour of *Lacticaseibacillus* bacteria in vitro. Further in vivo studies will be required to decipher whether these changes might impact positively their functionalities as normal resident of the gastrointestinal tract or as probiotics.

2. Results

2.1. Low Doses of Polyphenols Modulate Biofilm formation of *Lactobacillus* Bacteria without Affecting their Growth.

We investigated the ability of a panel of polyphenols belonging to various classes, stilbene (Resveratrol), flavonols (Quercetin, Catechecin), hydroxycinnamic acids (p-Coumaric acid, Chlorogenic acid, Caffeic acid) and hydrobenzoic acids (Ellagic acid, Shikimic acid, Protocatechuic acid), to modulate the biofilm formation by two *Lacticaseibacillus* bacteria: *Lacticaseibacillus paracasei* ATCC334 strain and *Lacticaseibacillus rhamnosus* GG strain. These strains have been well characterized for their property to form biofilm in vitro [17,31].

Polyphenols are usually added to bacteria or eukaryotic cells at concentrations in the micromolar range, however important discrepancies exist in the literature (from 1 μM to hundreds of μM) [32,33]. Thus, we first evaluated the impact of two doses of each polyphenol, in the micromolar range with a ten-fold difference: 30 μM and 300 μM , on the growth of both *Lacticaseibacillus* strains (Figures A1 and A2). For both strains, there were no significant effects of the nine

polyphenols at the low dose (30 μ M) on their growth curve, compared to the untreated culture, indicating that these polyphenols were well-tolerated by these bacteria at this low dose. Regarding the high dose of polyphenols (300 μ M), Resveratrol and, in a less extent, Quercetin altered the growth of both bacteria (Figure A1A,B and Figure A2A,B) indicating a potential deleterious effect of these polyphenols on the basal metabolism of the *Lacticaseibacillus* bacteria. As a consequence, the next experiences were performed with doses close to 30 μ M. Then, we measured the effects of the nine polyphenols on the biofilm formation by *L. paracasei* and *L. rhamnosus*. For this purpose, low dose of polyphenols (30 μ M) was added directly in the growth medium of bacteria and biofilm formation on polystyrene support was assessed 24 h later by enumerating biofilm-forming bacteria using a colony forming unit (CFU) assay on agar plates [16]. Three polyphenols: Resveratrol, Catechin and Ellagic acid, significantly stimulated the biofilm formation by *L. paracasei* ATCC334 strain compared to untreated bacteria, with the higher effect observed for the Resveratrol (143,6%) (Figure 1A). By contrast, Quercetin displayed an opposite effect with a reduction in the formation of biofilm by *L. paracasei* ATCC334 (Figure 1A). The other six polyphenols had no significant effects. Regarding the *L. rhamnosus* GG strain, no major positive effects of polyphenol were observed on biofilm formation (Figure 1B), suggesting a strain-dependent effects of Resveratrol, Catechin and Ellagic acid. Only slight, yet significant positive effect of Quercetin was observed. At the opposite, Resveratrol, p-Coumaric acid, Ellagic acid, Caffeic acid, Shikimic acid and Protocatechuic acid significantly tended to reduce the biofilm formation ability of *L. rhamnosus* GG (Figure 1B). Altogether, these results demonstrate potential strain-dependent effects of polyphenols on biofilm formation by *Lacticaseibacillus* and highlight a potential role of Resveratrol to markedly stimulate biofilm formation by *L. paracasei*. Thus, next experiments focused to better characterize this effect of Resveratrol. In order to confirm the potential strain-dependent effect of Resveratrol on biofilm formation by *Lacticaseibacillus* strains, we assessed the ability of this polyphenol to modulate biofilm formation by various strains from species belonging to the *L. casei* group (*L. paracasei*, *L. casei*, *L. rhamnosus* and *L. zae*) (Figure 1C). Beyond the *L. paracasei* ATCC334 strain, Resveratrol treatment also significantly stimulated, to the same extent, the biofilm formation by five other strains of the panel: *L. casei* BL23, *L. casei* VEL12204, *L. rhamnosus* ATCC7469, *L. rhamnosus* ATCC9595 and *L. rhamnosus* VEL12198. At the opposite, Resveratrol treatment can also reduce biofilm formation by some other strains, reinforcing the idea of a strain-dependent effect of this molecule. Of note, Resveratrol treatment

(30 μ M) did not markedly modify the growth curve of the tested bacteria compared to those obtained with untreated bacteria (Figure A3), except for the *L. paracasei* VEL12194 strain, for which a slight delay was observed upon Resveratrol treatment (Figure A3B in Appendix A).

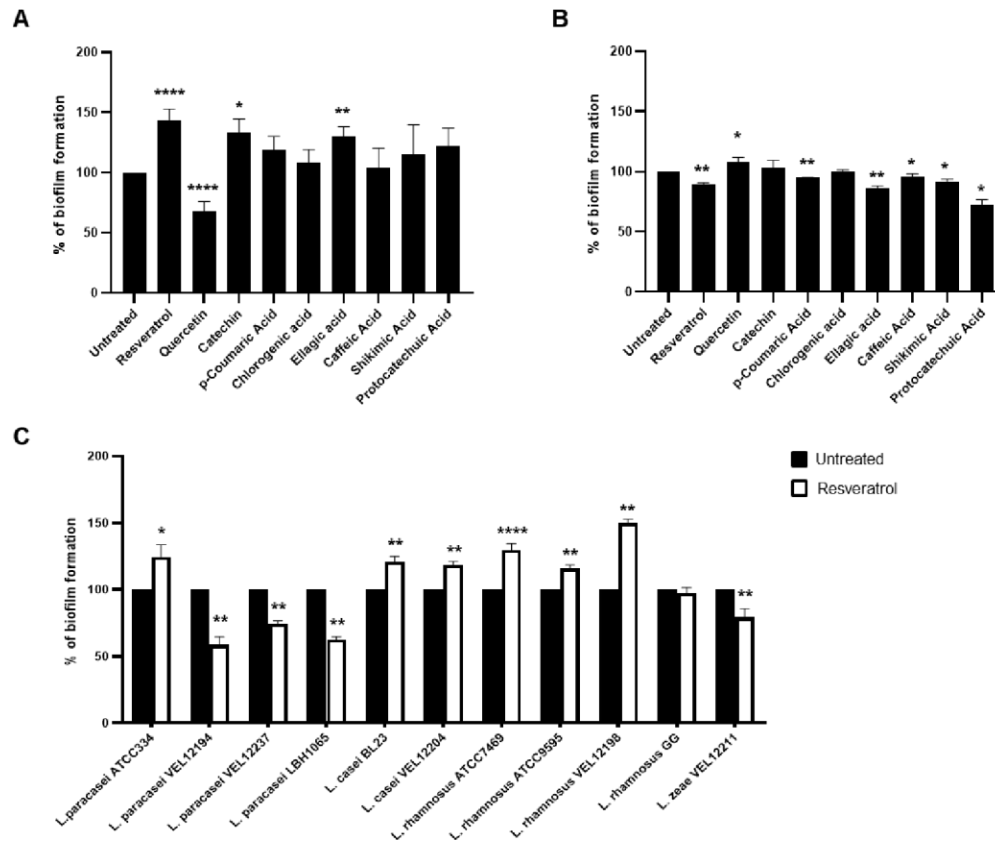


Figure 1. Low doses of polyphenols modulated biofilm formation of *Lacticaseibacillus* bacteria in a strain-dependent manner. (A) The ability of *L. paracasei* ATCC334 or (B) *L. rhamnosus* GG, untreated or incubated with 30 μ M of the indicated polyphenols, to form biofilms was measured after 24 h culture on a polystyrene support by enumeration on Man-Rogosa-Sharpe medium (MRS) agar plates. Data are expressed as mean percentage \pm standard error of the mean (SEM) of biofilm formation of at least three independent experiments, taken untreated bacteria value as 100%. * $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$ (versus untreated). (C) The ability of a panel of *Lacticaseibacillus* strains belonging to the *L. casei* group (name indicated below the x-axis), untreated (black bars) or incubated with 30 μ M of Resveratrol (white bars), to form biofilms was measured after 24 h culture on a polystyrene support by enumeration on MRS agar plates. Data are expressed as mean percentage \pm SEM

of biofilm formation of at least six independent experiments, taken untreated bacteria value as 100%. * $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$ (*versus* untreated).

2.2. Resveratrol Increased the Biofilm Formation by *Lactocaseibacillus paracasei* by Enhancing Bacterial Adhesion

The human large intestine is covered with a protective mucus layer composed predominantly of mucins proteins secreted by goblet cells [34]. Thus, we used mucin-coated polystyrene to mimic the intestinal intraluminal surfaces to visualize and to measure the ability of Resveratrol to enhance the biofilm formation by *L. paracasei* ATCC334 on a biotic surface during 24 h. We first visualized the formation of this biofilm by using the Syto9 probe, labelling all microorganisms in a population, and confocal laser scanning microscopy (Figure 2A). In the three conditions tested: untreated, Resveratrol 10 μM (Resv 10) and Resveratrol 50 μM (Resv 50), *L. paracasei* ATCC334 formed large flat biofilm structures, but biofilms formed upon Resveratrol treatment looks denser than those generated in the untreated condition (Figure 2A). To objectify these observations, we enumerated the number of living bacteria forming these biofilms at 24 h using the CFU assay. As shown in Figure 2B, a significant higher number of bacteria is retrieved from biofilm formed upon treatment with Resveratrol, with an effect peaking at a dose of 10 μM (167.8%), compared to those obtained from untreated bacteria (set as 100%). The formation of a biofilm can be schematically divided into four phases: (i) adhesion to the abiotic/biotic surfaces, (ii) microcolony formation corresponding to early development of a biofilm architecture, (iii) maturation of the biofilm and (iv) the dispersion (as depicted in Figure 2C) [18].

As microbial adhesion is an initial key step on biofilm formation, we analysed whether Resveratrol treatment can enhance the adhesion of bacteria to a mucin-coated support. Adhesion assay was performed by incubating *L. paracasei* ATCC334 for one hour at 37 °C on a mucin-coated polystyrene support. Adherent bacteria were counted by using the CFU assay (Figure 2D). In line with the increase in biofilm formation by *L. paracasei* observed in Figure 2B upon Resveratrol treatment, a significant increase in the adhesion of *L. paracasei* to the mucin support was observed in bacteria treated with 5 or 10 μM of Resveratrol, compared to untreated bacteria (Figure 2D). This suggests that Resveratrol might stimulate biofilm formation by promoting the adhesion of *L.*

paracasei to its support. However, higher doses of Resveratrol (25 and especially 50 μM) tended to reduce the adhesion of *L. paracasei* during this one-hour adhesion assay on mucin.

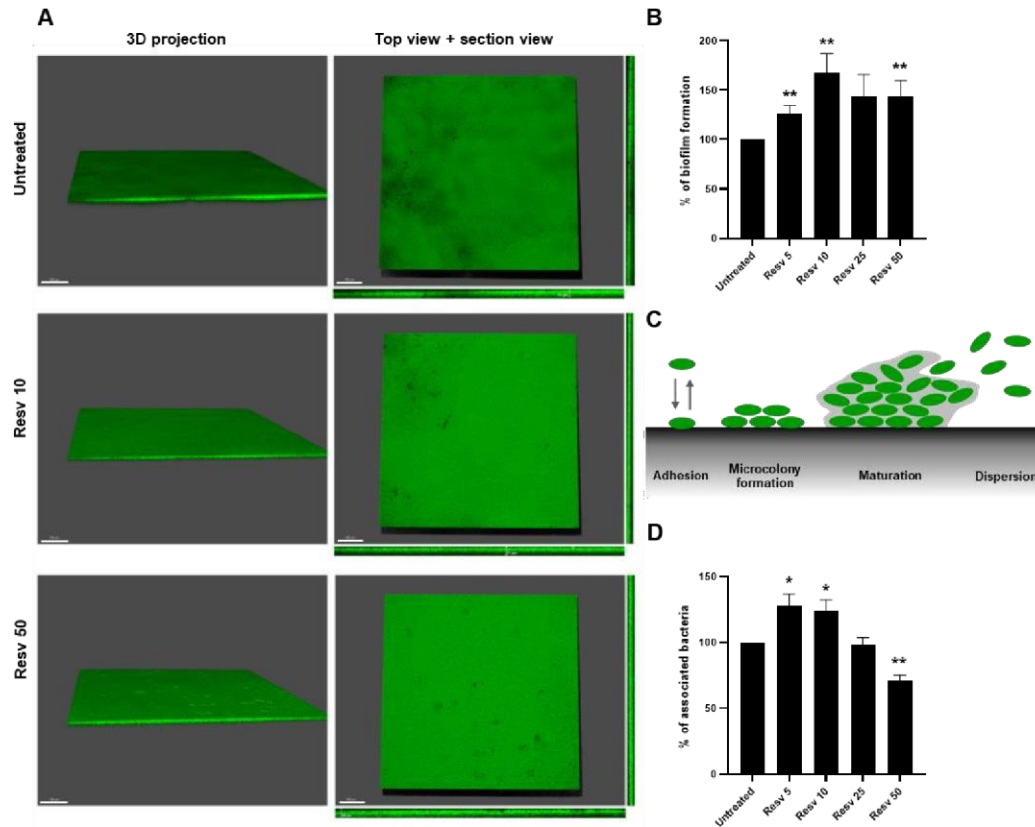


Figure 2. Resveratrol increased the biofilm formation by *L. paracasei* ATCC334 by enhancing its adhesion ability. **(A)** Confocal microscopy images of the biofilm formed by *L. paracasei* grown for 24 h in MRS medium alone (untreated) or in the presence of 10 μM (Resv 10) or 50 μM Resveratrol (Resv 50). The cells in the biofilms were stained with SYTO 9. 3D projections, top and section views are shown. **(B)** Biofilm formation was measured on a mucin-coated polystyrene support by enumeration on MRS agar plates. *L. paracasei* was cultured for 24 h in the absence (untreated) or presence of increasing doses of Resveratrol, from 5 μM (Resv 5) to 50 μM (Resv 50). Data are expressed as mean percentage \pm SEM of biofilm formation of at least three independent experiments, taken untreated bacteria value as 100%. ** $p < 0.01$ (versus untreated). **(C)** Schematic representation of biofilm formation stages. **(D)** Adhesion of *L. paracasei* to a mucin-coated polystyrene support after a 1 h incubation in the absence (untreated) or presence of increasing doses of Resveratrol, from 5 μM (Resv 5) to 50 μM (Resv 50). Adherent cells were enumerated on MRS agar plates and results are expressed as mean percentage \pm SEM of associated bacteria of at least three

independent experiments, taken untreated bacteria value as 100%. * $p < 0.05$, ** $p < 0.01$ (*versus* untreated).

2.3. Resveratrol Increases Adhesion of *Lacticaseibacillus paracasei* ATCC334 to Human Intestinal Epithelial Cells without Eliciting an Exacerbated Pro-Inflammatory Response

We next evaluated the effects of the same doses of Resveratrol on the ability of *L. paracasei* ATCC334 to adhere to two human intestinal epithelial cell (IEC) lines: HCT116 (Figure 3A) and HT29 (Figure 3B). For this purpose, Resveratrol was added to the cell culture medium of IECs, one-hour prior *L. paracasei* ATCC334 addition. In both cell lines, Resveratrol treatment significantly enhanced the adhesion of *L. paracasei* ATCC334, with a markedly higher effect for the adhesion to HT29 cells (Figure 3B). Interestingly, if the Resveratrol was added during the biofilm growth of *L. paracasei*, without addition of the polyphenols to host cells, the Resveratrol-treated bacteria remained able to better adhere to IECs compared to untreated bacteria, indicating that Resveratrol might act directly on bacteria to promote their adhesion (Figure 3C). Since an increase adhesion of bacteria to host cells might be detrimental by inducing a pro-inflammatory response [35,36], we checked whether Resveratrol, by promoting adhesion of *L. paracasei* to IECs, might trigger an exacerbated inflammatory response in basal condition (Figure 3D) or in inflammatory condition induced by a lipopolysaccharide (LPS) treatment (Figure 3E). Pro-inflammatory response was assessed by the measure, using ELISA assay, of the secretion of the prototypical pro-inflammatory cytokine IL-8 by HT29 IECs (Figure 3D,E). Interestingly, in basal condition, despite enhanced adhesion property to IECs, Resveratrol-treated *L. paracasei* ATCC334 did not induce more IL-8 upon challenge of IECs compared to untreated control bacteria (Figure 3). Similar observations were made under LPS-induced inflammatory response in HT29 (Figure 3E). Of note, we also checked whether the combination *L. paracasei* ATCC334 and Resveratrol did not induced an exacerbated inflammatory response in immune cells by monitoring the secretion of the pro-inflammatory cytokine TNF- α , using the J774 macrophage cell line (Figure A4A,B).

Thus, these results suggest that, even if Resveratrol enhanced adhesion properties of *L. paracasei* ATCC334 strain to host IECs, it did not elicit a pro-inflammatory response compared to the untreated condition.

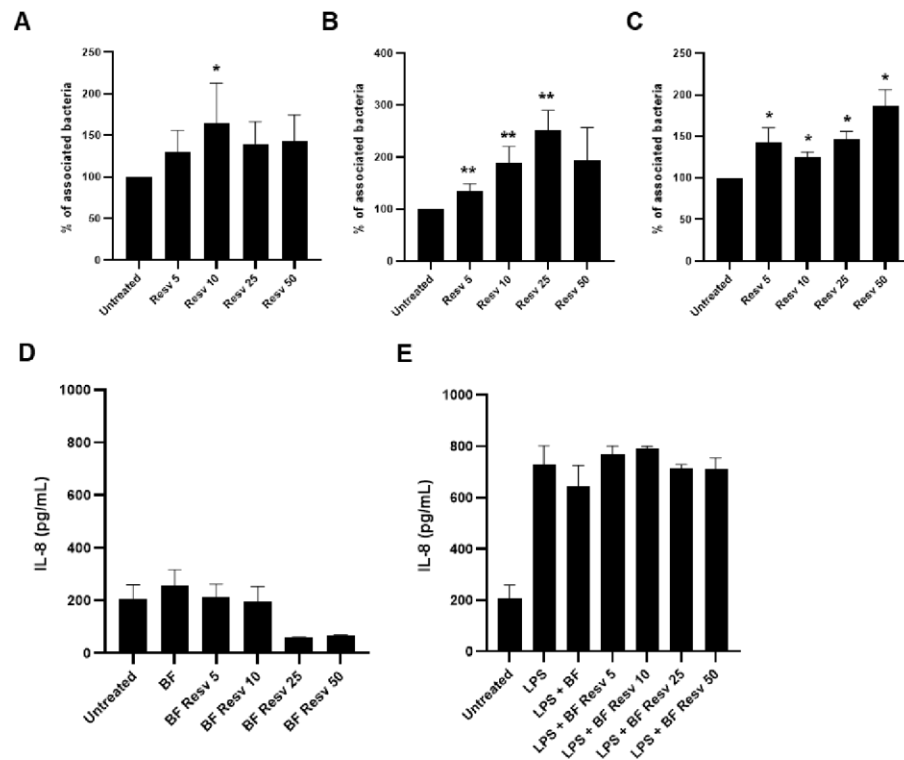


Figure 3. Resveratrol increased adhesion of *L. paracasei* ATCC334 to human intestinal epithelial cells (IECs) without eliciting an exacerbated pro-inflammatory response. (A) Adhesion of *L. paracasei* to HCT116 or (B) HT-29 IECs untreated or pre-treated for 1 h 30 min with increasing doses of Resveratrol, from 5 μ M (Resv 5) to 50 μ M (Resv 50). Adherent cells were enumerated on MRS agar plates and results are expressed as mean percentage \pm SEM of associated bacteria of at least three independent experiments, taken untreated bacteria value as 100%. * $p < 0.05$, ** $p < 0.01$ (versus untreated). (C) Adhesion to HT-29 IECs of 24 h biofilm grown *L. paracasei*, in the absence (untreated) or presence of increasing doses of Resveratrol, from 5 μ M (Resv 5) to 50 μ M (Resv 50). Results are expressed as in A and B panels. (D) HT-29 IECs were untreated or treated with biofilm-grown living *L. paracasei* at aMOI of 40 for 4 h. *L. paracasei* were grown under biofilm conditions in the absence (BF) or presence of increasing doses of Resveratrol, from 5 μ M (BF Resv 5) to 50 μ M (BF Resv 50).

IL-8 secretion (pg/mL) was determined by ELISA. Results are expressed as mean \pm SEM of at least three independent experiments. (E) HT-29 IECs were treated as in D but LPS treatment (100 ng/mL) was added concomitantly to *L. paracasei* treatment.

2.4. Resveratrol Changes Physico-Chemical Surface Properties of *Lacticaseibacillus paracasei* ATCC334 Strain

To elucidate the possible mechanism by which Resveratrol might enhance the adhesion properties of *L. paracasei*, and subsequent biofilm formation, we analyzed the surface properties of *L. paracasei* ATCC334 in basal condition and upon Resveratrol treatments. Indeed, the transition from a planktonic lifestyle to an attached state at a surface is a multifactorial process that is particularly determined by chemical and physical properties of the bacterial surface, that displays various electrical charges and hydrophobicity around the bacterial body depending on growth conditions [37]. Knowing the hydrophobic nature of Resveratrol and its ability to interact with numerous biological molecules [38], it might affect surface properties of *L. paracasei* and thereby the interactions of the bacteria with biotic and abiotic supports. To verify this hypothesis, we first performed a global analysis of bacterial surface charges by an electrophoretic mobility assay. As shown in Figure 4A, a significant reduction in the electrophoretic mobility of *L. paracasei* was observed upon treatment with 5 μ M Resveratrol (Resv 5) compared to the corresponding untreated bacteria. Even if nonsignificant, a similar trend was observed for higher doses of Resveratrol (Figure 4A). This negative shift in the electrophoretic mobility indicates that Resveratrol treatment increases negative charges at *L. paracasei* surface by revealing new functional groups such as carboxylates or sulfates. In addition, significant changes in conductivity were also measured in Resveratrol-treated bacteria, suggesting that Resveratrol modified their metabolism and the secretion of ions (minerals) which may contribute to the observed increase in the conductivity (Figure 4B).

Finally, we performed a microbial adhesion to solvents (MATS) assay to characterize the electron-donor/electron-acceptor properties of *L. paracasei* surface upon Resveratrol. In good accordance to the literature [39], untreated *L. paracasei* displayed a relative high affinity to the acidic solvent chloroform, indicating the basic nature of its cell surface (Figure 4C). This affinity tended to be increased by Resveratrol treatments, especially with doses of 10 μ M or higher, indicating that Resveratrol favored the basic nature of *L. paracasei* surfaces. Finally, we assessed by MATS the hydrophobicity of *L. paracasei* surface by measuring its affinity to the nonpolar solvent hexadecane

(Figure 4D). Regarding untreated *L. paracasei*, we confirmed the hydrophilic cell surface properties described in the literature for these bacteria [39], as indicated by the low affinity to hexadecane (less than 5 %) (Figure 4D). Interestingly, Resveratrol treatments induced an increase of about two-fold in the affinity of *L. paracasei* to hexadecane, demonstrating that Resveratrol rendered more hydrophobic the cell surface of the bacteria. This last result is particularly interesting since the hydrophobicity at bacterial surfaces was strongly associated with the ability of bacteria to adhere to abiotic and biotic supports, to form aggregates and to form biofilm [40–44].

Altogether, these results demonstrated that Resveratrol-treated *L. paracasei* displayed changes in the physicochemical properties of their surface, especially with a global increase in negative charges, a more basic nature and an increase in their hydrophobicity. These changes might largely contribute to the enhanced adhesion and biofilm formation abilities of Resveratrol-treated *L. paracasei*.

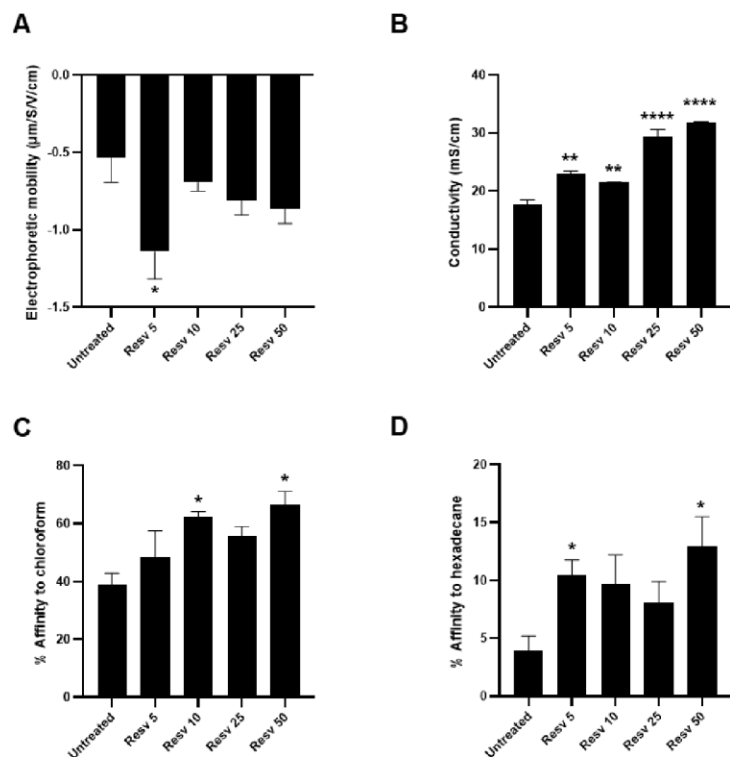


Figure 4. Resveratrol changed physico-chemical surface properties of *L. paracasei* ATCC334 strain. (A) Electrophoretic mobility of *L. paracasei* in absence (untreated) or presence of increasing doses of Resveratrol, from 5 µM (Resv 5) to 50 µM (Resv 50). Values (in µm/S/V/cm)

represent mean \pm SEM of at least five separate measures. **(B)** Measure of conductivity of *L. paracasei* in absence (untreated) or presence of increasing doses of Resveratrol, from 5 μ M (Resv 5) to 50 μ M (Resv 50). Values (in μ m/S/V/cm) represent mean \pm SEM of at least five separate measures. **(C)** MATS test, the percentage of adhesion of *L. paracasei*, in the absence (untreated) or presence of increasing doses of Resveratrol, from 5 μ M (Resv 5) to 50 μ M (Resv 50), to the acidic solvent chloroform or **(D)** to the non-polar solvent hexadecane was measured. Results are expressed as mean percentage of affinity \pm SEM of at least three separate experiments. * $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$ (*versus* untreated).

2.5. Resveratrol Promotes *Lacticaseibacillus paracasei* ATCC334 Aggregation.

The hydrophobicity of bacteria cell surfaces is also linked to aggregation, a bacterial lifestyle between the planktonic and biofilm states [45]. In addition, the formation of bacterial aggregates has been proposed to favor biofilm formation, notably by preparing bacteria to switching more rapidly to a biofilm-like phenotype [46]. To evaluate whether Resveratrol can promote *L. paracasei* aggregation, we treated for 1 h 30 min the *L. paracasei* ATCC334 strain with increasing doses of Resveratrol (from 5 to 50 μ M) and proceeded directly to microscopic examination of the living cultures. As illustrated by representative micrographs of each condition in Figure 5A, Resveratrol treatments significantly increased the number of *L. paracasei* bacteria forming aggregates (Figure 5B) and the size of these aggregates (Figure 5C). Aggregation of the bacteria upon Resveratrol treatment was confirmed by performing a sedimentation assay that consisted in measuring the evolution of turbidity during a short time (1 h 30 min) in a static culture [47]. Thus, if a treatment or a stress induced bacterial aggregation, a drop in optical density measured at 600 nm was observed, compared to those observed in a control culture. The significant reduction in turbidity at the top of the culture of Resveratrol-treated *L. paracasei* compared to the value obtained for untreated *L. paracasei* confirmed that aggregation occurred upon Resveratrol treatment (Figure 5D). To conclude, presumably as a consequence of the modification of *L. paracasei* surface properties (Figure 4), Resveratrol induced aggregation of *L. paracasei* and thereby might favor adhesion and biofilm formation (Figure 5E).

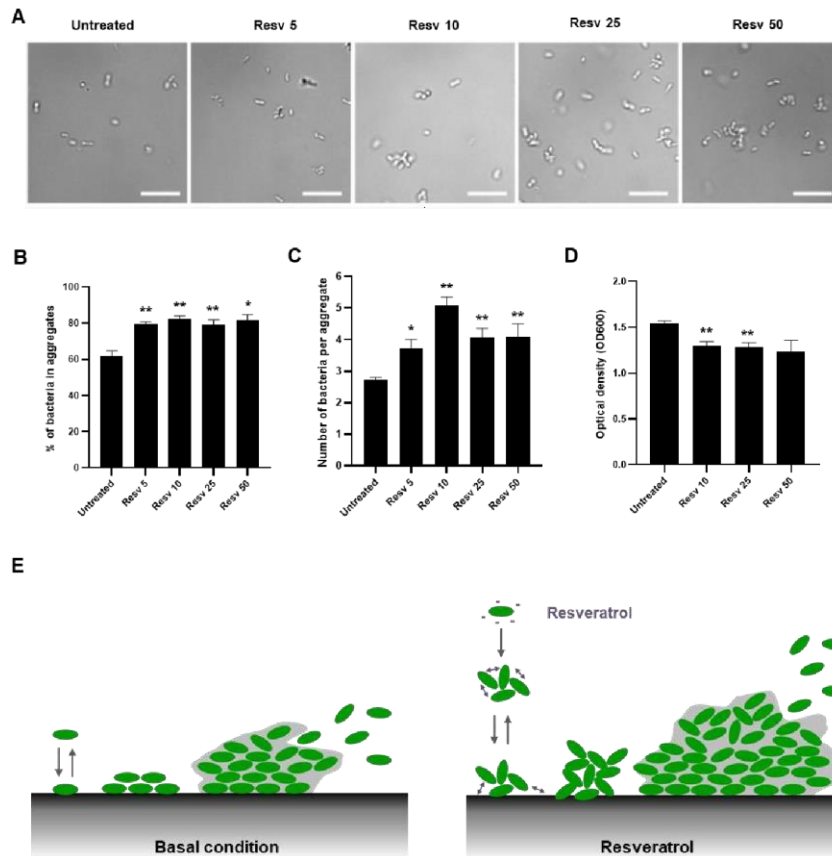


Figure 5. Resveratrol promoted *L. paracasei* ATCC334 aggregation. **(A)** Representative micrographs of live *L. paracasei* treated for 1 h 30 with increasing doses of Resveratrol from 5 μ M (Resv 5) to 50 μ M (Resv 50) and observed in phase contrast microscopy. In all images, the white scalebar = 10 μ m. For each condition, the percentage of bacteria \pm SEM forming aggregates is indicated in graph **(B)** and the mean number of bacteria per aggregate \pm SEM is shown in graph **(C)**. At least, 200 bacteria were counted per condition. * $p < 0.05$, ** $p < 0.01$ (*versus* untreated). **(D)** Sedimentation assay. Optical density at 600 nm (OD600) was measured on a static culture of *L. paracasei* untreated or treated with increasing doses of Resveratrol from 5 μ M (Resv 5) to 50 μ M (Resv 50), after 1 h 30 at 37 $^{\circ}$ C. Results are expressed as mean optical density at 600 nm \pm SEM of at least three separate experiments. ** $p < 0.01$ (*versus* untreated). **(E)** Schematic representation of the effect of Resveratrol treatment on aggregation, adhesion and biofilm formation by *L. paracasei*.

3. Discussion

Recently, the use of bacteria of the Lactobacillaceae family with the biofilm phenotype has shown to enhance their functionalities. A strategy to enhance the capacity of probiotic strains to form biofilm and consequently their colonization potential could be of first interest. In this study, we identified Resveratrol as an inducer of biofilm formation by *L. paracasei* ATCC334 strain. This effect is strain-dependent and relies on the enhancement of *L. paracasei* adhesion to abiotic and biotic surfaces, which represents the first step in biofilm formation. Resveratrol, by modifying negative charges and promoting a more basic nature and hydrophobicity at bacterial surface, enhanced *L. paracasei* aggregation and subsequently facilitated adhesion and biofilm development.

Resveratrol is more and more often regarded as a beneficial molecule in host–bacterial relationships. On the bacterial side, it is suggested that Resveratrol can have prebiotic-like effects since this polyphenol is able to increase the representation of beneficial bacteria, including those belonging to the Lactobacillaceae family, notably in the context of colitis and obesity [48–51]. *In vitro*, this modulation can be either positive for some bacterial species (*L. acidophilus*, *L. gasseri*, *L. ruminis*) or negative for some others (*Enterococcus faecalis*, *Escherichia coli* or *Yersinia pseudotuberculosis*), with sometimes strain-dependent effects [52]. In our study, a 30 μ M dose of Resveratrol has no effect on the growth rate of *L. paracasei* ATCC334 and *L. rhamnosus* GG, whereas a ten-fold higher dose (300 μ M) slows down the growth rate of both species. Resveratrol is also described to display potent antimicrobial activities against some bacterial species, notably by altering energy production, damaging DNA or by altering membrane integrity [53,54]. Thus, we can assume that, in the GI tract, Resveratrol, at a given concentration, might favor some bacterial species while inhibiting the growth of others, and thereby contributing to shaping gut-associated bacterial communities. Finally, Resveratrol can indirectly affect gut bacteria by modulating host processes that, in turn, can regulate bacteria. For instance, Resveratrol can modulate immune responses including processes involved in bacterial clearance, such as xenophagy [55,56].

Current knowledge of the precise mechanisms by which Resveratrol can enhance selectively the representation of Lactobacilli in the GI tract remains largely limited. The data presented in our study emphasize the positive role that Resveratrol can play on *Lacticaseibacillus* by enhancing their aggregation, adhesion, and biofilm formation abilities, presumably by modulating their

surface properties. Upon Resveratrol treatment, we observed at *L. paracasei* surface a global increase in negative charges, a more basic nature, and an increase in cell surface hydrophobicity. These results are in agreement with a previous study describing that Resveratrol can modify hydrophobicity on cell surface of Lactobacilli, either by increasing or decreasing it, depending on strains and dose of Resveratrol considered [57]. For instance, a 512 µg/mL dose of Resveratrol (corresponding to about 2243 µM, that is to say 44 times higher than the highest dose used in our study) increases hydrophobicity on cell surface of *L. paracasei* and *L. fermentum*, while decreasing it in *L. plantarum*. This strain-dependent effect of Resveratrol is also illustrated in our study, with an increased biofilm formation by some Resveratrol-treated *Lacticaseibacillus* strains, including the *L. paracasei* ATCC334 strain, whereas a decreased biofilm formation is observed for some others Resveratrol-treated strains of the same group, including three *L. paracasei* strains. Beyond Resveratrol effects on physico-chemical properties of bacterial cell surface, we could not exclude that Resveratrol might act indirectly by, for instance, modifying the production of exopolysaccharides (EPS) substances or by changing the expression profile of cell surface proteins (adhesins, pili) that can be, both of them, involved in adhesion processes [58–61]. A study using *L. acidophilus* NCFM strain has demonstrated that a 100 µg/mL dose of Resveratrol (corresponding to about 438 µM) stimulates adhesion to intestinal epithelial cells and increases the abundance of some proteins at bacterial surface (pyruvate kinase, 50S ribosomal protein L7/L12, elongation factor P) while decreasing some others (adenylosuccinate synthetase, and 6-phosphofructokinase) [59]. Even if the proteins identified are not belonging to the classical molecular determinants of bacterial adhesion due to their preferential intracellular localization, some of them are considered as moonlighting proteins, playing putative roles at bacterial surface [62]. Changes in the localization of these proteins might be facilitated by the fact that Resveratrol can affect bacterial membrane integrity or can create intracellular stress [53,54,63]. Thus, changes in physico-chemical properties of bacterial surface and modifications of expression levels of surface proteins seems to mediate Resveratrol effects on bacterial adhesion, however further investigations will be required to determine the relative importance of each mechanism. We could assume that these relative contributions of each mechanism might be dose- and strain-dependent and could explain the huge differences observed between various strains in response to Resveratrol treatment.

Since Resveratrol is still representing a major challenge for food and pharmaceutical industries due to its poor solubility, low bioavailability and possible adverse side effects, doses used to analyze Resveratrol effects *in vitro* should be consistent with *in vivo* reachable and tolerable concentration. Pharmacokinetics studies in rodent models demonstrated that in animals receiving *per os* Resveratrol doses from 2 to 240 mg/kg reached a micromolar range concentration in serum [32]. In human, largest tolerable doses used are about 5 g per day and allowed to reach also micromolar range concentration in plasma [38]. Thus, circulating concentrations of Resveratrol observed in rodent models and humans are entirely compatible with Resveratrol doses used in our present study. In addition, colon, by its direct exposition to diet, has been described as a target organ for Resveratrol with higher concentration achievable following oral administration, compared to those obtained in the plasma [64]. These data, demonstrating that Resveratrol can reach at relatively high concentration the colonic environment; suggest, that co-administration of Resveratrol with a probiotic might be effective. Of note, some inter-individual differences can be expected since gut microbiota has been described to convert and to metabolize Resveratrol. As an example, two bacteria in healthy humans, *Slackia equolifaciens* and *Adlercreutzia equolifaciens* have been identified as dihydroresveratrol producers from Resveratrol [65]. It is very likely that depending on the representation of these bacteria, and others involved in Resveratrol conversion, in the gut, Resveratrol availability might greatly differ from one individual to another. This observation can also be true more generally for all other polyphenols since bacteria can transform these compounds in many ways including ring fission, reduction, dihydroxylation, demethylation and decarboxylation [65].

To conclude, Resveratrol-treated *L. paracasei* bacteria display enhanced ability to adhere to abiotic and biotic surfaces. Knowing that this ability to adhere to intestinal epithelial cells is one of the criteria used in the selection of probiotic bacteria, formulation of bacteria with Resveratrol might offers an appealing strategy to ameliorate strain characteristics. Moreover, we demonstrated that this increased adhesion contributes to boosting biofilm formation by *L. paracasei*. This positive effect of Resveratrol on biofilm formation represents a novel finding since Resveratrol is essentially described in the literature for its inhibitory activities against biofilm derived from both Gram-positive and Gram-negative bacteria [53]. These inhibitory activities of Resveratrol on biofilm formation are often achieved at higher concentrations than those used in our study, reinforcing the idea that dose selection of Resveratrol used is of primary importance depending on

the expected effects and applications and should be carefully tested. Resveratrol dose should also be adapted to the probiotic bacterial strain considered (strain-dependent effects) and to the potential conversion of the molecule by the resident gut microbiota. For this purpose, further studies, using model organisms, will be required to ensure the *in vivo* feasibility of stimulating the functionalities of probiotics by polyphenols. An interesting challenge in the future will be to design and formulate new probiotics, eventually in association with active micronutrients such as Resveratrol, and tailored to integrate individual specific features (resident gut microbiota, clinical context, host genetic).

4. Materials and Methods

4.1. Bacterial Strains and Cell Culture

L. paracasei ATCC334, *L. paracasei* VEL12194, *L. paracasei* VEL12237, *L. paracasei* LBH1065, *L. casei* BL23, *L. casei* VEL12204, *L. rhamnosus* ATCC7469, *L. rhamnosus* ATCC9595, *L. rhamnosus* VEL12198, *L. rhamnosus* GG and *L. zae* VEL12211 strains were grown anaerobically without shaking at 37 °C in Man-Rogosa-Sharpe medium (MRS; Condalab) pH 5.8 (adjusted with acetic acid) for biofilm and planktonic cultures (as previously described [17]). *L. paracasei* VEL12194, *L. paracasei* VEL12237, *L. paracasei* LBH1065, *L. casei* VEL12204, *L. rhamnosus* VEL12198, and *L. zae* VEL12211 strains were kindly provided by L. Bermúdez-Humarán and P. Langella (Micalis Institute, INRA, Jouy-en-Josas, France) and previously characterized in [66]. HCT 116 cells (colonic carcinoma cells), HT-29 cells (colonic carcinoma cells) and J774A.1 macrophages were obtained from ATCC, cultured routinely in Dulbecco's Modified Eagle Medium (DMEM) GlutaMAX with 10% fetal bovine serum (FBS, Eurobio) and maintained at 37 °C in 5% CO₂ in air. All cell lines have been routinely tested for mycoplasma contamination using the PCR Mycoplasma Test Kit II (PromoKine). Stock solutions of trans-Resveratrol (Sigma), Quercetin (Sigma), Catechin (Sigma), p-Coumaric acid (Sigma), Chlorogenic acid (Sigma), Ellagic acid (Extrasynthese), Caffeic acid (Sigma), Shikimic acid (Sigma) and Protocatechuic acid (Sigma) were prepared in ethanol (50 mM). Bacteria or cells were treated with final concentrations of trans-Resveratrol ranging from 5 to 300 µM. For the other polyphenols, doses of 30 or 300 µM were used.

4.2. Growth Curves

Growth curves were performed by measuring Optical Density (OD) using a Tecan infinite 200pro microplate reader (Tecan), with Corning 48 flat bottom transparent polystyrol microplate, with lid and 200 μ L per well. Absorbance was measured each hour during 24 h at wavelength 600 nm, at 37 °C, with an orbital shaking (30 s) prior measurement. All strains were seeded at an initial OD of 0.05 (corresponding to 10^7 CFU/mL) in MRS pH 5.8 in absence (vehicle: ethanol) or presence of the indicated polyphenol at 30 μ M or 300 μ M, in triplicates.

4.3. Biofilm Formation Assay

24-well polystyrene plates (Costar 3524, Corning Incorporated) were coated with porcine mucin (10 mg/mL, Sigma) in distilled water (200 μ L/well; 4 °C, overnight). After discarding the mucin solution, wells were washed twice with a 150 mM NaCl solution and 1 mL per well of fresh MRS, supplemented or not (vehicle: ethanol) with the indicated polyphenol (at concentrations ranging from 5 to 50 μ M) and inoculated with 10^7 colony forming units (CFUs)/mL of a culture in stationary phase of *L. paracasei* ATCC334 or *L. rhamnosus* GG strains. Plates were incubated at 37 °C for 24 h. Cells attached to the well walls were quantified as described previously [16]. After incubation, the medium was removed from each well, and the plates were washed twice in a 150 mM NaCl solution to remove loosely attached cells. We added 1 mL of a 150 mM NaCl solution to each well before repeated pipetting to detach the biofilm, and serial dilutions of biofilm recovered suspension were spotted onto MRS agar plates. Each strain and/or condition was tested in at least three independent experiments, each with three biological replicates.

4.4. Adhesion Assay

HCT 116 or HT-29 intestinal epithelial cells were seeded 48 h prior adhesion assay at 4×10^5 cells/well in 24-well tissue culture plates with DMEM, 10% FBS. *L. paracasei* ATCC334 were cultured overnight in MRS pH 5.8, then washed twice in PBS and resuspended in MRS (adhesion to mucin) or DMEM (adhesion to intestinal epithelial cells) at 10^7 CFU/mL. Resveratrol (5 to 50 μ M) was added directly to MRS (adhesion to mucin, Figure 2D and resveratrol-treated biofilm, Figure 3C), or to DMEM (adhesion to IECs, 1 hour prior *L. paracasei* ATCC334 addition, Figure 2A,B). *L. paracasei* bacteria were added at 10^7 CFU per well (adhesion to mucin) or at multiplicity of infection (MOI) of 40. After 1 h 30 min, the wells were washed three times with pre-warmed PBS and bacteria were harvested by adding a solution of 1% Triton X-100 in PBS for 10 min. Total

bacteria adherent to the mucins/cells were quantified by serial dilution and plating on MRS agar plates. Results were expressed as mean percentage \pm standard error of the mean (SEM) of biofilm formation of at least three independent experiments, taken untreated bacteria value as 100%.

4.5. Confocal Laser Scanning Microscopy (CLSM)

The structure of *L. paracasei* biofilms was analyzed by confocal microscopy as described previously [16]. *L. paracasei* biofilm, in absence (vehicle: ethanol) or presence of Resveratrol (10 and 50 μ M Resveratrol) were formed as described in Section 4.3. Prior to image acquisition, each biofilm was fluorescently labelled with Syto9 probe (labelling all bacteria, Molecular probes), following manufacturer's instructions. After 10 min of incubation, the sample was placed on the motorized stage of a Leica TCS SP8 (LEICA Microsystems, France) at the DImaCell platform (<http://dimacell.fr/>), Dijon, France. All biofilms were scanned at 600 Hz at 20 \times magnification (HC PL APO CS2, 20x/0.75 DRY, NA: 0.75, Refractive Index: 1.00) water immersion objective lens with a 488 nm argon laser set at 0.7% intensity. Emitted fluorescence was recorded within the 500–538 nm range to visualize Syto 9 green fluorescence. Stacks of horizontal plane images (1024 \times 1024 pixels) with a z-step of 1 μ m were acquired for each biofilm. Three-dimensional projections and sections of a representative biofilm were reconstructed with LAS X software (LEICA Microsystems, France).

4.6. ELISA

HT-29 intestinal epithelial cells or J774-A1 macrophages were seeded 48 h prior adhesion assay at 4×10^5 cells/ well in 24-well tissue culture plates with DMEM, 10% FBS. *L. paracasei* ATCC334 were cultured overnight in MRS pH5.8, under biofilm condition, in presence of absence (vehicle: ethanol) of Resveratrol (5 to 50 μ M). Biofilms were then washed twice in PBS and resuspended in DMEM. *L. paracasei* bacteria were added to cells at a MOI of 40 for 4 h with or without a concomittant stimulation with Escherichia coli O127:B8 LPS (100 ng/mL, Sigma). All samples were analyzed in duplicate. After coincubation, cell supernatants were collected and frozen at -80 °C until further analysis. IL-8 (for HT-29 cells) and TNF- α (for J774-A1 cells) concentration in the supernatant were determined by an enzyme-linked immunosorbent assay (ELISA) (Biolegends, San Diego, CA, USA) following manufacturer's instructions.

4.7. Electrophoretic Mobility and Conductivity

The bacterial suspensions, cultivated in MRS medium and used in its stationary phase, were treated with increasing doses of Resveratrol, from 5 μM (Resv 5) to 50 μM (Resv 50) for 1 h 30 min, and centrifuged for 10 min at 7000 \times g. The pellets were resuspended in 1.5 mM of NaCl with a bacterial concentration of 1×10^5 cells/mL and then washed three times in 1.5 mM of NaCl. The measurements were performed using a ZetaCompact instrument (Cad instrumentation, Les Essarts-le-Roi, France) and electrophoretic mobility is expressed in $\mu\text{m}/\text{S}/\text{V}/\text{cm}$.

4.8. MATS

This partitioning method is based on the comparison between microbial cell affinity to a monopolar solvent and an apolar solvent. The monopolar solvent can be acidic (electron accepting) or basic (electron donating) but both solvents must have similar Lifshitz-van der Waals surface tension components. Chloroform, an acidic solvent which exhibits negligible basic character when pure, and hexadecane, a strongly basic solvent, were used in this study (Sigma, St Quentin Fallavier, France). *L. paracasei* ATCC334 bacteria were grown overnight in the absence of Resveratrol. Then, the bacterial cultures were exposed for 1 h 30 to different concentrations of Resveratrol (0 μM to 50 μM). According to Pelletier et al. [39], after a centrifugation of 10 min at 7000 \times g, bacteria were resuspended and washed three times in a 150 mM NaCl solution. After a last resuspension of bacteria, OD₄₀₀ was then measured and adjusted to 0.8. This solution (2.4 mL) was mixed by inverting and vortexed for 30 s with 0.4 mL of the indicated solvent. The mixture was allowed to stand for 15 min to ensure complete separation of the two phases before a sample (1 mL) was carefully removed from the aqueous phase and the optical density measured at 400 nm. The percentage of bound cells was subsequently calculated by: % adherence = $(1 - A/A_0) \times 100$ where A_0 is the optical density measured at 400 nm of the bacterial suspension before mixing and A is the absorbance after mixing.

4.9. Aggregation

The aggregation test was carried out with an overnight planktonic culture of the *L. paracasei* ATCC334 strain. OD₆₀₀ was measured and the appropriate Resveratrol concentration (0, 5, 10, 25, 50 μM) was achieved into each cuvette containing the bacteria at an OD₆₀₀ adjusted to 1. The OD₆₀₀ was then measured after a static culture of 1 h 30 min at 37 °C. After this, 10 μL of the pellet of

each cuvette was pipetted and inoculated onto coverslips under an optical microscope for visualization and the number of bacteria per aggregate was counted separately for each condition.

4.10. Statistical Analysis

Data are presented as the mean \pm standard error of the mean (SEM). Statistical analyses were performed with GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA). The non-parametric Mann and Whitney test was used to compare results between conditions. The p -values ≤ 0.05 were considered as statistically significant.

Author Contributions: Conceptualization, J.A.A., A.A.T., A.R., J.G. and P.L.; methodology, J.A.A., A.A.T., D.D.S.B., A.A., A.R., J.G. and P.L.; software, J.A.A. and A.A.T.; validation, A.R., J.G. and P.L.; formal analysis, J.A.A., A.A.T. and P.L.; investigation, J.A.A., A.A.T., A.H. and J.L.; resources, J.A.A., A.A.T. and J.L.; data curation, J.A.A., A.A.T., A.H. and P.L.; writing—original draft preparation, P.L. and J.A.A.; writing—review and editing, J.A.A., A.A.T., A.A., A.H., A.R. and J.G.; supervision, A.R., J.G. and P.L.; project administration, A.R., J.G. and P.L.; funding acquisition, A.R., J.G. and P.L. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

| | |
|-----------------|------------------------------------|
| CFU | Colony Forming Unit |
| CLSM | Cofocal Laser Scanning Microscopy |
| CO ₂ | Carbon Dioxide |
| DMEM | Dulbecco's Modified Eagle's Medium |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| FBS | Fetal Bovine Serum |
| GI | Gastro-Intestinal |
| IBD | Inflammatory Bowel Disease |
| IBS | Irritable Bowel Syndrome |
| IEC | Intestinal Epithelial Cell |
| IL-8 | Interleukin 8 |
| LBP | Live Biotherapeutic Product |
| LPS | Lipopolysaccharides |
| MATS | Microbial Adhesion To Solvents |
| MOI | Multiplicity Of Infection |
| MRS | Man-Rogosa-Sharpe Medium |
| NaCl | Sodium Chloride |
| NCFM | North Carolina Food Microbiology |
| NGP | Next Generation Probiotics |
| OD | Optical Density |
| PBS | Phosphate-buffered Saline |
| PCR | Polymerase Chain Reaction |
| SEM | Standard Error of the Mean |
| TNF- α | Tumor Necrosis Factor alpha |

Appendix A

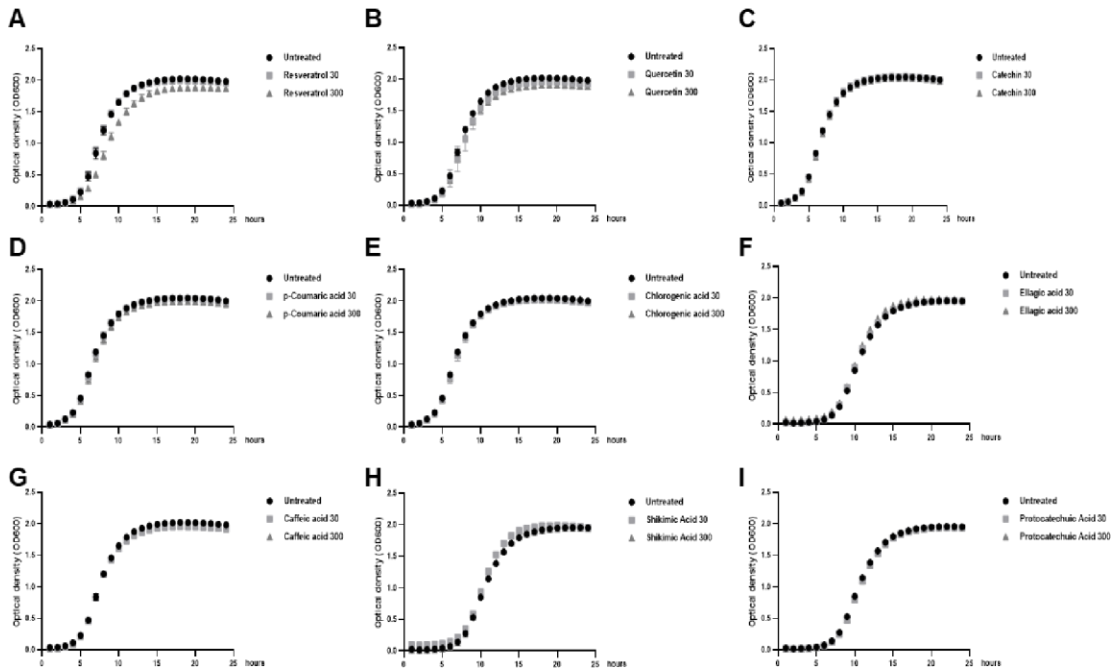


Figure A1. Growth curves of *L. paracasei* ATCC334 treated with various polyphenols. (A–I). Growth curves of *L. paracasei* ATCC334 untreated (black filled circle) or incubated with 30 μM (gray-filled square) or 300 μM (gray-filled triangle) of the indicated polyphenols in MRS medium at 37 °C. The OD₆₀₀ was monitored at 1 h intervals for 24 h. Values represent mean ± SEM of three independent experiments.

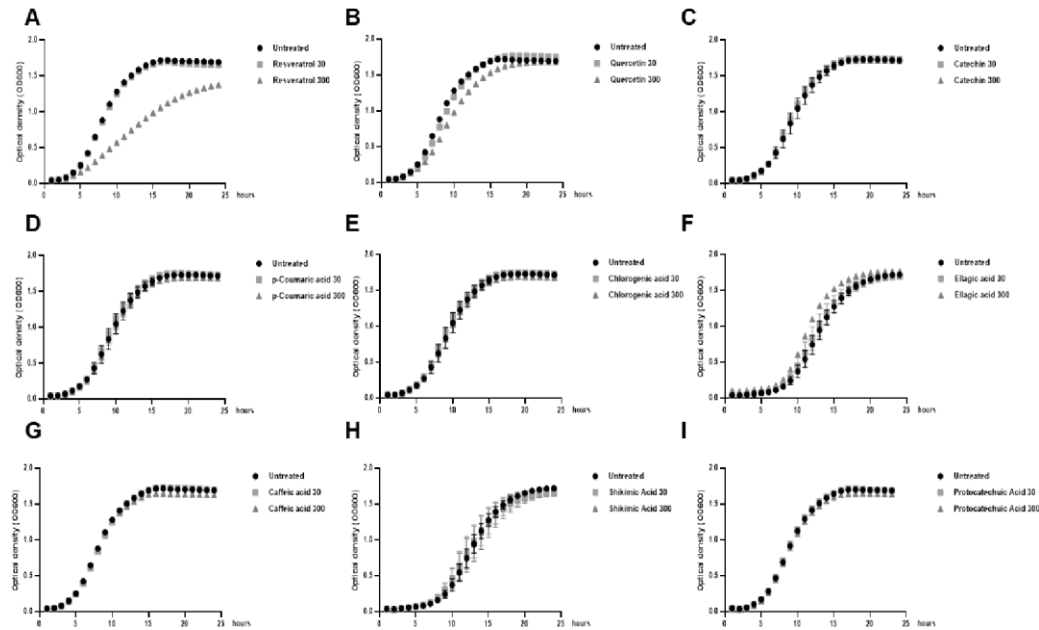


Figure A2. Growth curves of *L. rhamnosus* GG treated with various polyphenols. (A–I) Growth curves of *L. rhamnosus* GG untreated (black filled circle) or incubated with 30 μ M (gray-filled square) or 300 μ M (gray-filled triangle) of the indicated polyphenols in MRS medium at 37 °C. The OD₆₀₀ was monitored at 1 h intervals for 24 h. Values represent mean \pm SEM of three separate experiments.

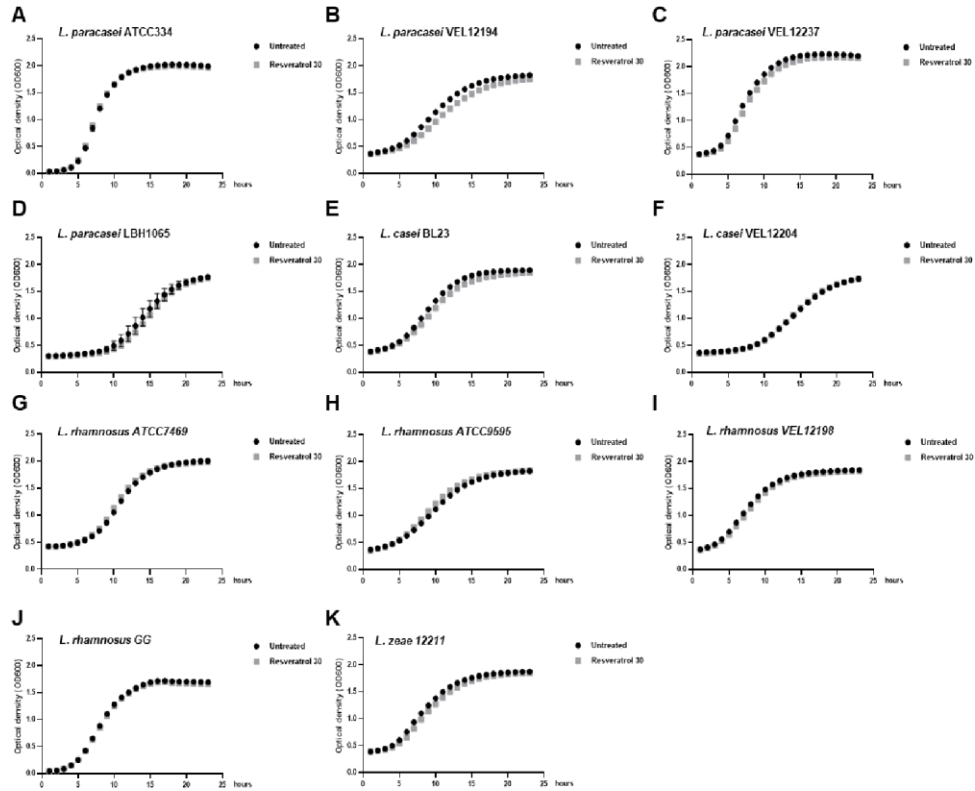


Figure A3. Growth curves of various *Lacticaseibacillus* strains with or without Resveratrol (30 μ M) (A–K) Growth curves of various *Lacticaseibacillus* strains (name of the strain indicated on each graph) untreated (black filled circle) or incubated with 30 μ M (gray-filled square) Resveratrol in MRS medium at 37 °C. The OD₆₀₀ was monitored at 1 h intervals for 23 h. Values represent mean \pm SEM of three separate experiments.

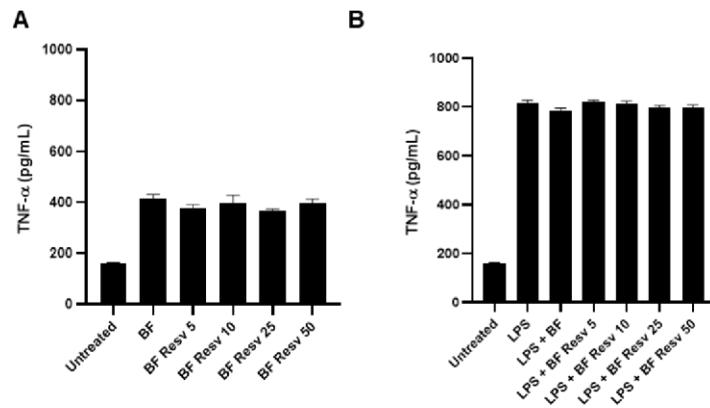


Figure A4. Combination of Resveratrol and *L. paracasei* did not elicit an exacerbated pro-inflammatory response. **(A)** J774 macrophages were untreated or treated with biofilm-grown living *L. paracasei* at a MOI of 40 for 4 h. *L. paracasei* were grown under biofilm conditions in the absence (BF) or presence of increasing doses of Resveratrol, from 5 μ M (BF Resv 5) to 50 μ M (BF Resv 50). TNF- α secretion (pg/mL) was determined by ELISA. Results are expressed as mean \pm SEM of at least three independent experiments. **(B)** J774 macrophages were treated as in A but LPS treatment (100 ng/mL) was added concomitantly to *L. paracasei* treatment.

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Paper 2

Paper 2: Modulation of the immunomodulatory properties of *Lactobacillus* strains by fasting.

-Article in processing-

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Abstract

The gut microbiota is essential to support intestinal homeostasis. In numerous socio-economically relevant human diseases, including inflammatory bowel diseases (IBDs), alterations in the composition and structure of the gut microbiota are observed and might contribute to the onset and/or progression of these diseases. Thus, there is an urgent need to identify and to develop innovative therapies aiming at restoring gut microbiota eubiosis and thereby intestinal homeostasis. Among existing approaches, dietary interventions and probiotic administration have demonstrated encouraging results *in vitro* and *in vivo* in model organisms, however efficiency of these approaches in human remains questionable. In this study, we investigated the combined effects of two interventions described separately to display immunoregulatory effects: fasting and lactobacilli administration. We demonstrated *in vitro* that fasting potentiates the ability of various lactobacilli strains to dampen LPS- or *Salmonella*-induced pro-inflammatory response in macrophages and intestinal epithelial cell lines. Using pharmacological inhibitor, we highlighted that amino acid deprivation seemed to be the key trigger explaining the immunoregulatory effects of fasting. Finally, we applied a fasting protocol *in vivo* to evaluate the beneficial combined effects of fasting and lactobacilli administration in a mice model of colitis, mimicking human IBDs. Altogether, the results presented in this study shed on light the positive potential of combining lactobacilli administration to host fasting.

Keywords: Fasting, Probiotics, Macrophages, Intestinal Epithelial cells, Inflammation, Colitis, *lactobacilli*, microbiome, ITAD.

INTRODUCTION

The gut microbiota is composed of hundreds of species of bacteria, archaea, fungi and protozoa, but also contained viruses that could shape the others microbial communities, especially the one that dominate qualitatively and quantitatively this microbiota, the bacteria [1]. These microorganisms actively participate in the maintenance of intestinal homeostasis, for example in facilitating the absorption of nutrients by enterocytes, modulating the immune system or by limiting the colonization of the gastrointestinal tract (GIT) by pathogens [2]. The beneficial effects of the gut microbiota on host homeostasis rely on a fine equilibrium between species constituting the resident microbiota and require a tolerogenic response from the gut-associated lymphoid tissue (GALT) to this resident microbiota, in order to avoid unwanted deleterious pro-inflammatory responses [3]. In various human disease states, a disruption of this balance has been observed, with modifications in the composition of the gut microbiota, usually called a dysbiosis and characterized by a drop in microbial diversity and an increase in pro-inflammatory species (i.e. pathobionts) [4]. These changes in the gut microbiota composition and its associated functions is presumed to contribute to the onset and progression of the diseases. This is well-exemplified in the context of inflammatory bowel diseases, such as Crohn's disease (CD), for which a decrease in gut microbial diversity is observed in patients, along with an increase in potentially aggressive species (e.g. Gammaproteobacteria including *Escherichia coli*) and decrease in pro-tolerogenic groups (*Bifidobacterium* spp, *Lactobacillus* spp or *Faecalibacterium prausnitzii* [5]. In a functional point of view, this dysbiotic gut microbiota associated to CD patients presents a decrease ability to produce Short Chain Fatty Acids (SCFA), such as butyrate, that are essential for host metabolism, a decrease in amino acid biosynthesis or an increase ability to produce oxidative stress. All these functional features might contribute to the CD onset and progression [5].

Different intervention strategies, including fecal microbiota transplantation (FMT) (i.e. whole microbial communities from a healthy donor), administration of probiotics (i.e. living microorganisms) or paraprobiotics (i.e. dead microorganisms), or even special nutrients, such as prebiotics, supporting the growth of beneficial microbes have been developed to act on the microbial communities of the GIT in order to attempt to restore homeostasis in host tissues (for review [6]). Among these strategies, FMT seems to be the more effective yet to restore gut

microbiota eubiosis by increasing microbial richness and allowing the reappearance of beneficial species on a long term in GIT of a receiver [7, 8]. FMT appears to be safe and well-tolerated by patients, however FMT applications are restricted by its dependency on healthy donors, collect of their fresh feces and the fact that it remains hard to define and verify that a given microbiota is a healthy one. Thus, efficiency of the other strategies, including probiotics, need to be improved in order to develop more convenient, safer and costless therapeutic options.

The current definition of probiotics is a “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host”, but new terms are also emerging to define new concepts in the field of probiotics, such as Live Biotherapeutic Product (LBP) or Next-Generation Probiotic (NGP) [9]. The latter one corresponds to strains identified on the basis of comparative microbiota analysis between healthy people and patients, and presenting potential health benefits (e.g. *Faecalibacterium prausnitzii* in the context of CD or *Akkermansia muciniphila* in the context of metabolic disorders [10, 11]). Even promising, these bacteria remain highly challenging to be producing and formulating at industrial scale and still should pass hard regulatory processes before thinking about their commercialization. Consequently, current market is still dominated by traditional probiotics with a long history of use and mostly belonging to the *Lactobacillus* spp and *Bifidobacterium* spp. genera. Of note, bacteria belonging to the extremely diversified *Lactobacillus* genus have been recently reclassified and subdivided into 25 genera taking into account their genetic, physiological and ecological features [12]. Thus, in this manuscript, the new nomenclature will be used, and the term “lactobacilli” will be employed to designate all organisms that were classified as *Lactobacillus* before.

Beneficial effects of Lactobacilli on host have been proposed to be mediated by various mechanisms, including pathogens antagonism, in some extent modulation of the gut microbiota composition, intestinal barrier strengthening or immunomodulation [13]. Lactobacilli produce several compounds that can influence the host's immune system such as components of the cell wall (e.g., peptidoglycan, lipoteichoic acid), DNA or exopolysaccharides. *In vitro* experiments and in model organisms, some Lactobacilli clearly exhibit strain-dependent immunomodulatory effects allowing to dampen excessive or prolonged inflammatory responses that can result in severe damage and dysfunction of host tissues [14, 15]. As an example, *Lactiplantibacillus plantarum* (*L. plantarum*) strains are able to downregulate pro-inflammatory responses, in a strain-

dependent manner, through the production of multiple compounds including exopolysaccharides, lipoproteins or lipoteichoic acids [16, 17, 18, 19, 20]. However, these bacteria displayed very limited effects in human in the treatment of inflammatory diseases such as IBDs [21]. Thus, further investigations are required to improve the efficiency of existing strains, including the development of innovative formulations or administration methods.

Cellular pathways governing the regulation of the innate and adaptive immune responses are intimately linked to metabolic pathways. As a consequence, there is a growing literature documenting how metabolic state of host (at cellular, tissue or whole-organism level) impacts on the immunity [22]. Metabolic status of the host is primarily influenced by nutrient content and nutrient timing, either directly or indirectly through effects on the gut microbiota. The main molecular pathways involved in immunometabolic signaling include the PI3K signaling pathway, the mTOR signaling pathway and the AMPK signaling pathway [23]. Among dietary intervention that could be considered to beneficially influence immune responses, controlled caloric restriction (CR) (i.e. decrease in total food intake) and intermittent fasting (i.e. eating patterns alternating eating and fasting periods) are well-documented to reduce low and high grade inflammation [24, 25, 26, 27, 28]. CR and fasting present additional beneficial effects, notably by improving metabolic parameters and counteracting several age-associated alterations and thereby promoting longevity [29, 30]. Mechanistically, these dietary interventions act on multiple signaling pathways included PI3K-, mTOR- and AMPK- associated signaling pathways. As an illustration, CR and Fasting can modulate inflammatory processes through the amino acid sensor GCN2 that in turn induces autophagy and represses inflammasome complexes activation, allowing to reduce intestinal inflammation [31]. Of note, autophagy is a ubiquitous eukaryotic cellular process mediating the lysosomal degradation of cellular components (organelles, proteins, lipids). It is activated by cells in response to various stimuli, including fasting, and is described to be closely intertwined with immune responses modulation [32].

In this study, we investigated in which extent the immunomodulatory properties of probiotics could be potentiated by modulating the nutrient intake *in vitro* using intestinal epithelial cell and macrophage cell lines and *in vivo* using an intermittent fasting model in mice. We demonstrated that short period of nutrient starvation allows to significantly potentiate the abilities of lactobacilli strains to downregulate pro-inflammatory responses in intestinal epithelial cells and macrophages.

These results were partially confirmed *in vivo* using an isocaloric twice-a-day (ITAD) feeding model in mice receiving concomitantly the administration of the *L. casei* strain BL23 and submitted to a chemically induced colitis (DSS). Compared to *ad libitum*-fed mice, ITAD-fed mice, or in a less extent *ad libitum*-fed mice receiving the *L. casei* strain BL23, are less sensitive to DSS-induced colitis, however co-administration of *L. casei* strain BL23 in ITAD-fed mice does not permit to obtain a synergistic effect of the two treatments as observed in *in vitro* models. Interestingly, ITAD feeding allows to modify the composition of the gut microbiome, notably by increasing the abundance of SCFA-producing species. Despite the mixed *in vivo* results obtained in this first report exploring the possible beneficial combination of fasting diet with probiotics, we are convinced that further investigations designed with modifications of the fasting protocol used or with other probiotic strains used in co-administration, could be of interest to explore in-depth this appealing low-cost combination.

RESULTS

Fasting modulates LPS-induced inflammatory response in macrophages.

The macrophages play critical roles in immune homeostasis and during pathogenic infection, notably through their immunoregulatory functions achieved by the release of the inflammatory mediators such as cytokines [33]. Thus, we started by investigating whether short fasting periods impact the inflammatory response generated by the murine macrophage cell line Raw 264.7 following a challenge by Gram-negative Lipopolysaccharides (LPS). Fasting was achieved by incubating cells in the nutrient-free medium EBSS (Earle's balanced salt solution) for 4 h. Fasted state of macrophages was checked by measuring the degree of autophagy activation in cells by immunofluorescence and RT-qPCR. As mentioned above, autophagic process is a cellular mechanism engaged during nutrient deprivation and its activation can be monitored by counting the number of autophagic vesicles per cell [34]. We visualized and counted these vesicles by immunofluorescence using antibodies against the autophagy-related proteins WIPI2 and LC3 that are present on autophagic vesicles at early or all times of the process, respectively (**Figure 1A-C**). A 4 h treatment was sufficient to significantly increase the number of vesicles positive for the early autophagy marker WIPI2 in fasted macrophages, in comparison to untreated macrophages, suggesting that autophagy was stimulated (**Figure 1A and B**). A similar trend, yet non-significant, was observed for the second autophagy marker LC3 (**Figure 1C**). Fasted state of cells upon the 4 h EBSS treatment was confirmed by measuring the mRNA expression levels of five autophagy-related genes, described to be upregulated upon activation of the process [35]. Expression of four of the five genes (*P62*, *Wip1*, *Lamp2a* and *Atg16l1*) was significantly increased in fasted macrophages in comparison to control macrophages, with the strongest upregulation observed for the *P62* gene (about 32-fold increased) (**Figure 1D**). Thus, a 4 h incubation of Raw 264.7 macrophages in EBSS is sufficient to induce a fasted state.

Next, we challenged Raw 264.7 macrophages with the LPS purified from the Gram-negative bacteria *Escherichia coli* during 6 h to induce a pro-inflammatory response. We monitored this response by measuring the mRNA expression levels of genes encoding pro-inflammatory cytokines (IL1- β , TNF- α , CCL3, CCL4 and MCP1) and an immunoregulatory cytokine (IL-10) (**Figure 1 E-J**). Macrophages were left in full medium or fasted for 4, 2 or 0 h prior LPS treatment (fasting was maintained during the 6 h LPS challenge). This three time points allow a gradual

modulation of the fasted state as measured by the expression level of the autophagy-related gene *P62* (**Sup Figure 1A**), without affecting cell viability. A 2 or 4 h fasting period before LPS stimulation allowed to markedly decrease the expression of the pro-inflammatory genes *Il1- β* , *Tnf- α* , *Ccl4* and *Mcp1* (**Figure 1E, 1F, 1H and 1I**), whereas the expression of the gene encoding the immunoregulatory cytokine *IL-10* is not affected and even significantly upregulated (about 2.5-fold) with the 2 h fasting period, compared to fed macrophages (**Figure 1J**). A similar, but milder, effect was observed when cells were starved concomitantly (0 h) with the LPS stimulation. In addition, incubating macrophages in minimal medium containing only 10% of DMEM (and 90% of EBSS) or in a less extent in serum-deprived medium, inducing a fasting state (**Sup figure 1B**), also allowed to reduce the expression levels of the genes encoding the cytokines *IL1- β* , *TNF- α* , *CCL4* and *MCP1*, in comparison to fed macrophages (**Sup figure 1C-G**). Altogether, these first results confirm in our experimental settings that fasting can modulate LPS-induced inflammatory responses and this modulation is determined by fasting time and intensity.

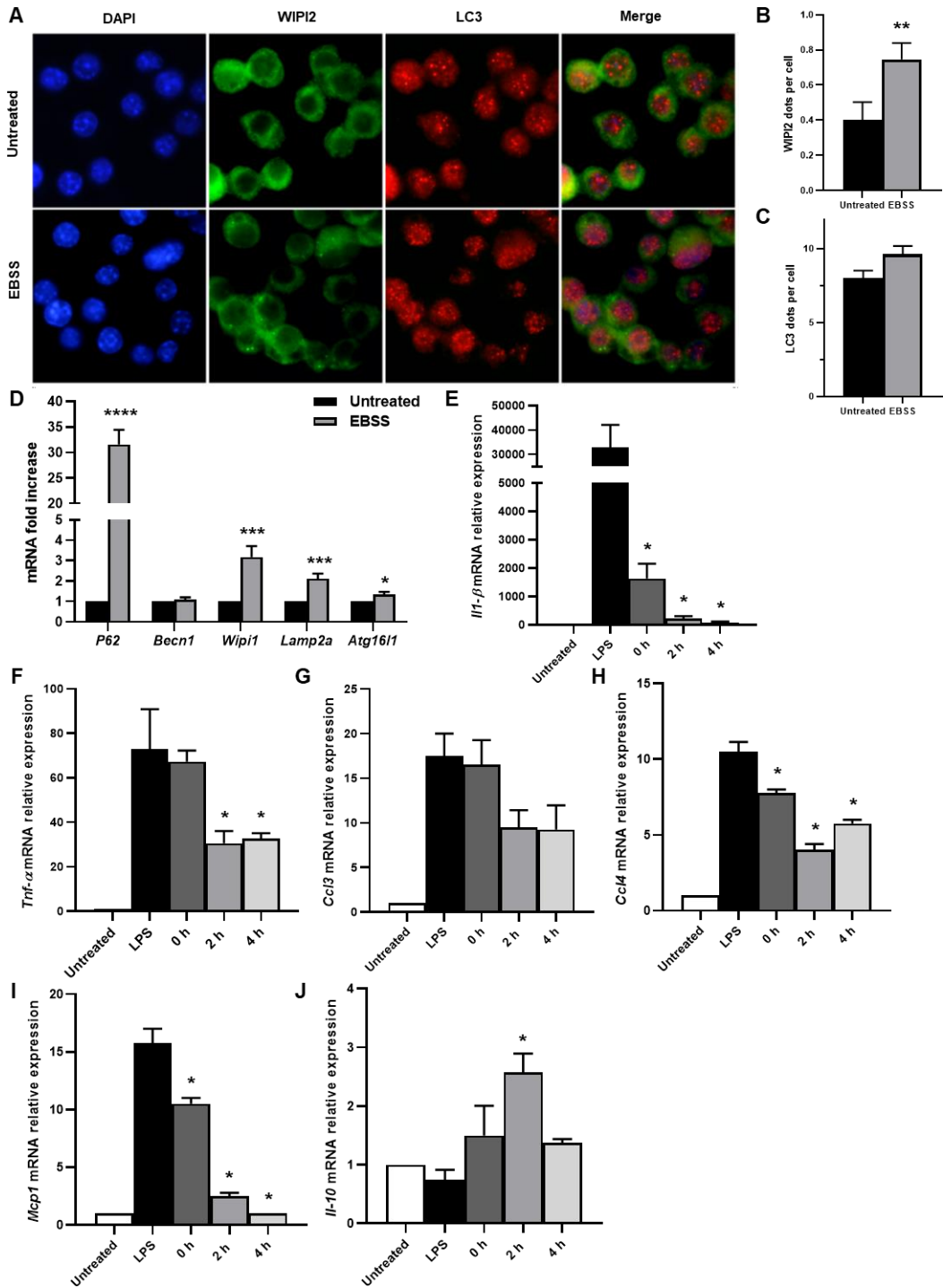


Figure 1: Fasting modulates LPS-induced inflammatory response in Raw264.7 macrophages.

(A) Representative images of Raw264.7 macrophages in full medium (untreated) or starved for 4 h in EBSS. Samples were processed for WIPI2 (green), LC3 (red) and nuclei (blue) staining. (B-C) Quantification of the number of (B) WIPI2 and (C) LC3 positive dots per cell. Results are as the number of positive dots per cell and each value is the

mean of at least three independent experiments \pm SEM. ** $p < 0.01$ (versus untreated). **(D)** RAW 264.7 macrophages were incubated in full medium (untreated, black bars) or starved for 4 h in EBSS (grey bars). The mRNA fold increase level of the autophagy-related genes *P62*, *Becn1*, *Wipi1*, *Lamp2a* and *Atg16l1* were measured by RT-qPCR (untreated cells taken as 1). The graphs show the mean \pm SEM of at least three independent biological experiments using two replicates each. * $p < 0.05$, *** $p < 0.001$ and **** $p < 0.0001$ (versus untreated). **(E-J)** RAW 264.7 macrophages were incubated in full medium and unstimulated (untreated, white bars) or stimulated with LPS (100 ng/mL) for 6 h in full medium (black bars) or in EBSS (0h, dark grey bars) or cells were incubated before (and during) LPS stimulation in EBSS for 2 h (average grey bars) or 4 h (light grey bars). The mRNA relative expression level of the pro-inflammatory cytokines **(E)** *Il1- β* , **(F)** *Tnf- α* , **(G)** *Ccl3*, **(H)** *Ccl4* and **(I)** *Mcp1*, and **(J)** the regulatory cytokine *Il-10* were measured by RT-qPCR (untreated cells taken as 1). The graphs show the mean \pm SEM of at least three independent biological experiments using two replicates each. * $p < 0.05$ (versus untreated).

Host cell fasting state potentiates the immunomodulatory properties of lactobacilli strains.

Since cellular pathways regulating the innate immune responses are intimately linked to metabolic pathways affected by fasting, we explored whether host cell fasting can modulate the immunomodulatory properties of lactobacilli upon LPS-induced inflammatory response. For this purpose, we fasted Raw 264.7 macrophages in EBSS or in EBSS containing 10% RPMI (*i.e.* full medium of this cell line) concomitantly to LPS stimulation. As previously shown in Figure 1 and Sup figure 1), these fasting conditions only induced a mild downregulation of the inflammatory response, allowing us to visualize a potential enhanced effect in the presence of the lactobacilli. The macrophages were treated 30 min before LPS stimulation by the *Lacticaseibacillus paracasei* (*L. paracasei*) strain ATCC334 alive, a bacterial strain described to present anti-inflammatory properties *in vitro* and *in vivo* [36, 37].

In our settings, the whole *L. paracasei* strain ATCC334 alive was able to beneficially modulated LPS-induced inflammatory response by decreasing the expression level the pro-inflammatory genes *Il1- β* and by stimulating the expression of the *Il-10* gene (**Figure 2A and 2F**), however the bacteria tended to increase the expression of the pro-inflammatory genes *Tnf- α* , *Ccl3*, *Ccl4* and *Mcp1* (**Figure 2B, 2C, 2D and 2E**). Interestingly, host cell fasting, either by incubation in EBSS or in 10% RPMI-EBSS, allowed to potentiate the immunomodulatory properties of *L. paracasei* strain ATCC334, notably by totally abrogating the expression of the pro-inflammatory IL-1 β gene,

but also by tending to reduce the expression of the *Tnf- α* , *Ccl3* and *Mcp1* genes, while supporting the expression of the *Il-10* gene (**Figure 2A-F**).

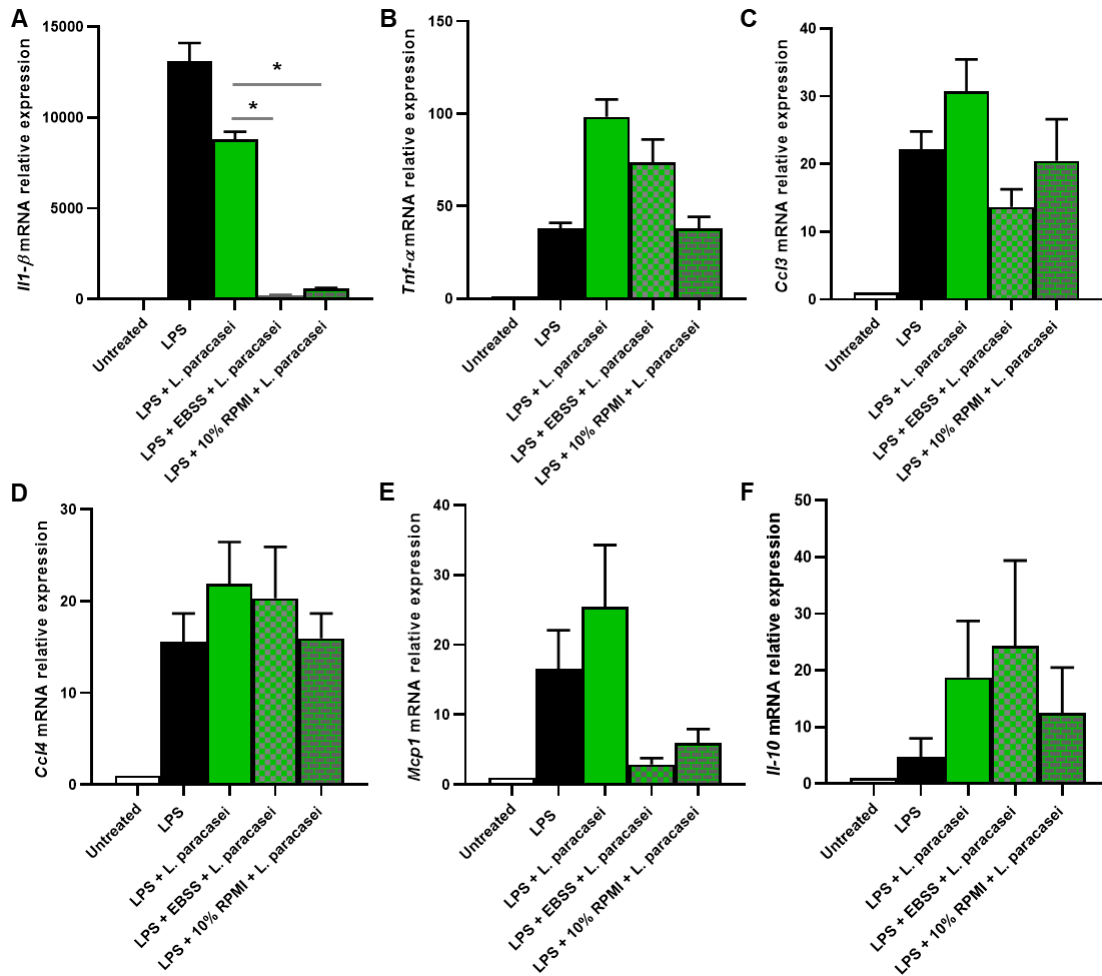


Figure 2: Fasting state of Raw 264.7 macrophages potentiates the immunomodulatory properties of *L. paracasei* strain ATCC334.

(A-F) RAW 264.7 macrophages were incubated in full medium and unstimulated (untreated, white bars) or stimulated with LPS (100 ng/mL) for 6 h in full medium in absence (black bars) or in presence of the *L. paracasei* strain ATCC334 (green bars). Alternatively, cells stimulated with LPS and incubated with *L. paracasei* strain ATCC334 were also concomitantly incubated in EBSS (green bars with grey squares) or in EBSS medium containing 10 % of RPMI (green bars with grey bricks). The mRNA relative expression level of the pro-inflammatory cytokines (A) *Il1- β* , (B) *Tnf- α* , (C) *Ccl3*, (D) *Ccl4* and (E) *Mcp1*, and (F) the regulatory cytokine *Il-10* were measured by RT-qPCR (untreated cells taken as 1). The graphs show the mean \pm SEM of at least three independent biological experiments using two replicates each. * $p < 0.05$ (versus LPS + *L. paracasei*).

These encouraging results supporting the idea that fasting can potentiate lactobacilli immunoregulatory properties and/or reducing their pro-inflammatory potential were strengthened by similar experiments in Raw 264.7 macrophages testing two other lactobacilli strains: *Lacticaseibacillus rhamnosus* (*L. rhamnosus*) strain GG and *Lacticaseibacillus casei* (*L. casei*) strain BL23. Both strains are described to present immunomodulatory properties [38, 39]. This feature was partially confirmed in our LPS-induced inflammatory response in Raw 264.7 macrophages, as shown by the important decrease in the expression of the pro-inflammatory gene *Il1- β* for both strains and the upregulation of the *Il-10* gene by the *L. casei* strain (**Figure 3A**), but both strains were unable to counteract the LPS-induced upregulation of the others pro-inflammatory genes tested. Combination of host cell fasting (EBSS) and *L. rhamnosus* strain GG or *L. casei* strain BL23 abrogated LPS-induced expression of the pro-inflammatory genes *Il1- β* and *Mcp1*, significantly reduced *Ccl3* mRNA level and tended to dampen *Tnf- α* and *Ccl4* genes expression (**Figure 3A-E**). Of note, fasting abrogated also the upregulation of the *Il-10* gene induced by *L. casei* strain BL23 (**Figure 3F**). Altogether, these results reinforced the idea that host immune cells in fasting state can potential anti-inflammatory properties of lactobacilli.

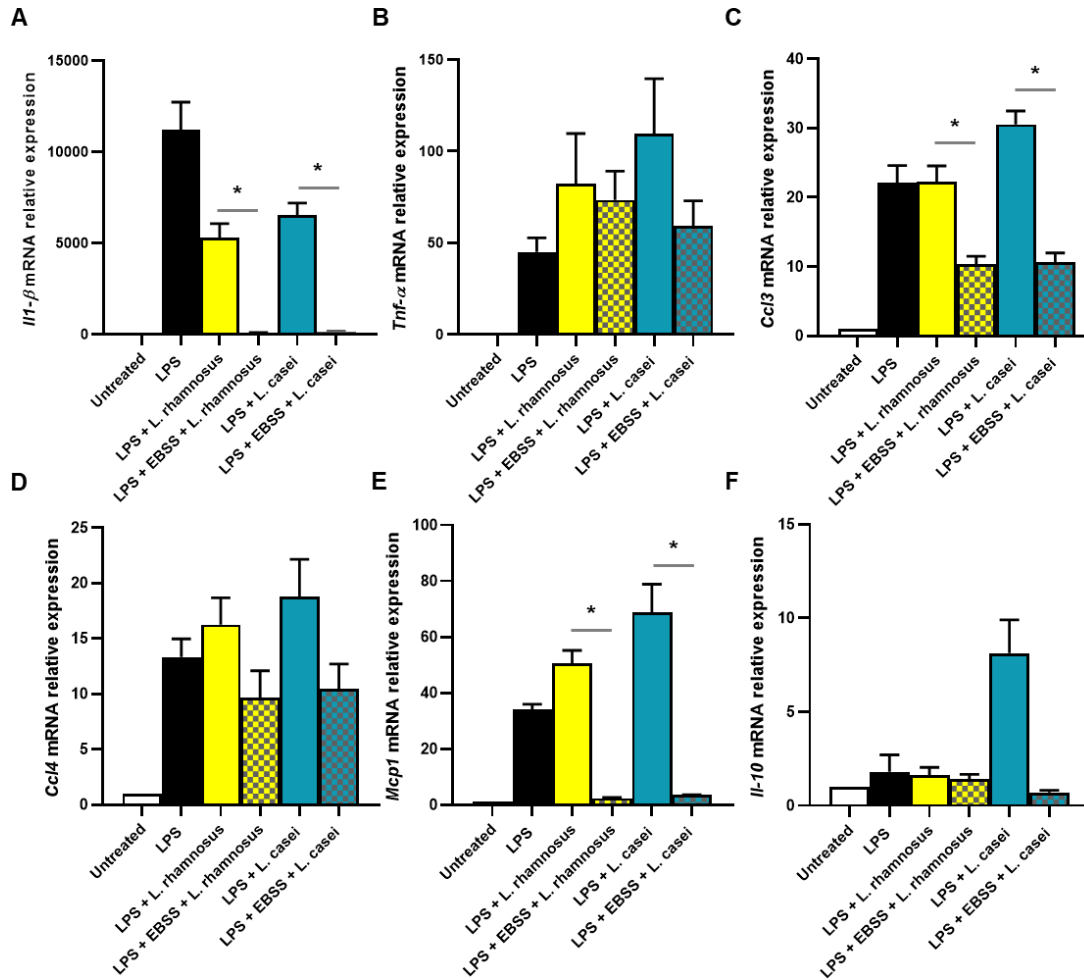


Figure 3: Fasting state of Raw 264.7 macrophages potentiates the immunomodulatory properties of two other lactobacilli strains: *L. rhamnosus* strain GG and *L. casei* strain BL23.

(A-F) RAW 264.7 macrophages were incubated in full medium and unstimulated (untreated, white bars) or stimulated with LPS (100 ng/mL) for 6 h in full medium in absence (black bars) or in presence of the *L. rhamnosus* strain GG (yellow bars) or *L. casei* strain BL23 (blue bars). Alternatively, cells stimulated with LPS and incubated with lactobacilli strains were also concomitantly incubated in EBSS (yellow bars with grey squares for *L. rhamnosus* strain GG and blue bars with grey squares for *L. casei* strain BL23). The mRNA relative expression level of the pro-inflammatory cytokines (A) *Il1-β*, (B) *Tnf-α*, (C) *Ccl3*, (D) *Ccl4* and (E) *Mcp1*, and (F) the regulatory cytokine *Il-10* were measured by RT-qPCR (untreated cells taken as 1). The graphs show the mean \pm SEM of at least three independent biological experiments using two replicates each. * $p < 0.05$ (versus LPS + lactobacilli)

Host cell fasting state and lactobacilli dampen the release of the pro-inflammatory cytokines TNF- α and IL1- β upon LPS stimulation.

To go further than transcriptional regulation of inflammatory-related genes by joint action of fasting and lactobacilli, we next evaluated their effects at protein level by measuring the release of two prototypical pro-inflammatory cytokines, TNF- α and IL1- β , by macrophages. High level of secreted TNF- α , as observed in the context of IBDs, could be detrimental for the gut tissue integrity because of its effects on tight junctions, pro-inflammatory cytokines production (e.g., IL-6 and IL-1 β) or on cellular viability [40]. IL1- β exerts pleiotropic effects on a wide range of cells and its overproduction is also associated with the onset of various human diseases, including IBDs [41]. However, even if murine Raw 264.7 macrophages have a functional and expressed *Il1- β* gene, these cells had been described to be deficient for the release of mature IL1- β , because of a lack of the cryopyrin inflammasome-associated protein ASC (Apoptosis-associated speck-like protein containing a CARD) necessary for the release of fully processed IL1- β [42]. Thus, next experiments were performed in another murine macrophage cell line, the J774 A.1, competent for IL1- β release. Similarly, to Raw 264.7 macrophages, a fasting state could be achieved in J774 A.1 macrophages by incubating them for 4 h in EBSS minimal medium as shown by the significant increase in the number of autophagic vesicles (positive for WIPI2 and LC3) in EBSS-incubated macrophages in comparison to control (**Figure 4A-C**). Beneficial combined effects of fasting and *L. casei* strain BL23 on the downregulation of pro-inflammatory genes *Il1- β* and *Tnf- α* expression seen in Raw 264.7 macrophages upon LPS stimulation were confirmed in this cell line (**Figure 4D-E**). At protein level, *L. casei* strain BL23 alone was able to totally abrogate the LPS-induced secretion of IL-1 β but has no effect on TNF- α release by macrophages (**Figure 4F-G**). Combined effect of *L. casei* strain BL23 with fasting permitted to abrogate both IL-1 β and TNF- α cytokines release induced by LPS, confirming that fasting state potentiates immunoregulatory properties of *L. casei* strain BL23. Interestingly, this effect was only found when using *L. casei* alive, and in less extent with its supernatant, but not with the heat-killed corresponding lactobacilli (**Sup figure 2A**), suggesting that these combined beneficial effects required living lactobacilli. Such combined effect of *L. casei* strain BL23 with host cell fasting state on TNF- α secretion induced by LPS was also observed with another *Lacticaseibacillus*, *L. paracasei* strain ATCC334. Finally, we demonstrated that more moderate fasting states, achieved by incubating macrophages in medium containing decreasing percentage of DMEM (from 100% DMEM to 0% DMEM, i.e. 100% EBSS)

also allowed to measure interesting effects of the combination of reduced nutrients concentration and the presence of the *L. casei* strain BL23 on the TNF- α cytokine release induced by LPS (**Figure 4H**). Indeed, even if *L. casei* strain BL23 alone or fasted state alone (in medium with 20 to 80% of DMEM) had no effects on LPS-induced TNF- α release, combination of both tended to reduce this secretion in a dose dependent manner, with a maximum effect in the medium containing 20% DMEM. To summarize, all the results presented above tends to demonstrate that combination of a fasted state in host cell and presence of a probiotic-like lactobacilli is more potent to dampen LPS-induced pro-inflammatory response than single intervention (fasting or lactobacilli alone).

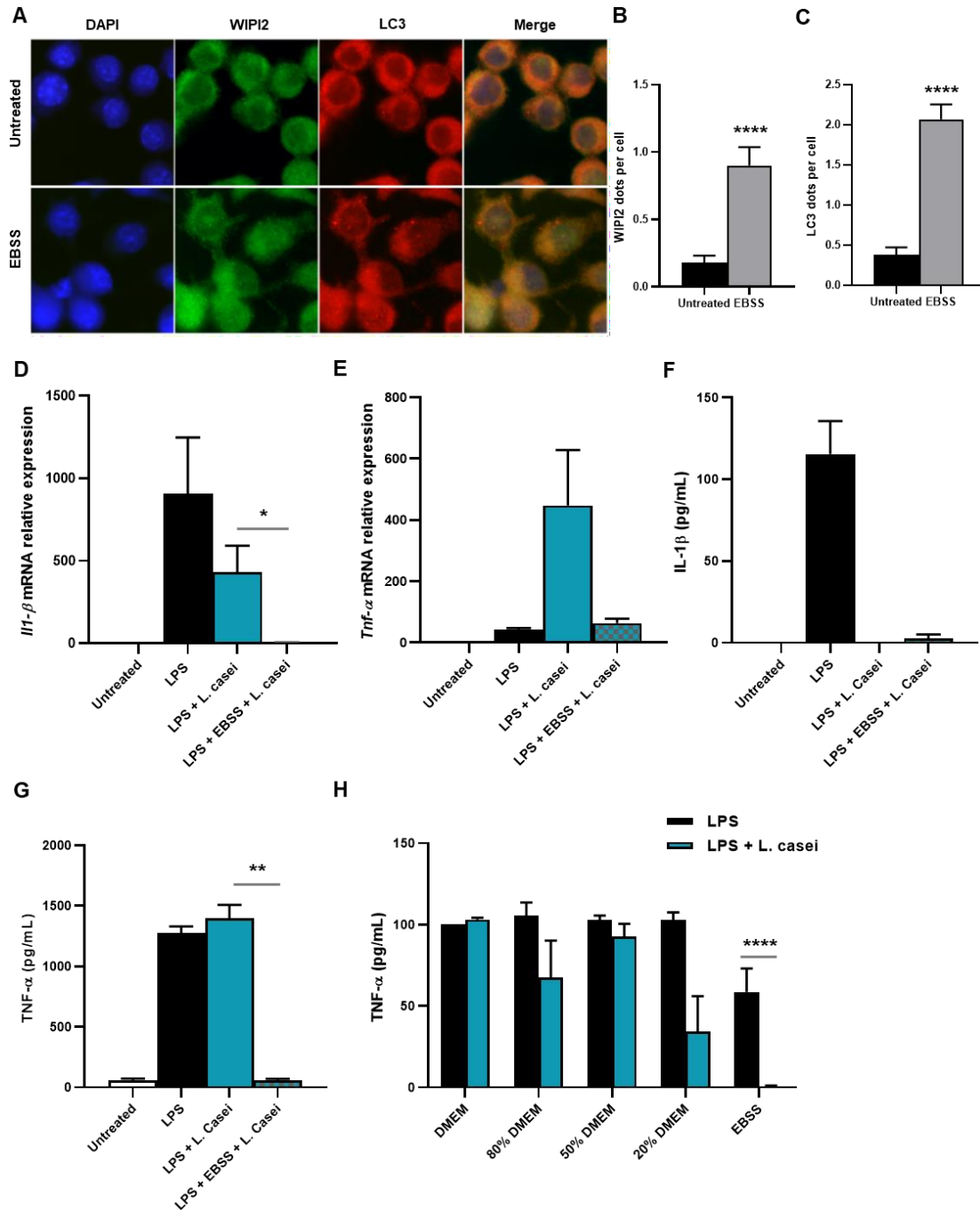


Figure 4: Fasting also modulates LPS-induced inflammatory response in J774 A1 macrophages.

(A) Representative images of J774 A1 macrophages in full medium (untreated) or starved for 4 h in EBSS. Samples were processed for WIPI2 (green), LC3 (red) and nuclei (blue) staining. (B-C) Quantification of the number of (B) WIPI2 and (C) LC3 positive dots per cell. Results are as the number of positive dots per cell and each value is the mean of at least three independent experiments \pm SEM. **** $p < 0.0001$ (versus untreated). (D-E) J774 A1 macrophages were incubated in full medium and unstimulated (untreated, white bars) or stimulated with LPS (100

ng/mL) for 6 h in full medium in absence (black bars) or in presence of the *L. casei* strain BL23 (blue bars). Alternatively, cells stimulated with LPS and incubated with *L. casei* strain BL23 were also concomitantly incubated in EBSS (blue bars with grey squares for *L. casei* strain BL23). The mRNA relative expression level of the pro-inflammatory cytokines **(D)** *Il1-β* and **(E)** *Tnf-α* were measured by RT-qPCR (untreated cells taken as 1). The graphs show the mean ± SEM of at least three independent biological experiments using two replicates each. * $p < 0.05$ (versus LPS + *L. casei*). **(F-G)** Same as in panels D and E, however **(F)** IL-1β and TNF-α secretion were determined by ELISA. Results are expressed in pg/mL as mean ± SEM of at least three independent experiments. ** $p < 0.01$ (versus LPS + *L. casei*). **(H)** J774 A1 macrophages were incubated in full medium (DMEM) or in medium containing decreasing percentage of DMEM (from 80% DMEM to 0% DMEM, *i.e.* EBSS) and stimulated with LPS alone (black bars) or with LPS in presence of *L. casei* strain BL23 (blue bars) for 6 h. TNF-α secretion was determined by ELISA. Results are expressed as percentage of TNF-α secretion ± SEM of at least three independent experiments (LPS stimulated cells in DMEM taken as 100%). **** $p < 0.0001$ (versus DMEM LPS).

Host cell fasting state and lactobacilli were also effective to dampen inflammatory response generated by intestinal epithelial cells.

Beyond their important roles in nutrients absorption and in forming a physical barrier, intestinal epithelial cells (IECs) have pleiotropic functions in the modulation of immune responses, notably by producing and secreting various immune mediators, including cytokines, in response to luminal bacteria [43, 44]. Among the immune mediators produced by IECs, Interleukin-8 (IL-8) is a prototypical pro-inflammatory cytokine produced by epithelial cells, associated to IBDs pathogenesis and involved, notably, in the recruitment of neutrophils at the site of infection [45, 46, 47]. In order to stimulate the production and secretion of IL-8 by the human intestinal epithelial cell line HT-29, we infected these cells by the Gram-negative pathogenic bacteria *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) strain C5, a bacteria known to induce IL-8 secretion by this cell line [48]. As expected, *S. Typhimurium* infection induced *IL-8* gene expression and triggered IL-8 secretion by HT-29 cells (**Figure 5A-B**). Pre-treatment of HT-29 cells with the *L. casei* strain BL23 was not sufficient to reduce this *Salmonella*-induced IL-8 production, however combination with cell fasting during infection was able to dramatically reduce IL-8 secretion by the infected cells (**Figure 5B**), without affecting mRNA expression level of the *IL-8* gene (**Figure 5A**). This result indicated that combination of *L. casei* and fasting act at post-transcriptional level (e.g., on protein synthesis or secretion) to reduce IL-8 release by infected cells. By contrast, in the same conditions, mRNA level of the gene encoding the IL-1β cytokine is

reduced by the combination of fasting and *L. casei* in comparison to cells left untreated or treated with *L. casei* alone (**Sup figure 3A**), suggesting that inflammatory mediators were not regulated in the same way by the combination of fasting/lactobacilli. Similarly to our previous observations in immune cells, moderate fasting states achieved by incubating IECs in medium containing decreasing percentage of DMEM (from 100% to 0% DMEM) also allowed to obtain interesting effects of the combination of reduced nutrients concentration and the presence of the *L. casei* strain BL23 on the IL-8 cytokine release induced by LPS (**Figure 5C**). This IEC cell line seemed to be more responsive to nutrient deprivation since a 50% reduction of DMEM is sufficient to strongly potentiate immunoregulatory properties of the *L. casei* strain BL23.

To gain molecular insights into how fasting state promoted the anti-inflammatory effects of lactobacilli on host cells, we hypothesized that stimulation of autophagy in host cells by fasting could promote anti-inflammatory effects as supported by an abundant literature [32, 49]. For this purpose, we treated HT-29 cells with two pharmacological inhibitors of autophagy: Bafilomycin A1 and Wortmannin, that block the process at late and early steps of the process, respectively [34]. Neither of them was able to inhibit the strong decrease in IL-8 secretion mediated by the fasting/*L. casei* combination, suggesting that autophagy is not involved in the present phenotype (**Figure 5D**). Alternatively to autophagy, studies have demonstrated that amino acid sensing can modulate inflammatory responses, especially in the context of the GIT, through the activation of the amino acid sensitive GCN2 kinase (General controlled non-repressed 2) [31]. As a result, an acute amino acid starvation is able to inhibit intestinal inflammation, notably through the suppression of inflammasome complexes activation. To investigate whether amino acid signaling could contribute to the immunoregulatory effects observed in our setting in immune and non-immune cells, we treated host cells with Halofuginone (HF). This drug mimics a deprivation in amino acids by inhibiting the prolyl-tRNA synthetase activity of the glutamyl-prolyl-tRNA synthetase, leading to the accumulation of uncharged tRNA (transfer RNA) [50]. This last event is a signal for cell indicating an amino acid insufficiency that trigger a particular integrated stress response called amino acid response (AAR) [51]. First, to check whether HF treatment of HT-29 cells induced the AAR (i.e., alerting cell of amino acid depletion), we measured the mRNA relative expression of two ATF4 (Activating Transcription Factor 4) target genes *CHOP* (C/EBP-homologous protein) and *GADD34* (Growth arrest and DNA damage-inducible protein). During amino acid deprivation, the transcription factor ATF4 is activated by the GCN2 kinase and increases the transcription of

its target genes, thus mRNA expression level of these genes are correlated with amino acid depletion sensed by the cell [52]. Treatment of cells for 4 h with HF (20 nM), and in a less extent EBSS treatment, was sufficient to induce a strong upregulation of both ATF4 target genes, indicating that HF has triggered AAR in HT-29-treated cells (**Figure 5E**). LPS-induced IL-8 secretion by HT-29 was significantly reduced in cells treated with 20 or 50 nM HF, suggesting that mimicking amino acid depletion is sufficient to recapitulate the immunomodulatory effects of fasting observed in our settings (**Figure 5F**). However, we were unable to observe a more important effect in combination with *L. casei* strain BL23. Similar results were obtained on the LPS-induced secretion of TNF- α by J774 A1 macrophages treated with HF (**Sup figure 3B-C**).

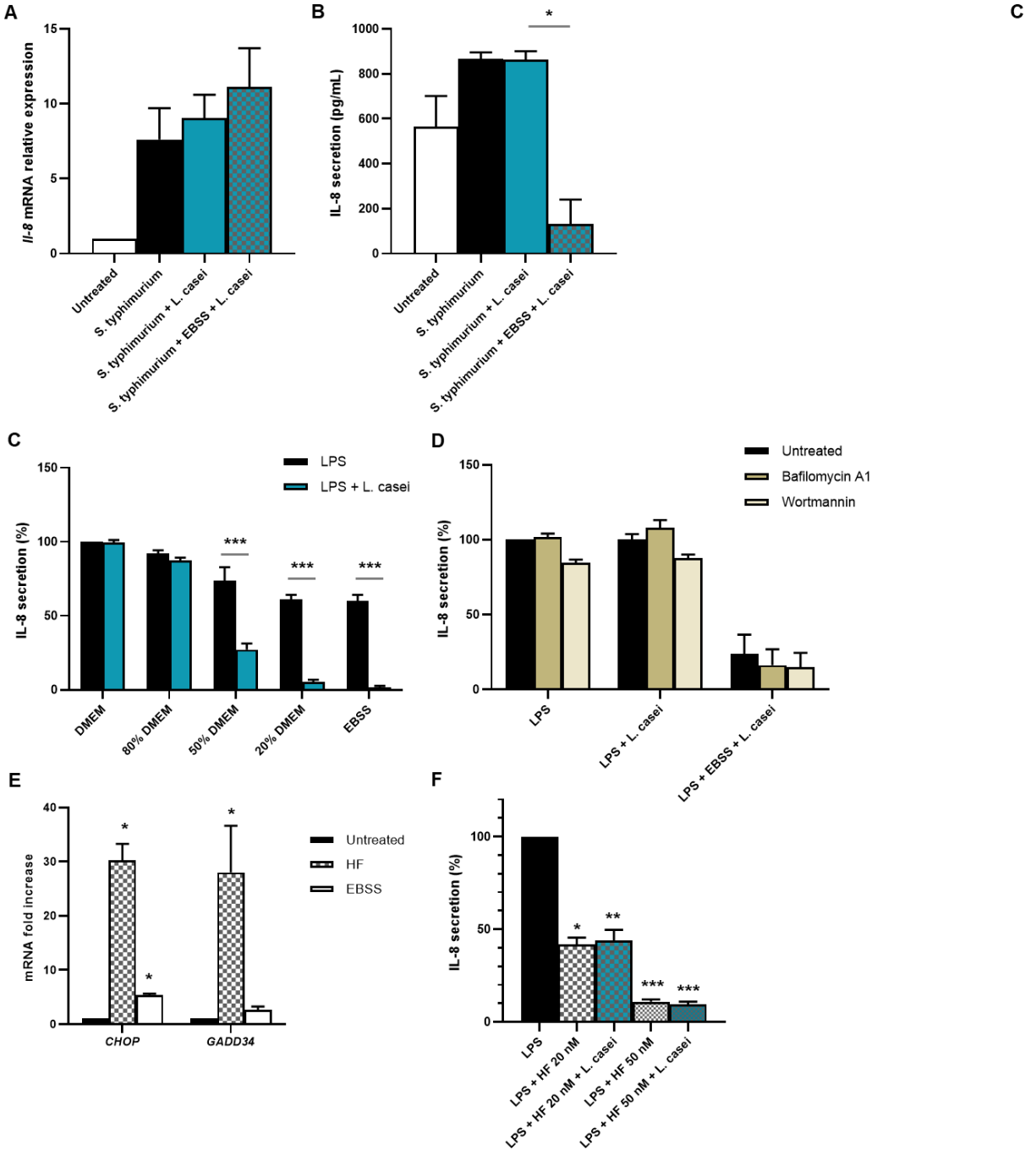


Figure 5: Host cell fasting state and lactobacilli were effective to dampen inflammatory response generated by intestinal epithelial cells.

(A-B) HT-29 cells were incubated in full medium and uninfected (untreated, white bars) or infected with *S. Typhimurium* in full medium in absence (black bars) or in presence of the *L. casei* strain BL23 (blue bars). Alternatively, infected cells and incubated with *L. casei* strain BL23 were also concomitantly incubated in EBSS (blue bars with grey squares for *L. casei* strain BL23). (A) The mRNA relative expression level of the pro-inflammatory

cytokines *Il8* was measured by RT-qPCR (untreated cells taken as 1). **(B)** IL-8 secretion was determined by ELISA. Results are expressed in pg/mL as mean \pm SEM of at least three independent experiments. * $p < 0.05$ (*versus* LPS). **(C)** HT-29 cells were incubated in full medium (DMEM) or in medium containing decreasing percentage of DMEM (from 80% DMEM to 0% DMEM, *i.e.*, EBSS) and stimulated with LPS alone (black bars) or with LPS in presence of *L. casei* strain BL23 (blue bars) for 6 h. IL-8 secretion was determined by ELISA. Results are expressed as percentage of IL-8 secretion \pm SEM of at least three independent experiments (LPS stimulated cells in DMEM taken as 100%). *** $p < 0.001$ (*versus* DMEM LPS). **(D)** HT-29 cells were untreated (black bars) or pre-treated with the autophagy inhibitors Bafilomycin A1 (100 nM, dark beige) or Wortmannin (100 nM, light beige). For each condition, cells were then stimulated with LPS (100 ng/mL) for 6 h in full medium in absence or in presence of the *L. casei* strain BL23. Alternatively, cells stimulated with LPS and incubated with *L. casei* strain BL23 were also concomitantly incubated in EBSS. IL-8 secretion was determined by ELISA. Results are expressed as percentage of IL-8 secretion \pm SEM of at least three independent experiments (LPS untreated cells taken as 100%). **(E)** HT-29 cells were untreated or incubated for 4 h with Halofuginone (HF, 20 nM, white with grey squares bars) or in EBSS (white bars). The mRNA relative expression level of the ATF4 target genes *CHOP* and *GADD34* were measured by RT-qPCR (untreated cells taken as 1). The graphs show the mean \pm SEM of at least three independent biological experiments using two replicates each. * $p < 0.05$ (*versus* untreated). **(F)** HT-29 cells were incubated in full medium and stimulated with LPS for 6 h in absence (black bar) or in presence of HF (20 nM, white bar with grey squares or 50 nM, white bar with smaller grey squares). These cells were also incubated with *L. casei* strain BL23 during LPS stimulation (blue bars). IL-8 secretion was determined by ELISA. Results are expressed as percentage of IL-8 secretion \pm SEM of at least three independent experiments (LPS stimulated cells taken as 100%). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (*versus* LPS).

Fasting protects mice against DSS-induced colitis but concomitant administration of *L. casei* strain BL23 does not potentiate this beneficial effect.

In order to assess *in vivo* whether fasting can potentiate the immunomodulatory effects of lactobacilli, we applied a feeding strategy that integrate periods of fasting between feeding windows based on a Isocaloric twice-a-day (ITAD) feeding strategy published recently by the Singh lab [30]. This dietary approach has been described to promote diverse metabolic benefits in various tissues, including a decrease in adiposity and an enhance muscle mass, and contributing to prevent age- and obesity-associated metabolic defects. However, the ability of this dietary intervention to modulate inflammatory response has never been investigated. C57BL/6J male mice were randomized between groups feeding *ad libitum* (food always available) and groups ITAD-fed for which food is available only during two feeding windows of 2 h: 8-10 h and 17-19 h (**Figure 6A**). As described in the literature, a 6 to 7 days period was necessary for the acclimation of mice

to ITAD feeding and after this period mice consumed almost the same amount of food than mice fed *ad libitum*, however a slight but significant reduction in the food intake was noticed for ITAD-fed mice in comparison to *ad libitum*-fed mice (**Figure 6B**). No difference was observed in the daily water intake between groups (**Figure 6C**). Despite the slight reduction in daily food intake of ITAD-fed mice, no significant differences were observed in the weight gain of mice between the two groups (**Figure 6D**). After a 17-day period of mice acclimation to ITAD feeding, a group of ITAD-fed mice and a group of *Ad-libitum*-fed mice received a daily administration (1×10^9 CFU in PBS) *per os* of the *L. casei* strain BL23 for 15 days (**Figure 6E**). After this 15-day prophylactic treatment with the *L. casei* strain, mice were subjected to an acute DSS- (Dextran Sodium Sulfate) induced colitis for 6 days by adding DSS at 2% in drinking water, followed by a 6-day recovery period (DSS removed from drinking water) (**Figure 6E**). This protocol is classically used to induce colitis in mice in order to reproduce some physiopathological features of human IBDs [53]. Thus, our experimental strategy was composed of the eight following mice groups (n=8 per group): **(i)** *Ad libitum*-fed, **(ii)** *Ad libitum*-fed + *L. casei*, **(iii)** *Ad libitum*-fed + DSS, **(iv)** *Ad libitum*-fed + *L. casei* + DSS, **(v)** ITAD-fed, **(vi)** ITAD-fed + *L. casei*, **(vii)** ITAD-fed + DSS and **(viii)** ITAD-fed + *L. casei* + DSS.

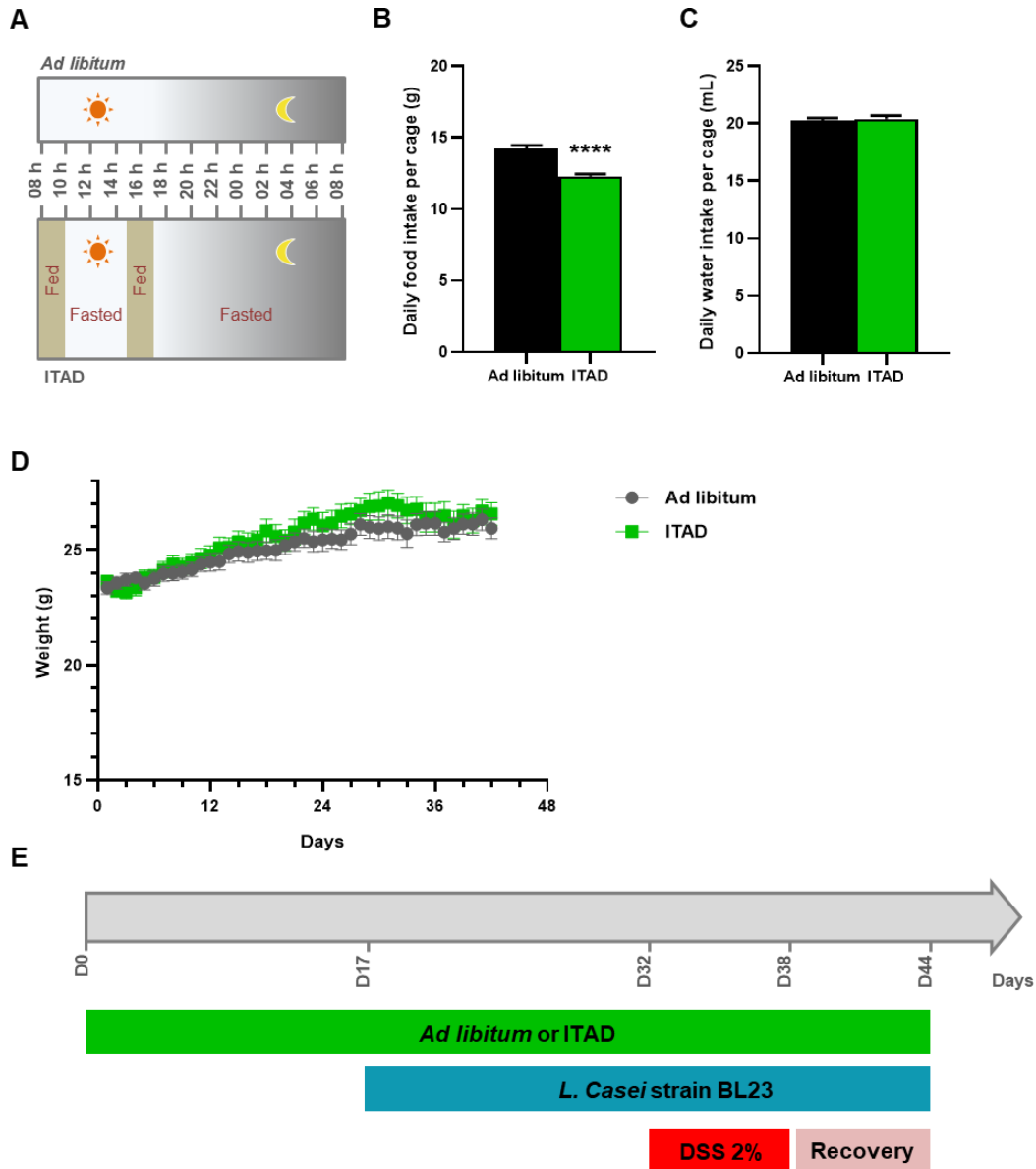


Figure 6: *In vivo* intermittent fasting in mice.

(A) The fasting strategy (isocaloric twice-a-day, ITAD) strategy in which mice were fed between 8 and 10 h and between 17 and 19 h, generating two periods of fasting over 24 h. Control mice were fed *ad libitum*, without restriction. (B) Measure of the daily food intake per cage, expressed in g, in *ad libitum*- (black bar, n=8) and ITAD-fed (green bar, n=8). **** p < 0.0001 (*versus ad libitum*) (C) Measure of the daily water intake per cage, expressed in mL, in *ad libitum*- (black bar, n=8) and ITAD-fed (green bar, n=8). (D) Weight curves of *ad libitum*- (grey curve, n=8) and ITAD-fed (green curve, n=8) mice over a 42-day period. The graphs show the mean weight per group expressed in g ± SEM. No significant differences were observed. (E) Experimental strategy based on the eight following mice groups (n=8 mice per group): (i) *Ad libitum*-fed, (ii) *Ad libitum*-fed + *L. casei*, (iii) *Ad libitum*-fed + DSS, (iv) *Ad libitum*-fed + *L. casei* + DSS, (v) ITAD-fed, (vi) ITAD-fed + *L. casei*, (vii) ITAD-fed + DSS and (viii) ITAD-fed + *L. casei* +

DSS. Administration of the *L. casei* strain (daily gavage at 1×10^9 CFU in PBS) started at day 17. Colitis induction by adding 2% DSS in drinking water started at day 32 for 6 days. Recovery period started at day 38 for 6 days. Mice were sacrificed at day 44.

During DSS-induced colitis, weight loss measurement is used as one of a hallmark of colitis severity [53]. Average daily weight loss was similar between the four colitis groups (*Ad libitum*-fed, *Ad libitum*-fed + *L. casei*, ITAD-fed, ITAD-fed + *L. casei*) during the 8th first days of the protocol (6 days 2% DSS + 2 recovery days), but a more pronounced weight decrease was observed for ITAD-fed mice receiving *L. casei* from day 9 to day 12 (only significant at day 12) in comparison to the other groups (**Figure 7A**). Of note, a slight significant decrease in weight loss was also notice for ITAD-fed mice in comparison to *Ad libitum*-fed mice at day 12. Thus, ITAD feeding did not permit to prevent DSS-induced weight loss and its combination with *L. casei* seemed to worsen the weight loss (**Figure 7A**). Evaluation of the disease activity index (DAI), combining score of stool consistency and bleeding, did not indicate significant differences between groups, but mice from the ITAD-fed group tended to have a lower DAI score (**Figure 7B**), suggesting a protective effect of the dietary intervention on this colitis parameter. Similarly to human IBDs, DSS-induced colitis was usually associated with an increased intestinal permeability, correlated with colitis severity [54]. Intestinal permeability assay using FITC-Dextran indicated that *L. casei* administration as well as ITAD feeding, or combination of both, were able to decrease intestinal permeability in comparison to ad libitum-fed control, suggesting that these interventions are beneficial against colitis (**Figure 7C**). Next, we assessed myeloperoxidase (MPO) activity on colon section. The MPO enzyme is abundantly produced by neutrophils and measure of its activity on colon section is a reliable approximation of neutrophil presence and therefore indicate inflammatory status of the tissue [54]. In both ITAD-fed mice groups (in presence or absence of *L. casei*) colonic MPO activity was similar to those measured in control mice without DSS, whereas in DSS-treated *ad libitum*-fed mice MPO activity was increased (**Figure 7D**). Finally measure of colon length retraction and mass loss of epididymal adipose tissue, two additional markers of colitis severity, did not reveal any significant differences between the four groups (**Figure 7E-F**). Altogether these results suggest that ITAD feeding seemed to confer protective effects on some key colitis parameters including stool consistency, bleeding, intestinal permeability restoration and limitation of neutrophils infiltration, however further experiments will be necessary to better characterize molecular effects of ITAD on colitis development. By

contrast to *in vitro* results combination of fasting (ITAD feeding) and lactobacilli did not confer superior beneficial effects than *L. casei* or ITAD alone.

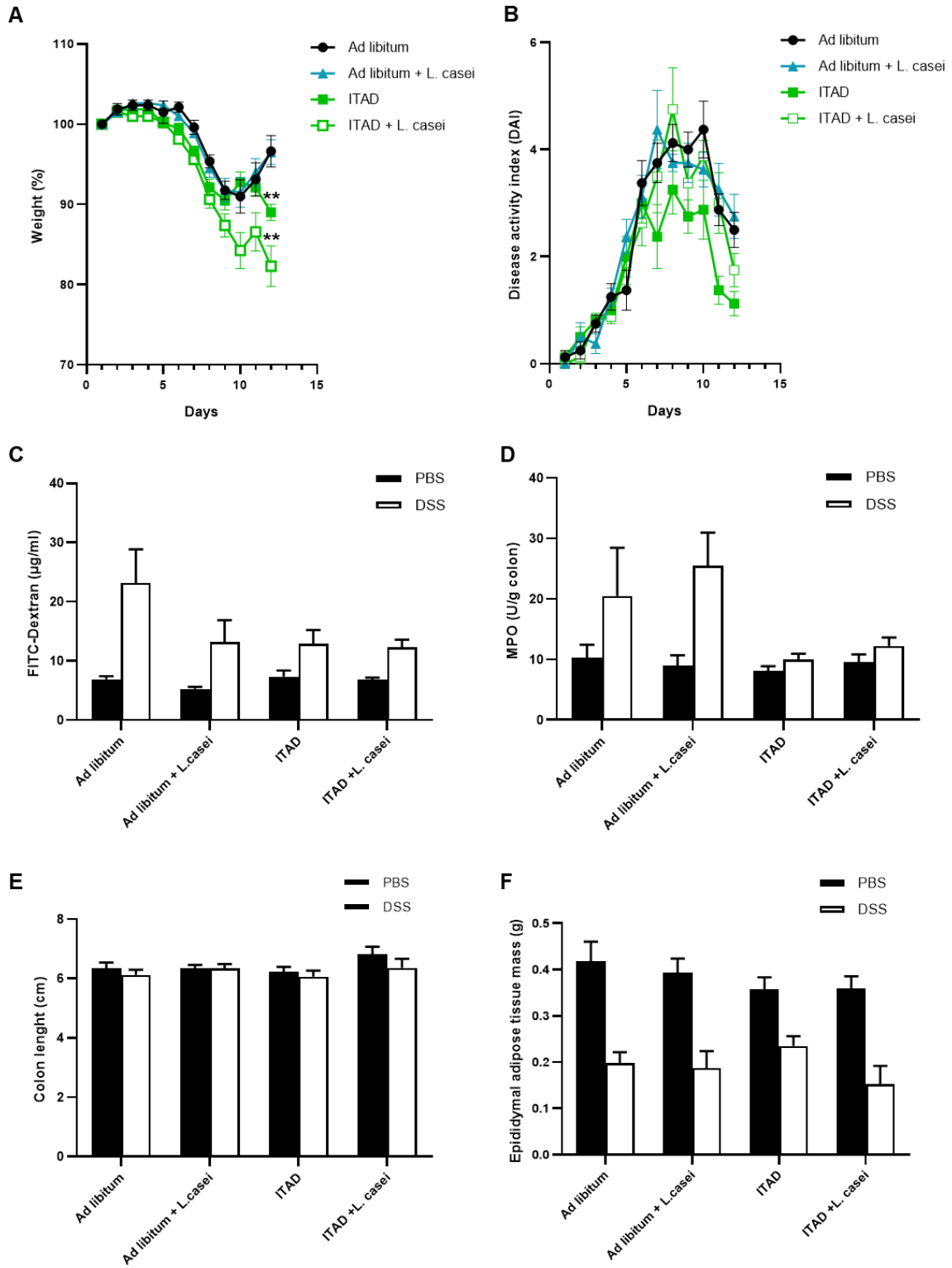


Figure 7: Fasting protects mice against DSS-induced colitis but concomitant administration of *L. casei* strain BL23 does not potentiate this beneficial effect.

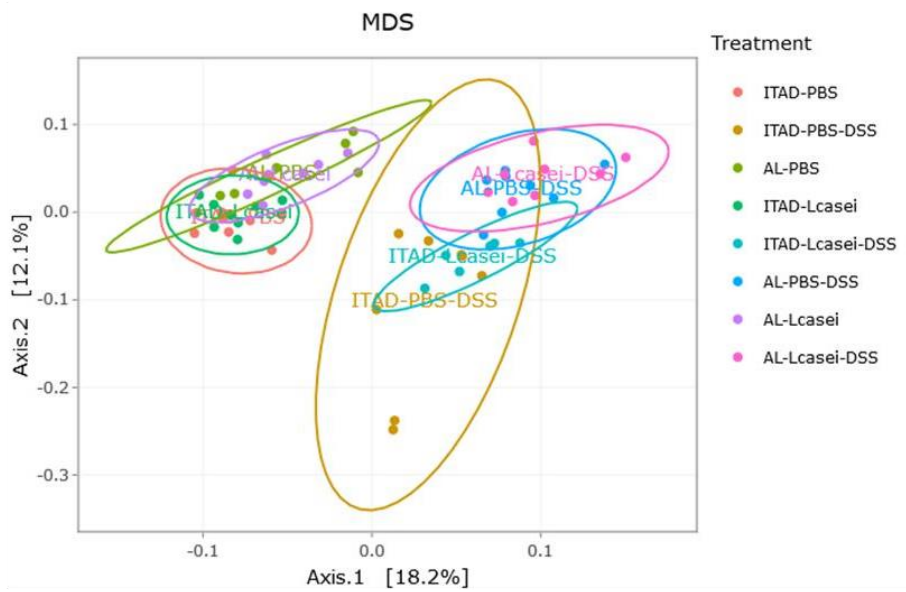
(A-B) Mice were fed *ad libitum* (black curves) or submitted to intermittent fasting (ITAD, green curve with filled square), and eventually orally administered with *L. casei* strain BL23 (blue curve for *ad libitum* mice and green curve with empty square for ITAD mice) 15 days before and during colitis induction. Colitis was induced by adding 2% DSS in drinking water for 6 days (D0-D6). Then, DSS was removed from drinking water and mice allowed to recover for 6 days (D6-D12). (A) Weight (initial weight taken as 100%, n=8 per group) and (B) DAI score including stool consistency and bleeding were monitored daily (n=8 per group). ** $p < 0.01$ (*versus ad libitum*). (C) FITC-Dextran levels in hemolysis-free serum, expressed as mean \pm SEM in $\mu\text{g/mL}$ from indicated mice groups, with DSS-induced colitis (white bars) or not (black bars) (n=8 per group). (D) Myeloperoxidase (MPO) activity measured on colon section and expressed as mean \pm SEM in U/g of colon. Data from the indicated mice groups, with DSS-induced colitis (white bars) or not (black bars) (n=8 per group). (E) Measurement of colon length (in cm) and (F) epididymal adipose tissue mass (in g) in the indicated mice groups, with DSS-induced colitis (white bars) or not (black bars) (n=8 per group).

Fasting, or its combination with *L. casei*, does not allow to counteract DSS-induced gut microbiome dysbiosis.

In patients with IBD, there are severe alterations in the structure and the composition of the gut microbiome. The common changes observed include: a reduced microbial diversity (α -diversity), an increased variability between individuals (β -diversity) and an increase in the facultative anaerobes (e.g. *Escherichia coli*) at the expense of obligate anaerobic (notably short-chain fatty acids (SCFAs) producers from the Ruminococcaceae and Lachnospiraceae families) [55, 56]. Similar alterations are often reported in the gut microbiome of mice challenged with a DSS-induced colitis [57, 58]. Since diet is a major driver shaping the gut microbiome composition [59], we investigated whether ITAD feeding (in combination or not with *L. casei*) impact gut microbiota composition in a basal state and whether this dietary intervention could limit DSS colitis-induced gut microbiome dysbiosis. For this purpose, we analyzed the fecal microbiome composition of the mice from the eight groups using 16S rRNA gene sequencing (Illumina sequencing technology). First, we measure the β -diversity between the 8 groups using multidimensional scaling analysis (MDS) of unweighted Unifrac distances (**Figure 8A**). This analysis revealed a significant clustering of samples according principally to the occurrence of the DSS colitis (17% of the variability, permanova, $p < 0.0001$) and secondary to the ITAD feeding (8.72% of the variability, permanova, $p < 0.0001$). Administration of the *L. casei* strain BL23 only accounted for 2.76% of

the variability (permanova, $p < 0.0045$). In good accordance with the literature [57, 58], the fecal microbiome of mice challenging with the DSS colitis (AL-PBS-DSS, AL-Lcasei-DSS, ITAD-PBS-DSS and ITAD-L.casei-DSS) exhibited a reduced α -diversity, as indicated by a decrease in the number of OTU observed and a decrease in the diversity indexes Chao1, Shannon and InvSimpson (**Figure 8B**). This decrease is particularly significant in the Ad libitum group (observed: $p = 4.45 \times 10^{-5}$, Chao1: $p = 4.23 \times 10^{-5}$, Shannon: $p = 0.00011$) and less significant in the ITAD-fed group (only Chao1: $p = 0.0261$) (**Figure 8B**). In basal condition, ITAD feeding tended to reduce α -diversity in comparison to *Ad libitum*-fed mice (Shannon: $p = 0.00385$ and InvSimpson: $p = 0.0305$), however no significant differences were observed upon DSS colitis between these two groups. Altogether these results shown that DSS colitis reduced diversity of the gut microbiome and this DSS-induced decrease is less pronounced in ITAD-fed mice, however these mice already displayed a basal lower bacteria diversity in their gut microbiome.

A



B

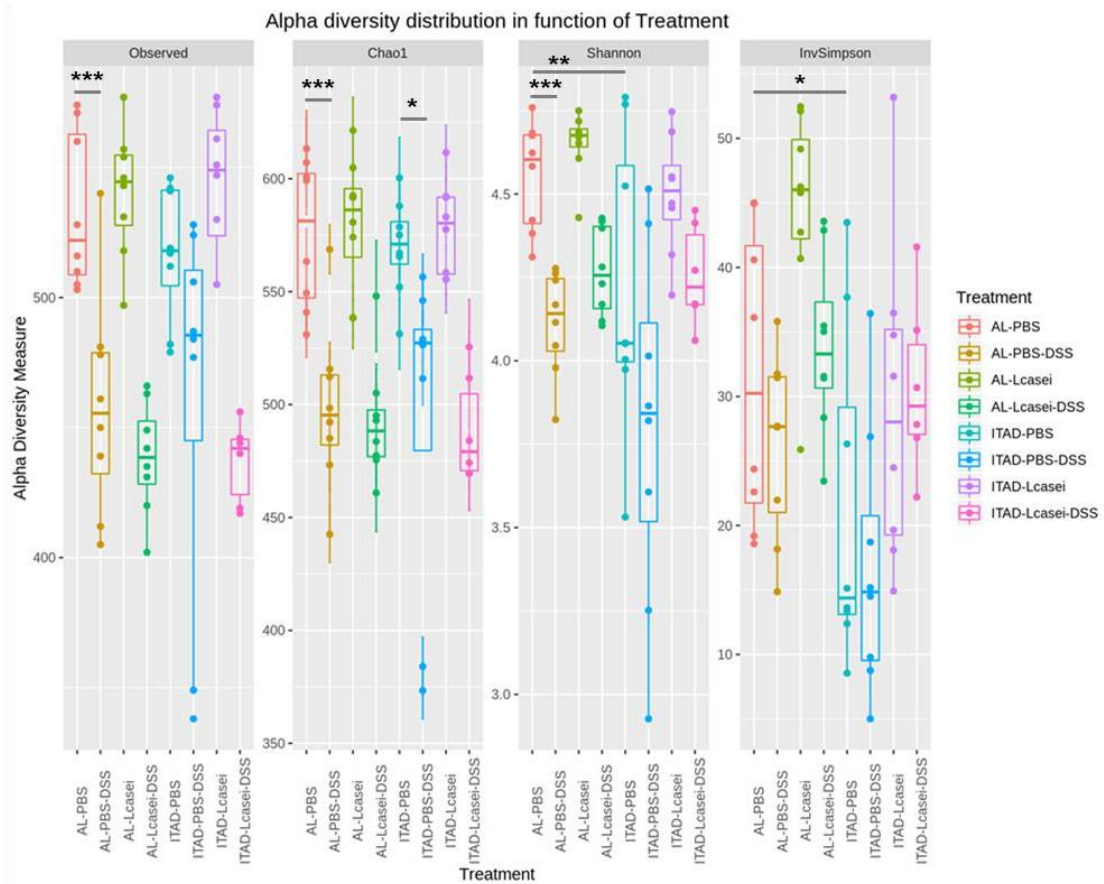


Figure 8: Effects of DSS-induced colitis, fasting and *L. casei* administration on α - and β -diversity of the gut microbiome.

(A) Analysis of β -diversity. Multidimensional scaling (MDS) plot of unweighted UniFrac distances showing distribution of fecal microbiome of the different mice groups (AL: *Ad libitum* and ITAD: Intermittent fasting). Fecal microbiome of mice submitted to the DSS colitis clustered together (17% of the variability, permanova, $p < 0.0001$) and then clustered dependently of the feeding mode (*Ad libitum* versus ITAD, 8.72% of the variability, permanova, $p < 0.0001$). (B) Analysis of α -diversity. Observed OTUs, Chao1, Shannon and InvSimpson indices of mice from the different groups (AL: *Ad libitum* and ITAD: Intermittent fasting) (ANOVA, significant results were only notified for *Ad libitum*/ITAD, *Ad libitum*/*Ad libitum*-DSS and ITAD/ITAD-DSS comparisons, * $p < 0.05$; ** $p < 0.01$, *** $p < 0.01$).

Gut microbiome analysis at the genus level revealed bacteria genera that were differentially abundant (defined as log₂-fold change > 1 or < 1) between *Ad libitum* group (AL-PBS) and ITAD group (ITAD-PBS) under basal condition (**Figure 9A**). These bacteria belonged mainly to the phyla Bacteroidetes and Firmicutes. Among the genera significantly less represented in ITAD-fed mice, compared to *ad libitum* mice, we mainly found bacteria from the genus *Turicibacter* (log₂-fold = -8,30, $p = 1,29 \times 10^{-15}$), *Lachnoclostridium* (log₂-fold = -3,96, $p = 1,58 \times 10^{-3}$), *Allistipes* (log₂-fold = -3,28, $p = 3,06 \times 10^{-3}$), *Colidextribacter* (log₂-fold = -2,99, $p = 1,43 \times 10^{-4}$), *Prevotellaceae* UCG-001 (log₂-fold = -2,13, $p = 7,87 \times 10^{-8}$) and *Odoribacter* (log₂-fold = -1,95, $p = 4,65 \times 10^{-6}$). By contrast, ITAD feeding strongly promoted the abundance of bacteria belonging to the genus *Lachnospiraceae* (NK4A136 group, log₂-fold = 39,21, $p = 1,95 \times 10^{-91}$) and in a less extent bacteria from the genera *Dubosiella* (log₂-fold = 6,19, $p = 8,75 \times 10^{-3}$), *Lactobacillus* (log₂-fold = 6,04, $p = 6,91 \times 10^{-4}$), *Fusobacterium* (log₂-fold = 3,70, $p = 1,03 \times 10^{-3}$), *Enterorhabdus* (log₂-fold = 2,28, $p = 1,05 \times 10^{-3}$) and *Parasutterella* (log₂-fold = 1,66, $p = 7,37 \times 10^{-4}$). Differential bacteria abundance observed between PBS groups and DSS colitis groups indicated that ITAD feeding tended to increase the number of OTUs for which DSS colitis affected their abundance (positively or negatively) (**Figure 9B-C**). In *ad libitum* feeding mice, DSS colitis induced significant decrease in the abundance of bacteria from 112 OTUs, while increasing the abundance of 44 OTUs (**Figure 9B**). Number of OTUs modified was slightly higher in ITAD feeding mice with a significant decrease in the abundance of bacteria from 120 OTUs and an increase in the abundance of 52 OTUs upon DSS treatment (**Figure 9C**). In both feeding groups, the bacteria belonging to the genus *Lachnospiraceae* were the most reduced in abundance upon DSS colitis (*ad libitum*: log₂-fold = -10,51, $p = 2,97 \times 10^{-24}$ and ITAD: log₂-fold = -8,88, $p = 4,23 \times 10^{-8}$). In basal condition, administration of *L. casei* strain BL23 only modified the abundance of a very few numbers of genera in *ad libitum*- and ITAD-fed mice (**Sup figure 4A-B**), however upon DSS-induced colitis, administration of *L. casei* modified the abundance of a greater number of bacteria, especially in

ITAD-fed mice (decrease abundance of 65 OTUs and increase abundance of 35 OTUs) compared to *Ad libitum*-fed mice (decrease abundance of 9 OTUs and increase abundance of 17 OTUs) (**Sup figure 4C-D**). Altogether these results indicated that ITAD feeding could modulate diversity and relative abundance of various bacterial groups. These modifications of the gut microbiome could participate to the beneficial effects of ITAD feeding during DSS-induced colitis. Interestingly, during colitis, ITAD feeding allowed that *L. casei* administration displayed more important effects on the gut microbiome composition, suggesting that combining fasting with lactobacilli administration might confer deeper effects on the resident microbiota.

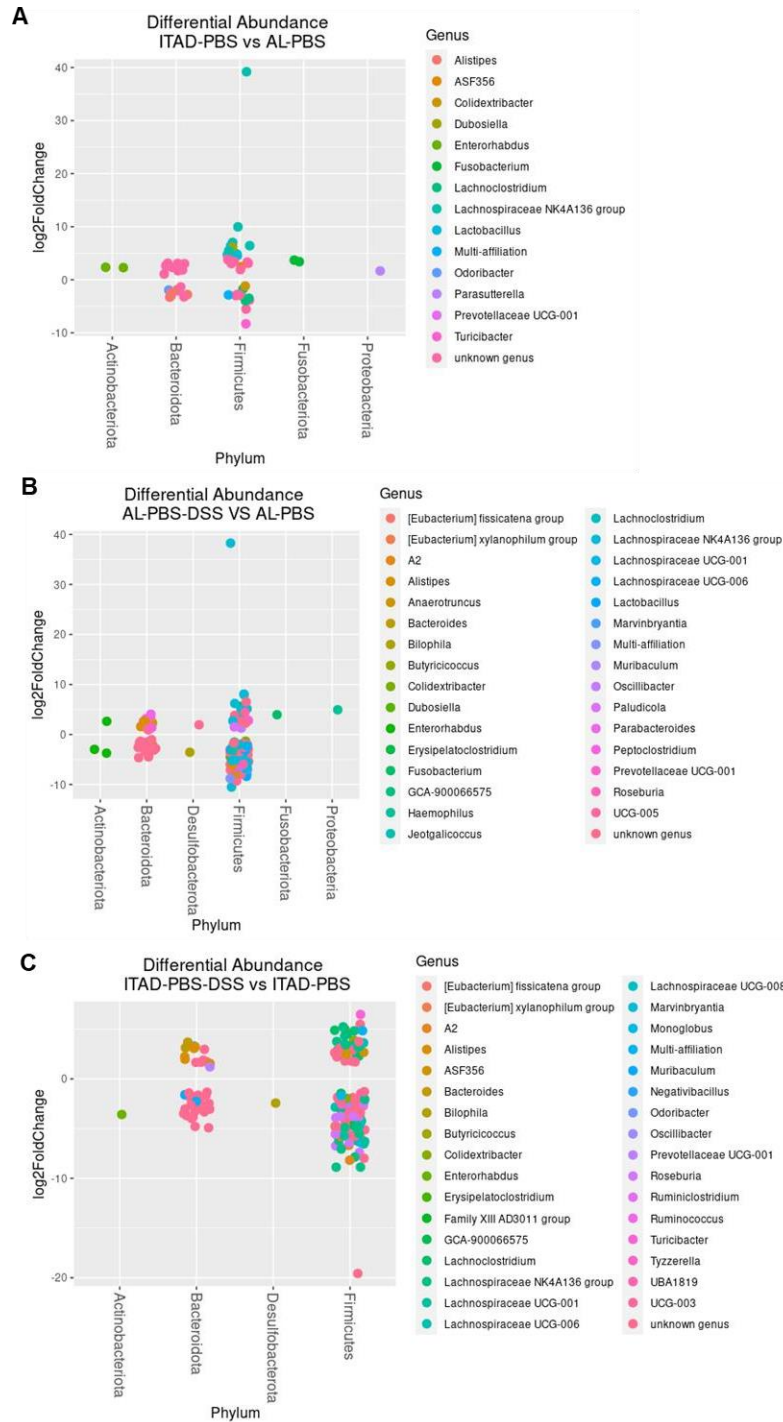


Figure 9: Bacteria genera significantly affected by ITAD feeding or DSS colitis.

(A-C) Log₂-fold change in abundance of OTUs associated with (A) ITAD feeding, (B) DSS colitis in *ad libitum* mice and (C) DSS colitis in ITAD mice. Key OTUs were identified by analysis with DESeq2 differential abundance analysis. For each analysis, genera are assigned to their corresponding phyla.

DISCUSSION

An increasing body of literature sheds on light the connections existing between metabolic pathways and immune signaling, contributing to the emerging research field called immunometabolism [22]. As a consequence, we could assume that qualitative, quantitative and temporal aspects of a diet can impact immune responses of an organism. Diet also shapes the composition and structuration of the resident gut microbiota, that reciprocally can modulate nutrients and secondary metabolites availability for the host [60]. The gut microbiota, by modulating nutrients availability and by stimulating directly the gut-associated immune system and intestinal epithelial cells plays a key role in the immune homeostasis of the GIT and contributes to its responsiveness during infections or non-infectious diseases [61]. Many human diseases, including IBDs, such as Crohn's disease or Ulcerative colitis, are associated with alteration of the equilibrium between the gut microbiota, the immune system and the GIT environment, leading to the deregulation of multiple key hosts signaling pathways [62]. Several dietary or therapeutic interventions have been tested to restore this equilibrium in the context of IBDs [63], however further investigations are needed to better understand in more detailed their molecular mechanisms and thereby improving their efficiency.

In this study, we investigated in which extent the immunomodulatory properties of lactobacilli could be potentiated by fasting *in vitro* using intestinal epithelial cell and macrophage cell lines and *in vivo* using an intermittent fasting model (ITAD) in mice. We demonstrated that fasting allowed to dampen LPS-induced pro-inflammatory response in two macrophage cell lines (Raw 264.7 and J774 A1) as shown notably by the reduction of expression and secretion of the prototypical pro-inflammatory cytokines IL1- β and TNF- α . These results were confirmed in the intestinal epithelial cell line HT-29, challenged by *Salmonella* Typhimurium infection or by LPS, in which fasting can reduce the expression and secretion of the pro-inflammatory cytokine IL-8. Interestingly, combining fasting of host cells (macrophages or IECs) with lactobacilli treatment (*L. paracasei*, *L. casei* and *L. rhamnosus*) results in most cases in a synergistic effect of both interventions, reducing more importantly the pro-inflammatory responses induced by LPS or the Gram-negative pathogen *S. Typhimurium*. Mimicking amino acid deprivation, by treating cells with the glutamyl-prolyl-tRNA synthetase inhibitor Halofuginone is sufficient to recapitulate the

effects of total cell starvation in EBSS minimal medium, suggesting that amino acid signaling plays a crucial role in this immunoregulatory effects of fasting. Indeed, key regulators of amino acid sensing in host cell have been linked to the modulation of inflammatory processes. A striking example is the GCN2 kinase. This kinase is a sensor belonging to the cellular integrated stress response (ISR), an evolutionary conserved mechanism allowing cells to cope with various stress, including amino acid starvation [31, 64]. Upon amino acid deprivation, GCN2 kinase is activated and phosphorylates eukaryotic initiation factor 2 α (eIF2 α), leading to global modifications of the cellular translation program, including preferential translation of some key genes such as the transcription factor ATF4 [65]. Phosphorylated eIF2 α is detected in many cellular contexts *in vitro* and *in vivo*, notably in intestinal dendritic cells, macrophages and epithelial cells under basal and inflammatory conditions [31], suggesting a crucial role for this signaling pathway in regulating inflammatory responses. In addition, mice knock-out for *Gcn2* in immune cells or in intestinal epithelial cells present an enhanced intestinal inflammation due to an uncontrolled activation of inflammasome complexes [31]. In our study, stimulation of the GCN2/eIF2 α /ATF4 pathway, through Halofuginone treatment, results in a marked decrease in the secretion of pro-inflammatory cytokines in intestinal epithelial cells and macrophages. Stimulation of this pathway is usually associated with stimulation of the autophagy pathway since some autophagy-related genes are ATF4 target genes [65]. In our study, autophagy does not seem to be involved since pharmacological inhibition of the process at early or late steps does not affect fasting immunoregulatory effects. Further study will be necessary to decipher the exact mechanism by which fasting in combination with lactobacilli can reduce pro-inflammatory responses. Use of culture medium depleted for single essential amino acids, known to be involved in the modulation of inflammatory responses, such as Tryptophan or Leucine, might be of interest to gain molecular insights [66, 67]. A deeper investigation on the activation status of the main signaling pathways involved in immunometabolic signaling (PI3K, mTOR and AMPK signaling pathways) might also be helpful to better characterize the combined beneficial effects of fasting/lactobacilli on inflammatory response [23]. For example, various TLR ligands (e.g. Pam3CSK4, Poly I:C or LPS) are able to stimulate the master metabolic regulator mTor [68], thus lactobacilli strains, by stimulating TLR through their surface-associated molecules might act on mTOR signaling, as suggested by a recent study [69].

Ability of probiotic strains to stimulate host signaling is usually a strain-dependent feature, depending on multiple factors and currently difficult to predict without experimental assays [14, 15]. In the present study, immunoregulatory properties of the three strains tested (*L. casei*, *L. paracasei* and *L. rhamnosus*) can be potentiated by host cell fasting, suggesting an effect conserved throughout strains. However, former *Lactobacillus* genus is a very heterogeneous bacterial group (now split into 25 new genera [12]) and many other probiotic strains belong to other genera (*Bifidobacterium*, *Bacillus* or *Streptococcus*) or to the fungal kingdom (*Saccharomyces*), thus interesting effects of probiotic combination with host cell fasting could be assessed for additional bacterial or fungal species to assess whether this beneficial effect is also observed for these species.

We tried to validate this phenomenon *in vivo* by applying an intermittent fasting protocol (ITAD) in mice [30]. This protocol has been previously described to promote metabolic benefits in various tissues, but its ability to modulate inflammatory response has never been assessed. In our study, ITAD feeding protects partially mice against DSS-induced colitis, notably by reducing intestinal permeability and neutrophils recruitment. The main advantage of this protocol is that fasting periods are relatively short in comparison to other published protocols that proposed fasting periods lasting 24h, with potential adverse effects [28, 70]. In human, an approaching protocol based on a diurnal intermittent fasting (applied during the Ramadan) with two meals per day led to significant decreases in plasma concentrations of the pro-inflammatory cytokines IL-1 β , IL-6, and IL-8 [71], however some discrepancies exist in the literature, mainly because of the heterogeneity of the protocols used [72]. By contrast with *in vitro* results, combination of ITAD with the *L. casei* strain BL23 does not result in a stronger protective effect. Further experiments, using alternative probiotic strains or temporal changes in the administration of the probiotics and/or feeding windows might be helpful to explore the potential benefits of this combined dietary approach *in vivo*. Interestingly ITAD or ITAD combination with *L. casei* administration impacts the gut microbiome composition in different ways, suggesting that these interventions (alone or combined) might be effective to modulate dysbiosis occurring during disease states, such as IBDs [55, 56]. In our study, *L. casei* strain BL23 is poorly able to modulate the gut microbiome composition during DSS-induced colitis in *ad libitum*-fed mice, while its administration in DSS-treated ITAD-fed mice led to more important changes in the gut microbiome composition, suggesting that ITAD feeding might create windows of opportunity in the gut microbiome to modify its composition with the final aim to restore its equilibrium.

To conclude, in this study we demonstrated *in vitro* and *in vivo* that fasting combined to lactobacilli administration could have potential beneficial effects on the regulation of immune responses (*in vitro*) or on the composition of the gut microbiome (*in vivo*). Further investigations are now necessary to gain molecular insights in these effects and to improve the efficiency of such interventions *in vivo* in the context of inflammatory diseases.

MATERIAL AND METHODS

Bacterial strains and cell culture.

L. paracasei ATCC3334, *L. casei* BL23 and *L. rhamnosus* GG strains were grown anaerobically without shaking at 37°C in Man-Rogosa-Sharpe medium (MRS; Condalab) pH 5.8 (adjusted with acetic acid). *Salmonella enterica* serovar Typhimurium strain C5 was grown in Tryptic Soy broth overnight at 37°C, under agitation.

The murine macrophages RAW 264.7 was cultured in RPMI medium 1640 (Gibco) supplemented with 10% fetal bovine serum and 1% antibiotics (Penicillin/streptomycin). The murine macrophage cell line J774A.1 and the human intestinal epithelial cell line HT-29 were cultured in Dulbecco's Modified Eagle Medium (DMEM) GlutaMAX supplemented with 10% fetal bovine serum (Eurobio) and 1% antibiotics (Penicillin/streptomycin; Eurobio). Cells were maintained in an atmosphere containing 5% CO₂ at 37°C. For experiments, cells were seeded in their corresponding medium without antibiotics. All cell lines were obtained from ATCC and have been routinely tested for mycoplasma contamination using the PCR Mycoplasma Test Kit II (PromoKine).

Antibodies and reagents.

For immunofluorescence experiments the following antibodies were used: rabbit polyclonal anti-LC3B (7543, Sigma) and mouse monoclonal anti-WIPI 2 (2A2, Millipore). Fluorescent secondary antibodies: Alexa Fluor-conjugated anti-rabbit IgG-568 and Alexa Fluor-conjugated anti-mouse IgG-488 were purchased from Invitrogen. Bafilomycin A1 (100 nM) and Wortmannin (100 nM) were purchased from LC laboratories. Halofuginone was purchased from Sigma.

LPS-induced inflammatory response and *Salmonella* infection.

HT-29, J774-A1 and Raw 264.7 cells were seeded 48 h prior LPS stimulation. When indicated, cells were treated 30 min before LPS stimulation by lactobacilli strains at a multiplicity of infection

(MOI) of 40. Fasting state was induced by washing cells two times in PBS and incubated cells in fresh EBSS (Sigma, or EBSS containing various percentage of DMEM as indicated). LPS stimulation was achieved by adding LPS from *Escherichia coli* O127:B8 (Sigma) at a final concentration of 100 ng/mL for 6 h.

Fluorescence microscopy

Cells were fixed for 10 min with PBS-4 % paraformaldehyde (PFA) and permeabilized and saturated for 20 min in PBS-10% FBS-0.1% saponin. Cells were incubated at room temperature for 2 h with the indicated primary antibodies and 1 h incubation with Alexa-fluor conjugated secondary antibodies and DAPI. Images were acquired using fluorescent microscope (Axiovision Zeiss). The number of LC3 or WIPI2 dots per cell were counted in at least 100 cells for each experiment. Each microscopy image is representative of at least three independent experiments.

ELISA

All samples were analyzed in duplicate. After cell treatments, cell supernatants were collected and frozen at -80°C until further analysis. IL-8 (for HT-29 cells), TNF- α (for Raw 264.7 and J774-A1 cells) and IL1- β (for J774-A1 cells) concentration in the supernatant were determined by an enzyme-linked immunosorbent assay (ELISA) (Biolegends, San Diego, CA) following manufacturer's instructions.

RNA isolation and qPCR analysis

Total RNAs were extracted using TRI reagent (Sigma). cDNAs were generated from 1 μg total RNAs using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The mRNA levels were determined by quantitative real-time PCR analysis using iTaq universal SYBR Green supermix (Bio-rad) and the primer sets listed in **Table 1** below. ΔCt values were calculated using the Ct values from the amplification of endogenous hypoxanthine-guanine phosphoribosyltransferase (Hprt) mRNAs. Quantitative real-time PCR was performed on the CFX96 PCR system (Bio-rad).

Table 1

| Species | Target mRNA | Forward primers | Reverse primers |
|---------------------|--------------------------------|-------------------------|------------------------|
| <i>Mus musculus</i> | <i>Tnf-α</i> | GGTGCCTATGTCTCAGCCTC | GCTCCTCCACTTGGTGGTTT |
| | <i>Il1-β</i> | GCCACCTTTTGACAGTGATGAG | GACAGCCCAGGTCAAAGGTT |
| | <i>Il-10</i> | TAAGTGCACCCACTTCCCAG | AAGGCTTGGCAACCCAAGTA |
| | <i>Ccl-3</i> | CGTGGAATCTTCCGGCTGTA | TACAAGCAGCAGCGAGTACC |
| | <i>Ccl-4</i> | TTCTGTGCTCCAGGGTTCTC | CTCACTGGGGTTAGCACAGA |
| | <i>Mcp1</i> | CTGCTGTTCACAGTTGCCG | GCACAGACCTCTCTTTGAGC |
| | <i>Atg16l1</i> | CGAATCTGGACTGTGGATGA | AGCAGGAACTTGGCAGAGAG |
| | <i>Wip1</i> | CTGCTTCTCTTCAACCAAGACT | ACGTCAGGGATTTCAATTGCTT |
| | <i>Becn1</i> | GGAAAAGAACC GCAAGGTGGTG | AAACTGTCCGCTGTGCCAGATG |
| | <i>P62</i> | GAGGCACCCCGAAACATGG | ACTTATAGCGAGTCCCACCA |
| | <i>Gadd34</i> | GACTCAAGCCAGAGTCCCTG | TAGAGGAATCTCGGGGTCTT |
| | <i>Atf3</i> | CCAGGTCTCTGCCTCAGAAG | CATCTCCAGGGGTCTGTTGT |
| | <i>Chop</i> | CCTAGCTTGGCTGACAGAGG | CTGCTCCTTCTCCTTCATGC |
| | <i>Hprt</i> | CAGTCCCAGCGTCGTGATTA | TGGCCTCCCATCTCCTTCAT |
| <i>Homo sapiens</i> | <i>IL-8</i> | TCCTGATTTCTGCAGCTCTGT | CCAGACAGAGCTCTCTTCCA |
| | <i>IL1-β</i> | GCCAATCTTCATTGCTCAAGTGT | GGTCGGAGATTCGTAGCTGG |
| | <i>CHOP</i> | AGCTGTGCCACTTTCCTTTC | CAGAACCAGCAGAGGTCACA |
| | <i>GADD34</i> | CTGTGATCGTTCTGGCA | GGAAGAAAGGGTGGGCATC |
| | <i>HPRT</i> | TTGCTTTCCTTGGTCAGGCA | ATCCAACACTTCGTGGGGTC |

Mice and fasting protocol in mice

Specific pathogen-free C57BL/6 mice (6–8 weeks old; Janvier, France) were maintained under normal husbandry conditions in the animal facilities of the National Institute of Agricultural Research (UEIERP, INRA, Jouy-en-Josas, France). All animal experiments began after 1 week of acclimation and were performed according to European Community rules of animal care and with authorization of the French Veterinary Services.

Fasting protocol was based on the Isocaloric Twice-a-Day Feeding (ITAD) protocol previously described [30]. Briefly, ITAD-fed mice were fed with the same amount of food as *ad libitum* mice but only during two 2 h windows (8-10 h and 17-19h). Both groups were group housed and

composed of age-matched male mice. After the feeding windows, residual food pellets, if present, were carefully collected and weighed at the end to evaluate daily food intake.

Induction of acute colitis and *L. casei* BL23 strain administration

The protocol of Dextran Sodium Sulfate (DSS)-induced acute realized was based on previous protocols [73]. Prior to colitis induction, mice were fed with 1×10^9 CFU in 200 μ l of *L. casei* strain BL23 or PBS alone daily for 15 days. At D0 colitis was induced by adding 2% (w/v) of DSS of a molecular weight of 36,000–50,000 kDa (MPBio) to the drinking water for 6 days. The mice were sacrificed at D12 (DSS recovery) after the DSS induction. For the recovery phase, DSS colitis induction was followed by 6 days of recovery with normal drinking water. Mice were monitored daily for weight loss, stool consistency, and fecal occult blood (Hemocult, Beckman Coulter). Disease Activity Index (DAI) has been calculated according to the protocol established by Cooper and colleagues [74]. Mice have been sacrificed by cervical dislocation.

Intestinal permeability *in vivo*

Intestinal permeability *in vivo* was assessed using fluorescein isothiocyanate-conjugated dextran (FITC–dextran 3000–5000 Da, Sigma–Aldrich) tracer as previously described [75]. Briefly, at the endpoint 0.6 mg/g body weight of FITC–dextran dissolved in PBS was administered to mice by oral gavage. To measure the presence of FITC–dextran in blood, 3.5 h after the gavage blood samples were recovered from the retro-orbital venous plexus and kept in dark at 4°C until analysis. Serum was separated by centrifugation and plasma FITC levels were determined using a fluorescence microplate reader (excitation 485 nm and emission 530 nm; Tecan, Lyon, France).

MPO activity.

MPO activity was measured using the method of Bradley et al. [76], modified as follows. Tissue samples were homogenized (50 mg/ml) in ice-cold 50-mM potassium phosphate buffer (pH 6) containing 5% hexadecyl trimethyl ammonium bromide (Sigma-Aldrich) and hydrogen peroxide. MPO is expressed in units per milligram of colon tissue.

Statistical analysis

Data are presented as the mean \pm standard error of the mean (SEM). Statistical analyses were performed with GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA). The non-parametric Mann and Whitney test was used to compare results between conditions. The p -values ≤ 0.05 were considered as statistically significant.

DNA extraction

A modified version of the protocol by Godon et al. [77] was used for DNA extraction. For each animal, 200 mg of frozen fecal sample were resuspended with a mix of 250 μ l of guanidine thiocyanate buffer (4 M guanidine thiocyanate 0.1 M Tris [pH 7.5] and 40 μ l of 10% N-lauroyl sarcosine 0.1 M phosphate buffer [pH 8.0]) and 500 μ l of 5% N-lauroyl sarcosine, and incubated 1 h at 70°C. One volume (750 μ l) of 0.1 mm diameter silica beads (Sigma) was added, and tubes were shaken 10 min at maximum speed of a Vibrobroyeur MM200 (Retsch, Germany). Tubes were vortexed and centrifuged 5 min at 14,000 rpm at 4°C. After recovery of the supernatant, 30 μ l of Proteinase K (Chemagic STARDNA BTS kit, Perkin Elmer, USA) were added and samples were incubated 10 min at 70°C at 250 rpm in Multi-Therm (Benchmark Scientific, USA), then 5 min at 95°C for enzyme inactivation. Tubes were centrifuged 5 min at 14,000 rpm at 4°C and supernatant were transferred in a deepwell. The plate was transferred on the nucleic acid workstation Chemagic STAR (Hamilton, Perkin Elmer, USA) and the extraction protocol was performed with Chemagic STAR DNA BTS kit (Perkin Elmer, USA) according to manufacturer instructions.

Primer design and library preparation

The V3-V4 hyper-variable regions of the 16S rDNA gene were amplified from the DNA extracts during the first PCR step using universal primers PCR1F_343 and PCR1_R784. PCR1F_343 CTTTCCCTACACGACGCTCTTCCGATCTACGGRAGGCAGCAG partial P5 adapter–primer (Nadkarni et al., 2002, PMID: 11782518); PCR1_R784 GGAGTTCAGACGTG-TGCTCTTCCGATCTTACCAGGGTATCTAATCCT partial P7 adapter–primer; PCR2_P5F AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC partial P5 adapter–primer targeting primer 1F (Luch et al., 2015, PMID: 26544955); PCR2_P7R CAAGCAGAAG-

ACGGCATAACGAGATNNNNNNGTACTGGAGTTCAGACGTGT partial P7 adapter including index-primer targeting primer 1R. PCR was performed using 2 U of a DNA-free Taq DNA Polymerase and 1x Taq DNA polymerase buffer (MTP Taq DNA Polymerase, Sigma-Aldrich, USA). The buffer was completed with 10 nmol of dNTP mixture (Sigma-Aldrich, USA), 15 nmol of each primer (Eurofins, Luxembourg) and Nuclease-free water (Qiagen, Germany) in a final volume of 50 μ l. The PCR reaction was carried out in a T100 Thermal cycler (Biorad, USA) as follows: an initial denaturation step (94°C for 10 min) was followed by 30 cycles of amplification (94°C for 1 min, 68°C for 1 min and 72°C for 1 min) and a final elongation step at 72°C for 10 min. Amplicons were then purified using magnetic beads CleanPCR (Clean NA, GC biotech B.V., Netherlands) in a 96 well format. Concentration of purified amplicons was controlled using a Nanodrop spectrophotometer (Thermo Scientific, USA) and a subset of amplicons size was controlled on a Fragment Analyzer (AATI, USA) with the reagent kit ADNdb 910 (35-1,500bp). Sample multiplexing was performed by adding tailor-made 6 bp unique indexes during the second PCR step at the same time as the second part of the P5F/P7R adapters to obtain primers PCR2_P5F and PCR2_P7R (Table 1). This second PCR step was performed on 50–200 ng of purified amplicons from the first PCR using 2.5 U of a DNA free Taq DNA Polymerase and 1xTaqDNA polymerase buffer. The buffer was completed with 10 nmol of dNTP mixture (Sigma- Aldrich, USA), 25 nmol of each primer (Eurofins, Luxembourg) and Nuclease-free water (Qiagen,Germany) up to a final volume of 50 μ l. The PCR reaction was carried out on a T100 Thermal cycler with an initial denaturation step (94°C for 10 min), 12 cycles of amplification (94°C for 1 min, 65°C for 1 min and 72°C for 1 min) and a final elongation step at 72°C for 10 min. Amplicons were purified as described for the first PCR reaction. Concentration of purified amplicons was measured using a Nanodrop spectrophotometer (Thermo Scientific, USA) and quality of a subset of amplicons (12 samples per sequencing run) was controlled on a Fragment Analyzer (AATI, USA) with the reagent kit ADNdb 910 (35-1,500 bp). Controls were carried out to ensure that the high number of PCR cycles (35 cycles for PCR 1 + 12 cycles for PCR2) did not create significant amounts of PCR chimera or other artifacts. The region of 16S rDNA gene to be sequenced has a length of 467 bp for a total amplicon length of 522 bp after PCR 1 and of 588 bp after PCR 2 (using the 16S rDNA gene of *E. coli* as a reference). Negative controls to assess technical background were included using Nuclease-free water (Qiagen, Germany). All libraries were pooled with equal amounts in order to generate equivalent number of raw reads for each library.

DNA concentration of the pool (no dilution, diluted 10x and 25x in EB + Tween 0.5% buffer) was quantified on a Qubit Fluorometer (ThermoFisher Scientific, USA). The pool at a final concentration between 5 and 20 nM was used for sequencing.

Illumina sequencing

The pool was denatured (NaOH 0.1N) and diluted to 7 pM. 15% PhiX Control v3 (Illumina, USA) was added to the pool as described in the Illumina procedure. 600 µl of this pool and PhiX mixture were loaded onto the Illumina MiSeq cartridge according to manufacturer instructions using MiSeq Reagent Kit v3 (2x300 bp Paired-end reads, 15 Gb output). FastQ files were generated at the end of the run (MiSeq Reporter software, Illumina, USA). The run quality was checked internally using PhiX control and each paired-end sequence was assigned to its sample using the multiplexing index. Sequences were processed using FROGS (Escudié et al., 2018, PMID: 29228191). Raw sequencing data quality was checked using FastQC and reads with a Phred qualityscore <30 was discarded. Chimeras and singletons were removed from the dataset. Quality control retained sequences with a length between 100 and 400 bp. Paired-end reads were merged using Vsearch. 16S rRNA Operational Taxonomic Units (OTUs) were assigned based on at least 99% sequence similarity to the lowest possible taxonomic rank against the SILVA Pintail 100-138 reference database. 100% of the sequences were affiliated. 14.22% of sequences were multi-affiliated at the species level and 2.80% of sequences were multi-affiliated at the genus level. Biodiversity of the samples (alpha diversity) was calculated with Chao1, Shannon and Inverse Simpson indexes and compared via ANOVA. Sequences were rarefied to an even depth of 19,580 sequences per sample to account for unequal sequencing depth across samples. Similarity between samples (beta diversity) was calculated with unweighted UniFrac distances. Permutational multivariate analysis of variance (PERMANOVA) was used to test for significant differences in microbiota composition in samples.

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Competing financial interests

The authors declare no competing financial interests.

Abbreviations

| | |
|-------|------------------------------------|
| AAR | Amino Acid Response |
| ASC | Apoptosis Associated speck line |
| ATF4 | Activating Transcription Factor 4 |
| AmpK | Activated Protein Kinase |
| Bp | Basepair |
| CFU | Colony Forming Unit |
| CHOP | C/EBP homologous protein |
| CD: | Dendritic cells |
| CR | Caloric Restriction |
| DMEM | Dulbecco’s Modified Eagle’s Medium |
| DNA | Deoxyribonucleic Acid |
| DSS | Dextran Sodium Sulfate |
| Db | Double Brin |
| DAI | Disease Activity Index |
| Dapi | Diamidino-2-Phenylindole |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| EBSS | Earle’s Balanced Salt |

| | |
|--------------|----------------------------------|
| FBS | Fetal Bovine Serum |
| FMT | Fecal Microbiota Transplantation |
| FITC | Fluorescein Isothiocyanate |
| GIT | Gastro-Intestinal Tract |
| GALT | Gut-associated lymphoid tissue |
| GCN2 | General Controlled Non-repressed |
| H | Hour |
| HF | Halofuginon |
| IBD | Inflammatory Bowel Disease |
| ITAD | Iso-Caloric-Twice-A-Day |
| IEC | Intestinal Epithelial Cell |
| ISR | Integrated Stress Response |
| IL-8 | Interleukin 8 |
| IL-10 | Interleukin 10 |
| IL-1 β | Interleukin-1 betta |
| LBP | Live Biotherapeutic Product |
| LPS | Lipopolysaccharides |
| MOI | Multiplicity Of Infection |
| MRS | Man-Rogosa-Sharpe Medium |
| Mtor | Mechanistic Target of Rapamycin |
| Mrna | Messenger RNA |
| MPO | Myeloperoxidase |
| ML | Milliliter |
| M | Molar |
| Mg | Milligram |
| nM | NanoMolar |
| Ng | Nanogram |
| N | Normality |
| NGP | Next Generation Probiotics |
| OUT | Operational Taxonomic Units |
| PBS | Phosphate-buffered Saline |

| | |
|---------------|---------------------------------|
| PFA | Paraformaldehyde |
| PH | Potential of Hydrogen |
| PI3K | Phosphatidylinositol-3-Kinase |
| PCR | Polymerase Chain Reaction |
| RPMI | Roswell Park Memorial Institute |
| SEM | Standard Error of the Mean |
| SCFA | Short Chain Fatty acid |
| TNF- α | Tumor Necrosis Factor alpha |
| TLR | Toll-Like Receptor |
| μ L | MicroL |
| $^{\circ}$ C | Degree Celsius |
| % | Percentage |

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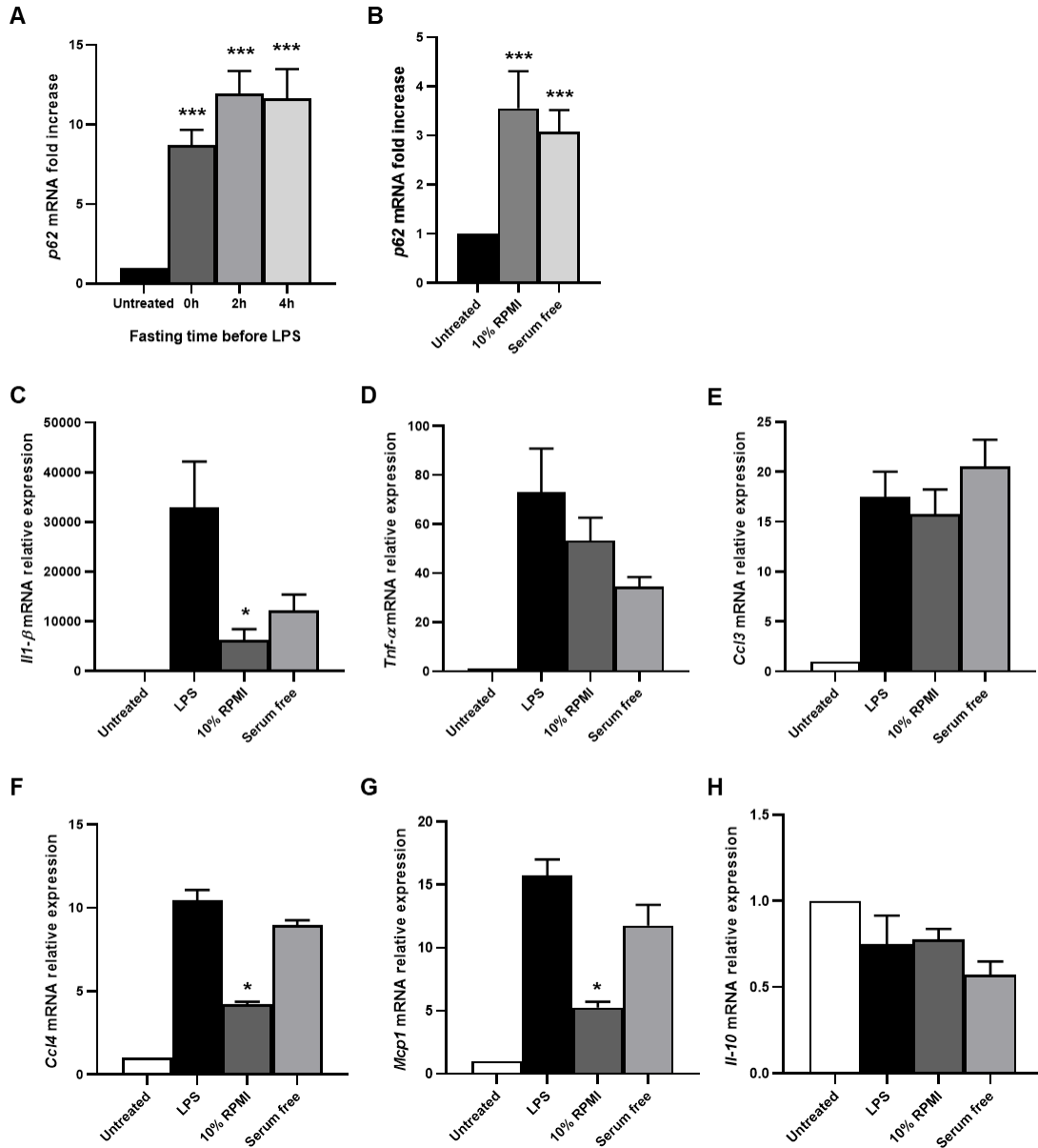
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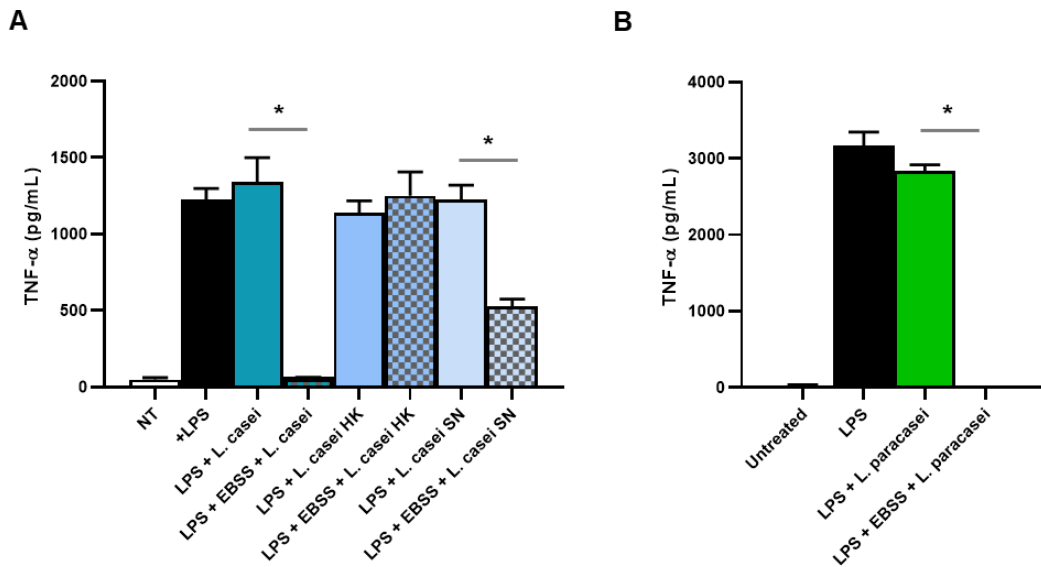
SUPPLEMENTARY FIGURES



Supplementary figure 1:

(A) RAW 264.7 macrophages were incubated in full medium (untreated, black bar) or in EBSS for 0h (dark grey bar), 2 h (average grey bar) or 4 h (light grey bar) before a 6 h period with LPS. The mRNA relative expression level of the autophagy-related gene *P62* was measured by RT-qPCR (untreated cells taken as 1). The graphs show the mean \pm SEM of at least three independent biological experiments using two replicates each. *** $p < 0.001$ (versus untreated). (B) RAW

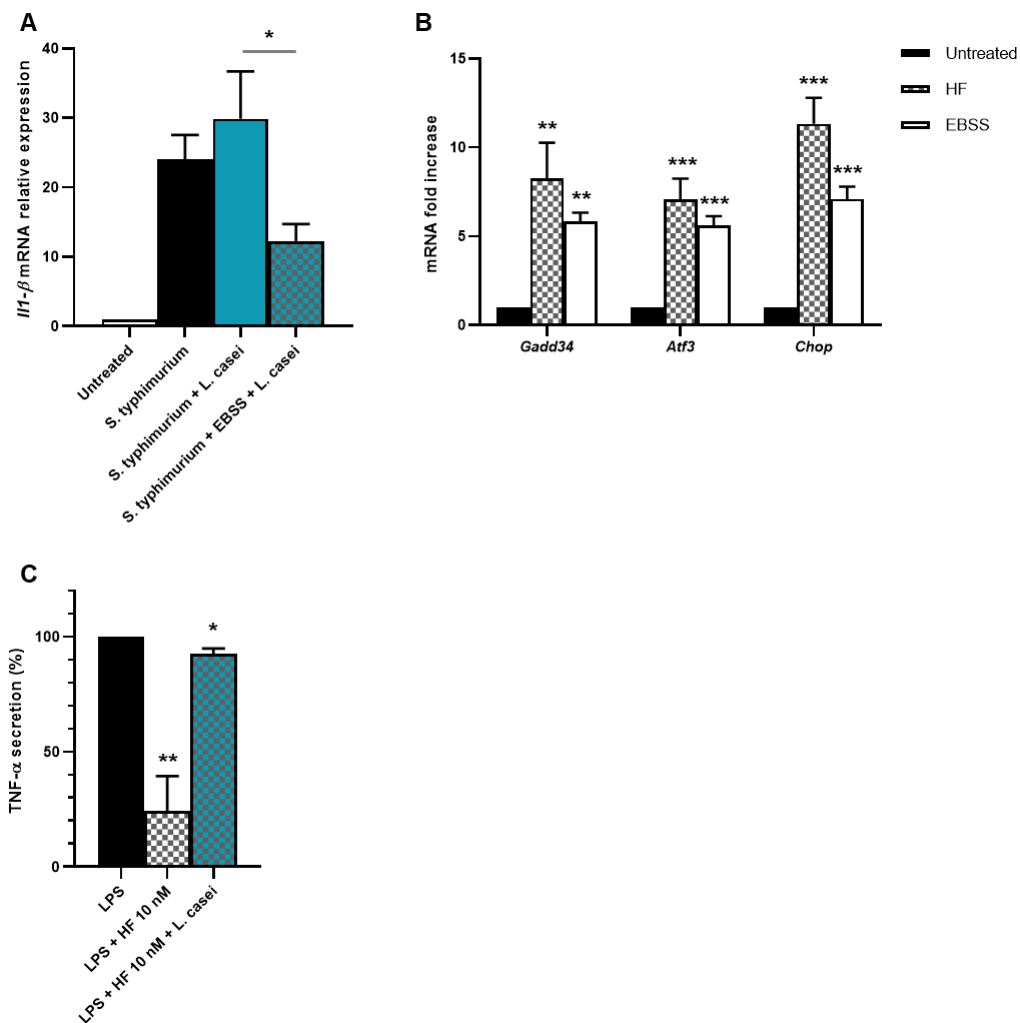
264.7 macrophages were incubated in full medium (untreated, black bar) or in EBSS medium containing 10 % of RPMI (dark grey bar) or in RPMI medium without FBS (light grey bar) for 6 h with LPS. The mRNA relative expression level of the autophagy-related gene *P62* was measured by RT-qPCR (untreated cells taken as 1). The graphs show the mean \pm SEM of at least three independent biological experiments using two replicates each. *** $p < 0.001$ (versus untreated). (C-G) RAW 264.7 macrophages were incubated in full medium and unstimulated (untreated, white bars) or stimulated with LPS (100 ng/mL) for 6 h in full medium in absence (black bars). Alternatively, cells stimulated with LPS were also concomitantly incubated in EBSS medium containing 10 % of RPMI (dark grey bar) or in RPMI medium without FBS (light grey bar). The mRNA relative expression level of the pro-inflammatory cytokines (C) *Il1- β* , (D) *Tnf- α* , (E) *Ccl3*, (F) *Ccl4* and (G) *Mcp1*, and (H) the regulatory cytokine *Il-10* were measured by RT-qPCR (untreated cells taken as 1). The graphs show the mean \pm SEM of at least three independent biological experiments using two replicates each. * $p < 0.05$ (versus LPS)



Supplementary figure 2:

(A) J774 A1 macrophages were incubated in full medium and unstimulated (untreated, white bars) or stimulated with LPS (100 ng/mL) for 6 h in full medium in absence (black bars) or in presence of the *L. casei* strain BL23 alive (blue bars), heat-killed (HK, average blue) or its supernatant (SN, light blue). Alternatively, cells stimulated with LPS and incubated with *L. casei* strain BL23 were also concomitantly incubated in EBSS (bars with grey squares). TNF- α secretion was determined

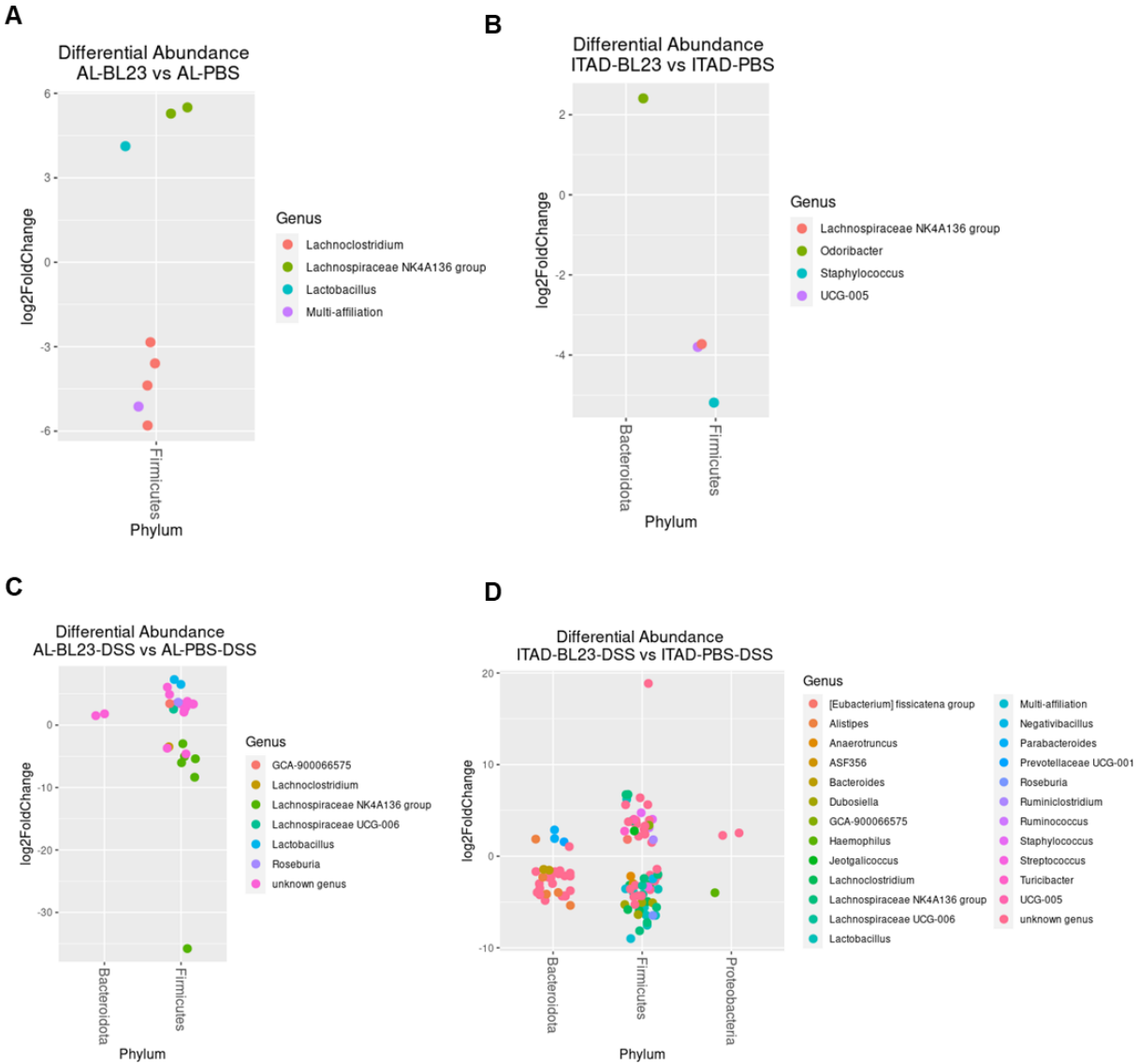
by ELISA. Results are expressed in pg/mL as mean \pm SEM of at least three independent experiments. * $p < 0.05$ (versus full medium for each condition). (B) (A) J774 A1 macrophages were incubated in full medium and unstimulated (untreated, white bar) or stimulated with LPS (100 ng/mL) for 6 h in full medium in absence (black bar) or in presence of the *L. paracasei* strain ATCC334 (green bar). Alternatively, cells stimulated with LPS and incubated with *L. paracasei* strain ATCC334 were also concomitantly incubated in EBSS (green bar with grey squares). TNF- α secretion was determined by ELISA. Results are expressed in pg/mL as mean \pm SEM of at least three independent experiments. * $p < 0.05$ (versus LPS + *L. paracasei*).



Supplementary figure 3:

(A) HT-29 cells were incubated in full medium and uninfected (untreated, white bars) or infected with *S. Typhimurium* in full medium in absence (black bars) or in presence of the *L. casei* strain

BL23 (blue bars). Alternatively, infected cells and incubated with *L. casei* strain BL23 were also concomitantly incubated in EBSS (blue bars with grey squares for *L. casei* strain BL23). The mRNA relative expression level of the pro-inflammatory cytokines *Il-1 β* was measured by RT-qPCR (untreated cells taken as 1). **(B)** J774 A1 macrophages were untreated or incubated for 4 h with Halofuginone (HF, 10 nM, white with grey squares bars) or in EBSS (white bars). The mRNA relative expression level of the ATF4 target genes *Gadd34*, *Atf3* and *Chop* were measured by RT-qPCR (untreated cells taken as 1). The graphs show the mean \pm SEM of at least three independent biological experiments using two replicates each. ** $p < 0.01$, *** $p < 0.001$ (*versus* untreated). **(C)** J774 A1 cells were incubated in full medium and stimulated with LPS for 6 h in absence (black bar) or in presence of HF (10 nM, white bar with grey squares). These cells were also incubated with *L. casei* strain BL23 during LPS stimulation (blue bars). TNF- α secretion was determined by ELISA. Results are expressed as percentage of TNF- α secretion \pm SEM of at least three independent experiments (LPS stimulated cells taken as 100%). * $p < 0.05$, ** $p < 0.01$ (*versus* LPS).



Supplementary figure 4:

(A-C) Log₂-fold change in abundance of OTUs associated with (A) *L. casei* strain BL23 administration in *Ad libitum*-fed mice, (B) *L. casei* strain BL23 administration in ITAD-fed mice, (C) *L. casei* strain BL23 administration in DSS-treated *Ad libitum*-fed mice and (D) *L. casei* strain BL23 administration in DSS-treated ITAD-fed mice. Key OTUs were identified by analysis with DESeq2 differential abundance analysis. For each analysis, genera are assigned to their corresponding phyla.

III. Discussion and Perspectives

Since alterations of the eubiosis state of gut microbiota are associated with a wide range of intestinal and extra-intestinal disorders (e.g. type I diabetes, rheumatoid arthritis, ankylosing spondylosis, IBDs, pulmonary disease, atopy, non-alcoholic fatty liver disease, obesity, atherosclerosis, colorectal carcinogenesis), modulation of the gut microbiota to improve health and to prevent or cure these disease states appears more and more as an appealing strategy. Intervention strategies on the gut microbiota include several approaches such as prebiotics, probiotics, antibiotic administration, FMT and eating behaviors such as fasting.

Within this line of thought, the general aim of the thesis was to explore dietary interventions in combination with probiotics that could be applied as novel strategies to modulate innate immune responses and to restore intestinal dysbiosis states. In this context, we gave interest in dietary and food supplements, mainly probiotics and micronutrients specifically phenolic compounds (i.e., resveratrol) on modulating the ability of probiotic strains belonging to the *Lactobacillus* genus to form mature biofilms and thereby to possibly enhance their beneficial properties within the gut targeting their adhesive capacities to human intestinal epithelial cells as well as their colonization properties. In parallel, we aimed to explore the impact of fasting on the ability of probiotic *Lactobacillus* bacteria to exercise their immunomodulatory activities and colonize the gastrointestinal tract.

Modulation of probiotic functionalities by polyphenols

Polyphenol compounds (PC) are more and more often regarded as a beneficial molecule in host–bacterial relationships. On host side, the molecular mechanisms of action underlying the response of host cell to polyphenols intake are rather complex since they appear to be multiple (via regulation of gene expression and (or) protein activity) and can occur at different body sites (gastrointestinal tract or peripheral organs) with potentially cell type-dependent effects ([García-Conesa, 2015](#)). On bacterial side, several recent studies have assessed the effects of polyphenols on the adhesion capacity of bacteria, including probiotic strains, and many of them were interestingly able to prove that PC can actually improve this capacity even during exposure to harsh conditions that mimic the GI tract environment. Mechanistically, Resveratrol was proven to modify physico-chemical properties of the bacterial surface in our study (**Article 1**) and thereby

enhance *L. paracasei* aggregation, subsequently facilitating adhesion and biofilm development. Likewise, in the study of de Souza E.L., Quercetin was proved to increase cell surface hydrophobicity of most of the tested *Lactobacillus* strains and increases or decreases in cell surface hydrophobicity varied in the presence of different Resveratrol concentrations among some strains (Dos Santos *et al.*, 2019). These results are in agreement with our study in (**Article 1**) describing that Resveratrol can modify hydrophobicity on cell surface of Lactobacilli, either by increasing or decreasing it, depending on strains and dose of Resveratrol considered.

What is also worth mentioning from a 2017 study, is that the presence of Resveratrol and other phenolic compounds like ferulic acid during bacterial growth stimulates adhesion of *Lactobacillus acidophilus* NCFM to mucin and human intestinal HT-29 cells, while other classes of polyphenols such as tannic acid improves adhesion only to HT-29 cells. Meanwhile, caffeic acid have very modest effect overall (Celebioglu *et al.*, 2018), which highlights the similar polyphenol-dependent effects obtained in our studies in (**Article 1**), describing the effects of a panel of polyphenols to stimulate biofilm formation by *lactobacilli* (Al Azzaz *et al.*, 2020, p. 334).

Could we generalize these effects upon all families and/or groups of polyphenols and *Lactobacillus* strains? The answer to this question, based on the previously described results, is perhaps “no” as for the strain-dependent effect of Resveratrol were illustrated in our study, with an increased biofilm formation by some Resveratrol-treated Lacticaseibacillus strains, including the *L. paracasei* ATCC334 strain, whereas a decreased biofilm formation is observed for some others Resveratrol-treated strains of the same group, including three *L. Paracasei* strains. Thus, a possible future assessment could be the setting up of an *in vitro* screening experiment containing the various existing polyphenol/probiotic strains in 96 wells plates confirming the probable mutual effect of the best candidates out of all the tested couples, with a further *in vivo* validation study using tiny animal models in Zebra Fish models before heading to a final pre-clinical confirmation study in mice models.

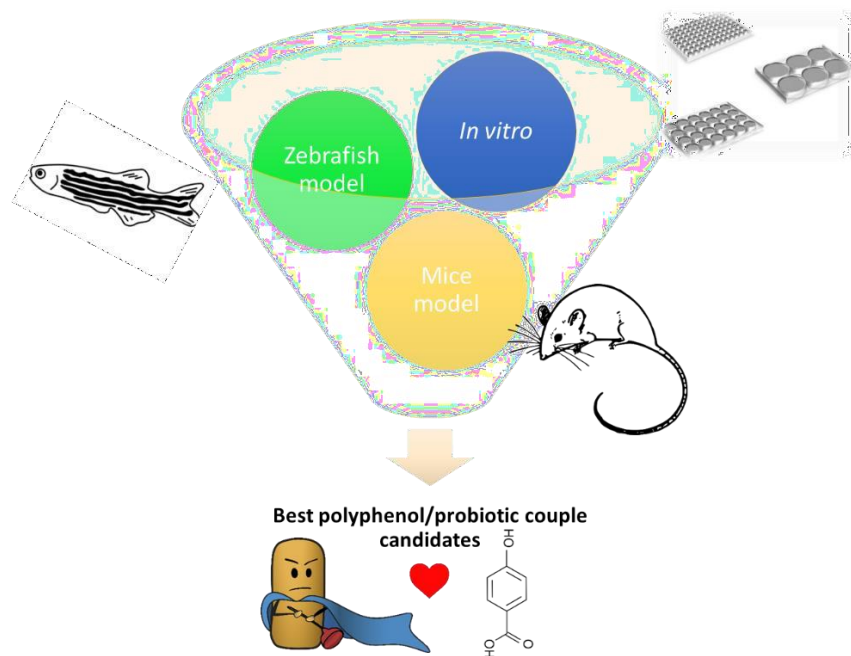


Figure 10: screening of various polyphenol/probiotic strains candidates in 96 wells plates in the detect of the ideal mutual effect.

Other functions could be the purpose of many future studies elucidating the crucial effects of polyphenols such as the physiological features of *lactobacillus*, their aggregation properties- which is an important feature of biofilm formation- and the survival and growth capacities of probiotic-like bacteria in the gut. Beyond the physical modulation of the interactions of bacterial cells with their neighboring cells or their microenvironment, the enhancement of probiotic strains metabolism features providing a growth advantage to these bacteria among complex microbial communities populating the human intestinal system, could actually be a quite interesting objective for later studies. Specifically, it is suggested that Resveratrol can have prebiotic-like effects since it is able to increase the representation of beneficial bacteria, including those belonging to the Lactobacillaceae family, notably in the context of disease states related to intestinal dysbiosis, such as colitis and obesity (Chen *et al.*, 2016; F. Li *et al.*, 2020). Similarly, according to the research conducted by Attri *et al.* (Attri *et al.*, 2018), a 10-day supplementation of polyphenols derived from sea buckthorn using *in vitro* simulated gut model, may increase the multiplication of lactic acid bacteria.

Depending on their concentration, polyphenols can act both as activators and inhibitors of bacterial multiplication and development (Othman *et al.*, 2019). In our study, growth of *Lactobacillus* strains tested (*L. paracasei* and *L. rhamnosus*) is globally not affected by various polyphenols with concentration up to 300 μ M whereas same polyphenols, in the same range of concentration, inhibits growth of some pathogenic bacteria, including quercetin, where several studies have found that it has a good inhibitory effect on the growth of pathogenic bacteria such as *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *Staphylococcus aureus*, *Escherichia coli*, *Proteus*, and *Aspergillus flavus* (Wang *et al.*, 2018; X. R. Qin *et al.*, 2009) Caffeic acid is another phenolic compound that demonstrated a pathogenic antimicrobial activity like the one it exerted against *S. aureus* clinical strains (Keřa *et al.*, 2018). Mechanistically, several studies showed a clear tendency for Gram-positive bacteria to be much more susceptible to the presence of polyphenols than Gram-negative bacteria (Tepe *et al.*, 2005). It is therefore interestingly believed that Gram-negative bacteria can be more resistant to the bactericidal activity of polyphenols due to the structure of their cell walls and the arrangement of their external membranes. This resistance results from the fact that the Gram-negative bacteria structure includes periplasmic space which is not present in Gram-positive bacteria. Moreover, the periplasmic space is rich in enzymes capable of degrading compounds demonstrating antimicrobial potential, permeating to the external environment (Mai-Prochnow *et al.*, 2016). The external membrane of Gram-negative bacteria cells is rich in lipopolysaccharides, creating a barrier that is practically impermeable for lipophilic molecules. Active components are able to bond with the cell surface in order to be subsequently transported inside. In parallel, depending on the pH of the environment and the presence of ions, plant polyphenols can act as substances with potent antioxidant properties that could be useful to protect probiotic strains associated with products during industrial processes like in the case of a chocolate product, rich in antioxidant polyphenols, where it has been evaluated as a potential protective carrier for oral delivery of a microencapsulated mixture of *L. helveticus* CNCM I-1722 and *B. longum* CNCMI-3470 (Possemiers *et al.*, 2010), interestingly, the coating of the probiotics in chocolate is an excellent solution to protect them from environmental stress conditions and for optimal delivery. Thus, combining functional food with polyphenols and *Lactobacillus* bacteria may not only protect food products against the development of undesirable and pathogenic microbiota, but may also have a positive effect on beneficial microbes associated with food matrix and human health.

Reaching this far, how could these *in vitro* obtained results be reproducible in other *in vivo* pre-clinical mice models with keeping the probiotic persistence during several weeks with a high rate of colonization? And in which health situation? Should it be conducted on basal state animal models as prophylactic treatment or on animals undergoing inflammatory diseases state? Could the inflammatory state potentiate somehow the polyphenols/probiotic mutual relationship effects? Something close to an *in vivo* experimental assay conducted upon an induced DSS colitis model that underwent a probiotic prophylactic treatment in the presence and/or absence of Resveratrol supplementation, would be a primary tool to reproduce the *in vitro* results in a first attempt.

Also, doses used to analyze Resveratrol effects *in vitro* should be consistent with those *in vivo* in reachable and tolerable concentration. Many other factors should be taken into consideration before establishing a complete reasonable study upon these molecules. For instance, many dietary polyphenols have been utilized for prevention of colitis in rodent models (Martin and Bolling, 2015). Despite the considerable number of positive studies on polyphenols for IBD treatment in rodents, few polyphenols have been examined in human intervention studies of colitis, like green tea polyphenols (GTP) for example, where it was proved that the modifying effects of GTP on DSS-induced acute colitis depends upon its dosage and the expression of proinflammatory cytokines (Kim *et al.*, 2010). Given these limitations, data from polyphenols in rodent models suggests merit for pursuing additional clinical studies for prevention of colitis which demands a greater need to understand how targeted polyphenol delivery systems could be used to optimize treatment for colitis. Given this, a combinational formulation of both probiotics and polyphenols targeting an ideal delivery to the gastro intestinal tract could also represent a promising strategy like the one developed recently in our laboratory, through a CPB-formulated probiotic bacteria displaying an ability to reduce the severity of a DSS-induced colitis mouse model, with a decrease in colonic mucosal injuries, less inflammation, and reduced weight loss compared to DSS control mice (Heumann *et al.*, 2020).

Nevertheless, the recent decades of research in the microbiome and immunology fields have revealed the importance of the interindividual variability in gut microbiota composition in promoting health and causing disease.

The effectiveness of polyphenols here consists in reaching the target location, i.e., phospholipid cell membranes, or in altering the synthesis of intracellular compounds, i.e., enzymes, ATP or

intracellular proteins (Silhavy *et al.*, 2010). Now in order to understand the health effects of consuming polyphenols, it is essential to know their fate in the body once ingested in order to determine whether or not they reach the suspected target tissues. It is therefore very important to know that the bioavailability of polyphenols varies widely from molecule to another. Some polyphenols are well absorbed through the intestinal barrier and are found in the blood, whereas some others are rapidly conjugated and excreted, limiting their bioavailability. Bioavailability is on its turn also affected by many physicochemical factors such as structure of the polyphenol, its polarity, its molecular mass, its state (crystalline vs amorphous), its digestibility by gastrointestinal enzymes and its absorption in enterocytes (Rechner *et al.*, 2002).

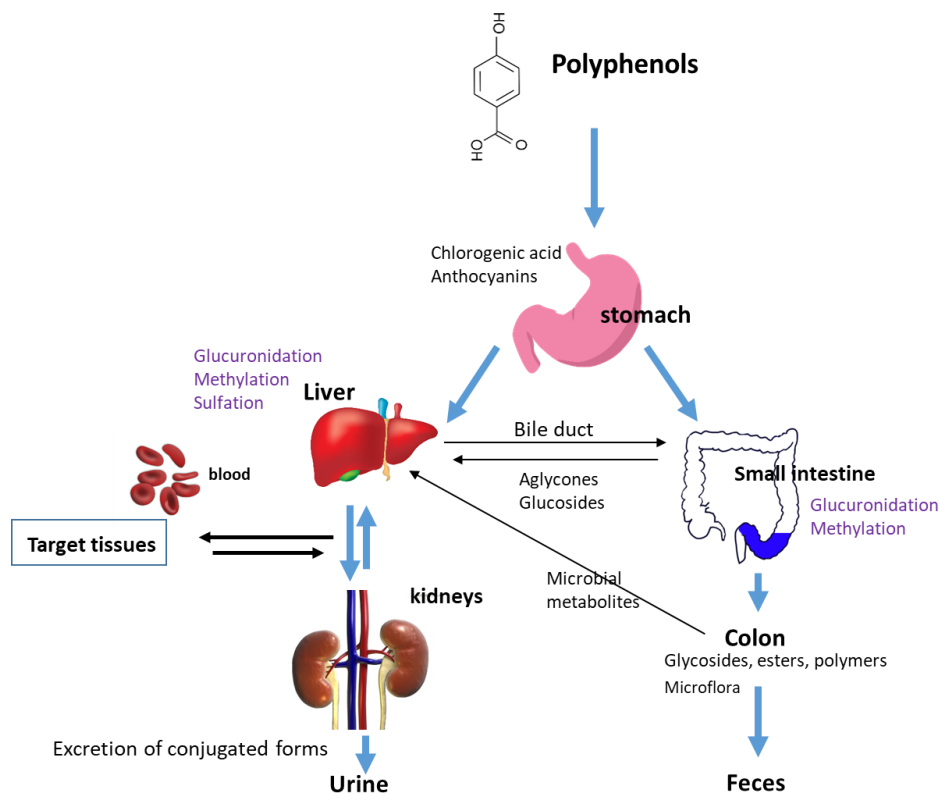


Figure 11: Polyphenol bioavailability

Polyphenols which are not absorbed in the small intestine reach the colon, where the microbiota can convert them to primary metabolites, able to reach the systemic circulation where they undergo additional transformation into secondary metabolites. The microbiota thus can increase the bioavailability and pharmacokinetic properties of some polyphenols throughout their biochemical modification, just like in the case of quercetin in two studies, where the combination of quercetin-

3-glucoside or α -glucosyl rutin with fructooligosaccharides (FOS) increased the concentration of quercetin and O-methylated quercetin conjugates in plasma of a rat animal model, in comparison with the plasma concentrations in rats treated with quercetin-3-glucoside or α -glucosyl rutin alone (Matsukawa *et al.*, 2009; Campos *et al.*, 2012). This suggests that FOS may effectively decrease the degradation of quercetin via the modification of the intestinal microbiota and therefore, increase the bioavailability of the parent compound, quercetin. Herein, dietary fiber showed a promise in modifying the bioavailability and physiological functions of polyphenols by changing components of the microbiota (Kawaba *et al.*, 2019). Or on the opposite to that, the gut microbiota can rather inactivate or reduce the bioavailability of a polyphenol through its extensive catabolism. Although many polyphenol metabolites are bioactive, they may not be active in the same exact manner as the parent compounds and would generally have reduced function relative to parent compounds (Luca *et al.*, 2020). Since the gut microbiota's composition of each individual is unique and is influenced by genotype, physiological state, diet and lifestyle, we could assume that polyphenols bioavailability and their potential beneficial effects will automatically vary widely from an adult individual to another based on the biochemical transformation catalyzed by the resident gut microbiota. In addition, it is largely known that the gut microbiome becomes quite unstable in elderly people, displaying exacerbated inter-individual variations (Jeffery *et al.*, 2016). These facts reinforced the idea that dietary approaches aiming to modulating the gut microbiota should be personalized based on specific features of the target population. However, the categorization and identification of individuals responsive to a given polyphenol beneficial properties is not a trivial matter. Which explains the urge need to develop new and better strategies to improve the delivery of polyphenols to their target sites and cells, thus maximizing their chance of being more effective in a large number of people. As an example, a recent study formulated the polyphenol curcumin in a self-nano micellizing solid dispersion to enhance the effectiveness of this polyphenol in the context of Alzheimer's disease (Parikh *et al.*, 2018).

This rapid transformation of polyphenols by the gut microbiota complicates also *in vivo* animal models investigating the molecular targets of polyphenols as they may not reflect the metabolite(s) presented to cells or tissue in humans since the gut microbiota composition of laboratory animal models greatly differ from their human counterparts in term of richness, diversity and dominant species (Turner *et al.*, 2018).

And to complexify this further, the food context seems to effectively modulate the polyphenol's interactions with the gut microbiota. Polyphenols interact with food nutrients or ingredients that influence their bioavailability. Interactions with proteins generally decrease this bioavailability, as do associations with dietary fibers, unlike lipids which seem to improve it (Rechner *et al.*, 2002) (Rechner *et al.*, 2002). Beyond the bioavailability function, some macronutrients could act on optimizing other points, like in this study where several sugars were examined and presented a similar trend of protective effect regarding polyphenol degradation whereas fructose displayed the highest efficacy in the case of epigallocatechin gallate (EGCG) (Hanuka Katz *et al.*, 2020). Others suggested that the protective effect of different sugars (fructose, sucrose, glucose, mannose, and galactose) on EGCG was due to a combination of several mechanisms: decreased oxygen solubility, chelation of transition metal ions and scavenging of reactive oxygen species.

The very same idea was discussed in detail in (**Chapter 3**) whereas the focus was held onto the effect of ingested nutrients on the functionalities of probiotic-like bacteria rather than phenolic compounds. This could be the chance to connect the three concepts for that polyphenols represent a part of multi-component systems in food production, with interactions that can affect their stability, activity, and bioavailability and hence influence on their activities into the host and their accessibility to the gut microbiota.

The purpose of supplementations is usually to increase the daily intake or the Nutritional Value Daily (VNR) of polyphenols. To date, there are still no recommendations concerning the VNR, or even the maximum tolerated daily dose, since the effects. The harmful effects of polyphenols in the event of overconsumption have been poorly studied but existing available studies point out adverse effects of high consumption of some polyphenols: A striking example is the ability of polyphenols to interact with nutrients before their absorption, resulting in the inhibition of their uptake by the enterocytes. This phenomenon has been notably observed for thiamin, folic acid and iron, leading for the latter one to a risk of anemia in consumers (Cory *et al.*, 2018). In addition, some polyphenols belonging to the isoflavones group have been described to affect steroid hormones (e.g., estrogen). In theory, polyphenol supplementation should not be considered if the population was consuming a diet enriched in fruits and vegetables where they are present in relatively high concentration. Do we still need to extract or synthesize these molecules in order to transform them into an additive food and incorporate them into food products in concentrations

exceeding those already present in natural products (fortified products, food supplement food, etc.)? Many questions could be great open platforms for future investigations.

On the other hand, the stability of the polyphenolic compounds represents as well, a wealthy subject to invest research on in this matter for that it is significantly, and structure dependently affected in many studies whereas Fructose was spotted, in the same previously cited study ([Hanuka Katz *et al.*, 2020](#)), to enhance the stability of most of the identified polyphenols, possibly, at least partially, due to a decrease in solubilized oxygen. The lack of correlation between the total antioxidant capacity and polyphenol stability may be attributed to the fact that the extract contains more unidentified polyphenols, wherein every polyphenol has a different contribution to the antioxidant capacity, and the fact the polyphenol degradation products may have a significant contribution to this capacity. Also, a large fraction of the published information on shelf-life stability of polyphenolic compounds focuses on the whole food product, making fundamental conclusions regarding non-enzymatic degradation kinetics and the effect of structure in the presence of additional common components more difficult and product-specific. The complexity arises from matrix effects (and how it changes during shelf-life) and the (residual) enzyme activity.

Hence, the proposed benefits of polyphenols, either as protective/prophylactic substances or as therapeutic molecules, may be achieved by the consumption of a natural polyphenol-enriched diet, by their use as food supplements, or with formulations as pharmaceutical drugs/nutraceuticals. An interesting challenge in the future could be to design and formulate new probiotics, eventually in association with active micronutrients such as Resveratrol, and tailored to integrate individual specific features (resident gut microbiota, clinical context, host genetic). However, poor regulatory constrictions of commercial polyphenol supplements and nonpharmaceutical formulations remain a concern for their safe use, as it is in the case of medicinal pomegranate products for cancer ([Vlachojannis *et al.*, 2015](#)).

Last, but not least, these issues open a vast window to additional studies proposing new hypotheses in relation with the consumption and metabolism of polyphenols and their health benefits. One idea could be the possibility to incorporate the concept of chrono-nutrition, i.e., the study of the interactions between biological rhythms, metabolism, and nutrition, into the research area of polyphenols and health ([Arola-Arnal *et al.*, 2019](#)). This concept of chrono-nutrition is present in the second research work of this thesis by applying the ITAD protocol to mice. It seems reasonable

that biological rhythms which are present in all organisms (plant and animals) and include both circadian and seasonal rhythms may influence the human responses to the intake of dietary polyphenols. This research should combine the bidirectional understanding of the influence of biological rhythms on the plant production and composition in polyphenols as well as on the metabolic and responsive capacity of the consumers. In this sense, this brings up another unexplored section suggesting that factors affecting the biological clock, such as gene polymorphisms of the core clock machinery and seasonal changes in the light–dark cycle, and the ideal timings of incorporating such bioactive molecules in our diet exert a marked influence on physiological activity. This way, chrono-nutrition becomes another interesting factor that contributes to human interindividual variability in response to polyphenols and that surely needs further and extensive investigation. This also highlights the need for further human clinical trials with better designs to understand interindividual variability and to improve the consistency and relevance of the effects in humans. It additionally shows the difficulty but, also the importance, of understanding the metabolism and mechanisms of action of these compounds and the interest in translating this knowledge into improved technologies to enhance the efficiency of the application of polyphenols for human health and disease.

Fasting acting on Probiotic modulation

Food intake is a complex, periodic behavior through which an organism draws the nutritional resources necessary for survival from its environment. Hunger and satiety are psychophysiological states which, respectively, trigger or inhibit food consumption. Homeostatic or non-homeostatic mechanisms help to determine the periodic triggering of food intake. The size of meals is determined by an interaction of sensory factors (quality and variety of food stimulation), gastrointestinal signals, and neuroendocrine responses to the arrival of nutrients in the digestive tract (Bellisle *et al.*, 2005). Nutrients, by regulating cell metabolism, could modulate a wide range of cellular response, including immunity. Thus, immunity is intimately linked to the cellular metabolism.

Inflammation is a body's natural response to aggression. However, when this is inappropriate in term of intensity or duration, it can be the cause of pathological phenomena such as tissue damage, fibrosis or carcinogenesis. Numerous regulatory pathways can modulate, directly or indirectly, the

inflammatory responses. Among them, the regulatory pathways of cell metabolism appear more and more clearly involved in the control of the inflammatory status of numerous immune cells. A growing field of research, called immunometabolism, could reveal the pathophysiological mechanisms behind the development of many diseases. With the westernization of eating habits and the high consumption of high-energy foods, excess calorie intake is frequently observed in the world population. It is thought to be responsible for establishing a chronic systemic inflammatory state (Lumeng *et al.*, 2011), linked to the development of metabolic diseases with an inflammatory component, such as type 2 diabetes or atherosclerosis (Haslam *et al.*, 2005). Excess calorie intake is also implicated in the occurrence of inflammatory and autoimmune diseases (Manzel *et al.*, 2014). Conversely, fasting or low-calorie diets have been shown to have a protective effect against these diseases (Mattison *et al.*, 2012). However, the mechanisms by which reduced calorie intake modulates systemic inflammation are still poorly understood.

In one study, immune cells from CR treated mice produced less IL-1 β , IL-6 and TNF- α compared to those from HFD control mice (J. Wang *et al.*, 2013). These results agree with previous reports on CR in both humans (Arguin *et al.*, 2012) and animals (Williams *et al.*, 2007; Kirchner *et al.*, 2012). Other comparable studies investigating nutrient deprivation suggest that CR is able to decrease inflammation in several experimental models. For example, CR normalized TNF- α and IL-6 serum levels in old mice up to young mice levels (Spaulding, Walford and Effros, 1997) and promoted a youthful transcriptional profile that includes downregulation of inflammatory pathways in rats and middle-aged humans (Mercken *et al.*, 2013). This result stands somehow in agreement with a part of our *in vitro* results in (Article 2) where nutrient starvation of immune cells (J774A1 and THP1 macrophages) and intestinal epithelial cells (HT-29) lowered the pro-inflammatory cytokine secretion profile including the TNF α in both HT-29 human intestinal epithelial cell lines and J774A1 immune murine cell lines as well as the HT-29 human secreted IL-8. However, the originality of the work of this thesis stands thus in the potentiated immunomodulatory properties observed after the supplementation of *Lactobacillus* strain (*Lactobacillus casei* BL23, *Lactobacillus Rhamnosus* GG, *Lactobacillus casei* ATCC334). Probiotic immunomodulatory properties were also observed in some studies where selected probiotics have been indeed shown to modulate immune responses and inflammatory biomarkers in human Dendritic cells. More profoundly, results indicated that *Bifidobacterium breve* CNCM I-4035 affected the intestinal immune response, whereas its supernatant exerts anti-inflammatory

effects that are mediated by dendritic cells. Similarly, *Lactobacillus paracasei* CNCM I-4034 was shown to decrease pro-inflammatory cytokines and chemokines in human intestinal DCs that were challenged with *S. typhi* CECT 725. Other selected probiotics also exhibited *in vitro* anti-inflammatory properties. Mechanistically, both probiotic strains and CFS reduced the expression of pro-inflammatory cytokines via an action principally mediated by TLRs (Plaza-Díaz *et al.*, 2017).

Controversially, other studies showed opposite results wherein in some studies, glucose-deprived cells, revealed up-regulation of multiple cytokines and chemokines, including IL-6 and IL-8, in response to starvation stress (Püschel *et al.*, 2020). In this study, starvation-induced cytokines were cell type-dependent, and they were also released from primary epithelial cells from different cell lines (HeLa, A549, H460, and SW900) (Püschel *et al.*, 2020). This opens the door to another research perspective towards the assessment of other cell types and/or probiotic-like strains that could act on the immune response in synergy with cell starvation since strain dependent effects play a vital role in deciding to what level this co-administration could influence the modulation of the intestinal inflammation towards a better homeostatic state.

Another kind of combinational experimental trials evoking these two main keys of the thesis from another perspective, is an earlier *in vivo* study investigating the effects of probiotics on nutritional status and gut mucosal atrophy after fasting on groups of rats whereas feeding efficiency was marked greater in the probiotic group than in the control one suggesting that probiotic treatment belonging to *Bifidobacterium lactis* BL and *Streptococcus thermophilus* for 3 days enhances the recovery of nutritional status and lessen gut mucosal atrophy after fasting (Dock *et al.*, 2004). This study highlights the possibility of a probiotic treatment to accelerates and help attending a better recovery from a gut-related atrophy after a fasting regimen, which opens the eye on the interesting mutual relationship between these two concepts despite the experimental dissimilarity observed from a study to another. In other studies, significant changes of the rat fecal microbiota that arise rapidly in young growing animals after short-term administration of a CR diet were noted. As in particular, an increase of *Lactobacillus* was noted significant after 8 weeks of CR treatment and its relative abundance was considerably higher in CR vs AL fed animals after 36 weeks of dietary intervention (Fraumene *et al.*, 2018). Taken together, this data suggest that *Lactobacillus* intestinal colonization is hampered in fed young rats compared to CR fed ones, while health-promoting CR

diet intervention enables the expansion of this genus rapidly and persistently up to adulthood, which could thus act indirectly on the modulation of the immune response in the gut.

To get back on track, our *in vitro* observations were partially confirmed in *in vivo* experiments using the ITAD protocol feeding strategy in DSS-colitis induced mice models where fasting diet allows to decrease the disease activity index and the inflammatory marker MPO, but also to strengthen the intestinal barrier integrity. By contrast to *in vitro* results, the combination of a supplementation in *Lactobacillus casei* strain BL23 with the fasting diet in mice does not improve colitis symptoms (compared to the effects observed for the fasting diet alone). However, for practical reasons, this feeding protocol consists of test mice eating the same amount of food as ad libitum (Ad-lib) controls, albeit they eat their food at two 2 h windows early and late in the diurnal cycle wherein eating occurs between 8 and 10 a.m. (feeding window 1) and between 5 and 7 p.m. (feeding window 2), such that food consumed at these two diurnal windows equals the food consumed by Ad-lib mice in 24 hr (Martinez-Lopez *et al.*, 2017). This protocol poses questionable behavior of fed mice in such a way that mice are rather nocturnal animals since it is a diurnal rodent, that is, its activity is mainly nocturnal. Thus, the mouse will spend its days resting. Waking them up or disturbing them at such times can be detrimental to her health and make her aggressive, suggesting that lighting conditions may be even more important than we assume. This could interestingly open the door towards a novel *in vivo* protocol wherein periods of feeding are reversed to specific night shifts if possible. Another solution could be to create two reversed artificial window cycles mimics in the animal laboratory to put animal models in the ideal periods of flexibility in receiving treatments perhaps this could potentiate complementary or additional effects upon starvation with or without probiotic co-administration. The goal of this perspective is to offer new guidelines to aid in the design, analysis and interpretation of studies of feeding behavior in mice.

Of note, the anti-inflammatory effects of probiotics have been also demonstrated in experimental models wherein probiotic supplementation was shown to provide protective effects during spontaneous and chemically induced colitis by downregulating the production of inflammatory cytokines or by inducing regulatory mechanisms in a strain-specific manner. In a study, chronic colitis was induced by two DSS treatment cycles with a 10-day rest period. The probiotic supplementation was started after the first DSS treatment cycle and continued until the end of the

experiment (Zheng *et al.*, 2016). *L. rhamnosus* NutRes 1, but not *B. breve* NutRes 204, rapidly and effectively improved the DSS-induced bloody diarrhea during the resolution phase. However, an increased expression of TLR2, TLR6, chemokine ligand 2, IL-1 β , TNF- α , and IL-6 was found in DSS-treated mice with *L. rhamnosus* supplementation.

On the formulation-based level, capsules with *bifidobacteria*, *lactobacilli*, and *Streptococcus thermophilus* DSM24731 were administered to mice exposed to 5, 10, and 15 cycles of DSS. Interestingly, a probiotic mixture lessened the disease activity index score and colon inflammation after these cycles and reduced simultaneously the histological alterations and the incidence of colonic dysplastic lesions in the three considered periods. In addition to that, the probiotic mix reduced the TNF- α , IL-1 β , IL-6 production, and increased IL-10 levels in colon tissue in the three periods assayed (Talero *et al.*, 2015). Additionally, in rats treated with DSS for seven consecutive days, the probiotic mixture exhibited anti-inflammatory properties, including reducing the disease activity index, which stands in part with our *in vivo* results. Of note, in the evaluation of probiotic properties, animal studies seem to be more extensively used than cell models (Plaza-Díaz *et al.*, 2017).

Two factors left are still open to discuss in this matter, that is, the ideal way to incorporate probiotic-like strains supplementations to the mice's nutrition and in which period of the day this should be done for that the feeding rhythm could also constitute an important factor affecting results for that some specific periods are proven to hold more fruitful outcomes in comparison with others according to several studies. Traditionally, supplements whether to be pharmaceutical drogues or additives are usually auto or co-administrated for mice models. Yet, the time of administration remains quite unexplored from the point of view study design and promising effects and whether it should be done during a fast-induced regimen or a regular one.

Also, a related worth asking question that often arises is whether these protocols could get applied on human models as novel clinical studies. As a matter of fact, in spite of the remarkable health benefits on diverse levels including the extension of health-span and lifespan in multiple organisms (Colman *et al.*, 2009; Mattison *et al.*, 2012), humans still adhere poorly to fasting and CR (Moreira *et al.*, 2011), and while fasting is unlikely easy on some people following religious or regional reasons, it is an uneasy concept and quite a difficult challenge not to break the fast on many others making it a little complicated to implement such restrictions in our lives, which makes the human

clinical studies somehow discouraging, unpractical and primarily restricted to epidemiological studies. Fortunately, this ethical and experimental limitation has motivated the search for sustainable approaches to extend health-span beyond their exclusive application on traditional classic animal models like Nematode whereas studies have shown that the reduction of their food source can effectively extend their lifespan as the restriction increases to starvation (Wei *et al.*, 2008, p. 9), similar to the mammalian model system. Alternate healthy feeding strategies, including the in vogue intermittent fasting which involves alternating intervals of extreme caloric reduction with periods of normal eating (Anson *et al.*, 2003; Heilbronn *et al.*, 2005; Varady *et al.*, 2009) and time-restricted feeding (Chaix *et al.*, 2014) each mimic the effects of CR. Such studies of various types of fasting (less than 24 h) have shown reduced levels of pro-inflammatory cytokines, such as IL-1 β , IL-6 or TNF- α (Faris *et al.*, 2012). Besides, since fasting activates autophagy, it is conceivable that dietary interventions mediate their benefits, in part, through autophagy. Still, the integrative physiology of autophagy and its ability to upgrade metabolic correction in a dietary intervention model remains vastly unexplored. Nevertheless, fasting-mimicking intervention (Brandhorst *et al.*, 2015) could also be a part of the list by using some medicines known as caloric restriction mimetics (CRM) whereas gerontologists and biologists are still attempting to develop drugs to mimic the beneficial effects of caloric restriction without the need for diet limitations such as Resveratrol, Rapamycin, Metformin and Halofuginone (Lee *et al.*, 2013). However, it is important to note that there is evidence showing adverse side-effects of the use of these active molecules like the susceptible exposure to some diseases and the ineffectivity in some species making them uncertain treatments as true CRMs. Cutting off a certain nutrient of the diet could also represent a new strategy modulating intestinal homeostasis such as lipids, proteins and carbohydrates as detailed in (Chapter 2) of the thesis. For example, like the starvation mode that involves cutting off carbohydrates from the diet and referred to as ketogenic diet. This could open an eye towards another perspective underlying the importance of nutrients in inflammation modulation and treating dysbiosis cases in a way wherein we investigate the crucial role of some specific nutrients. For example, like testing the effects of a single amino acid deficiency where current evidence suggests that the relative roles of the GCN2 and mTORC1-mediated responses to amino acid starvation may differ depending on the specific amino acid(s) that is (are) deficient and the particular tissue (Anthony *et al.*, 2004). like Glutamine, Leucine and Methionin.

On the flip side, early results have found that some types of fasting may have negative effects on aspects of health and some potential harms. In some cases, severely limiting calories can be dangerous for people with certain conditions, such as diabetes and people under medication. On a long-term, it could cause nutrient deficiency side effects and may thus lead to malnourishment- if taken to an extreme- particularly through vitamin deficiency and muscle loss (Roger Collier et al.,2013).

IV. Conclusion

Currently, there is still a lack of effective strategies to correct dysbiotic states of the gut microbiota that are associated with numerous complex diseases. Intestinal dysbiosis-related diseases seem to be multifactorial dysregulations on various standards, resulting in profound effects on the host whether on the metabolic, immunologic or compositional level and that in terms of correction, is strongly dependent of various key points including the type of population enrolled in the study (healthy individuals, individuals at risk, or patients with active disease at enrollment), the dysbiosis state itself at baseline, the probiotic or control prevention (preventive, treatment) along with other combinational interventions (diet, caloric restriction), and the potential expected outcomes (restoration, alteration or improvement) each related to the exact signaling pathway associated after the intervention, for that each disease has its own signaling pathway which mainly include P38 MAPK, JNK MAPK, PI3K/Akt, NF-kB signaling pathways. As consequence, this poses a substantial burden on human health throughout the world. Based on the numerous single-related targets in the disease network, multi-target design strategy begins to constitute a crucial direction to seek for enhanced therapy, and multi-target treatments seem to have the ability to regulate more targets than single-target ones, affecting the disease network with more potency. In the disease network resulting through a form of intestinal dysbiosis, several targets involving mTOR or not, could implicated with others, and their respective signaling pathways could cross with others, contributing together to the development of disease progression.

Future researchers must also be aware of the various factors, such as genetics, diet and environmental factors, which impact the formation of gut dysbiosis. This knowledge, along with

the continuing work of identifying the gut microbiota present in humans, additional future research protocols in this field should be able to become closer in successfully intervening against dysbiosis and its associated diseases and in increasing the effectiveness of treatment.

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