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Effect of gut microbiota from children with autism spectrum disorder (ASD) on behavior and ASD-related biological markers in germ-free mice

Léa Roussin

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Léa Roussin. Effect of gut microbiota from children with autism spectrum disorder (ASD) on behavior and ASD-related biological markers in germ-free mice. Human health and pathology. Université Paris-Saclay, 2023. English. NNT : 2023UPASB024 . tel-04099821

HAL Id: tel-04099821

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Effect of gut microbiota from children with autism spectrum disorder (ASD) on behavior and ASD-related biological markers in germ-free mice

Effet du microbiote intestinal d'enfants atteints de troubles du spectre autistique sur le comportement et les marqueurs biologiques liés à ces troubles chez la souris axénique

Thèse de doctorat de l'université Paris-Saclay

École doctorale n° 581 : agriculture, alimentation, biologie, environnement, santé (ABIES)

Spécialité de doctorat : Sciences de la nutrition

Graduate School : Biosphera. Référent : Faculté des sciences d'Orsay

Thèse préparée dans l'UMR **Micalis Institute** (Université Paris-Saclay, INRAE, AgroParisTech), sous la direction de **Laurent NAUDON**, Chargé de Recherche (HDR), et sous la co-direction de **Sylvie RABOT**, Chargée de Recherche (HDR)

Thèse soutenue à Paris-Saclay, le 29 mars 2023, par

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Keywords : Sciences du comportement, Microbiote intestinal, Physiologie, Neurosciences

Abstract : Les troubles du spectre autistique (TSA) sont des troubles neurodéveloppementaux multifactoriels caractérisés par des comportements répétitifs et des difficultés dans l'interaction sociale et la communication ; ils s'accompagnent parfois d'anxiété, de déficiences cognitives et de symptômes gastro-intestinaux (GI). Des facteurs génétiques, épigénétiques et environnementaux sont impliqués dans leur pathophysiologie. De nombreuses études montrent une composition distincte du microbiote intestinal chez les personnes atteintes de TSA par rapport aux personnes neurotypiques, ainsi que dans les modèles murins de TSA par rapport aux animaux WT. Bien que l'origine de cette altération du microbiote soit inconnue et qu'elle puisse être influencée par les préférences alimentaires restrictives ou les anomalies GI de certaines personnes atteintes de TSA, elle pourrait aggraver les symptômes de ces troubles. En effet, des données cliniques et précliniques montrent que cette altération du microbiote est en interaction avec le système immunitaire et peut jouer un rôle dans le développement de plusieurs des symptômes et des déficiences fonctionnelles observés chez les individus atteints de TSA, comme une inflammation et une perméabilité intestinale excessive, une altération du métabolisme du tryptophane et une neuroinflammation accrue.

Par conséquent, nous avons étudié l'effet chez des souris axéniques d'une transplantation du microbiote fécal (TMF) d'enfants atteints de TSA sur le comportement et les marqueurs cérébraux, immunitaires et GI liés aux TSA. Nous avons choisi deux groupes distincts d'enfants atteints de TSA comme donneurs de microbiote : un groupe sans symptômes gastro-intestinaux (groupe A) et un groupe présentant des symptômes gastro-intestinaux (groupe AG), à savoir une constipation sévère et chronique. Pour les deux groupes de TSA, les enfants donneurs témoins étaient leurs frères et sœurs neurotypiques. Cela limite l'effet potentiel des facteurs génétiques et environnementaux sur la composition du microbiote entre les groupes de donneurs TSA et neurotypiques.

Nous avons étudié l'effet de la TMF chez deux lignées de souris qui présentent des différences génétiques et comportementales, à savoir les lignées BALB/c et C57BL/6. Dans les deux lignées, la TMF des groupes TSA a entraîné une diversité alpha et composition distinctes du microbiote intestinal chez les souris, par rapport à la TMF des groupes frères et sœurs. Bon nombre de ces différences étaient spécifiques à la lignée, mais, une proportion réduite de *Prevotellaceae* a été trouvée de façon constante dans les deux lignées de souris ayant reçu la TMF du groupe AG, par rapport à la TMF de leurs frères et sœurs. Chez les souris C57BL/6, la TMF du groupe A a diminué l'activité locomotrice et la TMF du groupe AG a augmenté les comportements répétitifs et altéré la mémoire spatiale, par rapport à la TMF de leur frères et sœurs. Ces altérations se sont accompagnées d'une diminution de la proportion de populations de lymphocytes T pro-inflammatoires dans la rate et d'une augmentation de l'expression des gènes impliqués dans les jonctions serrées dans l'intestin des souris ayant reçu la TMF des groupes A et AG par rapport celles des frères et sœurs. Chez les souris BALB/c, il n'y avait pas de différence de comportement entre les groupes de souris, mais la TMF des groupes A et AG a réduit le nombre de neurones sérotoninergiques dans le noyau du raphe, par rapport à la TMF des frères et sœurs. De plus, dans cette lignée, la TMF du groupe A a réduit les marqueurs d'inflammation dans le côlon, par rapport à la TMF des frères et sœurs.

Cette étude apporte de nouvelles données sur l'effet de la TMF de personnes atteintes de TSA sur des souris, soulignant l'importance du bagage génétique des animaux receveurs et la prise en compte des symptômes GI dans les études sur l'axe microbiote-intestin-cerveau.

Titre: Effect of gut microbiota from children with autism spectrum disorder (ASD) on behavior and ASD-related biological markers in germ-free mice

Mots clés : Behavioral science, Gut microbiota, Physiology, Neurosciences

Résumé: Autism spectrum disorder (ASD) is a multifactorial neurodevelopmental disorder characterized by repetitive behaviors and impairments in social interaction and communication. It is sometimes accompanied by anxiety, cognitive impairments and gastro-intestinal (GI) symptoms. Genetic, epigenetic and environmental factors are involved in ASD pathophysiology. Many studies report a distinct gut microbiota composition in people with ASD compared to typically developing people, as well as in genetic and environmental murine models of ASD compared to WT animals. While the origin of those microbiota alterations is unknown, and might be influenced by the restrictive dietary preferences or GI abnormalities of some individuals with ASD, they could worsen ASD-related symptoms. Indeed, clinical and pre-clinical data show that those microbiota alterations are at interplay with the immune system and may play a role in the development of many of the symptoms and functional impairments observed in individuals with ASD such as an excessive gut inflammation and permeability, altered serotonin and kynurenin pathways, and increased neuroinflammation.

Therefore, we investigated the effect in germ-free (GF) mice of a fecal microbiota transplantation (FMT) from children with ASD on ASD-related behavior and ASD-related brain, immune and GI parameters. We chose two distinct groups of children with ASD as microbiota donors: one group without GI symptoms (group A) and one group with GI symptoms (group AG), namely a severe and chronic constipation. For both ASD groups, control children donors were their TD siblings. This limits the potential effect of genetic and environmental factors on microbiota composition between ASD and TD donor groups.

We investigated the effect of FMT in two mouse strains that present genetic and behavioral differences, i.e. the BALB/c and C57BL/6 strains. In both strains, FMT led to distinct gut microbiota alpha-diversity and composition in ASD-recipient mice, compared to TD sibling-recipient mice. Many of those differences were strain-specific but, remarkably, a decreased proportion of *Prevotellaceae* was found consistently in both mouse strains in AG-recipients, compared to the TD sibling-recipient mice. In C57BL/6 mice, FMT from group A decreased locomotor activity and FMT from group AG increased repetitive behaviors and impaired spatial memory, compared to FMT from TD siblings. Those alterations were accompanied by a decreased proportion of pro-inflammatory T lymphocyte populations in the spleen and an increased expression of genes involved tight-junction function and regulation in the gut of both A and AG group-recipient mice compared to sibling-recipient mice. In BALB/c mice, there was no difference of behavior between ASD recipient mice and sibling-recipient mice, but FMT from both groups A and AG reduced the number of serotonergic neurons in the raphe nuclei, compared to FMT from TD siblings. In addition, in this strain, FMT from group A reduced markers of inflammation in the colon, compared to FMT from siblings.

This study brings more data on the effect of FMT from individuals with ASD on GF mice, highlighting the importance of the genetic background of the recipient animals and consideration of GI symptoms in studies on the microbiota-gut-brain axis.

ACKNOWLEDGMENTS

I would like to thank my supervisors Laurent and Sylvie, for their support during those three and a half years of PhD (and the internships before that!). You have been really implicated in my work and very attentive and helpful during the ups and downs of the project. I have learned a lot and I am really glad to have been your student.

I would also like to thank everyone else in the Amipem team, led by Philippe Gérard. I have been really happy to be a part of this team for my PhD, and before that, for my first ever internship and my master's internship, there is a reason I kept coming back! More specifically, I would like to thank Elise for your help with the animal experiments and in the lab, Catherine for your help and advice in general and for leading me through the SCFAs analyses, Magali for the 16S rRNA sequencing analysis and showing me many lab techniques, and Vivien and Sandy for your advice and precious help in the lab. Plus, I would like to thank Christine Véronique, Xu fei and Mélanie, who have always been ready to help and give me advice when needed.

Thank you to all the students that have participated in the project during their internships, Ines, Elisa, Victor, Sandra, Viviana and Charlotte. You have been a great help during this project, and I was glad to work with you.

I also am very grateful to the team in Anaxem, who have helped me through the animal experiments and provided everything needed to carry them out successfully. More specifically, Anne for teaching me to work with animals and in isolators, and helping many times with my experiment, Fatima for installing and repairing the brand new isolators and for your help when I had issues with them, Aurélie for always finding solutions to my problems, and answering my many questions, and Claire, Joanne, Amanda and Camille for all your help with the mice and preparing the materials for my isolators.

Thank you to our collaborators in EBRIS, who welcomed me at the very beginning of my PhD for the crucial task of collecting the donor's samples. It was nice to have such a great view on the Amalfi coast during the not so glamorous task of aliquoting stool samples! More specifically, thank you Ilaria for coordinating the collect, and Chicha for helping me during my stay and for taking care of the last few samples left after I was gone.

I also want to thank our collaborators in Utrecht University, the team led by Aletta Kraneveld, for their participation in the experiment and their welcome during my stay there. I felt very welcomed and had a great stay. Thank you, Paula, Naïka and Lucía, for your advice, help, and answers to my many questions, and for leading most of the immunohistochemistry experiments with the help of Johana, Patricia, Ioana and Paula.

Thank you also to our collaborators in Tampere University, Reija, Karoliina and Jake, for the bioinformatics and correlation analyses and for welcoming me in Finland. I had a very nice time, and it was nice to exchange on the analyses in real time.

I would also like to thank Marie-Laure Michel and Aurélie Magniez of the Probi-hôte team here in Micalis, that advised and helped me for the flow cytometry protocol and more specifically Marie-Laure for the analysis.

Thank you to all the members of my jury for accepting to be a part of it, and for taking the time to travel to Paris, from quite far away for some, to attend the defense.

Finally, thank you to my family and friends, for being here and reassuring when I was frustrated with my issues with the experiments, and during the writing of this thesis.

DISSEMINATION AND TEACHING

PUBLICATIONS

1st author publication:

- Léa Roussin, Naïka Prince, Paula Perez-Pardo, Aletta D. Kraneveld, Sylvie Rabot and Laurent Naudon. **Role of the Gut Microbiota in the Pathophysiology of Autism Spectrum Disorder: Clinical and Preclinical Evidence.** *Microorganisms* 2020, 8, 1369. <https://hal.inrae.fr/hal-03245982/document>

Other publications:

- Jacopo Troisi et al. 2020 **Genome, Environment, Microbiome and Metabolome in Autism (GEMMA) Study Design: Biomarkers Identification for Precision Treatment and Primary Prevention of Autism Spectrum Disorders by an Integrated Multi-Omics Systems Biology Approach** *Brain Sciences* 2020, 10 (10), pp.743. <https://hal.inrae.fr/hal-03276479>
- Lucía N. Peralta-Marzal, Naika Prince, Djordje Bajic, Léa Roussin, Laurent Naudon, Sylvie Rabot, Johan Garssen, Aletta D. Kraneveld and Paula Perez-Pardo. **The Impact of Gut Microbiota-Derived Metabolites in Autism Spectrum Disorders** *International Journal of Molecular Science* 2021. 22(18):10052, <https://www.mdpi.com/1422-0067/22/18/10052/htm>

POSTER, PRESENTATIONS

Poster: FENS 2022 9th-13th July 2022

Presentations: -October 2020 and 2021: Presentation of my project to students from the Master "Biodiversity Genomics and Environment"

-February 2021: Participation in "Skype a Scientist". Online presentation of my project to middle school students.

-October 2021: Presentation at the Optinutribrain Symposia

-April 2022: Presentation at the doctoral days of the ABIES graduate school

-November 2022: Presentation at the Beneficial microbes conference (9th edition)

-December 2022: Presentation at the "Young researcher symposia" of FHU PaCeMM

Other: I was interviewed about the GEMMA project by the communication department of Paris Saclay University to be included in the 20th issue of "L'Edition" (Scientific vulgarization Journal of Paris Saclay University) that came out in January 2023.

TEACHING

-In 2021 I supervised tutorials (12h) and practicals (24h) for bachelor students of Paris Saclay University (Versailles-St-Quentin campus).

-I was part of the organizing committee of the second edition of the summer school "From Gut to brain: how our microbiome influences our emotions and behaviors" that took place from the 23rd to the 27th of august 2021.

SUPERVISING

During my PhD, I participated in the supervising of the internships of bachelor and master students: Ines Haddam and Elisa Gry (2nd year of masters), Sandra Letort (1st year of masters), Viviana Bisceglia and Victor Gomez (3rd year of bachelor) and Charlotte Rossignol (2nd year of engineering school)

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ABBREVIATIONS

4-EPS	4-Ethylphenylsulfate	DCM	Dichloromethane
5-HIAA	5-hydroxyindoleacetic acid	DMEM	Dulbecco/Vogt modified Eagle's minimal essential medium
5-HT	5-hydroxytryptamin (Serotonin)	DMSO	Dimethylsulfoxyde
5-HT1A	5-HT receptor 1-A	DNA	Deoxyribonucleic acid
A	"ASD" donor group	DO	Displaced object
AADC	aromatic acid decarboxylase	DOPAC	3,4-dihydroxyphenylacetic acid
ADAM	A disintegrin and metalloproteinase	ECs	Enterochromaffin cells
ADOS	Autism diagnostic observation schedule	ENS	Enteric nervous system
AG	"ASD+GI" donor group	EPM	Elevated plus maze
ASD	Autism spectrum disorder	EtOH	Ethanol
ASV	Amplicon sequence variant	FCS	Fetal calf serum
BBB	Blood brain barrier	FITC-SA	Fluorescein isothiocyanate-sulfuric acid
BDNF	Brain derived neurotrophic factor	FMT	Fecal microbiota transplantation
BEV	Bacterial extracellular vesicle	FOS	Fructo-oligo saccharide
BSA	Bovine serum albumin	FST	Forced swim test
CMA	Cow's milk allergy	GABA	Gamma-aminobutyric acid
CNS	Central nervous system	GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
CT	Cycle threshold	GEMMA	Genome, Environment, Microbiome and Metabolome in Autism
CTF	Corrected total fluorescence	GF	Germ free
CV	Conventional	GFAP	Glial fibrillary acidic protein
DA	Dopamine	GI	Gastrointestinal
DBE	Dibenzyl ether	GSRS	Gastrointestinal Symptoms Rating Scale

HPA	Hypothalamic-pituitary-adrenal axis	PCR	Polymerase chain reaction
HVA	homovanillic acid	PFA	Paraformaldehyde
IBA1	Ionized calcium-binding adapter molecule 1	PFC	Prefrontal cortex
IDO	Indoleamine-2,3-dioxygenase	PVN	Paraventricular nucleus
IFN	Interferon	QA	Quinolinic acid
IL	Interleukin	qPCR	Quantitative polymerase chain reaction
ILC3	Innate lymphoid cells	REFS	Recursive ensemble feature selection
KA	Kynurenic acid	RNA	Ribonucleic acid
KO	Knock-out	ROS	Reactive oxygen species
KYN	Kynurenin	RPMI	Roswell Park Memorial Institute Medium
LCL	Lymphoblastoid cell lines	S-A	Siblings of "ASD" donor group
LPS	Lipopolysaccharide	S-AG	Siblings of "ASD+GI" donor group
MHFD	Maternal high fat diet	SCFAs	Short chain fatty acids
MHPG	3-methoxy-4-hydroxyphenylglycol	SERT	Serotonin transporter
MIA	Maternal immune activation	SFB	Segmented filamentous bacteria
MLCK	Myosin light chain kinase	SPF	Specific pathogen free
NDO	Non displaced object	TD	Typically developing
NE	Norepinephrin	TNF	Tumor Necrosis Factor
NMDA	N-methyl-D-aspartate	TPH1	Tryptophan hydroxylase 1
NR	NMDA receptor	TPH2	Tryptophan hydroxylase 2
NSFT	Novelty suppressed feeding test	Trp	Tryptophan
OF	Open-field	VPA	Valproic acid
OMV	Outer membrane vesicle	WT	Wild-type
PBS	Phosphate buffered saline	ZO-1	Zona-occludens 1
PCoA	Principal component analysis		

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Remarks:

-This thesis refers to concepts associated with analysis of the gut microbiota. These concepts are explained in detail in Annex 1 p.246

-A lexicon of clinical scales used for assessment of gastro-intestinal and behavioral symptoms of ASD that are mentioned in this thesis is available in Annex 2 p.251

-The general introduction was redacted using as a basis the published literature review titled: "Role of the gut microbiota in autism spectrum-disorder: clinical and preclinical evidence" that I wrote during the first year of my PhD. The original paper can be found in Annex 5 p.257

1 GENERAL INTRODUCTION:

1.1 THE MICROBIOTA-GUT-BRAIN AXIS

The interconnection between the gut and the brain has been studied since the 19th century, although a more intricate understanding of the bidirectional communication between the enteric nervous system (ENS) and central nervous system (CNS) only appeared in the late 20th century. In parallel, research on the gut microbiota and how it can impact health has also appeared in the second half of the 20th century [1, 2]. However, it is only quite recently that the role of the gut microbiota in gut-brain communication has been truly considered. The first experimental report came from a paper published in 2004, by Sudo et al. in which the authors reported hypothalamic-pituitary-adrenal axis (HPA) axis overactivation after acute stress in germ-free (GF) mice compared to specific pathogen free (SPF) mice (i.e. conventional), which was partially reversed by colonization of the GF animals with SPF microbiota [3]. Since then, starting in 2011, more studies on GF, or antibiotic-treated animals, were published. They mostly confirmed those first observations and showed that a lack, or depletion, of microbiota could also impact behavior (locomotion, anxiety-like behavior, resignation, social behavior, spatial memory, fear extinction behavior), blood brain barrier (BBB) permeability, neuroinflammation and various neurotransmitter systems (serotonin, dopamine (DA), noradrenaline, glutamate, oxytocin) in the brain and/or the gut. Plus, those defects were sometimes corrected, at least partially, by recolonization of the animals [4–22]. In parallel, gnotobiotic animals (GF mice colonized with a controlled bacterial population) showed that specific bacterial groups, species or metabolites/compounds like indole or lipopolysaccharide (LPS), could have an impact on the brain (microglial maturation, brain immune response, BBB permeability) and behavior (mobility, resignation, anxiety-like behavior, social behavior) [13, 14, 16,

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18, 23–26]. Plus, a few studies have transferred behavioral phenotypes and brain alterations to mice through microbiota transfer from human patients suffering from major depressive disorder [10, 27–30], Parkinson's disease [31] and autoimmune encephalitis [32].

Based on animal studies using GF, antibiotic-, or probiotic-treated mice, a few mechanistic pathways have been proposed as to how the bacteria in the gut can impact the brain (Fig 1) [33].

Firstly, there has been some evidence that the anxiolytic effect of probiotic treatments in mice was revoked after vagotomy, which implies that the vagus nerve was necessary in this effect [25, 34, 35]. However, in other studies, vagotomy did not prevent the detrimental effect of GF status or antibiotic treatment, or the beneficial effect of recolonization [5, 12, 13], which implies that there are other ways of communication besides the vagal pathway. One of those is the immune system. Indeed, it is known that bacterial metabolites, or components of dead bacteria, can influence immune homeostasis. They can alter blood cytokine levels and thus affect the brain either directly through the blood circulation, or by creating a general inflammatory state, that can impact nervous transmission to the brain. Bacterial compounds can also stimulate the secretion of neuropeptides by enterochromaffin cells (ECs) in the gut, which could impact the brain either through the vagus nerve, or directly through the bloodstream. Finally, bacterial products could also pass directly in the bloodstream and may reach the brain (particularly when gut and/or brain permeability is increased) [33] (Fig 1). Recently, the role of BEVs (bacterial extracellular vesicles) and OMVs (outer membrane vesicles) in this communication has been brought into light. Indeed, those vesicles are capable of transporting and delivering RNA, DNA and protein cargo to the brain via circulation as they can cross epithelial barriers and more specifically the BBB. They could explain the effect of peripheral LPS exposure on brain inflammation, as free LPS cannot cross the BBB, but OMVs carrying LPS on their surface can. Studies on OMVs or BEVs derived from patients with Alzheimer's disease, or specific bacteria (*Lactobacillus plantarum*, *Paenacaligenes hominis*), show that they can impact memory, neuroinflammation, BBB permeability, brain derived neurotrophic factor (BDNF) and neurotrophin-4 levels or tau phosphorylation, either through direct passage to the brain or vagal nerve stimulation [36].

While the research on the gut microbiota brain axis started off with research on anxiety and depression, its implication in neurodegenerative disorders, other psychiatric pathologies and neurodevelopmental disorders is now more and more studied. In this introduction, I will describe the clinical and preclinical studies that have investigated the gut brain axis in autism spectrum disorder (ASD).

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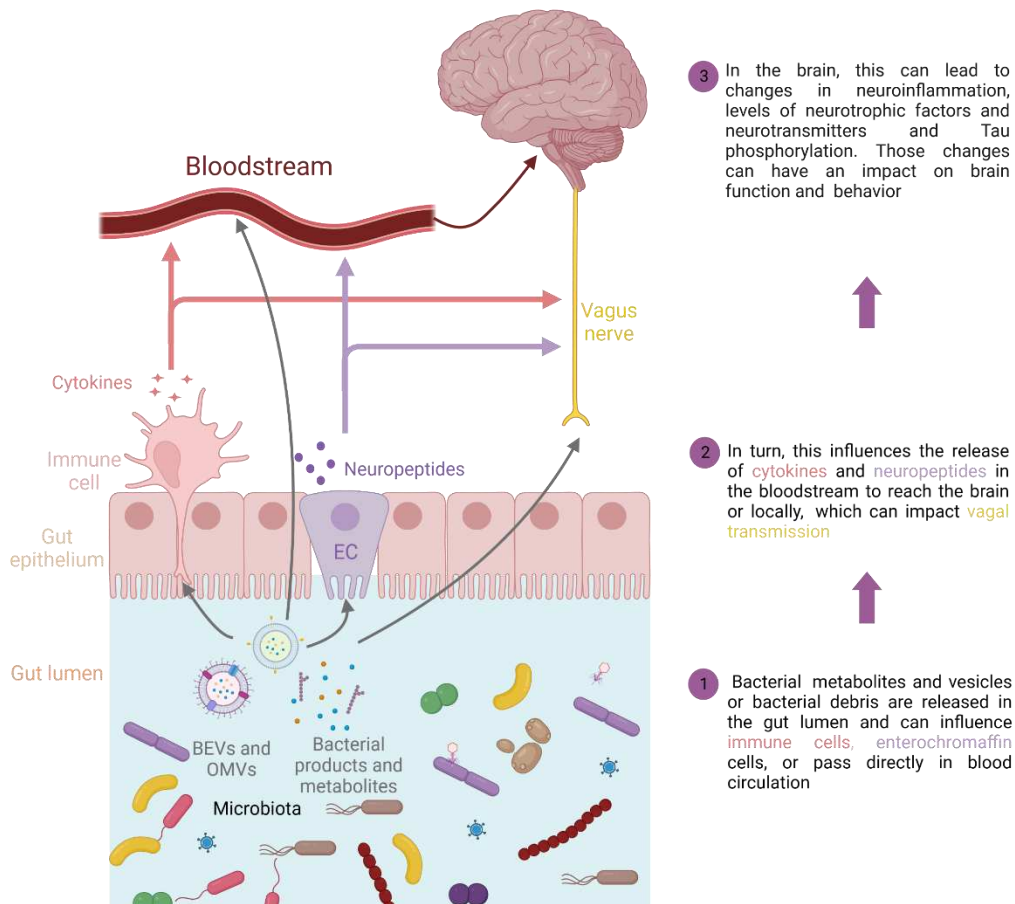


Figure 1: Schematic representation of the different communication pathways between the gut microbiota and the brain. Figure created on Biorender.com and inspired by a figure from Rabot et al. (2017) [33]

1.2 AUTISM SPECTRUM DISORDER (ASD)

ASD is one of the most prevalent neurodevelopmental disorders, characterized by impairment in social behavior and communication, and prevalence of repetitive and stereotyped behaviors and interests. It is often accompanied by increased or decreased sensory sensitivity and can also involve cognitive impairments and anxiety disorders [37].

As implied by the term "spectrum", individuals with ASD can present wildly different symptoms with varying severity. Plus, there is no defined biomarkers for ASD, as it is a multifactorial disorder, and can only be reliably diagnosed through behavioral examination by a health professional at 18-24 months of age at the earliest. Although the behavioral diagnosis of ASD has improved in the past

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decade, it is still hard to characterize, and many people with ASD (especially those who are verbal and show no cognitive impairment) are diagnosed late in life [37–39]. ASD is also considered to be two to three times more prevalent in males than females. However, this ratio might be biased by the fact that many diagnostic tools often underdiagnose females [40].

In the last 50 years, the prevalence of ASD has tremendously increased, and continued to do so in the last two decades, when diagnostic tools became more efficient. Prevalence was considered to be around 1 in 150 in the United-States in 2000, and is now estimated at 1 in 59 in the US, 1 in 89 in the European Union and 1 in 97 worldwide [41–45].

In the early 1990s, ASD was believed to be due at 90% to genetic factors and highly heritable due to the high risk for siblings of individuals with ASD. More recent studies described a genetic heritability of around 50%. However, these data are difficult to assess accurately, as the genetic variants responsible for ASD are also associated with other neurodevelopmental disorders. The same studies have proven that environmental factors also play an important role in this disorder and can in part explain such an increase in the prevalence of ASD [46–50]. Thus, it is interesting to investigate how external factors, such as nutrition, medication, or other environmental factors that could cause inflammation, oxidative stress or endocrine disruption could impact ASD symptoms. Since those factors are known to also impact the microbiota, it is possible that one way of action of those factors on ASD is through microbiota modification.

It is important to point out that the prevalence of gastrointestinal (GI) symptoms is four-times higher in individuals with ASD than in typically developing (TD) individuals. Those GI abnormalities are often characterized by increased gut permeability and abnormal immune function in the gut, that correlate with ASD severity [51–53]. Interestingly, Luna et al. (2017) reports that children with ASD with functional GI disorders show a distinct gut microbiota and immune signature compared to TD children with the same GI symptoms. This study also found a correlation between the ASD-specific dysbiosis (more specifically the increase in species from the *Clostridiales* order) and GI symptoms (inflammation and abdominal pain) [54]. Correlations between microbiota and GI symptoms (constipation) have also been observed in other studies on individuals with ASD [55, 56]. Finally, in Wang et al. (2020), increased blood levels of zonulin (a marker of increased intestinal permeability) were corrected by probiotic treatment with an improvement in ASD symptoms [57]. This suggests that these GI symptoms are an integral part of ASD pathophysiology and are in interaction with the gut microbiota and the immune system.

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1.3 CLINICAL AND PRECLINICAL EVIDENCE FOR INVOLVEMENT OF THE GUT MICROBIOTA IN VARIOUS ASPECTS OF ASD

1.3.1 Gut microbiota composition and Changes in Bacterial Metabolites in ASD

1.3.1.1 Clinical Evidence

In 2000, Sandler et al. [58] hypothesized that dysbiosis due to antibiotic treatment in young children was involved in the apparition of regressive autism observed in some of those children. They postulated that this was due to colonization of neurotoxin-producing bacteria and started a clinical trial on 18 children with a vancomycin treatment with the aim of eliminating these bacteria. They observed improvement in the behavior of those children during the treatment, but it did not last after stopping the treatment. Although the association between early-life antibiotic treatment and ASD has not been confirmed since then [59], this princeps study proved the existence of a causative link between modulation of the gut microbiota composition and behavior, in a subset of children with ASD. In 2012, the same team published a summary of their research on the subject, highlighting a dysbiosis in children with ASD. One genus, *Desulfovibrio* was present in 50% of children with ASD, some of their siblings, but never in unrelated controls. The proportion of *Desulfovibrio* correlated with severity of ASD symptoms [60].

Since then, many research teams have been investigating the gut microbiota of children with ASD, and most of them have observed a difference of composition compared to TD individuals. Three meta-analyses compared these data in an attempt to identify specific bacterial taxa with a consistent pattern of change across studies. The first one [61] analyzed 9 papers, the second [62] studied 17 papers including 8 from the 9 papers reviewed in [61] and the third [63] analyzed 18 papers including 7 of the ones included in the first study [61] and 12 of the ones included in the second study [62]. All three meta-analyses reported a decrease in *Bifidobacterium* and two of them [61, 62] an increase in *Faecalibacterium* and *Clostridioides* in children with ASD despite high interstudy heterogeneity. Only Iglesias-Vasquez et al. (2020) [62] reported differences at the phylum level, such as higher Bacteroidota/Firmicutes ratio in children with ASD, or elevated relative abundance of Proteobacteria. The three meta-analyses present discrepancies, as Xu et al. (2019) [61] reported a decrease of *Bacteroides* and *Parabacteroides* in children with ASD when Iglesias-Vázquez et al. (2020) [62] reported an increase of both those genera. Plus, Andreo-Martinez et al. (2021) observed a decrease in *Streptococcus*, which was not observed in the other two

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papers [63]. Furthermore, Xu et al. (2020) [61] observed a lower abundance of *Akkermansia* in children with ASD when the other two studies [62, 63] reported no difference. However, the observations of Xu et al. (2020) and Iglesias-Vasquez et al. (2020) [61, 62] might be disputable, as Andreo-Martinez et al. (2021) reanalyzed those data with a different method, which they claim mitigates biases, and found that none of the described differences were statistically significant [63].

Around the same time, two systematic reviews were also published by Ho et al. (2020) [64] and Bezawada et al. (2020) [65] who compared 26 and 28 studies, respectively, including 14 and 16 of the studies included in any of the previously mentioned meta-analyses. Both underlined the heterogeneity of results among studies and reported a few consistent results. They pointed out that many studies observed an increase in some *Clostridioides* species, and a lower proportion of *Bifidobacterium*. Bezawada et al. (2020) [65] also reported that the *Sutterella* genus was found to be more abundant in children with ASD in many studies.

Overall, those meta-analyses and reviews confirm the presence of a dysbiosis in ASD but highlight very heterogeneous results among studies. These could be due to methodological differences, but also to the fact that the different cohorts come from multiple countries with different lifestyles and dietary habits. In addition, the age groups of the children recruited in the different studies vary significantly, with some including children as young as 2 years old, an age at which the gut microbiota is not completely stabilized [66]. Despite this, there seems to be a rather consistent increase of *Clostridioides*, which is considered to be a putative harmful genus, and a decrease in *Bifidobacterium* which is considered beneficial. Surprisingly, however, two of the meta-analyses [61, 62] report an increase in *Faecalibacterium* in individuals with ASD, when the only known species from this genus, *Faecalibacterium prausnitzii*, is considered a beneficial bacterium with anti-inflammatory properties [67].

One other factor to consider is the impact of alimentation on the microbiota. In a recent study, Yap et al. (2021) analyzed the microbiota of 99 children with ASD through a metagenomic analysis, compared to two control groups: one composed of TD siblings of some of the ASD group, and one composed of unrelated TD individuals. The authors used age, sex, and dietary preference as covariates and did not find any differences in microbiota composition between ASD and control groups, aside from reduced relative abundance of *Romboutsia timonensis* in the ASD group. α -Diversity (see Lexicon in Annex 1 p.247) had a positive correlation with dietary diversity and a negative correlation with Bristol scores but no correlation with behavioral symptoms (Autism Diagnostic

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Observation Schedule (ADOS) and Social Responsiveness Scale) suggesting that the differences in microbiota composition observed in some ASD cohorts could be due to the fact that children with ASD often have food aversion or sensitivity which leads to a less diverse diet. Besides, behavioral scores were positively correlated with dietary diversity [68]. However, in a study of a similar scale, Chen et al. (2022), also included dietary preference as a covariate (as well as GI symptoms) and did find multiple differences in microbiota composition (assessed by 16S rRNA sequencing) up to family level between the ASD and control groups, some correlating with behavior [69].

It is interesting to point out that, among the different studies previously mentioned in this part, the control groups differed in their constitution. They were of three types, either composed of only siblings of children with ASD, only of unrelated individuals or of both siblings and unrelated individuals. In the studies with both types of control groups, the sibling group often seemed to have a different microbiota profile compared to unrelated individuals, and was sometimes closer to the "ASD profile" [70–74]. This is not surprising considering the influence of genetics and environment on gut microbiota composition. Unpublished work from Luna et al. presented at the 74th Annual Meeting Society of Biological Psychiatry Chicago (May 2019) [75] reported a significant difference between the microbiota of TD siblings of children with ASD and unrelated controls. This team also reported differences in the composition of the microbiota depending on the presence or absence of GI symptoms. This has been confirmed in a recent study that observed differences in β -diversity and relative abundance of some phyla (notably increased abundance of *Faecalibacterium*) in young children with ASD and GI symptoms compared to children with ASD and no GI symptoms [76].

Overall, the variability between studies can be explained by heterogeneity in composition of the ASD and control groups, and whether the authors took GI symptoms and dietary preferences into account. Only an increase in the number of studies and a better standardization of recruitment protocols and study design could address this issue. Longitudinal studies that taking into account environmental factors such as diet or treatments would also allow a better characterization of ASD-related microbiota modulations.

In an unpublished report presented at the 9th Beneficial Microbes conference, Peralta-Marzal et al. used a machine learning approach called Recursive ensemble feature selection (REFS) in 3 independent public datasets of microbiota composition from cohorts of children with and without ASD. They used REFS on

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one of the datasets (117 samples) to identify 26 relevant features (Amplicon sequence variants (ASVs) that allow most differentiation between TD and individuals with ASD) (see lexicon for ASVs in Annex 1 p.246) then tested those features on the 2 other datasets (223 samples), in which they could identify which subject was ASD or TD with good accuracy solely based on the microbiota composition. This suggests that, despite the confounding impact of diet or other factors, there is still a potential for finding ASD specific taxa through machine learning approaches. In all three datasets, there was a consistent increase in ASVs from *Clostridiaceae* and *Anaerospironacter*, and a decrease in *Butyricoccus*. It would be interesting to apply this method to a greater number of datasets in the future.

The existence of a different gut microbiota in many individuals with ASD compared to TD individuals is now well-accepted, but its relationship with the disorder and its potential impact on them is still not understood. For this reason, researchers have been focusing on bacterial metabolites that are differentially modulated in children with ASD. Different teams have found an increase in urinary *p*-Cresol, a bacterial metabolite derived from tyrosine, in young children with ASD [77–80]. These teams hypothesized that this increase could be due to a higher level of *p*-Cresol producing bacteria such as *Clostridioides difficile*. Polyethylene glycol treatment to mobilize gut in children with ASD and chronic constipation improved behavioral symptoms at 6 months and modulated urinary *p*-Cresol levels, although this was not correlated with the behavioral improvement [81]. Interestingly, fecal levels of *p*-Cresol have also been found to be increased in children with ASD by Kang et al. in two different studies [79, 82]. Although the increase of urinary and fecal *p*-Cresol in young children with ASD has been observed several times, there is little evidence so far explaining the mechanisms underlying this increase (aside from gut motility). While recent *in vivo* and *in vitro* studies showed that *p*-Cresol could alter neurite growth, synaptic density, neuronal activity, dopaminergic transmission and neuroinflammation [27, 83–85], there is still little understanding on the effect of elevated *p*-Cresol in ASD.

Short chain fatty acids (SCFAs) are considered to be key actors of the microbiota-gut-brain axis, and their involvement in multiple neurological disorders has been increasingly described [86]. In the context of ASD, some studies have reported altered fecal levels of SCFAs in children with ASD, but with great diversity in results [52, 57, 76, 87–89]. Adams et al. (2011) reported a decrease in total SCFAs in stool of individuals with ASD [52] while Wang et al. (2012) [87] reported an increase. Butyrate levels were found to be decreased in the stools of individuals with ASD in Liu et al. (2019) and Wang et al. (2020) [57, 88] but were increased in another

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study [76]. Both Deng et al. (2022) and Liu et al. (2019) found increased levels of valerate in the stools of individuals with ASD [76, 88]. Fecal propionate levels were increased in children with ASD according to two studies [76, 87] but found to be decreased in Wang et al. (2020) [57]. Jones et al. (2022) found elevated acetate in very young children that had just been diagnosed with ASD in a cohort of at-risk children [56]. Finally, Averina et al. (2020) [89] found decreased expression of genes related to production of butyrate in the metagenome of children with ASD.

As of now, there is still little clinical evidence of the impact of those SCFAs differences in ASD pathophysiology; most of the evidence comes from animal studies and will be discussed later in this review. Interestingly however, patients suffering from propionic acidosis, a genetic disorder characterized by an accumulation of propionate, present neurodevelopmental delay, and there is a very high prevalence of ASD (21%) in patients with this disease [90]. Propionate can increase oxidative stress, thus influencing mitochondrial activity. Mitochondrial dysfunction has been reported in many individuals with ASD and believed to play a role in its pathophysiology [91]. Frye et al. (2016) [92] showed that lymphoblastoid cell lines (LCL) derived from individuals with ASD had a different response to propionate than LCL from control subjects, especially in an oxidative environment, where propionate induced an overproduction of ATP and mitochondrial dysfunction. In a similar *in vitro* study, using butyrate, Rose et al. (2018) [93] found that LCL derived from individuals with ASD responded differently to butyrate than LCL from control subjects. In LCL from controls, butyrate decreased mitochondrial respiration when it did not significantly alter it in LCL from individuals with ASD and increased it in LCL from individuals with ASD with mitochondrial dysfunction.

Dysregulation of other gut microbiota metabolites, such as ones involved in oxidative stress, mitochondrial function, or neuro-immune regulation, has also been described in individuals with ASD [82, 94]. However, more research is still needed to fully understand the impact of those dysregulations on behavioral and GI symptoms of ASD.

1.3.1.2 Preclinical Evidence

Because of the multifactorial aspect of ASD, several murine models have been developed. Some are genetic models, like the Shank3 or Shank3B KO, NL^{3R451C}, PCDH9 KO, Fmr1 KO and 15s11-13 CNV models, based on extinction or mutation of genes known to be involved in some cases of ASD in humans [95–100] or the BTBR mouse strain, considered an idiopathic ASD model, based on its behavioral phenotype [101]. There are also many environmental models either based on a

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challenge during gestation that are known to be risk-factors for ASD in humans (maternal high-fat diet (MHFD), maternal immune activation (MIA), maternal exposure to valproic acid (VPA)) or during life (cow's milk allergy (CMA), early-life exposure to VPA) [102–105]. All those ASD models have been classified as such because they present altered behaviors related to ASD symptoms (social interaction and communication deficits and/or stereotyped behaviors). Interestingly, a growing number of studies reported GI symptoms in some of these models, like those observed in individuals with ASD. More precisely, increased intestinal permeability was found in Shank3 KO, BTBR and MIA mice [106–109], and abnormal cytokine profiles, or increased markers of inflammation and oxidative stress have been found in the gut of BTBR, MIA and MHFD mice [107, 109–113]. Plus, an increase of myeloperoxidase expression (marker of inflammation) was found in the ileum of VPA mice [114]. In the NL3^{R451C} mouse model, Hosie et al. (2018) [115] reported a faster transit associated with an increase of the number of nitric oxide producing myenteric inhibitory neurons in the jejunum. Similarly, Sharna et al. (2020) found increased number of those neurons in the caecum of this mouse model [116], when the opposite was observed in the colon of BTBR mice [108].

Interestingly, many studies also found gut microbiota dysbiosis in those models, detailed in Tables 1 and 2 (p. 54 and 58). Although the nature of the dysbiosis is very different among the different models, there are a few similarities. Firstly, a decrease in α -diversity has been described in Shank3 KO, BTBR, MIA and MHFD mice and VPA rats [108, 110, 117–120]. However, other studies in Shank3B KO, NL3^{R451C}, BTBR, MIA and VPA mice did not observe any change in α -diversity [25, 107, 109, 115, 121–123]. Most of the studies included in Tables 1 and 2 that assessed β -diversity observed a difference between controls and model animals, at the exception of 5 of them [99, 109, 122, 124, 125]. At the phylum level, an increase in *Bacteroidota* and a decrease in *Firmicutes* was observed in BTBR and MIA mice, and in VPA rats [108, 118–120]. This is in agreement with the elevated *Bacteroidota/Firmicutes* ratio in individuals with ASD described in a meta-analysis previously mentioned [62]. However, other studies reported an increase of *Firmicutes* in Shank3 KO, MIA and MHFD mice, and VPA mice and rats, which, except in Shank3 KO mice, was accompanied by a decrease in *Bacteroidota* [106, 110, 121–124]. The increase in *Proteobacteria* reported in individuals with ASD was not seen in ASD mice models except in Shank3 KO mice by Sauer et al. (2019) [106]. Plus, a decrease in this phylum was observed in BTBR mice by Coretti et al. (2017) [107]. At lower taxonomic levels, as in individuals with ASD, a decrease in *Lactobacillus* has been observed in MIA and Shank3 KO mice [109, 117] as well as a decrease in *Lactobacillus reuteri* in Shank3 KO, Shank3B KO and BTBR mice [25, 117] and a decrease in *Lactobacillus brevis* and *Lactobacillus ruminis* in Shank3 KO

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mice [117]. However, *Lactobacillus* was increased in BTBR and MIA mice [107, 123]. The *Prevotella* genus, which has been found to be decreased in individuals with ASD [126], was also decreased in Shank3 KO mice [117], MIA mice and VPA mice and rats [122, 124] but was increased in BTBR mice and in a different study in MIA mice [107, 118, 119]. Although changes in proportion of *Clostridioides* species seem to be recurrent in individuals with ASD, they were only observed in BTBR mice by Newell et al. (2016) [118], who found decreased and increased levels of various *Clostridioides* species in cecal contents and feces, respectively. This study underlies important differences between cecal and fecal composition, which must be taken in consideration, as most of the studies cited only assessed microbial composition of feces. Plus, most studies used only male mice; however, among the few that used both male and female animals, most observed strong sex-related differences in microbiota composition [107, 117, 120–122]. Both of those criteria should be considered in future studies.

In a recent systematic review, Alamoudi et al. (2022) have compared the microbiota composition in 13 clinical studies on individuals with ASD and in 7 of the preclinical studies in animal models mentioned in the previous paragraph. They have found only a few common alterations between studies on individuals with ASD and at least one of the animal model studies: at phylum level a decrease in *Verrucomicrobia*, and a modulation of *Firmicutes* (increased or decreased depending on the study). At the genus level, increased proportions of *Clostridioides*, *Bilophila*, *Dorea*, and *Lactobacillus* were found as well as a decrease in *Blautia* [127].

Overall, the bacterial alterations observed vary between studies and models, and only partially reflect the alterations observed in individuals with ASD. Nonetheless, the occurrence of microbiota alterations in multiple genetic and environmental rodent models of ASD is a strong indicator of the implication of the microbiota-gut-brain axis in ASD pathophysiology. As previously mentioned, GF rodents present impaired social interaction [7, 9, 13, 22] and thus have been proposed as an environmental ASD model [20]. The presence of those alterations in GF animals and the fact that they can be rescued by recolonization reinforces the hypothesis of a role of the microbiota in regulating those behaviors [13, 20, 22].

Interestingly, there are also reports of altered bacterial metabolites levels in different ASD models, similar to what is observed in individuals with ASD. In the MIA model, alterations in several serum metabolites have been observed; in particular, 4-ethylphenylsulfate (4-EPS), a metabolite that is derived from the bacterial metabolite 4-ethylphenol, was found to be drastically increased. 4-EPS is derived from tyrosine and is structurally close to *p*-Cresol. Interestingly, a probiotic

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treatment with *B. fragilis* NCTC 9343 restored normal serum levels of 4-EPS in the MIA model and ameliorated anxiety-like behavior, but did not have an effect on social or repetitive behaviors [109]. A whole range of intestinal bacteria can catabolize tyrosine into *p*-Cresol, but there is less literature on the bacterial production of 4-EPS [128]. Therefore, the reasons behind such an increase in this model are currently not known. Interestingly, a 4-week *p*-Cresol treatment impaired social behavior and increased repetitive behavior in wild-type (WT) mice [85] and in BTBR mice [84], in which it also induced hyperactivity and an increase in anxiety-like behavior. *p*-Cresol treatment also impaired excitability of dopaminergic neurons in the ventral tegmental area of WT mice, a circuit involved in the social reward system [129], and increased the levels of DA and related metabolites in various brain regions of BTBR mice [84]. In Bermudez-Martin et al. (2021), the effect of *p*-Cresol on behavior in WT mice seemed dependent on its effect on microbiota composition, as fecal microbiota transplantation (FMT) from *p*-Cresol treated mice to WT mice induced similar behavioral impairments, and, in contrast, FMT from WT mice to *p*-Cresol-treated mice restored normal social behaviors [130]. Another way that *p*-Cresol might affect brain function in the context of ASD is through its effect on the regulation of microglial activation. In a recent study, Zheng et al. (2022) observed elevated *p*-Cresol in VPA mice, accompanied by variation in ADAM10/ADAM17 ratio, which are metalloproteinases involved in tumor necrosis factor α (TNF- α), Interleukin-6 (IL-6) and TNF- α receptor-1 cleavage, which could play a role in microglial activation. *p*-Cresol treatment in murine microglial cell cultures (BV2 cells) decreased the expression of ADAM10 and ADAM17 and decreased LPS-induced TNF- α and IL-6 production in those cells [131].

The implication of SCFAs has also been investigated in ASD models. In the BTBR model, Golubeva et al. (2017) [108] reported decreased levels of acetate and isobutyrate, but increased levels of butyrate, in the caecum. In addition, increased levels of cecal butyrate have been observed in male VPA mice [121]. However, Cristiano et al. (2022) observed reduced serum concentration of butyrate in BTBR mice. Interestingly, in this study, administration of butyrate in mothers during pregnancy and lactation reduced repetitive behavior and restored normal social behavior in BTBR offspring. Butyrate treatment of the mothers also reduced the expansion of cerebellar cortex normally present in BTBR offspring, but this effect did not last into adulthood. Finally, the authors observed abnormalities in firing patterns and plasticity (long-term potentiation) of Purkinje cells in BTBR mice but not in BTBR offspring whose mother had received butyrate [132]. In another study, peripheral butyrate injection decreased some repetitive behaviors and improved social deficits in BTBR mice [133].

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There has also been reports of an effect of propionate on ASD-related behaviors, as its administration in the brain of rats impairs social behavior and cognitive functions and increases repetitive behaviors [134–136]. In addition, similarly to what is observed in the VPA model, mother exposure to propionate induces social deficits in the offspring [137]. Finally, acute peripheral treatment with propionate in rats induced social interaction impairment, as well as fewer neurons, an increased number of glial cells and differences in glial cell morphology in the amygdala [138]. Those observations have been since then repeated in other studies and propionate treated rats have been described as an environmental ASD model in recent studies [139–141].

1.3.2 Influence of the Gut Microbiota on Immune System Dysregulation in ASD

1.3.2.1 Clinical Evidence

1.3.2.1.1 *General inflammation*

Immune system impairments such as higher blood levels of pro-inflammatory cytokines, dysfunctional immune cells or presence of autoantibodies targeting brain proteins, have been observed in many children with ASD, and in their mothers during pregnancy and post-partum. Interestingly, studies have shown that those increases in pro-inflammatory cytokines can correlate with the severity of some behavioral symptoms [142–144]. Many clinical studies report higher prevalence of ASD following bacterial or viral infection during pregnancy, which could lead to an inflammatory environment in the placenta and amniotic fluid. It has been hypothesized that those infections, whether they occur before or after birth, could play a crucial role in ASD pathogenesis, as they can influence important neurodevelopmental mechanisms, like microglial maturation and synaptic pruning [144].

The gut microbiota and the immune system are intrinsically linked. It is accepted that a major constitutive function of the immune system is to control the microbiota and reinforce the intestinal barrier. In turn, the microbiota also has a direct effect on the immune system, as bacterial metabolites or compounds can influence differentiation of immune cells, or regulate their activity, not only in early postnatal development but throughout the lifespan. The mammalian immune system has co-evolved with the establishment of the microbiota, to reach a symbiotic relationship. However, this relationship can become deleterious depending on genetic background, environmental challenges or changes in nutrition [145, 146].

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Because of those observations, it has been hypothesized that one way of action of the gut microbiota in ASD was through its action on the immune system, more specifically on the balance of T cells populations. T cells subtypes are the results of CD4+ T cells differentiation, which is orchestrated by the levels of cytokines in a complex system of activation and inhibition (Fig 6).

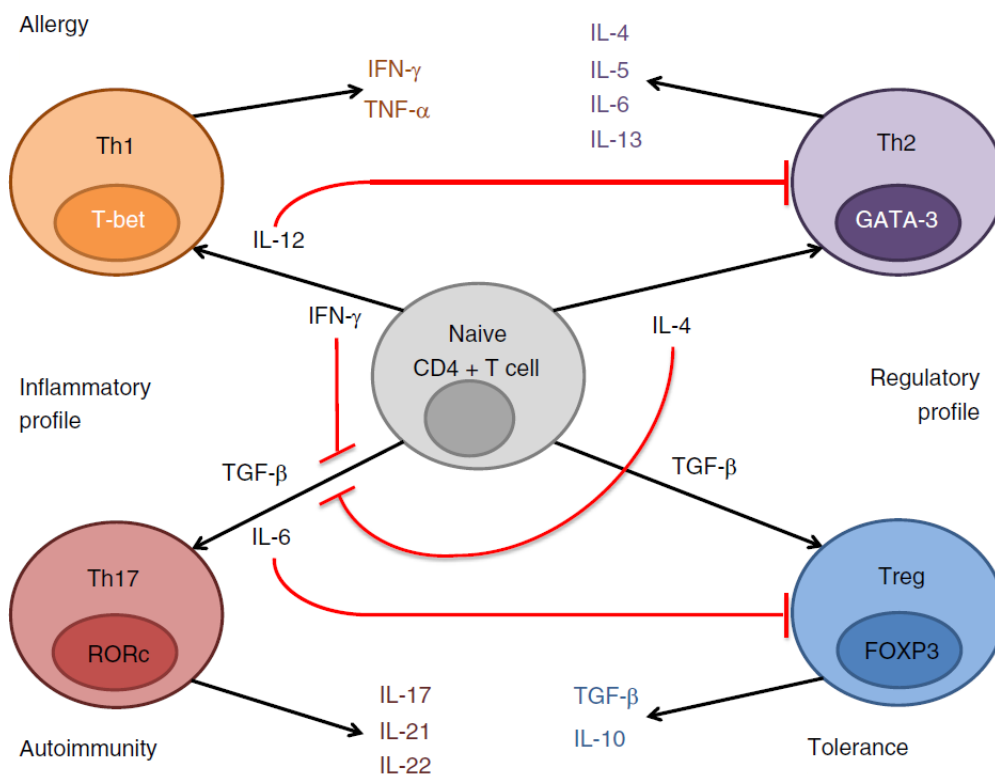


Figure 2: Schematic of T cell differentiation depending on cytokine environment and cytokines production profile of different T cell subtypes. An increased proportion of Th1 and Th17 leads to an inflammatory profile that has been linked to autoimmunity and allergy whereas the Th2 and Treg cells are associated with a more regulatory profile to avoid too much inflammation. Figure modified from Figueiredo et al. (2016) [147].

Th1 and Th17 cells are pro-inflammatory and can be involved in autoimmunity. On the contrary, Treg cells are anti-inflammatory and play a protective role against autoimmunity. Disruption of Th1, Th2, Th17 and Treg balance, characterized by changes in Th1/Th2, Th17/Treg, and Th1/Treg ratios has been linked to the pathophysiology of many autoimmune diseases, and could also be involved in ASD [147–152]. Although differentiation is mostly driven by immune signals such as chemokines or cytokines, the Treg/Th17 balance specifically could be influenced by an altered microbiota. Indeed, differentiation into Treg can be induced by some species of *Clostridiales* and by *Bacteroides fragilis*, whereas the

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differentiation into Th17 cells can be induced by some segmented filamentous bacteria (SFB) [153]. More specifically, *in vitro* and *in vivo* studies show that some secondary bile acids, which are produced by bacterial species of the human microbiota, can modulate Treg and Th17 differentiation [154, 155]. Finally, there is some evidence that OMVs derived from probiotics can influence Treg proliferation [36, 156]. There is less evidence of an impact of microbiota on differentiation into Th1, although an *in vitro* study by Park et al. (2015) showed that acetate and propionate could not only influence T cell differentiation into Th1, but also influence IL-10 production by T cells depending on the cytokine environment [157]. Overall, those findings show that microbiota and microbiota derived metabolites can play a central role in regulation of T cell populations.

Interestingly, in Rose et al. (2018) [158], pro-inflammatory cytokines were elevated in the serum and gut of individuals with ASD, and this elevation was higher in children with ASD and GI symptoms than in children with ASD and no GI symptoms. In addition, another study by the same team characterized circulating effector T cell populations in individuals with ASD with or without GI symptoms in comparison to TD individuals. They found increased levels of IL-17 positive CD4+ and CD8+ T cells in individuals with ASD compared to controls suggesting a more inflammatory profile, and this increase was even stronger in individuals with ASD with GI symptoms. The levels of interferon (IFN) γ were also increased in individuals with ASD with GI symptoms compared to individuals with ASD without. Furthermore, they found decreased regulatory T cells in both ASD groups compared to TD, and a decrease in Treg/Th17 ratio in individuals with ASD with GI symptoms [159]. These observations have been completed by the fact that some of the bacterial species altered in individuals with ASD appear to be associated with overproduction of IFN and pro-inflammatory cytokines in the gut. Indeed, a correlation was found between fecal levels of *Faecalibacterium* and increased levels of genes involved in type I IFN and IFN- γ signaling in immune cells of children with ASD compared to TD-unrelated controls [160]. Type I IFN signaling induces antimicrobial programs and is involved in regulation of innate and adaptive immunity, but also in autoimmune diseases [161]. In addition, Luna et al. (2017) [54] reported the existence of a correlation between levels of multiple bacterial species in children with ASD and GI symptoms, and elevated levels of various cytokines in their blood. Finally, in Liu et al. (2021), single nucleotide variants found in individuals with ASD and associated with immune response were correlated with the abundance of specific bacterial species (identified through a metagenomic analysis). Plus, elevated blood cytokine levels also correlated with bacterial species and bacterial metabolites in those individuals [162].

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1.3.2.1.2 Neuroinflammation:

Microglia are the immune cells of the brain, and, in a healthy environment, they play a role of sentinels to locate neuronal damage. However, in the presence of inflammatory cytokines, microglia enter an “activated” state and change in function and morphology to resemble macrophages. Under those conditions, astrocytes, which usually play a role in regulation of synaptic function and neurotransmission, can also become activated, and go under morphological changes into a state called astrogliosis (Fig 3). Microglial activation and astrogliosis have been found in many neurological diseases and disorders and reflect an increased inflammatory state in the brain. Some studies reported increased microglia activation and astrogliosis in ASD [163].

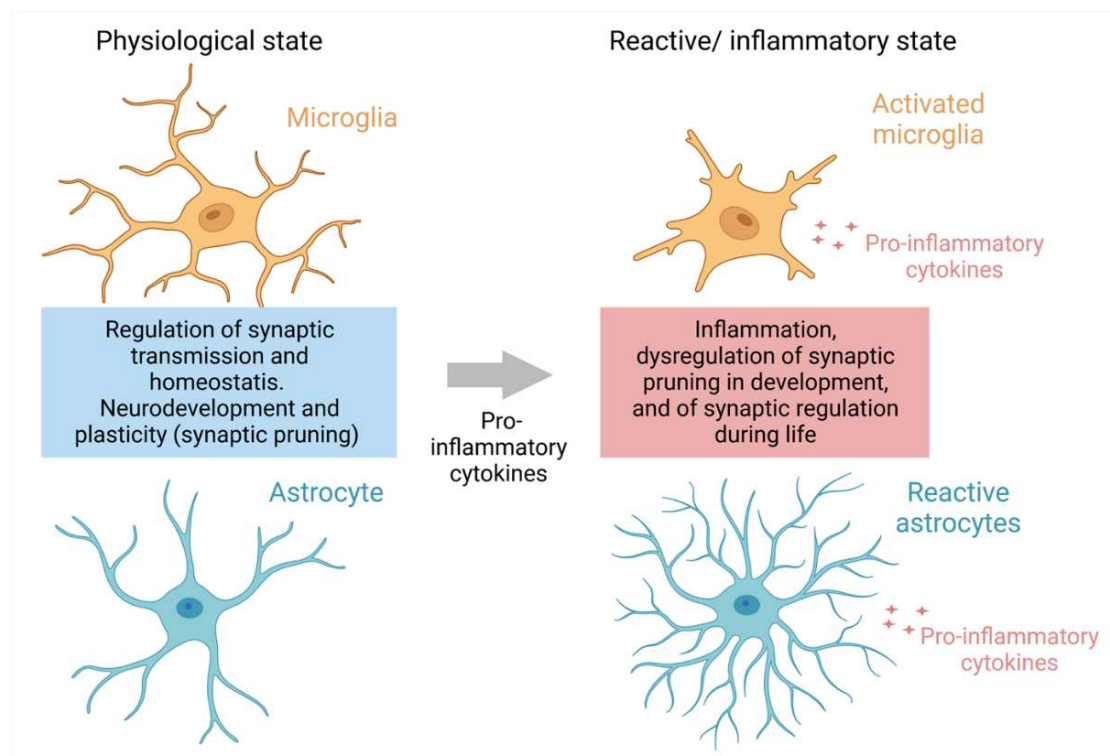


Figure 3: Functions of microglia and astrocytes in a physiological state, which can become deleterious when they are over-activated. Figure created on Biorender.com

Indeed, *post-mortem* observation of the brains of individuals with ASD revealed an increased number of glial fibrillary acidic protein (GFAP)-positive cells and increased GFAP protein levels, which are markers of astrogliosis. Plus, increased markers of reactive microglia and astrocytes were found in various brain regions, but most notably in the cerebellum. Similarly to what has been observed in the

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blood of individuals with ASD; increased levels of many pro-inflammatory cytokines were found in the brain and cerebrospinal fluid of individuals with ASD *post-mortem* [164, 165]. Suzuki et al. (2013) [166] used positron emission tomography and observed more reactive microglia in various brain regions of individuals with ASD compared to controls, most strikingly in the cerebellum. Those neuroglial alterations are believed to play a role in ASD pathophysiology, as microglia and astrocytes are involved in neurodevelopment, in part via synaptic pruning. In physiological conditions, synaptic pruning consists in reinforcement of important connections and removal of redundant connections by phagocytosis.

This process plays a crucial role in wiring the brain during development and is involved in plasticity during life but could be deleterious if overly activated (Fig 3). Thus, a reactive state of microglia and astrocytes in development and throughout life in ASD may result in changes in neuronal morphology and connectivity, which could contribute to behavioral and cognitive alterations [167]. To our knowledge there are no clinical studies that link those neuroinflammatory defects to the altered microbiota in ASD. Only animal studies, as described below, provide evidence for a crucial role of a complex microbiota in microglial maturation and function.

1.3.2.2 Preclinical Evidence

First, studies on GF animals proved that the microbiota is important for maturation of the immune system and helps maintain immune homeostasis [168] but also microglial development and function [11, 16]. Indeed, Erny et al. (2015) [16] showed that absence of microbiota from birth (GF mice) or depletion during life (SPF mice treated with antibiotics) led to immature microglia exhibiting a blunted response to LPS challenge. This was reversed by co-housing with SPF mice with a complex microbiota. Interestingly, a normal microglial phenotype was also restored by the administration of a cocktail of SCFAs in the drinking water. Another study found many differences in gene expression in the microglia of GF mice or antibiotic treated mice (to a lesser extent) compared to SPF, in embryonic and adult brains, in a sex-dependent manner. Plus, they found increased microglial density and excessive ramifications in embryonic brains of GF mice, but not antibiotic treated mice, in comparison to SPF mice [11]. Finally, Rothhammer et al. (2018) demonstrated therapeutic effects of a tryptophan-derived bacterial metabolite, 3-indoxylsulfate, on microglia- and astrocyte-related neuroinflammation in a mouse model of multiple sclerosis [169]. Those results proved that a complex microbiota and its metabolites are necessary for microglia maturation and can influence microglia and astrocyte function both during development and throughout life.

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As previously mentioned, an immune challenge during pregnancy in the MIA model results in a dysbiosis in the offspring along with altered communication, social and repetitive behaviors and cortical defects similar to those seen in individuals with ASD [103, 119, 170]. MIA offspring also display immune alterations similar to those of some individuals with ASD, such as an increase in IL-6 and IL-17 pro-inflammatory cytokines in the blood, and higher proportion of Th17 cells [109, 171]. Interestingly, in Hsiao et al. (2013) [109], a probiotic intervention with *Bacteroides fragilis* NCTC9343 was sufficient to restore normal IL-6 levels. Kim et al. (2017) [171] demonstrated that a vancomycin treatment in MIA mothers during the whole gestational period prevented Th17 dysregulation in mothers and the appearance of behavioral and cortical alterations in offspring. The authors suspected that the vancomycin treatment induced a depletion of SFB which can induce T cell differentiation into Th17. They observed no MIA-induced behavioral phenotypes in mouse strains lacking SFB, and in consequence producing less Th17. Gavage of those mice with SFB was sufficient to restore MIA-induced phenotypes in the offspring. These data demonstrated that the presence of SFB in the gut, and the consequent Th17 differentiation, was necessary to induce behavioral and cortical abnormalities in MIA offspring [171]. Finally, Tartaglione et al. (2021) found that the increased levels of TNF- α observed in the cerebellum of MIA offspring were correlated negatively with social behavior, and the relative abundance of Firmicutes and positively with the relative abundance of Bacteroidota [123].

It is interesting to note that immune dysregulations can also be observed in genetical or environmental models of ASD that are not related to an immune challenge. Plus, these dysregulations are often associated with bacterial modifications.

In BTBR mice, an enhanced inflammatory response to LPS challenge has been observed, and basal colonic levels of TNF- α and IL-6 were elevated, which correlated with some of the alterations in bacterial composition [107, 172]. In models based on deletion or mutation of the Shank3 gene, systemic increase of IL-6 and IL-17 have been observed, as well as more GFAP-positive cells, which is a marker for astrogliosis. Interestingly, treatment with *L. reuteri* MM4-1A could lower IL-17 levels in this model [106, 117].

In the MHFD model, an increase of peripheral IL-6 was observed, at basal level and following LPS challenge compared to normal diet offspring. Plus, MHFD offspring display more circular microglia with shorter branch length, which could suggest microglial activation [173]. In the same model, increased colonic levels of IL-1 β , IL-6 and TNF- α in adult MHFD offspring has been observed [110] as well

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as an increase in intestinal levels of IL-17 due to a higher proportion of innate lymphoid cells 3 (ILC3) in the intestinal lamina propria of the offspring [113]. In this last study, the authors treated pregnant mice with antibiotics to obtain offspring with depleted microbiota. The offspring was then transplanted with gut microbiota from either MHFD or control mice of the same age. They observed a higher proportion of ILC3 in offspring colonized with MHFD microbiota compared to offspring colonized with microbiota from controls. This result proved that the effect on ILC3 cells was dependent on the microbiota [113].

Finally, in the VPA model, various studies reported an increase of microglial density in various brain regions, and a LPS challenge induced overproduction of IL-6, IL-1 β and TNF- α in the brain and in the spleen [114, 174–176]. Plus, in this model, markers of neuroinflammation were found to be increased in the dorsal hippocampus associated with marked changes in microbiota composition in the intestinal tract [114, 121]. As previously mentioned, VPA mice and rats also have a disturbed microbiota with elevated butyrate production [120, 121]. Butyrate is often considered a beneficial SCFA in gut-brain axis regulation, and has been found to enhance intestinal permeability, but decrease BBB permeability in GF mice and promote anti-inflammatory responses [8, 86]. However, de Theije et al. (2014) [114, 121] proposed that the microbiota changes and the elevated butyrate levels they observed in the caecum of VPA mice could be associated to increased intestinal inflammation through modulation of the mucus composition. This lead would be interesting to pursue in order to gain a better understanding of the link between elevated butyrate and inflammation in this model.

Overall, those preclinical results show that immune challenges either during pregnancy or throughout life lead to ASD-like behaviors in rodents, and that this effect can be microbiota-dependent. Those observations, and the fact that immune dysregulations are present in many ASD models and often correlate with microbiota changes and altered behaviors, imply the existence of a microbiota-immune system-brain relationship that could be part of the pathophysiology of ASD. However, most of the evidence is still based on preclinical research and there is a need for more clinical research on the subject.

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1.3.3 Influence of the Gut Microbiota on Dysregulation of Tryptophan Metabolism in ASD

1.3.3.1 Clinical Evidence

Tryptophan (Trp) cannot be produced by the body and only comes from dietary consumption. Dysregulations of the Trp metabolism in ASD have been described. However, their implication in the disorder is still unclear. Dietary Trp is the precursor for serotonin (or 5-hydroxytryptamin (5-HT)) and kynurenine (KYN), whose pathways have been shown to be dysregulated in ASD [177]. KYN is produced mostly in the liver from Trp by indoleamine-2,3-dioxygenase (IDO), that is activated in presence of pro-inflammatory cytokines. KYN can then cross the BBB and, in the brain, be transformed into two derivatives, kynurenic acid (KA) in astrocytes or quinolinic acid (QA) in activated microglia. KA is neuroprotective and reduces excitotoxicity via inhibition of the N-methyl-D-aspartate (NMDA) receptors, whereas QA is an agonist of those receptors and thus overly activate which can have neurotoxic effects [177, 178] (Fig 4).

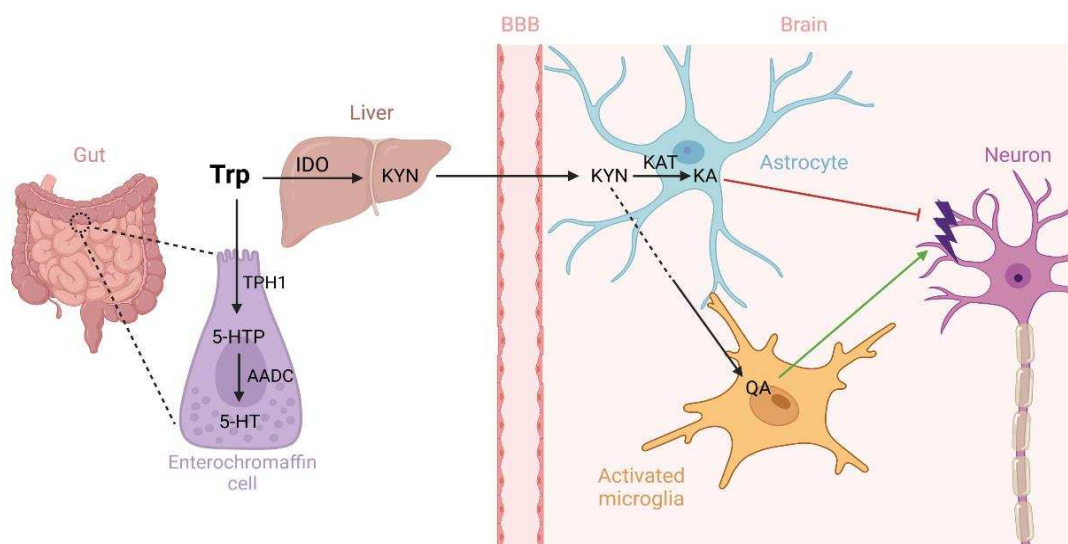


Figure 4: Schematic of Trp metabolism and the impact of KYN derivatives in the brain. Trp in the gut is transformed into 5-HT in ECs. In the liver, Trp can be transformed into kynurenin by IDO, activated in presence of pro-inflammatory cytokines (IL-6 and IL-1) and TNF- α . Kynurenin can cross the BBB and, in astrocytes, be transformed into kynurenic acid (KA) by kynurenin aminotransferase (KAT). In activated microglia, through a few intermediate steps, KYN can be transformed into quinolinic acid (QA). QA activates neuronal NMDA receptors, which can lead to excitotoxicity. KA inhibits those receptors, thus limiting this overactivation. Figure created on Biorender.com

In addition, KA could facilitate astrocyte differentiation into a protective state while QA can trigger astrocyte activation [179, 180]. Thus, the dysregulation of KYN pathway can also be involved in neuroinflammation.

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Serum of individuals with ASD presents lower KA concentration [178, 181], higher KYN/KA ratio and higher QA concentration [178]. Decrease of KA and increase of QA in the serum might reflect similar changes at the central level, thus leading to increased excitotoxicity, which may be involved in ASD pathophysiology.

The other main derivative of Trp is 5-HT. Ninety-five percent of 5-HT circulating in the body is produced by the gut ECs through the action of the rate-limiting enzyme tryptophan hydroxylase 1 (TPH1) and of the aromatic acid decarboxylase (AADC) (Fig 4). It plays a crucial role in regulation of GI functions. In the brain, 5-HT is produced via tryptophan hydroxylase 2 (TPH2) which is present predominantly in the raphe nuclei and plays an important role in various brain functions such as mood, sleep or appetite regulation. Furthermore, both central and peripheral 5-HT play a role in pre- and postnatal neurodevelopment, thus their dysregulation has been hypothesized to be involved in ASD pathophysiology [182, 183]. A few studies report central alterations of 5-HT in individuals with ASD [184–186]. However, most of the evidence towards dysregulation of the 5-HT metabolism in ASD comes from the fact that increased blood levels of 5-HT have been widely observed in individuals with ASD since the 1960s, being found in more than 25% of them [183, 187]. While this increase could be due to increased uptake by platelets or decreased breakdown, it could also be due to increased 5-HT release by ECs in the gut [183, 188]. One study has found a small positive correlation between severity of GI symptoms and whole blood 5-HT levels in individuals with ASD [189]. Interestingly, Luna et al. (2017) [54] found decreased levels of Trp and elevated levels of the 5-HT metabolite 5-hydroxyindoleacetic acid (5-HIAA) in rectal tissue of individuals with ASD with GI dysfunction, and those modulations correlated with the increase or decrease of some bacterial species in the gut microbiota of those individuals. Interestingly, Wang et al (2020) found elevated plasmatic levels of 5-HT and 5-HIAA in individuals with ASD, as well as decreased KYN. All of those alterations were restored after 108 days of a daily synbiotic treatment (mix of *Bifidobacterium infantis* Bi-26, *Lactobacillus rhamnosus* HN001, *Bifidobacterium lactis* BL-04, *Lactobacillus paracasei* LPC-37 and fructo-oligo saccharides (FOS)), which was not the case in children treated with placebo [57]. To our knowledge, this is the only study that assessed the effect of a probiotic treatment on the Trp pathway in individuals with ASD. However, it has been investigated in a few other studies in healthy subjects or patients with various pathologies. In a study by Kato-Kataoka et al. (2016) [190], daily intake of a fermented drink containing *L. casei* Shirota for 8 weeks prevented the elevation of plasmatic Trp of healthy subjects before a stressful examination period. In another study on adults suffering from functional constipation, a long-term administration (105 days) of *L. reuteri* DSM-17938 led

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to lower plasmatic levels of 5-HT [191]. Finally, daily administration of probiotics for 8 weeks, either the *L. helveticus* R0052/*B. longum* R0175 mix or *L. plantarum* 299v, resulted in a decrease in serum KYN/Trp ratio and a decrease in serum KYN levels, respectively, in patients suffering from depressive disorders [192, 193].

While these studies provide evidence that probiotic treatments can influence the Trp pathway, it is still unclear if the changes of microbiota in ASD are involved or not in the Trp alterations observed in individuals with ASD.

1.3.3.2 Preclinical Evidence

Multiple preclinical studies have proved that the microbiota can influence Trp metabolism. Clarke et al. (2012) [194] have observed increased plasmatic Trp and a decreased plasmatic KYN/Trp ratio in male and female GF mice, the latter being restored by gut colonization with SPF microbiota. Plus, two separate studies found that GF mice had lower colonic levels of 5-HT and lower colonic expression of TPH1 mRNA, compared to SPF mice or mice, colonized with microbiota from healthy human donors [195, 196]. The study from Yano et al. (2015) [196] found an increased expression of the 5-HT transporter gene, *Slc6a4*, in the colon of GF mice, which they hypothesize to be a compensatory response to the deficit in 5-HT synthesis. Interestingly, colonization of GF mice at postnatal day 42 with spore-forming bacteria from either SPF mice or healthy human donors restored colonic and serum levels of 5-HT and normal TPH1 and *Slc6a4* gene expression in the colon. The other study, from Reigstad et al. (2015) [195], found that *in vitro* stimulation of human-derived ECs with acetate or butyrate could induce TPH1 expression. Overall, those results show that certain types of bacteria from the gut microbiota and their metabolites can influence Trp metabolism along the 5-HT and KYN pathways.

Interestingly, BTBR, MIA, CMA and VPA mouse models of ASD all show impaired 5-HT metabolism. MIA and VPA mice present increased serum 5-HT [109, 124] and CMA mice have increased 5-HT but decreased 5-HIAA in the ileum [105]. A decrease of intestinal 5-HT was also found in BTBR mice, as well as an increase in 5-HT/5-HIAA ratio [108]. In the VPA model, de Theije et al. (2014) [114] observed decreased 5-HT levels in the ileum associated with fewer ECs. The authors also observed alterations in 5-HT metabolism in the brain, such as a decrease of 5-HT and increase in 5-HIAA/5-HT ratio in the prefrontal cortex (PFC) and amygdala. Plus, MIA mice present increased expression of the 5-HT_{2A} receptor in the frontal cortex [170]. However, it is still unclear if those central alterations are influenced by intestinal 5-HT. As previously mentioned, microbiota alterations were

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described in all those models. Interestingly, in VPA, BTBR and MIA mice, the 5-HT alterations correlated with some of the observed microbiota changes [108, 114, 170].

Although most evidence towards an influence of the gut microbiota on alteration of neurotransmitter systems in ASD is focused on 5-HT, there has been sporadic evidence on involvement of the gut microbiota in other neurotransmitter systems related to ASD, particularly the GABAergic (gamma-aminobutyric acid) and glutamatergic systems. Clinical studies have found alterations in central or peripheral levels of GABA or glutamate, or altered expression of their receptors in the brain of individuals with ASD [80, 197–199]. Some bacteria of the human gut microbiota are capable of producing GABA, which could be one of the ways that the microbiota impacts the gut-brain axis [200]. Interestingly, Kang et al. (2018) [79] found lower levels of GABA, as well as lower GABA/glutamate ratio in the feces of children with ASD. However, the authors found no correlation between these changes and the microbiota modulations observed in these individuals. Another recent study looked at gene expression in the metagenome of individuals with ASD and found a decrease in genes related to GABA production [89].

More evidence comes from animal studies, as decreased expression of GABA receptors has been observed in the hippocampus of Shank3 KO mice. Interestingly, this alteration correlated with *L. reuteri* levels in the microbiota of those mice, and *L. reuteri* MM4-1A treatment partly restored those expression levels [117]. Similarly, Khattaf et al. (2022) found decreased expression levels of GABA and GABA receptor in brain homogenates of propionate treated rats, which was corrected by a 3-week treatment with *Bifidobacterium infantis* [141].

There is still little evidence of the implication of the microbiota in those GABA and glutamate alterations in ASD, but those first results provide a promising avenue to pursue.

In conclusion to this first part, it is now well-accepted that individuals with ASD have a disturbed microbiota, with altered metabolic activity. Increasing evidence shows that those disruptions can influence the immune system and Trp metabolism, both in the periphery and in the brain. Thus, the gut microbiota may have an influence on neurodevelopment and brain function during the life of individuals with ASD. These new findings have prompted many teams to test whether interventions on the gut microbiota could have beneficial effects on GI symptoms, brain function and behavior in ASD.

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1.3.4 Clinical and Preclinical Interventions Targeting the Gut Microbiota

1.3.4.1 Probiotic Intervention Studies for ASD Symptoms

Recently, a few interventional clinical studies and more interventional preclinical studies have been published, bringing evidence that modulation of the gut microbiota can influence ASD-related behaviors, as well as some elements explaining the underlying mechanisms of this effect.

1.3.4.1.1 Clinical Studies

As previously mentioned, one of the first studies to establish a link between gut microbiota dysbiosis and ASD was published in 2000 by Sandler et al. [58], who observed behavioral improvement of children with ASD during a vancomycin treatment, showing that modification of gut microbiota can induce changes in behavioral symptoms. However, those effects did not persist after the treatment, and a long-term antibiotic treatment is anyway not feasible. Thus, researchers have started to investigate the potential role of probiotic treatments in ASD. Multiple studies have investigated the effects of a probiotic treatment in children with ASD [57, 74, 201–206]. Only some of these studies analyzed the behavior of the children [74, 201–203] and among those, 3 have reported behavioral improvement in children with ASD after probiotic administration [57, 204, 206, 207]. In an open-label study by Shaaban et al. (2017) [208], 30 children with ASD were given a 3-month, daily treatment with a patented probiotic mixture (composed of strains of the species *Lactobacillus acidophilus*, *Lactobacillus rhamnosus* and *Bifidobacterium longum*) which induced an improvement in communication, sociability and cognitive awareness, characterized by a decrease in the Autism Treatment Evaluation Checklist score. In Liu et al. (2019) [209], *Lactobacillus plantarum* PS128 was given to 36 children for 4 weeks in a placebo-controlled trial. The authors did not observe an improvement in behavioral scores using different diagnosis scales, but saw a decrease in anxiety behavior, hyperactivity, and opposition/defiance behaviors. They propose that the effects of the treatment could have been stronger if it had been administered for a longer period. However, the same probiotic strain was tested in a randomized double-blind placebo-controlled study by Kong et al. (2021) for 3 months as a sole treatment and for 3 additional months in combination with a nasal oxytocin spray, but no significant differences in behavior was observed. In another double blind randomized controlled trial [206], young children with ASD received a commercial probiotic mix (Vivomixx®) or placebo daily for 6 months. When all children were included in the analysis, there was no significant difference in the change in ADOS calibrated severity (ADOS-CSS) score of the children between probiotic and

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placebo groups. However, in a secondary analysis, the authors separated children with and without GI symptoms in each treatment group and saw a significant decrease in ADOS-CSS scores only in children without GI symptoms who received the probiotic. In the GI group, the probiotic treatment had some effects on GI symptoms. These results highlight again the importance of taking GI symptoms into account in interventional studies. Indeed, an hostile gut environment (inflammation, reactive oxygen species (ROS) production, altered mucus composition) could limit bacterial implantation and thus the potentially beneficial effects of probiotic treatments or FMT [210].

In the previously mentioned placebo-controlled trial by Wang et al. (2020), the probiotic mix+ FOS treatment, but not the placebo, improved behavioral symptoms (Autism Treatment Evaluation Checklist), after 60 days of treatment compared to baseline. Plus, 60 days of treatment also ameliorated GI symptoms (6-item GI severity index) and decreased the elevated plasmatic zonulin observed in individuals with ASD. These behavioral and GI improvements were maintained or even increased after 108 days of treatment [57]. Finally, in a randomized double-blind placebo-controlled study with group crossover, 61 children with ASD received a 3-month treatment with a probiotic mix (*Lactobacillus fermentum* LF10, *Lactobacillus salivarius* LS03, *L. plantarum* LP01 and 5 strains of *B. longum* DLBL) or placebo. Probiotic treatment had significant effects compared to placebo and ameliorated diarrhea and abdominal pain, as well as communication (Vineland adaptive behavior scale) and disadaptive behaviors, assessed by Psycho-educational profile [207].

Overall, considering the variations in the probiotic choice, group size, duration of treatment and behavioral assessment tools, these results are not yet sufficient to establish a beneficial effect of probiotic interventions on behavior in ASD. Only a few of the existing studies are properly controlled and randomized without experimental bias, as detailed in a recent meta-analysis, where out of 24 studies, only 3 could be included in the meta-analysis according to their criteria, which did not allow to conclude on a positive effect of probiotic treatment on ASD symptoms [211]. However, the use of probiotics could be an interesting lead of treatment or preventive measures as suggested by numerous preclinical studies showing an effect of probiotics on behaviors related to ASD, which will be detailed in the following section. Since each probiotic species or strain could have a different influence on ASD symptoms, only an increased number of studies could allow to identify specific beneficial probiotic strains.

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1.3.4.1.2 Preclinical Studies

As previously mentioned, there are multiple murine models of ASD, genetic or environmental, that present altered behaviors relative to ASD. As many of those models also have impaired GI function and gut microbiota, as previously described, many research teams have wondered if modulating the microbiota composition using a probiotic treatment could improve the altered behaviors of those models. One of the first groups to publish such a study was Sarkis Mazmanian's group, who in a study previously mentioned, tested the effect of *B. fragilis* NCTC 9343 in the MIA mouse model [109]. They observed an improvement in anxiety-like behavior, stereotyped behavior, communication, and cognitive function but no impact on social behavior. MIA mice also presented elevated serum levels of 4-EPS, which was restored to control values by the probiotic treatment. In parallel, a chronic systemic administration of 4-EPS to naïve mice induced an anxiety-like behavior. Other studies have assessed the effect of probiotic treatment on the MIA model, through treatment either of the mothers [212] with a symbiotic mix (*Bifidobacterium bifidum* and *Bifidobacterium infantis*, *Lactobacillus helveticus* and FOS), or of the offspring with *Parabacteroides goldsteinii* MTS01 [111]. In both studies, the treatment reduced anxiety-like behavior and improved social behavior in the MIA offspring. In Wang et al. (2019) probiotic treatment of the mothers also improved depressive-like behavior and prevented decrease of the number of parvalbumin neurons and associated decrease in GABA/Glutamate ratio in the PFC in the adult offspring [212].

Probiotic treatments have also been used in other ASD models : Buffington et al. (2016) [20] showed that gut concentration of *L. reuteri* was decreased in the MHFD mouse model, and that treatment with *L. reuteri* MM4-1A ameliorated social behavior in those mice. Based on this observation, another team showed improvement of social and repetitive behavior in the Shank3 KO genetic model of ASD, following treatment with *L. reuteri* MM4-1A [117]. This has been further explored by Sgritta et al. (2019) [25] who found that *L. reuteri* MM4-1A treatment improved social behavior in the VPA environmental model, the BTBR idiopathic model and the Shank3B KO genetic model. They also reported that administration of *L. reuteri* MM4-1A improved social behavior of GF mice, proving that this bacterium could act on its own. Interestingly, the same study also demonstrated that the effect of *L. reuteri* MM4-1A in Shank3B^{-/-} mice was dependent on the vagus nerve, as treatment with this bacterium was inefficient in vagotomized Shank3B^{-/-} mice. This work also brought a very thorough mechanistic explanation of the probiotic effect, showing that it was dependent on the presence of oxytocin receptors in the ventral tegmental area, which is involved in social interaction-induced neuronal plasticity [25]. A different strain of *L. reuteri* (*L. reuteri* RC-14®)

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was also tested in BTBR mice, in which it reduced repetitive self-grooming behavior, improved social interaction and modified ultrasonic vocalizations. In this study, the authors also tested the effect of a prebiotic, oligofructose-enriched inulin, and the symbiotic of the two. They induced the same behavioral effect as the probiotic alone, at the difference that the prebiotic given alone worsened social interaction. Plus, *L. reuteri* RC-14® (with or without inulin) decreased intestinal permeability [213].

Two other studies have tested probiotic treatments in BTBR mice: one tested the effect of kefir [214] and the other tested two probiotic strains (*L. salivarius* HA-118 and *Lactobacillus rhamnosus* HA-114) [215]. Both kefir and the two probiotic strains reduced the social novelty deficit in BTBR mice, and kefir also decreased repetitive behavior (marble burying test) [214, 215].

A few papers have assessed the effects of probiotic on propionate treated rats [139–141]. Only one study saw an effect of the treatment on behavior, but most of them reported effects on the brain. Indeed, in Abuaish et al. (2021), a 30-day treatment with *B. longum* BB536 reduced the social interaction deficit (3 chamber test) and the brain expression levels of BDNF that were increased in propionate treated rats [139]. In Alghambi et al. (2022), *Lactobacillus paracasei* and Protexin® (commercial mixture of probiotics) both rectified the α -melanocyte stimulating hormone (α -MSH) decrease caused by propionate and, in Khattaf et al. (2022), Protexin® and two other probiotic treatments (*B. infantis* and *Lactobacillus bulgaricus*) restored the propionate-induced decrease of GABA in the brain [140, 141].

Two recent studies have also tested probiotics on VPA models, either in the offspring of VPA treated mice [216] or in adult WT rats treated with VPA [217]. The first study tested *L. plantarum* ST-III-fermented milk and the other a probiotic mixture composed of 4 species of *Lactobacillus* or *Bifidobacterium*. Both studies found that the probiotic treatment restored the social interaction deficit observed in the model animals. In Mintál et al. (2022), the authors also assessed the effect of a 4-week antibiotic treatment during adulthood on WT rats. This led to impaired social behavior similar to the alterations observed in the VPA model, which was reversed by the probiotic treatments [217].

Finally, another study in WT mice found that treatment of mothers with triclosade (a biocide) induced increased anxiety-like behavior and hyperactivity during the OF test in male and female offspring. Treatment also impaired social behavior in males but ameliorated it in female offspring. Finally, it increased grooming frequency during the OF, but only in males. The authors then specifically selected

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male and female mice with low scores in the social interaction test, and tested the effect of a two weeks treatment with *L. plantarum* ST-III. Treatment restored the deficits in social behavior in male mice and reduced anxiety-like behavior, and repetitive grooming in female mice [218].

Among all the studies previously mentioned, 6 investigated the effect of the probiotic treatments on microbiota composition [109, 213, 214, 216, 218, 219]. Three observed an increase of α -diversity [215, 216, 219], one observed a decrease [213], and two studies did not see an effect of the treatment on α -diversity [109, 214]. Only two studies observed differences in β -diversity after probiotic treatment, which reflects different bacterial profiles between groups in those studies [215, 216]. All six studies observed effects of the probiotic treatments on relative abundance of some bacterial taxa (up to the family level in most cases), which were found to be correlated with behavior in three studies [213, 214, 218].

A few of those studies [111, 140, 215] observed variations in serum or brain markers after treatments, some of which correlated with behavior [215]. Some studies also observed an effect of probiotic treatments on inflammation. The probiotic treatment reduced the levels of pro-inflammatory cytokines in the colon of animals in Lin et al. (2022), in the serum and brain of mothers that received the probiotic and in the fetal brains of their offspring in Wang et al. (2019) [111, 212]. Treatment also decreased the levels of regulatory T cells in BTBR mice in Van de Vouw et al. (2021) [214]. However, Pochakom et al. (2022) found an increase of some pro-inflammatory cytokines in the gut of VPA treated animals after probiotic treatment, which shows that the probiotic could have increased gut inflammation in this case [215].

Overall, this recent preclinical and clinical data provides good arguments on the potential effect of specific probiotic treatments on ASD related behavior in and on the mechanistic functioning of the microbiota-gut-brain axis in the context of this disorder. In the coming years we should see more studies published, owing to large-scale projects involving longitudinal surveys of children at risk for ASD and intervention trials with probiotics, for example, the European-funded GEMMA project [220] and two other prospective trials [221, 222].

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1.3.4.2 FMT Studies

1.3.4.2.1 Clinical Studies

To our knowledge, there are very few clinical studies exploring the impact of FMT on ASD symptoms. FMT is most commonly used as treatment of *Clostridioides difficile* infections, where it seems very efficient [223]. Aside from that, a few controlled studies have proven that FMT could have a therapeutic effect in patients with irritable bowel syndrome, colonic administration of the FMT yielding seemingly better results than oral capsules [224]. A pioneer study from 2012 and a few more recent controlled studies in metabolic diseases (metabolic syndrome, obesity, type-1-diabetes or non-alcoholic fatty liver disease) have shown efficacy of FMT in reducing weight, insulin resistance, liver fat accumulation, or halting the decrease of insulin production [225–227], while other studies did not see an improvement of any metabolic parameters [228, 229]. Depending on the study, the “placebo” control can be a probiotic treatment, a placebo capsule or autologous FMT (FMT using own feces).

In addition, a few open-label studies have also started to investigate the impact of FMT in various neurological disorders [230]. While those results are promising, there is a need for more large-scale controlled longitudinal studies.

In 2017, Kang et al. published an open label study in which they performed FMT in 18 individuals with ASD (7–16 years old) and comorbid GI symptoms [231]. Eighteen weeks after FMT, the team observed an increase of α -diversity (Faith’s phylogenetic diversity index), and changes in the relative abundances of 3 bacterial genera (*Bifidobacterium*, *Prevotella*, and *Desulfovibrio*). They also reported an amelioration of GI symptoms (based on the Gastrointestinal Symptoms Rating Scale (GSRS)) and of ASD-related behavioral symptoms, assessed according to clinical and parental based scales (Parent Global Impressions–III, Vineland Adaptive Behavior Scale II, Childhood Autism Rating Scale, Social Responsiveness Scale, and Autism Behavior Checklist). The same team published a follow-up study 2 years later, and the previously observed ameliorations in GI and behavioral symptoms had been maintained or even increased [232]. In a recent study on the same cohort, a metagenomic analysis of the microbiota composition mostly confirmed the changes observed by 16S sequencing in the first study, 10 days after FMT, although after two years most of those changes were no longer significant [233].

In two other papers on the same cohort, the authors compared the plasmatic or fecal metabolic profiles of the 18 children with ASD to that of 20 TD age-matched controls, before and after the FMT. Fecal and plasmatic metabolite profiles were

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both impacted by FMT and, interestingly, the profiles shifted to be closer to that of TD individuals in both studies [82, 234]. In Kang et al. (2020), plasmatic *p*-Cresol, that was increased in the ASD group as previously mentioned, was lowered after FMT [82]. Finally, Nirmalkar et al. (2022) observed variation in relative abundance of microbial genes after FMT, including some involved in regulation of oxidative stress by bacteria, and the overall expression profile was closer to TD after 2-years [233].

In a bigger scale open-label study, 40 children with ASD and GI symptoms (3-17 years) received weekly FMT for 4 weeks from one healthy TD donor. Behavioral and GI symptoms were assessed at the end of treatment and 4 and 8 weeks after the end. FMT improved behavior as indicated by a significant decrease of Childhood autism rating scale, social responsiveness scale and autism behavior checklist scores. FMT also improved GSRS and Bristol stool scale scores reported by parents, decreased 5-HT and GABA and increased DA concentrations in the serum. Those modulations were maintained after 8 weeks except for the change in DA. Interestingly, Bristol scores were positively correlated with GABA levels and negatively with 5-HT levels. The authors also analyzed the microbiota composition of the children at the different time points and saw no effect of treatment on α -diversity and did not report on any changes in specific bacterial taxa. However, they then compared the bacterial population in "responder" vs "non-responder" children (defined as children with less than 50% reduction in average GSRS scores after treatment) and saw distinct β -diversity between those groups, as well as increased relative abundance of *Eubacterium coprostanoligenes* in "non-responder" children. Plus, the relative abundance of this genus negatively correlated with GSRS and serum concentration of GABA [235].

Those results offer a promising lead on the efficacy of FMT for amelioration of behavior and GI symptoms in ASD, but it needs to be further investigated in controlled studies.

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1.3.4.2.2 Preclinical Studies

The use of ASD mice models in preclinical studies is necessary for a more mechanistic understanding of the impact of FMT on microbiota, GI symptoms, other ASD-related markers, and ASD-like behavior. Preclinical research has brought evidence of a potential therapeutic role of FMT in many neurological disorders, including ASD [230].

In Goo et al. (2021) Fmr1 KO mice received daily FMT from WT mice for 4 weeks, which ameliorated the deficits in social novelty and memory (Y maze and novel object tests), and reduced, but did not completely normalize, TNF- α and Iba-1 (microglial marker) concentrations in the brain [100]. As previously mentioned, GF mice present impaired social interaction [20, 22]. Interestingly, in Buffington et al. (2016) [20], colonization of GF mice at weaning with microbiota from SPF mice normalized anxiety-like and social behaviors, while colonization with microbiota from MHFD mice did not. It is interesting to point out that the effects of the FMT did not appear when it was done at 8 weeks of age, highlighting the existence of a critical time window during which FMT in initially GF mice can impact behavior. Those results show that FMT from healthy mice can improve ASD-related behaviors.

Conversely, FMT from an ASD mouse model can induce behavioral deficits in healthy mice. Indeed, Saunders et al. (2020) performed FMT from adult MIA mice or from control mice into WT mice whose microbiota had been depleted by antibiotic treatment. The mice that received MIA microbiota showed impaired performances in the object recognition test compared to mice that received microbiota from controls [170].

Interestingly some studies have also investigated the effect of FMT from human donors to mice. Firstly, Chen et al. (2020) performed FMT in the MIA mouse model, using pooled stools from three healthy human donors. In this model FMT reduced repetitive behavior and anxiety-like behavior but had no effect on social behavior [119]. This shows that healthy microbiota from humans can improve behavior in a mouse model, but more studies have investigated how the microbiota from human individuals with ASD could impact mice behavior.

In Qi et al, WT female pregnant rats received a pooled microbiota suspension from 8 donors with ASD. The authors compared the offspring (oFMT) of those animals to WT rats that received no FMT (oCTR) and to offspring of VPA treated rats (oVPA). oFMT showed delayed growth indicators, impaired social behavior and increased repetitive behaviors compared to oCTR, in a similar way to what was observed in oVPA vs. oCTR. Serum levels of 5-HT were increased in oFMT

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(and oVPA) compared to oCTR, while levels of GABA and norepinephrine were decreased. Finally, oFMT showed decreased α -diversity of the gut microbiota, distinct β -diversity compared to oCTR and oVPA, as well as increased relative abundance of *Actinobacteria* and other variations in relative abundances at genus level [125]. Similarly, Avolio et al. (2022) tested the effect of FMT from children with ASD or unrelated TD children in SPF mice (6 weeks old) after microbiota depletion through treatment with Polyethylene Glycol. The authors also compared those animals to the offspring of VPA treated mothers. FMT from individuals with ASD induced increased anxiety-like behavior and impaired social behavior and memory in a similar way as in VPA animals. The authors also observed increased levels of TNF- α in the small intestine and differences in post-translational modifications (decreased methylation levels) in brain of the mice that received FMT from ASD adult individuals, compared to those that received FMT from TD individuals [236]. However, those studies do not allow to conclude that those behavioral alterations are due specifically to the ASD microbiota, as they could simply be due to receiving human microbiota. It is possible that same effects could be observed with FMT from TD individuals.

A few studies, however, have compared the effect of FMT from ASD or TD individuals in mice.

Firstly, in Sharon et al. (2019) [237], GF female and male mice were colonized at weaning with microbiota from individuals with ASD with GI symptoms and TD children as controls (individual donors to individual mice). Their offspring (named oASD and oTD, respectively) showed decreased social interaction and increased stereotyped behavior in oASD compared to oTD. This was accompanied by alternate splicing for many genes in the PFC including a few that are known to be involved in some cases of ASD or other neurodevelopmental disorders. The team also observed a correlation between behavioral differences and the higher or lower quantity of specific bacteria in oASD mice microbiota compared to oTD. Finally, they highlighted some metabolomic differences in colonic content of oASD mice compared to oTD mice, particularly a decrease in 5-aminovaleric acid and taurine, which could rescue some behavioral defects when administered to BTBR mice. We cannot rule out that those differences might be due, at least in part, to the effect of the microbiota on the mothers and not directly on the offspring. However, other studies have assessed the direct effect of FMT from ASD or TD donors on mice.

In Gonzales et al. (2021), 7 weeks old antibiotic treated SPF mice received FMT from 12 adults with ASD or 10 TD individuals (individual donors to individual mice). FMT from individuals with ASD reduced intestinal permeability in the

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proximal colon and decreased levels of various proteins (S100 β , GFAP, Synapsin, β III-tubulin) in glial cells in the colon compared to FMT from TD individuals. FMT from individuals with ASD also decreased expression of IL-1 β and TNF- α in the colon. However, the authors did not report on any behavioral assessment [238]. Finally, in Xiao et al. (2021), 3 weeks old GF mice received pooled microbiota from 5 children with ASD or 5 unrelated TD children. Mice that received the microbiota from children with ASD showed impaired social behavior and increased anxiety-like and stereotyped behaviors compared to mice that received microbiota from TD children. FMT from children with ASD also induced changes in the protein levels of TPH1, TPH2, serotonin transporter (SERT) and serotonin receptor 1-A (5-HT1A) in the colon and the brain of the animals [239].

Overall, these results show that FMT from donors with ASD, either in SPF mice with depleted microbiota or in GF mice, can impact microbiota composition, gut metabolites, behavior, neurotransmitter levels and brain and gut markers of inflammation and permeability, in a different way than FMT of microbiota from TD donors.

The clinical and preclinical studies presented in this part suggest that FMT, could be a promising approach to improve behavior and GI symptoms in individuals with ASD. However, more clinical studies need to be done to reinforce this hypothesis. More preclinical studies are also necessary to gain more insight into the mechanisms by which FMT can induce systemic and neuronal changes leading to behavioral improvement.

1.4 CONCLUSION

Despite discrepancies between studies, the data presented in this review converge to conclude that individuals with ASD exhibit an abnormal microbiota composition, with disturbed activity. Whether these alterations are involved in the onset or development of ASD or occur as a consequence of it (due to dietary preferences and/or other factors), a growing body of research suggests that they may aggravate the behavioral symptoms and biological signs of ASD.

This led to the use ASD animal models to try to elucidate the mechanisms underlying the involvement of the gut microbiota in this disorder. To date, the preclinical studies conducted in rodents have particularly shown that alterations in the microbiota could increase systemic, gut, and brain inflammation, gut permeability and disrupt the Trp metabolism. With the evidence detailed in this introduction we can propose a model of how those parameters and microbiota could be at interplay in ASD (Fig 5):

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A disturbed microbiota leads to disturbed production of bacterial products and vesicles (BEVs/OMVs), which can influence 5-HT release by ECs and pass in the blood circulation. Bacterial vesicles can pass through the BBB and lead to microglial activation. Plus, the presence of some bacterial products or vesicles in the blood can induce general immune dysregulation, notably lead to more T cell activation and differentiation into pro-inflammatory T cells profiles (Th1 or Th17). In the gut, this would lead to increased inflammation, which can alter gut epithelium integrity and cause excessive intestinal permeability (leaky gut). These phenomena will allow even more bacterial products, or even bacterial cells, to escape from the luminal compartment, worsening the general inflammatory state. In this inflammatory state, Trp is increasingly derived into KYN, which can pass through the BBB and lead to neurotoxicity and astrocyte activation through QA production in activated microglia (Fig 5).

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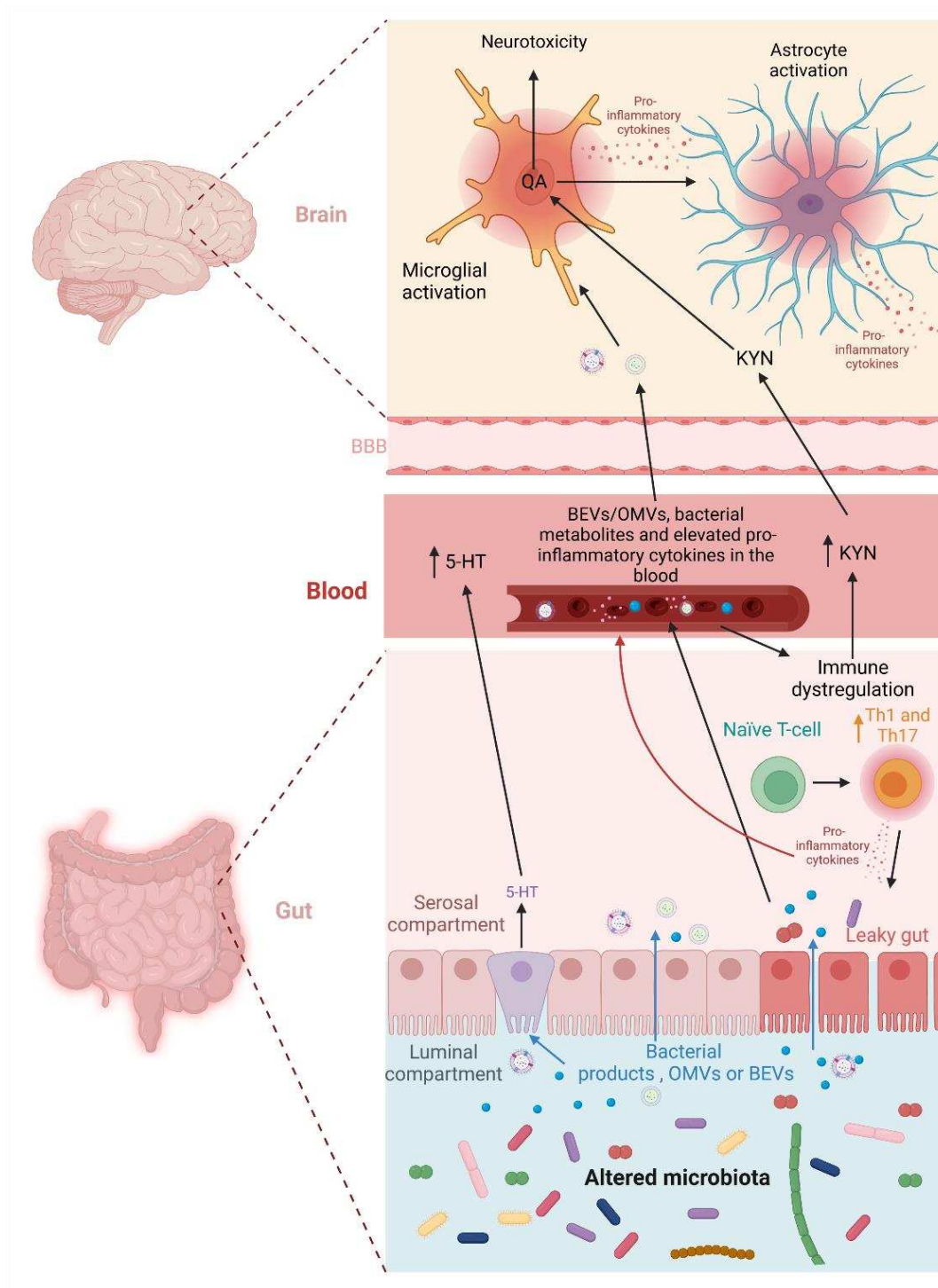


Figure 5 : Summary schematic showing the potential impact of a disturbed gut microbiota on various parameters in the gut, systemic circulation, and brain in ASD (Figure created with Biorender.com).

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Table 1 :Microbiota modulations in genetic models of ASD. N.S = Not specified. * ARISA = automated ribosomal intergenic spacer analysis

Model	Sex	Age	Sample	Method	Difference in Microbiota Compared to Controls	Ref.
Shank3 KO	F/M	8 weeks	Feces	16S rRNA seq (V4 region) RT-qPCR	<p>α-diversity: ↓</p> <p>β-diversity: Difference between groups</p> <p>Class level: ↓<i>Bacilli</i></p> <p>Order level: ↓<i>Lactobacillales, Rhodospirillales, Rickettsiales and Turicibacteriales</i></p> <p>Family level: ↑<i>Veillonellaceae</i>; ↓<i>Lactobacillaceae, Bacteroidaceae, Acetobacteriaceae, mitochondria and Turicibacteriaceae</i>; ↑<i>Veillonellaceae</i></p> <p>Genus level: ↓ <i>Lactobacillus, Coprococcus, Bacteroides, Acetobacter, Turicibacter</i> and <i>Prevotella</i>;</p> <p>↑<i>Veillonella</i> in males ↓ in females</p> <p>Species level: ↓ <i>Lactobacillus reuteri, Lactobacillus brevis, Lactobacillus ruminis</i> in both male and female;</p> <p>↓ <i>Veillonella parvula</i> and <i>Veillonella dispar</i> in females, ↑<i>V. dispar</i> in males</p>	[117]
	N.S	10 weeks	Feces	16S rRNA seq (V3-V5 regions)	<p>No assessment of diversity</p> <p>Phylum level: ↑<i>Actinobacteria</i> and <i>Firmicutes</i>; ↓ <i>Proteobacteria</i></p> <p>Absence of <i>Verrucomicrobia</i>; Presence of <i>Deferribacteres, Chlamydiae</i> and <i>Tenericutes</i></p> <p>Order level: ↑ <i>Bifidobacteriales</i> and <i>Eggerthellales</i></p> <p>Genus level: ↑<i>Asaccharobacter, Eggerthella, Enterorhabdus</i> and <i>Paraeggerthella</i></p> <p>Species level (closest homology to OTUs): ↑ <i>B. pseudolongum, Asaccharobacter WCA-131-CoC-2, Eggerthella YY7918</i> and <i>Enterorhabdus caecimuris</i>.</p>	[106]

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Model	Sex	Age	Sample	Method	Difference in Microbiota Compared to Controls	Ref.
Shank3B KO	M	Adult	Feces	16S rRNA seq (V4 region)	<p>α-diversity: No changes</p> <p>β-diversity: Difference between groups</p> <p>Bacterial modulations were not detailed except for:</p> <p>Species level: ↓ <i>L. reuteri</i></p>	[25]
NL3 ^{R451C}	M	9 weeks	Feces	ARISA *	<p>α-diversity: No changes</p> <p>β-diversity: Difference between groups at 3 weeks of age (not at 9 weeks)</p> <p>Bacterial modulations were only detailed at OUT level:</p> <p>OTUs levels: ↑ OTUs from <i>Lachnospiraceae</i> family, ↓ OTUs from <i>Candidata</i> phylum</p>	[115]
15s11-13 CNV	M	Adult	Feces	16S rRNA seq (V1-V2 regions)	<p>α-diversity: ↓</p> <p>β-diversity: No difference</p> <p>No difference observed at higher taxonomic levels than species.</p> <p>Species level (closest homology to OTUs): ↓ <i>Clostridioides hathewayi</i>, <i>Oscillibacter valericigenes</i>, <i>Barniesella viscericola</i>, <i>Anaerotruncus colihominis</i>; <i>Roseburia inulinivorans</i>, <i>Fusibacter paucivorans</i>, <i>Clostridioides clostridioforme</i>, <i>Clostridioides sp.</i>, <i>Ruminococcus sp.</i>, <i>Desulfocurvus vexinensis</i>, <i>Hydrogenoanaerobacterium saccharovorans</i>, <i>Prevotella sp.</i></p>	[99]

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Model	Sex	Age	Sample	Method	Difference in Microbiota Compared to Controls	Ref.
BTBR	M	7 weeks	Feces and cecal content	16S rRNA seq	<p>α-diversity: ↓</p> <p>β-diversity: Modulated in cecal content only</p> <p>Phylum level: ↑ <i>Bacteroidota</i> in cecal content</p> <p>Family level: ↓ <i>Enterobacteriaceae</i> both cecal and fecal</p> <p>Species level: ↑ <i>A. Muciniphila</i>, <i>Lactobacillus</i> spp., <i>Roseburia</i> spp., <i>Clostridium leptum</i>, <i>Prevotella</i> spp.</p> <p>↓ <i>Clostridioides</i> cluster XI both cecal and fecal</p> <p>In cecal content only, ↑ <i>Methanobrevibacter</i> spp.; ↓ <i>Clostridium coccoides</i> and <i>Clostridioides</i> cluster I</p> <p>In feces only, ↑ <i>C. coccoides</i> and <i>Clostridioides</i> cluster I; ↓ <i>Methanobrevibacter</i> spp.</p>	[118]
	F/M	12 months	Feces	16S rRNA seq (V3-V4 region)	<p>α-diversity: No changes</p> <p>β-diversity: Difference between groups</p> <p>Phylum level: ↑ <i>Proteobacteria</i> and TM7 in female</p> <p>Genus level: ↑ <i>Bacteroides</i> and <i>Parabacteroides</i>; ↓ <i>Dehalobacterium</i> in both male and female.</p> <p>In females only, ↑ <i>Prevotella</i>, <i>Coprobacillus</i>, <i>Sutterella</i>, <i>Akkermansia</i>, and unclassified genera of <i>Desulfovibrionaceae</i> and <i>Enterobacteriaceae</i> families; ↓ <i>Oscillospira</i> and unclassified members of TM7 and <i>Rikenellaceae</i> families</p> <p>In males only, ↑ <i>Lactobacillus</i>, <i>Coprobacillus</i> and unclassified genus of the <i>Helicobacteraceae</i> family; ↓ <i>Dehalobacterium</i>, <i>Ruminococcus</i> and <i>Desulfovibrio</i></p> <p>Species level: N.S</p>	[107]

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Model	Sex	Age	Sample	Method	Difference in Microbiota Compared to Controls	Ref.
BTBR	M	14 weeks	Cecal content	16S rRNA seq (V3-V4 region)	<p>α-diversity: ↓</p> <p>β-diversity: Difference between groups</p> <p>Phylum level: ↑ <i>Verrucomicrobia</i>, <i>Bacteroidota</i>; ↓ <i>Firmicutes</i> and <i>Cyanobacteria</i></p> <p>Genus level: ↑ <i>Akkermansia</i>, <i>Bacteroides</i>, <i>Bilophila</i>, <i>Enterorhabdus</i> <i>Intestinomonas</i> and S24-7;</p> <p>↓ <i>Odoribacter</i>, <i>Parabacteroides</i>, <i>Rikenella</i>, <i>Blautia</i>, <i>Coprococcus</i>, <i>Bifidobacterium</i>, <i>Desulfovibrio</i>, <i>Lachnospiracae_Incertae Sedis</i> and RC9 gut group</p>	[108]
	M	Adult	Feces	16S rRNA seq (V4)	<p>α-diversity: N.S</p> <p>β-diversity: Difference between groups</p> <p>Bacterial modulation were not detailed except for:</p> <p>Species levels: ↓ <i>L. reuteri</i></p>	[25]

General introduction

Table 2: Microbiota modulations in environmental models of ASD. All studies used mice except for Liu et al. (2018) [120] and Gu et al. (2020) [122] who used rats. N.S = Not specified.

Model	Sex	Age	Sample	Method	Difference in Microbiota Compared to Controls	Ref
MIA	F/M	Adult	Feces	16S rRNA seq (V3-V5 region)	<p>α-diversity: No changes</p> <p>β-diversity: Difference between groups</p> <p>Bacterial modulations were only detailed at OUT level:</p> <p>↑OTUs from the <i>Alphaproteobacteria</i> and <i>Bacili</i> classes, <i>Bacteroidales</i> order and <i>Prevotellaceae</i>, <i>Lachnospiraceae</i> and <i>Porphyromonadaceae</i> families</p> <p>↓ OTUs from the <i>Actinobacteria</i> phylum, <i>Gammaproteobacteria</i>, <i>Mollicutes</i> and <i>Erysipelotrichi</i> classes and <i>Ruminococcaceae</i>, <i>Erysipelotrichaceae</i> and <i>Aligenaceae</i> families</p>	[109]
	M	Adult	Cecal content	16S rRNA seq	<p>No assessment of diversity</p> <p>Family level: ↑ <i>Ruminococcaceae</i>, <i>Porphyromonadaceae</i>, <i>Aerococcaceae</i> and <i>Erysipelotrichaceae</i></p> <p>Genus level: ↑ <i>Candidatus</i></p>	[170]

Table continues on the next page.

General introduction

Model	Sex	Age	Sample	Method	Difference in Microbiota Compared to Controls	Ref
MIA	N.S	Adult	Feces	16S rRNA seq (V3-V4 region)	<p>α-diversity: ↓</p> <p>β-diversity: Difference between groups</p> <p>Phylum level: ↑ <i>Bacteroidota</i> and <i>Verrucomicrobia</i>; ↓ <i>Firmicutes</i></p> <p>Genus level: ↑ <i>Prevotella</i>, <i>Prevotella_other</i>, <i>Akkermansia</i> and a genus of S24-7 family; ↓ <i>Oscillospira</i>, <i>Ruminococcus</i>, <i>Bacteroides</i>, <i>Dehalobacterium</i>, <i>Desulfovibrio</i>, <i>Lactobacillus</i>, and members of the <i>Clostridiales</i> order and <i>Rikenellaceae</i>, <i>Lachnospiraceae</i> and <i>Ruminococcaceae</i> families.</p> <p>Species level: ↑ F16 and OTUs from the <i>Bacteroidales</i> order, <i>Clostridiaceae</i>, <i>Enterobacteriaceae</i> and S24-7 familie and <i>Akkermansia</i> and <i>Prevotella</i> genera</p> <p>↓ OTUs from the <i>Clostridiales</i> order, <i>Ruminococcaceae</i> and <i>Rikenellaceae</i> families and <i>Ruminococcus</i>, <i>Bacteroides</i>, <i>Dehalobacterium</i>, <i>Desulfovibrio</i>, <i>Oscillospira</i> and <i>Odoribacter</i> genera</p>	[119]
	F/M	4 and 17 weeks	Feces	16S rRNA seq (V3-V4 regions)	<p>No assessment of diversity</p> <p>Phylum level: ↑ Firmicutes/<i>Bacteroidota</i> ratio</p> <p>Family level: ↑ <i>Bacteroidaceae</i>, <i>Cyclobacteriaceae</i>, <i>Cytophagaceae</i>, <i>Lactobacillaceae</i>, <i>Lentimicrobiaceae</i> and <i>Sphingobacteriaceae</i> ↓ <i>Akkermansiaceae</i>, <i>Clostridiaceae</i>, <i>Erwiniaceae</i>, <i>Kiloniellaceae</i>, <i>Mycoplasmataceae</i>, and <i>Rhizobiaceae</i></p> <p>Genus level: ↑ <i>Bacteroides</i>, <i>Lactobacillus</i>, <i>Nitritale</i>, <i>Paludibacter</i>, <i>Parabacteroides</i>, <i>Ruminococcus</i>, <i>Sporocytophaga</i>, and <i>Turicibacter</i>; ↑ <i>Desulfotomaculum</i>, <i>Lentimicrobium</i> in females only; ↑ <i>Fretibacter</i> in males only. ↓ <i>Akkermansia</i>, <i>Anaerofilum</i>, <i>Anaerotruncus</i>, <i>Anaerocolumna</i>, <i>Butyricicoccus</i>, <i>Faecalicatena</i>, <i>Fodinicurvata</i>, <i>Flintibacter</i>, <i>Phoceae</i>, <i>Intestinibacillus</i>, <i>Tyzzarella</i> and <i>Ureaplasma</i>.</p>	[123]

Table continues on the next page.

General introduction

Model	Sex	Age	Sample	Method	Difference in Microbiota Compared to Controls	Ref
MIA	M	3-5 weeks	Feces	16S rRNA seq (V4 region)	<p>α-diversity: ↓ β-diversity: No difference Phylum level: ↑ <i>Firmicutes</i>, ↓ <i>Bacteroidota</i> Family level: ↑ <i>Lachnospiraceae</i> ↓ <i>Coriobacteriaceae</i>, <i>Erysipelotrichaceae</i> Genus Level : ↑ <i>Parabacteroides</i>, <i>Enterococcus</i>, <i>Desulfovibrio</i> ↓ <i>Prevotella</i> Species level : ↑ <i>Bacteroides ovatus</i>, ↓ <i>Parabacteroides distasonis</i>, <i>Faecalibacterium prausnitzii</i></p> <p>Note: the authors made a choice to include only the taxa that are known to be modulated in ASD or IBD, hence, there might be other differences that have not been detailed here.</p>	[124]
VPA	F/M	4 weeks	Feces	16S rRNA seq (V3-V5 region)	<p>α-diversity: No changes β-diversity: No difference Phylum level: ↑ <i>Firmicutes</i>; ↓ <i>Bacteroidota</i> Genus level: ↑ Uncultured genus of <i>Erysipelotrichales</i>, uncultured genera of the <i>Bacteroidales</i> and <i>Desulfovibrionales</i> orders</p>	[121]

Table continues on the next page.

General introduction

Model	Sex	Age	Sample	Method	Difference in Microbiota Compared to Controls	Ref
VPA	M/F	8 weeks	Feces	16SrDNA seq (V3-V4 region)	<p>α-diversity: ↓</p> <p>β-diversity: Difference between groups</p> <p>Phylum level: only in males ↑ <i>Bacteroidota</i>; only in female, ↑ <i>Actinobacteria</i></p> <p>Class level: Only in males ↑ <i>Bacteroida</i>, <i>Alphaproteobacteria</i>; ↓ <i>Coriobacteria</i></p> <p>Family level: ↑ <i>Eubacteriaceae</i>, <i>Rikenellaceae</i> and <i>Staphylococcaceae</i>; ↓ <i>Enterobacteriaceae</i></p> <p>Genus level: ↑ <i>Anaerofustis</i>, <i>Proteus</i>, <i>Staphylococcus</i>, and <i>Allobaculum</i></p> <p>Only in females ↑, <i>Bifidobacterium</i>, <i>Odoribacter</i> and <i>Candidatus Arthromitus</i></p> <p>Species level: ↑ <i>Ruminococcus flavefaciens</i>, OTUs from the <i>Clostridiales</i> order and the <i>Ruminoccus</i> and S24-7 genera.</p>	[120]
	M	3-5 weeks	Feces	16SrDNA seq (V4 region)	<p>α-diversity: ↓</p> <p>β-diversity: No difference</p> <p>Phylum level: ↑ <i>Firmicutes</i> ↓ <i>Bacteroidota</i></p> <p>Family level: ↑ <i>Lachnospiraceae</i>, <i>Rikenellaceae</i>, <i>Peptostreptococcaeae</i> ↓ <i>Coriobacteriaceae</i>, <i>Erysipelotrichaceae</i></p> <p>Genus Level : ↑ <i>Parabacteroides</i>, <i>Enterococcus</i>, <i>Dorea</i>, <i>Megasphaera</i>, <i>Desulfovibrio</i>, ↓ <i>Oscillospira</i>, <i>Prevotella</i></p> <p>Species level: ↑ <i>Bilophila wadsworthia</i> ↓ <i>Parabacteroides distasonis</i></p> <p>Note: the authors made a choice to include only the taxa that are known to be modulated in ASD or IBD, hence, there might be other differences that have not been detailed here</p>	[124]

Table continues on the next page.

General introduction

Model	Sex	Age	Sample	Method	Difference in Microbiota Compared to Controls	Ref
VPA	F/M	3 weeks	Feces	16SrDNA seq (V3-V4 region)	<p>α-diversity: No difference β-diversity: No difference Phylum level: ↑ <i>Firmicutes/Bacteroidota</i> ratio Genus level: ↑ <i>Candidatus, Eubacterium, Desulfovibrio</i> ↑ <i>Ruminococcus, Intestinimonas, Eubacterium, Bacteroides</i> in males only ↑ <i>Acetatifactor</i> in female only. ↓ <i>Prevotella, Phascolarctobacterium</i> in males and females. ↓ <i>Corynebacterium, Jeotgalicoccus, Oscillibacter</i> in males only. ↓ <i>Catabacter, Escherichia, Enterorhabdus, Anaerofustis, Sellimonas, Marvinbryantia, Intestinimonas</i> and genera from <i>Erysipelotrichaceae, Ruminococcaeae, Coriobacteriaceae</i> and <i>Lachnospiraceae</i> families in females.</p>	[122]
	M	4 weeks	Feces	16SrDNAseq	<p>α-diversity: ↓ β-diversity: Difference between groups Phylum level: ↑ <i>Firmicutes</i> Genus Level: ↑ <i>Lactobacillus</i></p> <p>Note: the authors made a choice to include only the taxa that were also modulated in rats that received microbiota from ASD patient, hence, there might be other differences that have not been detailed here</p>	[125]
MHFD	M	Adult	Feces	16SrDNA seq (V4 region)	<p>α-diversity: ↓ β-diversity: Difference between groups No detail of the changes in bacterial taxa</p>	[20]

Table continues on the next page.

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Model	Sex	Age	Sample	Method	Difference in Microbiota Compared to Controls	Ref
MHFD	N.S	3 and 8 weeks	Feces	16SrDNA seq (V3-V4 region)	<p>α-diversity: ↓</p> <p>β-diversity: Difference between groups</p> <p>Phylum level: ↑ <i>Firmicutes</i> and <i>Verrucomicrobia</i>, ↓ <i>Bacteroidota</i></p> <p>Family level: ↑ <i>Peptostreptococcaceae</i></p> <p>Genus level: ↑ <i>Streptococcus</i>, <i>Akkermansia</i> ↓ <i>Lachnospiraceae_incertae_sedis</i></p>	[110]
	N.S	1 week	Feces	16SrDNA seq	<p>No assessment of diversity</p> <p>Phylum level: ↑ <i>Firmicutes</i></p> <p>Class level: ↑ <i>Betaproteobacteria</i>, ↓ <i>Gammaproteobacteria</i></p> <p>Genus level: ↑ <i>Lactococcus</i>, ↓ <i>Escherichia</i></p>	[113]

2 PART I- EFFECT OF GUT MICROBIOTA FROM CHILDREN WITH ASD ON BEHAVIOR AND ASD RELATED BIOLOGICAL MARKERS IN GF MICE

2.1 OBJECTIVES-STUDY DESIGN

The evidence presented in this introduction suggests a causality between the microbiota alterations observed in individuals with ASD and some of their behavioral, brain GI and immune symptoms. The GEMMA project (Genome, Environment, Microbiome and Metabolome in Autism) aims to further investigate this causality, and the mechanisms behind it. This project includes a clinical arm, which aims to study the contribution of microbiota, as well as genomic, environmental and metabolomic factors via the immune system, in the development of ASD. It consists in following the evolution of those parameters in newborns that already have a sibling with ASD, to see if any modulation of these markers precede the diagnosis of ASD in a subset of those children [207] (Fig 6).

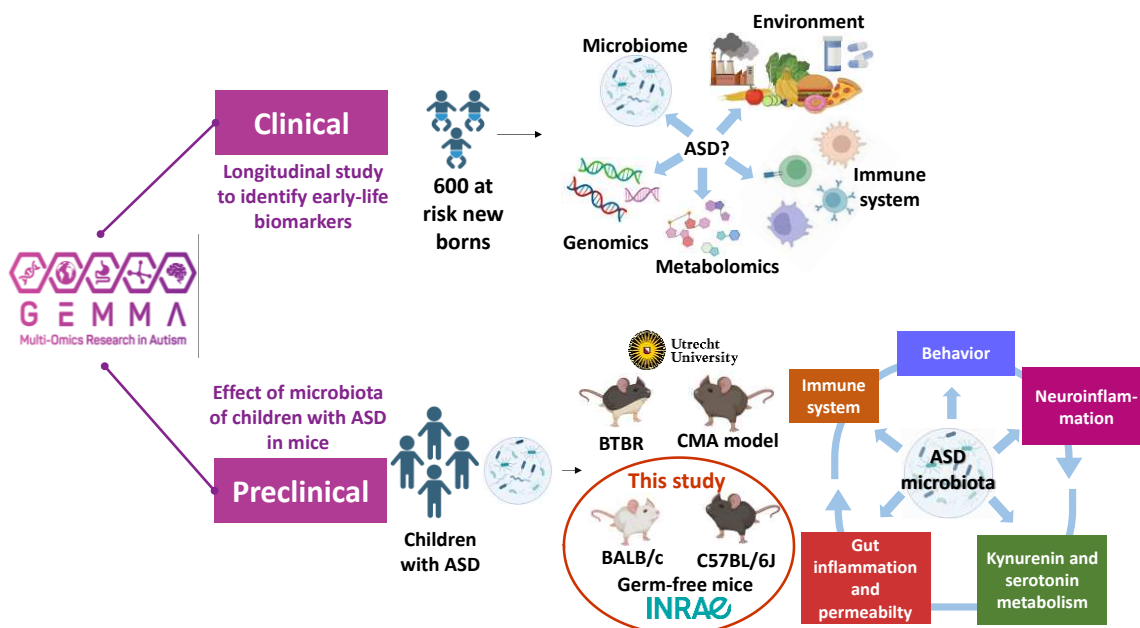


Figure 6 : Representation of the principal aims of the two main arms of the GEMMA project. Clinical arm: various biological parameters (including the microbiome), are monitored during 3 years in 600 at risk newborns (= who have a sibling with ASD) to identify early life biomarkers in the subset of those children who will be diagnosed with ASD. Preclinical arm: aims to assess the effect of FMT from children with ASD on various mice models. This thesis reports only on the part carried out in INRAe, on GF mice.

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The GEMMA project also includes preclinical arm, which aims to study the effect of microbiota from individuals with ASD on ASD-related behaviors and markers in mice models, through a mechanistic approach. This preclinical part is carried out both in Utrecht University and in Micalis Institute in INRAe, in a close collaboration (Fig 6).

In INRAe, the FMT experiment was carried out on GF mice. We studied the impact of gut microbiota from children with ASD, with or without GI symptoms, or from their TD siblings, in two different strains of GF mice (BALB/c and C57BL/6J). We investigated the impact of this transfer on ASD-like behaviors and ASD related biological markers (Fig 7).

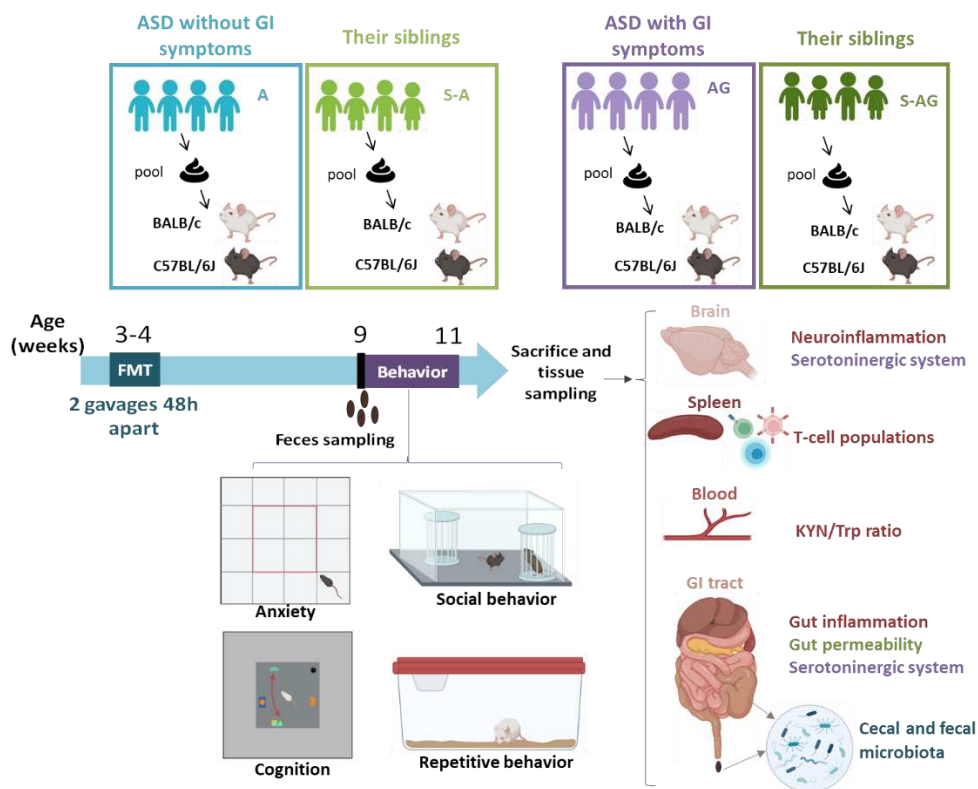


Figure 7: Study design of the FMT experiment on GF mice. BALB/c and C57BL/6J GF mice received pooled microbiota from 4 distinct donor groups at 3-4 weeks of age. Their feces were sampled at 9 weeks of age before the start of behavioral testing. Mice were killed at 12 weeks of age and their blood, brain, spleen, gut segments and cecal content were collected for further analyses: cecal and fecal gut microbiota composition (blue), gut permeability (green), systemic, gut and brain inflammation (red), gut and brain serotonergic system (purple).

The use of GF mice allowed us to be certain that the microbiota of the mice was exclusively the one that we transferred. Plus, by keeping the mice in

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isolators for the entire duration of the experiment, we ensured that there was no impact of environmental microbes on the transferred microbiota. The GF model has its limitations, as the absence of microbiota from birth leads to several alterations, some of which might not be rescuable by FMT after weaning. However, when using SPF or conventional mice, the original microbiota of the animals needs to be depleted through antibiotic treatment and/or bowel cleansing, but there is a possibility of “leftover” microbes. Plus, the long-term implantation of the microbiota might not be as close to that of the original inoculum than it is when using GF mice [240].

The choice of 2 distinct ASD groups was based on the knowledge that the composition of the microbiota of children with ASD is different depending on the presence or absence of GI symptoms. Plus, by choosing siblings as controls, we could ensure that those children are genetically close to the individuals with ASD, live in the same environment and are likely to have more similar diets. Thus, the differences in microbiota between ASD and TD groups are more likely to reflect ASD-specific differences. We have also decided to pool the microbiota of the 4 children in each group. The use of pooled microbiota for FMT from human to mice has been applied in studies on ASD [125, 236, 239] and depression [27, 30]. In other studies, microbiota from individual donors was given to individual mice (or to subgroups of mice), to avoid mixing microbiota ecosystems [237, 238]. However, this approach can bring heterogeneity in results and variable factors for statistical analysis (different donors could yield different effects). Pooling limits this heterogeneity and removes the possibility of a “donor effect” as all mice receive the same microbiota.

We have chosen to test this FMT in two different mouse strains in order to highlight a potential effect of the genetic background, and because previous FMT experiments from ASD donors to GF mice have used either the BALB/c or the C57BL/6J strain. Those two strains have notable behavioral differences due to distinct genetic backgrounds from inbreeding. The most commonly observed difference is a more “emotive behavior” of BALB/c mice in tests of anxiety and depressive-like behaviors, compared to C57BL/6J mice [241]. In addition, studies on different strains of GF mice or rats report different behavioral alterations compared to SPF animals (detailed in part 2 of this thesis) [4, 6, 7, 9, 10, 12–15, 20–22], which shows that the implication of the microbiota in regulation of behavior is different depending on the genetic background.

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Mice received the pooled microbiota by oral gavage right after weaning, which allows good engraftment of microbiota [240], while being early enough that it could still impact brain and behaviors. Indeed, studies show that some microbiota modulations need to occur in an early time window to have an impact on behavioral or brain parameters [3, 12, 20, 21]. As a successful colonization by some bacterial species may require other bacterial species as first colonizers [242], fecal samples were administered twice, at a 48-h interval.

The animals were tested at adult age in behaviors that are altered in most ASD models (social and repetitive behaviors) as well as in a test of anxiety and a test of cognition (spatial memory), as children with ASD often have co-morbid anxiety and/or cognitive impairments.

We formulated the following hypotheses concerning the effect of FMT from our human donors on these animals:

- The composition and/or activity of the implanted microbiota will differ between the ASD and sibling groups.
- Mice receiving microbiota from individuals with ASD will show impaired behavior in the various tests compared to mice that received microbiota from their siblings.
- Mice receiving microbiota from individuals with ASD will have impairments in the various physiological parameters that we know altered in individuals with ASD and suspected to be impacted by the microbiota (immune system, GI permeability and inflammation, KYN and 5-HT pathways, neuroinflammation) compared to mice receiving microbiota from their siblings.
- The microbiota from children with ASD and GI symptoms might have a stronger effect on behavior and other markers, notably those associated with gut health.
- There could be strain-specific effects of the transferred microbiota.

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2.2 MATERIALS AND METHODS

2.2.1 Donors and fecal samples

Donors were children with ASD and their neurotypical siblings. They were recruited in families with no history of ASD by the University Hospital of Naples (Italy), in collaboration with EBRIS (European Biological Research Institute of Salerno, Salerno, Italy). Detailed information on the children with ASD are indicated in table 3. The donors were divided into four groups as follows: group "A" (6-9 years old, males) consisting of children with ASD without associated gastrointestinal disorders; group "S-A" (3-14 years old, 1 female and 3 males) consisting of their neurotypical siblings; group "AG" (7-10 years old, males) consisting of children with ASD with associated gastrointestinal disorders; group "S-AG" (3-13 years old, 2 females, 2 males) consisting of their neurotypical siblings.

Group	Patient	GI symptoms	Bristol score	DSM-5 score	Medications (currently)	Nutritional/probiotics supplements	Food Allergy
A	1	None	5	3	Antipsychotic : Risperidone; Anticonvulsant : Topiramate	No	No
A	2	None	4	2	No	No	No
A	3	None	4	2	Stimulant : Methylphenidate	No	No
A	4	None	5	3	No	No	No
AG	5	Constipation	1	3	No	Neurax Bio (<i>L.plantarum</i> +Magnesium) Periodically	No
AG	6	Constipation	2	3	Antipsychotic : Risperidone; Anticonvulsant : Valproate	No	No
AG	7	Constipation	3	1	No	No	No
AG	8	Constipation	1	1	Anticonvulsant : Valproate	No	No

Table 3: GI and ASD scores of the donors from A and AG groups and medications at the time of sampling. Explanation of DSM-5 scores and Bristol stool scale are available in Annex 2 p. 252.

None of the children had received antibiotics within the two months preceding fecal collection. Fecal samples were collected in November of 2019. Families collected at home, using a collection device provided by MaatPharma (Lyon, France) (Fig 8).

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Samples were stored in the refrigerator until transfer to EBRIS (European Biology Research Institute of Salerno, Italy) laboratory the same day or the next day. Each sample was then carefully homogenized and filtered by mixing manually in the collection pouch for several minutes and diluted 5-fold in a cryoprotective diluent designed to optimize the revivification of the microbiota (MaatPharma) [243, 244], aliquoted into 50 x 2 mL cryotubes with seal-fitted screw caps, and stored at -80°C (Fig1). These samples were used for fecal microbiota transplantation experiments, and samples of each group were pooled before inoculation (as previously described [27, 30, 239]).

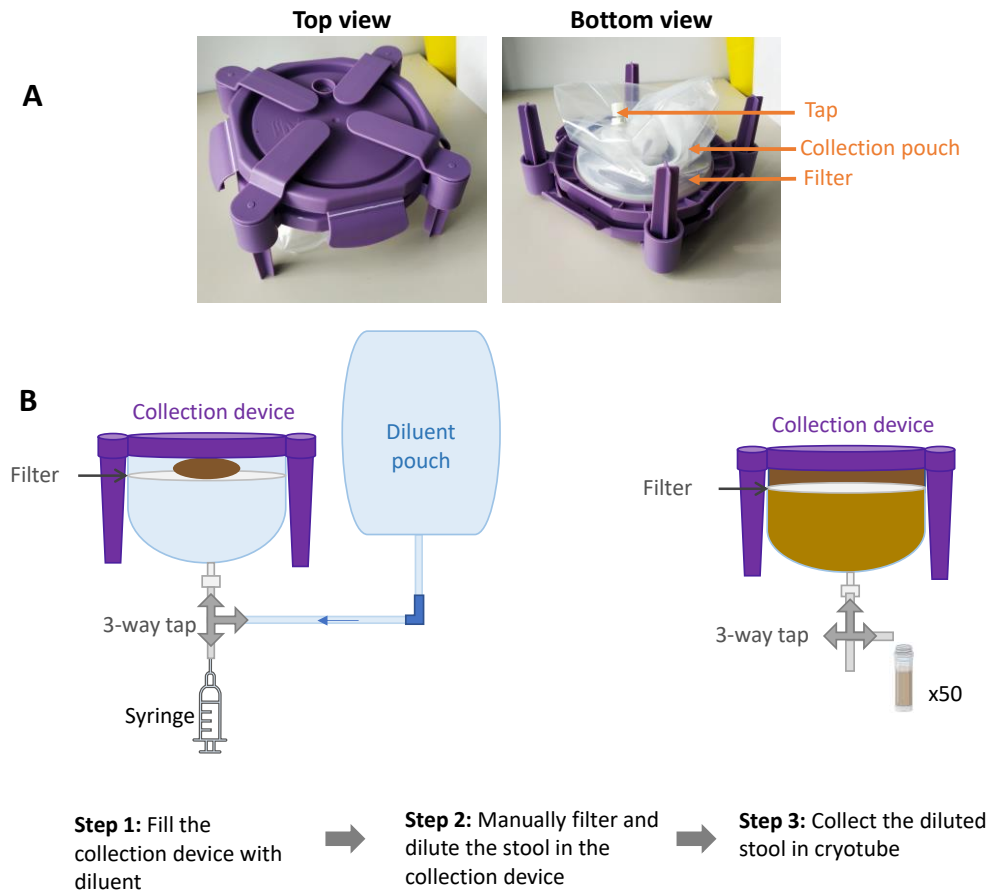


Figure 8: Stool collection device A) Picture of the MaatPharma collection device. B) Schematic of the filtration and homogenization method

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2.2.2 Animals

Experimental procedures were carried out in accordance with EU Directive 2010/63/EU for animal experiments. They were approved by the Ethics Committee of the INRAe Research Center at Jouy-en-Josas (Comethea C2EA-45) and authorized by the French Research Ministry (APAFIS#22637-2019101715102827 v2).

Each group of mice (named S-A, A, S-AG or AG according to which pool of donor feces they received) was composed of 10-13 male GF BALB/cAnNTac or C57BL/6JCrI mice bred and maintained in separate sterile isolators at the Anaxem GF animal facility (INRAe, Micalis Institute, Jouy-en-Josas, France). For each mouse strain, the experiment was performed in two cohorts of 5-8 mice. The animals were born in breeding isolators and transferred to the experimental isolators at 21-28 days of age. The experimental isolators were flexible-film isolators separated in a living compartment and a behavior compartment linked together by a closed airlock. The living compartments were fitted with DPTE® aseptic transfer systems (Getinge, Les Ulis, France) to connect containers (Getinge, Les Ulis, France) to allow entry of sterile consumables and transfer GF animals into the isolators (Fig 9). Upon entry into the experimental isolators, and 48h later, each mouse received 100 µL of pooled donor samples by oral gavage. Individual aliquots in seal-fitted screw caps tubes were entered into the isolator through a port, where they were left to thaw at room temperature for 30 min while the outside of the tube was exposed to a peracetic acid solution (Diluted 1:10 from a 38 % solution) for sterilization. The pool was prepared in the isolator by mixing 1.5 mL of each sample in a 40 mL tube. The pool was then homogenized and administered with a 1 mL syringe (Terumo, Japan) fitted with a flexible alimentation probe (Ecimed, Boissy St-Leger, France). The remainder of the pool was spread on the coats of the animals and in the cage, so that they remained exposed to the microbiota by grooming throughout the day. Animals were housed in standard cages (32x15x12 cm) of 3-5 mice, with sterile bedding (BK; SAFE, Augy, France), wooden chew sticks, a transparent plastic cylinder, paper towel and nesting material as environmental enrichment. The bedding was changed weekly. Mice had access to autoclaved water acidified with hydrochloric acid (pH 5.5) and γ-irradiated standard diet (R03-40; SAFE) *ad libitum*. They were exposed to an artificial light of 100 lux (with a 12-h light/dark cycle) and a temperature between 20 and 24°C. During the experiments with BALB/cAnNTac mice, the mice showed significant aggressive behavior among themselves, and we therefore separated them into

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cages of 2, or even isolated them in the most extreme cases. In each group, 4 or 5 additional male mice of the same strain that received the same FMT, used as unknown mice in the social interaction test were housed in the same isolators as the test mice and in the same conditions, but their cages were kept separated from those of the test mice to avoid contact with each other.

2.2.3 Behavioral tests

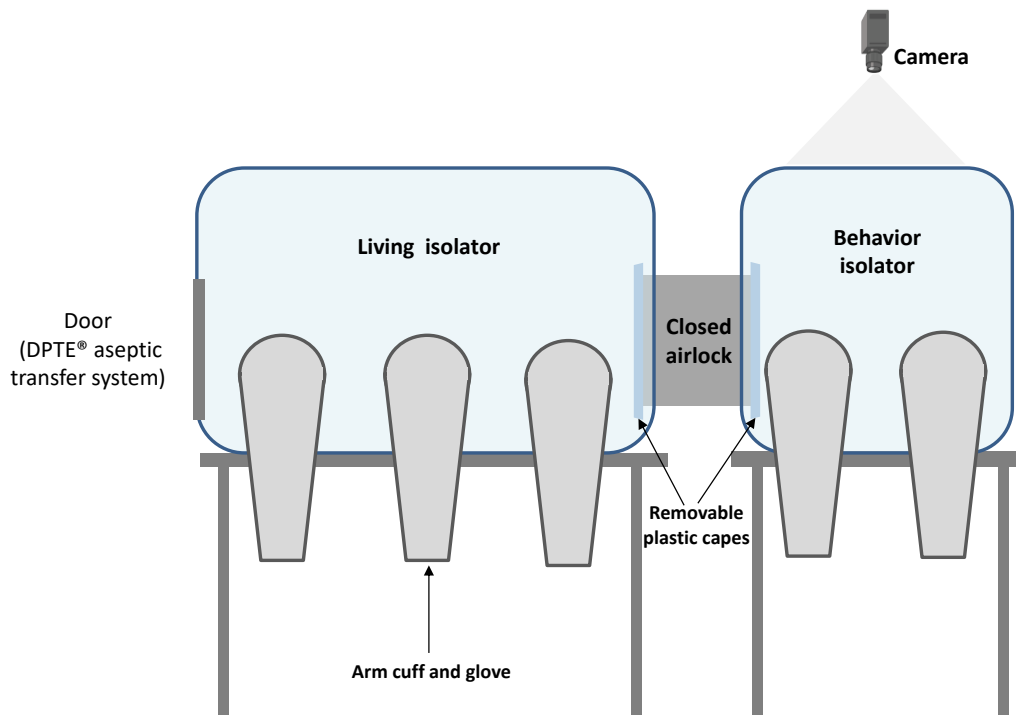


Figure 9: Diagram of a pair of isolators. The arm cuffs and gloves allow manipulation of the animals inside the isolators.

All behavioral tests were performed on mice at 9 weeks of age in the behavioral compartment of the isolator in the morning (after 9 am) up to early afternoon (no later than 3pm). The tests were performed by experimenters that had been regularly involved in handling of the mice throughout the protocol so that the animals were used to them. To avoid experimental bias, behavioral tests were conducted simultaneously for the following pairs: S-A and A group, S-AG, and AG group, by two experimenters. The animals from each group were evenly distributed between the two experimenters. Figure 10 details the calendar of the behavioral experiments.

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FMT experiment

Week 1							Week 2						
Mon	Tue	Wed	Thu	Fri	Sat	Sun	Mon	Tue	Wed	Thu	Fri	Sat	Sun
Open-field and Spatial object recognition							Social interaction and novelty			Self-grooming			

Figure 10: Calendar of the behavioral tests

2.2.3.1 Anxiety-like behavior: Open field test

The test was conducted in a square open field (OF) of 45x45 cm and a 20 cm height with opaque walls. The mouse was placed in the bottom right corner of the OF and filmed for 5 min by a camera placed above the isolator. The videos were analyzed with the ANY-maze software (Stoelting Co., Dublin, Ireland). To define the zones, the surface of the OF was divided into 25 equal squares and the 9 central squares constituted the central zone. The "corner" zones were made up of one square (Fig 11). We analyzed the time spent and the distance traveled in each of the zones (periphery, center, and corners), as well as the total distance travelled and speed. It is considered that the anxiety of a mouse is inversely proportional to the time spent in the center (anxiogenic zone), and proportional to the time spent in periphery, especially in the corners. If a mouse did not visit at least 2 corners and/or travelled a total of less than 2 meters during the test, it was excluded from the analysis.

Reference: Adapted from Prut et al. (2003) [245]

2.2.3.2 Spatial memory: Spatial object recognition test

This test was conducted immediately after the OF test, the latter being the first of the 5 phases of this test. The duration of each phase was 5 min separated by a 3-minute interval during which the mice returned to their home cages. Visual cues (printed sheets of contrasting colored shapes) were placed on the outside of the four walls of the isolator. The first phase (P1) (OF test previously described) allowed the mice to accustom themselves to the test environment. During the next 3 phases (P2-P4), a Lego® plate (25 x25 cm) was placed in the center of the OF, on which were placed 5 Lego® objects that differed in shape, size, and color and were between 4 and 7 cm high and 1 or 2 cm wide (ToyPro, Nederweert, The Netherlands). Each object was 1 cm from the edge of the plate and 6-8 cm from each other (Fig 12). The mouse was placed at the center of the OF and was left to explore the

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environment and the objects. For phase 5 (P5) the position of two objects was switched (always the same two objects, referred to as “displaced objects” (DO)) (Fig 11). The video was analyzed with the ANY-maze software and the time the animal spent in contact (snout contact) with each object was measured for P4 and P5. A mouse with a normal spatial memory should have a preference for DO compared to non-displaced objects (NDO) in phase 5. We measured the recognition index (RI): **RI=(DO.P5-All.P4)-(NDO.P5-All.P4)**. With **DO.P5**=average time spent interacting with displaced objects in the test phase (P5), **NDO.P5**= average time spent interacting with non-displaced objects in the test phase (P5) and **All.P4**=average time spent interacting with any object in the last training phase (P4).

All.P4, represents the interaction time with objects for which the mice should have no preference. Thus, if a mouse has a preference for DO and no preference for NDO in P5, (DO.P5-All.P4) should be a positive number and (NDO.P5-All.P4) close to 0. Hence (DO.P5-All.P4)-(NDO.P5-All.P4) is proportional to DO preference.

If a mouse did not interact with all the objects in phase 4 and/or had a total interaction time of less than 3 s in phase 5 it was excluded from the analysis.

Reference : Adapted from Leonibus et al. (2007) [246]

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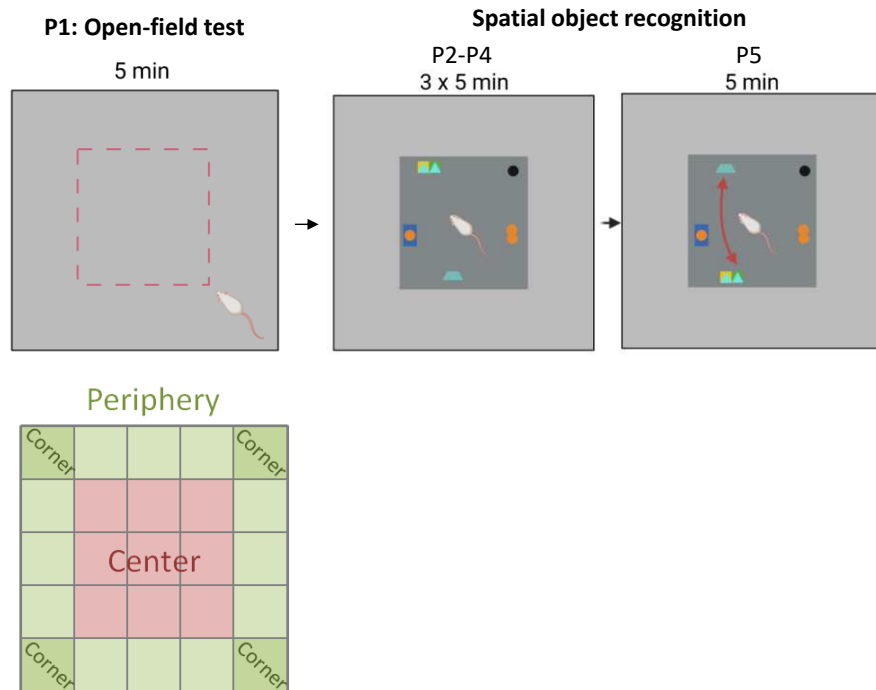
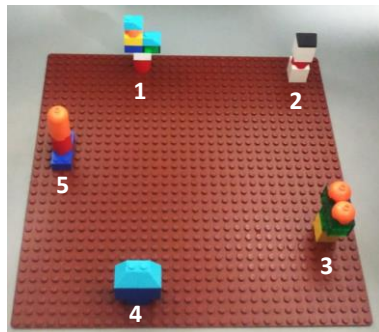
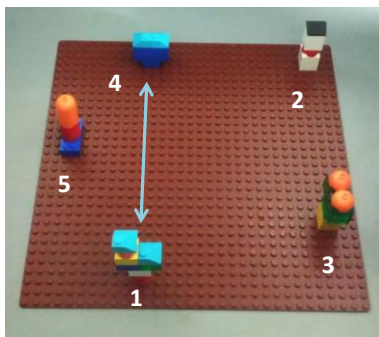


Figure 11: Schematic of the OF test spatial object recognition test. Figure created on Biorender.com

Disposition of the objects on the plate (25 x 25 cm):



Phases 2 to 4



Phase 5

Sizes of the objects (at the biggest points):

Object 1:
H= 7 cm W= 2 cm



Object 2:
H= 6 cm W= 1 cm



Object 3:
H= 6 cm W= 2 cm



Object 4:
H= 4 cm W= 2 cm



Object 5:
H= 6 cm W= 2 cm



Figure 12 : Pictures of the objects and their disposition for the spatial object recognition test

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2.2.3.3 Social behavior: Social interaction and novelty

These tests also took place in the OF, in which two transparent perforated plexiglass cylinders were placed in opposite corners, 5 cm from the edge of the OF. The cylinders were 10 cm in diameter and 14.5 cm high, with an opaque plexiglass cover, and placed on top was a smooth cylinder of the same diameter and 8 cm high to prevent the animals from climbing on the top of the cylinders. The test was filmed from above. In a first phase of habituation, the mouse was placed for 5 min in the center of the OF with the two empty cylinders. Without removing the tested mouse, an unknown mouse was put in one of the two cylinders for the social interaction phase. After 5 min, a new unknown mouse was put in the other cylinder for the social preference phase. The videos were analyzed manually (in a blinded setting) to measure the time spent interacting with each of the cylinders (interaction with the snout). It is considered that a mouse with normal social behavior should interact more with the cylinder containing the mouse during the social interaction phase (called "Mouse cylinder" in the rest of this manuscript), and with the cylinder containing the newly added mouse (called "Unknown mouse" cylinder in the rest of this manuscript) in the social novelty phase.

Mice that did not interact with one of the cylinders during the habituation phase were excluded from the analysis.

Reference: Adapted from Crawley et al. (2007) [247]

2.2.3.4 Repetitive behavior: Self-grooming assessment

Mice were placed in an empty clean cage with a small amount of bedding that did not allow them to dig and filmed from the side for 10 min. Videos were analyzed manually in a blinded setting. The parameters measured were the total grooming time, the total number of grooming bouts, the number of incomplete and complete grooming bouts, and the latency to first grooming. A complete grooming bout was defined by the passage from snout/head grooming to body grooming, staying at least two seconds on each part. An incomplete grooming bout was defined as grooming only one part of the body, even if it lasted several seconds. The average of each parameter from both experimenters was used in the statistical analysis. It is considered that a mouse with repetitive behaviors would present an increase in the number of grooming bouts, particularly incomplete ones, and/or a decreased duration of each grooming bout, and decreased latency

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to first grooming.

Reference: Adapted from McFarlane et al. (2008) [248]

2.2.4 Microbiota analysis

2.2.4.1 Analysis of fecal and cecal microbiota composition

Two fresh fecal pellets per mouse were collected 6 weeks after FMT to analyze the microbiota composition at the start of the behavioral tests. Pellets were collected in the morning and stored at -80°C . At euthanasia (9 weeks after FMT) half of the cecal contents were also stored at -80°C for this analysis. DNA was extracted from fecal and cecal samples using the QIAamp® PowerFecal® Pro DNA Kit (Qiagen, Germany) following the provided protocol. The concentration and purity of the DNA was checked by nanodrop (Fisher Scientific, Strasbourg, France). The DNA was then diluted to obtain a concentration of about $100\text{ ng}/\mu\text{L}$, then amplified by PCR using the Phanta Max Super-Fidelity Kit (Vazyme, Nanjing, China) and 16S V3-V4 region primers (see sequence below).

Forward primer: 5' ACG GRA GGC AGC AG 3'

Reverse primer: 5' TACCAGGGTATCTAATCCT 3'

Amplicon size was checked on 1 % agarose gel in TAE 1X with Midori Green Advance (Nippon Genetics Europe, Düren, Germany) and 1 kb ladder (New England Biolabs, Evry- Courcouronnes, France). The expected bands were 457 bp. Amplicons were transferred on MicroAmp™ Optical 96-Well Reaction Plate (Applied Biosystems, Fisher Scientific) and sent for quality control, sequencing library construction and Illumina sequencing to the @BRIDGE platform (INRAE, Jouy-en-Josas, France) for BALB/c mice and to the GenoToul GeT-Biopuces platform (Toulouse, France) for C57BL/6J mice. The sequences were analyzed using R combining dada2 v.1.26 [249] and FROGS 4.0.0 [250] workflows. Adapters were removed (cutadapt v. 3.5) and forward and reverse reads were filtered (dada2 filterAndTrim function; truncation length: 200bp). The error model was then calculated using the learnErrors function. Then, the dada2 core sample inference algorithm was executed. Forward and reverse reads were merged with a minimum overlap of 20 bp. The resulting sequences were saved in a sequence table using makeSequenceTable function. Chimeras were detected using the vsearch tool according to FROGS v4.0.0 guidelines. Reads were assigned to ASVs with dada2. ASVs with global abundance lower than 0.005 % were removed

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from the following analysis with FROGS filters. The ASVs in the sequence table were then assigned to species using FROGS affiliation with silva 138 [251]. 16S rRNA amplicon sequencing bioinformatic analysis was then performed on those ASVs on R (v4.2.2) with package Phyloseq (v1.42.0) and Phyloseq.extended (v0.1.1.9).

2.2.4.2 Short chain fatty acid (SCFAs) analysis

At euthanasia, one half of the cecal content was stored at -80°C for this analysis. SCFAs were extracted by diluting the cecal contents in Milli-Q water (2 mL/g of content). After vortex stirring and incubation for 2 h at 4°C, samples were centrifuged (15 min, 15 000 g, 4°C) and the supernatants were transferred to tubes containing phosphotungstic acid (10 % of the sample volume) and incubated overnight at 4°C. After centrifugation (15 min, 15 000 g, 4°C), the supernatants were transferred to empty tubes and kept at -20°C (1 week maximum). Before gas chromatography analysis, samples were centrifuged again (30 min, 15 000 g, 4°C). Between 50 and 200 µL of sample supernatant or standards (10mM mixed SCFAs) were put in chromatography flasks with ¼ volume of internal standard (2-ethylbutyrate (Supelco, Sigma-Aldrich)) and analyzed with a gas chromatograph (Agilent 7890B, Agilent Technologies, Les Ulis, France) equipped with a split-splitless injector, a flame-ionisation detector, and a fused silica capillary column (15 m x 0.53 mm x 0.5 µm) (Supelco, Sigma-Aldrich). The carrier gas (H₂) flow rate was 10 mL/min, and the temperature curve was 100°C for 10 min followed by an increase from 100 to 180°C at a rate of 20°C/min and 2 min hold. The detector temperature was 240°C. The peaks obtained were integrated using OpenLAB Chemstation software (Agilent Technologies).

Each sample was analyzed in duplicate, and the average value was used. Response coefficient (R_c) for each SCFA was determined using the chromatogram of the standards with the following formula: **R_c = (A_{IS}/A_{SCFA}) x (C_{SCFA-Std} / C_{IS})**. With **A_{IS}**: Area under internal standard peak; **A_{SCFA}** : Area under SCFA peak; **C_{SCFA-Std}**=SCFA concentration in the standard; **C_{IS}**: Internal standard concentration. Which allowed to calculate SCFA concentration in the samples: **C_{SCFA} = (A_{SCFA}/A_{IS}) x C_{IS} x R_c**. This concentration was normalized by the dilution factor of 1.25 and the multiplier (supernatant weight/sample weight). Measures of acetate, propionate, butyrate, and branched and long chain fatty acids (isoSCFAs + valerate + caproate) were expressed in % of total SCFAs for statistical analysis.

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2.2.5 Flow cytometry quantification of T cell populations in the spleen

At euthanasia, spleens were collected and kept on ice in Roswell Park Memorial Institute (RPMI) medium until processing during the same day. Spleens were then crushed on a 70 μ M cell strainer, rinsed with 10 mL of RPMI, centrifuged (1400g, 5 min, 4°C) and resuspended in 2 mL of red blood cell lysis buffer (1X) (eBiosciences, Fisher Scientific) for 4 min on ice. The reaction was stopped by adding 5 mL of RPMI+Fetal calf serum (FCS) (5 %). The cells were centrifuged again (1400 g, 5 min, 4°C) and the pellet was transferred to 2 mL of RPMI+FCS (5 %). The cells were then counted on a Kova slide (Pierron Arreguemines, France) after staining the dead cells with Trypan blue (1:100 dilution) (Corning, NY, USA). Approximately 5×10^6 cells per well were then plated in a 96-well plate (round shaped wells). Plates were centrifuged to remove the supernatant and then labeled for cell viability using viability dye (eBiosciences, Fisher Scientific) 15 min at 4°C in the dark, then incubated with CD16/CD32 20 min at 4°C in the dark to block nonspecific interactions, then incubated with the surface antibody mix (see table 1 below) for 20 min at 4°C in the dark. For all these steps, the antibodies were diluted in PBS Fluo (Phosphate Buffered Saline (PBS) 1X + 2 % FCS +0.01 % azide). The cells were then washed and incubated in Fox P3 Fix/Perm buffer (eBiosciences, Fisher Scientific) for 45 min at 4°C in the dark and washed in 1X permeabilization buffer (eBiosciences, Fisher Scientific). Cells were then incubated in the intracellular antibody mix (see table 2 below) diluted in 1X permeabilization buffer (eBiosciences, Fisher Scientific) for 30 min at room temperature in the dark and washed twice before being resuspended in 100 μ L of PBS formalin (PBS 1X +1 % formalin). The cells were kept at 4°C in the dark until the next day. Just before analysis, cells were centrifuged (1400 g, 5 min) and then resuspended in 100 μ L of PBS Fluo before being measured by flow cytometry (LSRFortessa™ X-20, BD biosciences, San Jose USA) and analyzed using the FLOJo™ Software (BD-Biosciences).

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Target	Type of marker	Marker	Fluorochrome	Supplier	Dilution
T-lymphocytes (general)	Surface	CD4	BV510	Biologend (San Diego, CA)	1/40
		CD69	PE/Cy7	Fisher Scientific (Strasbourg, France)	1/40
Th1	Intracellular	Tbet	Alexa Fluor 647	Biologend (San Diego, CA)	1/40
Th2	Surface	T1/ST2	FITC	MD Biosciences Bioproduct (Oakdale, MN)	1/20
	Intracellular	Gata 3	eFluor 450	Fisher Scientific (Strasbourg, France)	1/20
Th17	Intracellular	RoRyT	Alexa Fluor 647 (APC)	BD biosciences (Heidelberg, Germany)	1/40
Treg	Intracellular	FoxP3	FITC	Fisher Scientific (Strasbourg, France)	1/40

Table 4: Antibodies used for T-lymphocyte analysis by flow cytometry.

2.2.6 Immunohistochemistry

Immunohistochemistry analyses were all done in Utrecht University (collaborator on the GEMMA project). I participated in the protocols during my stay there in November 2021, but most of the staining and image processing/analysis has been performed by our collaborators in Utrecht University (more precisely, Naïka Prince, Lucía Peralta Marzal, Johana Jarkulischová, Patricia Alves Soares, Ioana Matei, Paula Pallarola Martínez) and coordinated by Paula Pérez Pardo and Aletta Kraneveld.

2.2.6.1 Intestine

-Pre-processing: At the end of the experiment ileum and colon were collected. The different parts were washed with PBS 1X, then opened lengthwise and rolled into Swiss rolls (Fig 13). The Swiss rolls were placed in histology cassettes. Tissues in cassettes were fixed for 24 hours in 4 % paraformaldehyde and then kept in 70 % ethanol at 4°C before being sent to Utrecht University where they were embedded in paraffin blocks. The blocks were then cut in 0.5 µm slices and mounted on histology slides (4 slides per roll and 4-5 slices per slides).

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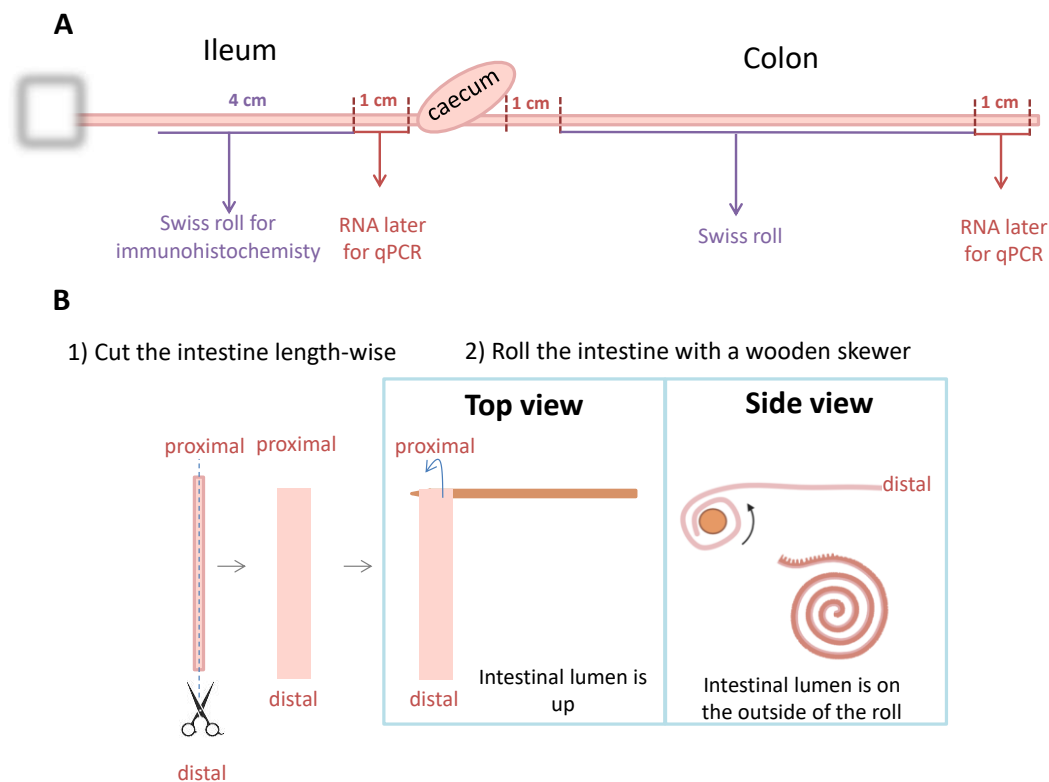


Figure 13 : Intestinal sections A) Intestinal sections collected for immunohistochemistry and qPCR B) Schematic of the swiss roll.

-Immunostaining: Immunostainings of 5-HT, e-cadherin and GFAP were performed on sections of Swiss rolls of ileum and colon.

Primary antibodies: Rabbit anti GFAP (Dako, 1 :1000); Mouse anti e-cadherin (BD Biosciences; 1 :200); Rabbit anti 5-HT (Sigma; 1 :10000)

Secondary antibodies: Goat anti-rabbit AlexaFluor 594; Goat anti mouse AlexaFluor 488 (Thermofisher 1:200)

The sections were deparaffinized by immersion in 100 % xylene (2x 2 min) followed by rehydration in a series of ethanol (EtOH)/water solutions (100 % EtOH 2 times 10 min, 90 % EtOH, 70 % EtOH, 50 % EtOH, MilliQ water, each for 5 minutes). For antigen retrieval, slides were incubated in sodium citrate buffer 0.01M pH6 (Sigma-Aldrich) which was brought to a boil. Slides were allowed to cool for 1 h and rinsed with PBS 1X. Blocking was done using 3 % bovine serum albumin (BSA), 3 % normal goat serum, 0.1 % Tween 20 in PBS 1X as blocking buffer (BSA – Sigma-Aldrich, normal goat serum –Agilent;

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Tween 20 – BioRad, Hercules, CA) 1 h at room temperature followed by overnight incubation with primary antibodies diluted in blocking buffer. The sections were then washed in PBS 1X with 0.1 % Tween 20 for 5 min on a horizontal shaker. Secondary antibodies were diluted in freshly prepared PBS 1X with 0.1 % BSA and 0.1 % Tween 20 and the sections were incubated with those antibodies in dark boxes for 1 h at room temperature, followed by three consecutive washes with PBS 1X with 0.1 % Tween 20 for 5 min on a horizontal shaker. The sections were mounted in ProLong® Gold Antifade mounting media reagent with DAPI (Cell Signaling Technology, Leiden, The Netherlands) for DNA counterstaining.

-Imaging: Images were acquired on a Leica TCS SP8 confocal microscope equipped with a 25x water objective (HCX IRAPO L). Laser power was set to 5.50 for the 405 nm and at 1.50 for the 598 nm. Images had a size of 1024x1024 pixels with a pixel size of 604.99 nm, a pixel dwell time of 600 ns, a zoom factor of 0.75. Six images of five consecutive villi in the ileum or 8 consecutive crypts in the colon were acquired and analyzed. For the analysis of GFAP and e-cadherin the integrated density was quantified in the regions of interest: epithelial cell layer (E-cadherin) of 5 villi or 8 crypts, and submucosal tissue layer (GFAP) under those villi /crypts, using the ImageJ software [252]. For GFAP, the background subtraction rolling ball radius was set at 25 pixels. The integrated densities of the stained areas in each picture were first corrected for background using the following formula to obtain the corrected total fluorescence (CTF): **CTF = integrated density- (area X mean grey value background)**. The mean CTF was calculated by averaging the CTFs of six regions of interest from each sample. For the analysis of 5-HT, cells were counted manually by two separate experimenters in 5 consecutive villi in the ileum and 8 consecutive crypts in the colon. The counts of both experimenters were averaged and used for statistical analysis.

2.2.6.2 Brain

-Pre-processing: The left hemisphere of the brain was removed entirely (except the olfactory bulb). The hemisphere was then placed in a 2mL Eppendorf tube containing 1mL of 4 % paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) at room temperature for 24 h and then kept in PBS 1X + 0.02 % sodium azide (Sigma-Aldrich) at 4°C until further processing (Utrecht University).

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-Immunostaining: Whole hemispheres were transpised (IDisco method) and stained for TPH2 and ionized calcium-binding adapter molecule 1 (Iba1).

Primary antibodies: Goat anti-TPH2 (Everest biotech (Bicester, United-Kingdom, 1:1000) and Rabbit anti-IBA1 (Fischer Scientific, 1:1000)

Secondary antibodies: Donkey anti-goat Alexa Fluor 647 (Fischer Scientific, 1:500), Donkey anti-rabbit Alexa Fluor 594 (Fischer Scientific, 1:500)

The brains were dehydrated in successive baths of 20 %, 40 %, 60 %, 80 %, and two times 100 % methanol for 1 h at room temperature. They were then incubated in dichloromethane (DCM) (66 %) and methanol (33 %) at room temperature with agitation overnight. They were then incubated in methanol (95 %) and H₂O₂ (5 %) for 24 h at 4°C, then rehydrated in successive baths of water and methanol at 80 %, 60 %, 40 %, 20 %. Then they were washed in PBS 1X with added TritonX-100 (2 %) (PTx.2) for 1 h at room temperature. Samples were incubated 2 days at 37°C in permeabilization buffer (400 mL PTx.2 + 11.5g glycine + 100 mL dimethylsulfoxide (DMSO)) then 2 days at 37°C in blocking buffer (42 mL PTx.2 + 3 mL donkey serum + 5 mL DMSO), then 5 days at 37°C with primary antibodies diluted in PTwH (PBS 1X + 0.2% Tween-20 with 10 µg/mL heparin) with added 5 % DMSO and 3 % donkey serum. After 5 washes of 1 h with PTwH, they were incubated with secondary antibodies (same solvent as above) for 5 days at 37°C in the dark. The samples were then dehydrated by consecutive 1 h baths of 20 %, 40 %, 60 %, 80 % and 100 % methanol followed by a second 100 % methanol bath overnight. The samples were then incubated for 3 h at room temperature in 2:1 DCM: methanol, then twice for 15 min at room temperature in 100% DCM. Samples were stored in dibenzyl ether (DBE) until imaging.

-Imaging: Images were acquired on an UltraMicroscope II Light sheet microscope (LaVision BioTec, Bielefeld, Germany) equipped with Olympus MVX10 zoom body (Laser powerset 80 %; sheet width 50 %; exposure time 200, dynamic focus set on the recommended value). For the analysis of Iba1 positive cells (microglia) in the PFC, the zoom factor was set to 6.3x, and 561 images with a step size of 2.0 µm were taken, with the dynamic focus being automatically adjusted every 3 to 4 steps. 100 images in TIF format were cropped to a size of 1024x1024 pixels, converted into stacks, subtracted for background, and adjusted for brightness and contrast using ImageJ. Background subtraction rolling ball radius was set at 25 pixels. Brightness

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and contrast were individually adjusted. Microglial cell count and morphology were computed with 3DMorph software 80 in MATLAB, using stacks of 100 images spaced 2 μm apart from each sample. For the analysis of TPH2 positive cells (serotonergic cells), the zoom factor was set to 2.5x, and 401 images were taken with a step size of 2.5 μm , the dynamic focus being automatically adjusted every 7 steps. The images in TIF format were converted into Imaris Image Files using Imaris File Converter 9.7.0 and analyzed using the spot analysis algorithm in Imaris 9.7.2 software, with the cell size of 12.02 μm being constant. The threshold (using the "quality" option) was individually adjusted, and faulty detection of fibers and blood vessels was adjusted for each image. The number of spots corresponding to the TPH2 positive cell count in the raphe nuclei was then recorded from the statistics tab in the Imaris software.

2.2.7 RT-qPCR

2.2.7.1 RNA extraction

At euthanasia, intestinal sections (Fig 13) were put in 100-200 μL of RNA later® (Sigma-Aldrich) at 4°C for 24 h before being stored at -80°C. Brain dissections (PFC, hippocampus) from the right hemisphere were dipped into isopropanol before being flash-frozen on dry ice and kept at -80°C. Total RNA extraction was performed using RNeasy Plus mini kits (Qiagen). First, samples were put in 600 μL of lysis buffer (573 μL RLT Plus buffer (Qiagen), 4 μL dithiothreitol 1 M (Sigma-Aldrich), 3 μL reagent DX (Qiagen)) with a stainless-steel bead (Qiagen) and homogenized at 1800 rpm for 2 min in a grinder (Powteq GT300, Grosseron, Couëron, France) in racks that had been previously frozen at -80°C. Samples were centrifuged at 5000 g for 3 min at room temperature and the supernatants were transferred to new cryotubes. The supernatants were then frozen at -80°C or used directly for extraction following the provided protocol. No more than the equivalent volume of 20 mg of tissue was used to avoid clogging of the columns. Quality of the RNA eluates was checked on a 1 % agarose gel with 6X GelRed® Prestain Buffer with Blue Tracking Dyes (Biotium, Fremont, CA) and RNA concentration and purity was checked on nanodrop (Fischer Scientific). RNA eluates were frozen at -80°C until further use.

2.2.7.2 Reverse transcription and qPCR

Reverse transcription of 1 μg of the RNA samples was performed using the High-Capacity cDNA reverse transcription kit (Applied Biosystems, Fisher

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Scientific) and the cDNA solution obtained was diluted (1:7 for the intestine and 1:5 for the brain) and stored at -20°C. For IL-6, IL-10 and TNF- α , as the concentration in the initial samples was below threshold of the Real-Time Polymerase Chain Reaction (PCR) System, pure rDNA samples were pre-amplified using TaqMan™ PreAmp Master Mix (Applied Biosystems, Fisher Scientific) and the resulting amplicons were diluted 1:9. Quantitative PCR (qPCR) was performed on diluted cDNA samples (or amplicons) using Taqman Mastermix and TaqMan™ Gene Expression Assay (FAM or VIC) (detailed in table 4) in MicroAmp™ Optical 96-Well Reaction Plate and analyzed using StepOne™ Real-Time PCR System (Applied Biosystems, Fisher Scientific) using the following program: 2 min at 50°C; 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Ct were normalized by subtracting cycle threshold (CT) of the housekeeping gene (GAPDH or β -actin) and $2e^{-\Delta\Delta CT}$ was calculated (using the average of all the animals for normalization as there is no “control group”) and used for statistical analysis.

Intestine			Brain		
Marker	Reference	Dye	Marker	Reference	Dye
GAPDH	Mm99999915_g1	VIC	β -Actin	Mm02619580_g1	FAM
TPH1	Mm01202614_m1	FAM	BDNF	Mm04230607_s1	FAM
Occludin	Mm01349279_m1	FAM	5-HT1AR	Mm00434106_m1	FAM
Claudin-2	Mm00516703_s1	FAM	SERT	Mm00439391_m1	FAM
MLCK	Mm00653039_m1	VIC	GFAP	Mm01253033_m1	FAM
ZO-1	Mm01320638_m1	FAM			
IL-6	Mm00446190_m1	FAM			
IL-10	Mm01288386_m1	FAM			
TNF- α	Mm00443258_m1	FAM			

Table 5: Details of the primers used for qPCR (TaqMan™ Gene Expression Assay (Applied Biosystems, Fisher Scientific))

2.2.8 Analysis of KYN/Trp ratio (ELISA)

At the end of the experiment whole blood from the trunk was collected into MiniCollect® 0.5/0.8 mL CAT Serum Sep Clot Activator tubes (Greiner Bio-One, Madrid, Spain), left to coagulate at room temperature for 2-4 h and centrifuged at 15 000 g for 10 min. Serum was aliquoted into two tubes and frozen at -80°C. Seric concentrations of kynurenine and tryptophan were measured using L-Kynurenine and Tryptophan ELISA kits (Immusmol,

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Bordeaux, France) following the provided protocol. Samples were analyzed in duplicate and optical density (OD) was measured using a multi-detection microplate reader (Infinite m200; Tecan, Männedorf, Switzerland) and concentration was extrapolated from the standard curve.

2.2.9 Statistical analysis

Some of the data did not follow normal distribution (Shapiro-Wilk test) or had unequal variances between groups (Fischer test). Therefore, for consistency in the statistical analysis, we decided to compare all data using non-parametric tests and to represent them on graphs as individual values with median. Comparisons between two groups (S-A vs A or S-AG vs AG) were performed using a Mann-Whitney test. Comparisons to a theoretical value (for social behavior test) were performed using the Wilcoxon signed rank test. The level of significance was set at $p < 0.05$. Calculations were performed with the GraphPad Prism software (version 7.03, La Jolla, CA, USA). Outliers were identified using the "Identify outliers" function in the GraphPad Prism software with a ROUT value of 1%. All values identified as outliers were removed from the statistical analysis.

Statistical analysis for 16S sequencing data was performed on R was performed on R (v4.2.2) with package Phyloseq (v1.42.0) and phyloseq.extended (v0.1.1.9). Mann-Whitney test was used to compare between two groups, and Adonis test to test for group and cohort effects in the principal component analysis for β -diversity.

2.2.10 Spearman correlation

This analysis has been done by collaborators of the GEMMA project in Tampere university (Finland), Karoliina Salenius, Jake Lin and Reija Autio.

To evaluate associations between the gut microbiome and behavior, Spearman correlation analysis was used separately for the two mice strains (BALB/C and C57BL/6J). Two analyses were done, one including all mice of a strain regardless of the group and one done separately for each group (separating "A vs S-A" and "AG vs S-AG"). The microbiome variables selected were Shannon, Chao and Inverse Simpson abundance metrics as well as the microbiota composition at phylum level, taken from fecal or cecal microbiota data. The same Spearman correlation method was further utilized for the integration of the gut permeability, immune and brain markers with caecum metabolites and microbiome, all measured from

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samples taken after euthanasia. In the analysis including all mice regardless of group, p-values were adjusted with the Benjamini-Hochberg method to correct for the multiple comparisons. In the analysis separated by groups, the correlations with p-value<0.05 in either ASD, siblings or both were selected for correlation comparison analysis to identify which of these significantly differed between the ASD and sibling groups. The comparisons were made using Fisher's r to z-transformation and z-test and the resulting p-values were adjusted with the Benjamini-Hochberg method to correct for the multiple comparisons. In all analyses p-values p<0.05 were considered statistically significant. Statistical analyses were performed in R (version 4.2.2).

Remark: Immunohistochemistry data was not included in the correlation analysis, as there were sometimes many missing samples due to technical issues, and because part of the data is being reanalyzed.

2.3 RESULTS-MICROBIOTA COMPOSITION AND FERMENTATION ACTIVITY

2.3.1 BALB/c

2.3.1.1 Microbiota composition (Fecal and Cecal)

2.3.1.1.1 Fecal microbiota

We analyzed the composition of the fecal microbiota 6 weeks after FMT, i.e., right before the behavioral tests, to assess composition of the implanted microbiota in each group, before it could be altered due to the stress of the tests. Firstly, we assessed the α -diversity, which was increased in AG mice compared to S-AG both in number of observed ASVs ($p=0.008$) and Chao1 index ($p=0.009$) but not according to Shannon or InvSimpson index (Fig 14 A-B). Regarding the β -diversity, principal component analysis (PCoA) showed that there was a distinct microbiota composition between S-A and A ($p=0.001$ $R^2=0.30$), and S-AG and AG groups ($p=0.002$ $R^2=0.17$), according to Unifrac distances (Fig 14 C-D). There was also a significant cohort effect in A and S-A groups ($p=0.007$ $R^2=0.087$) but, this effect is negligible, as its effect size (R^2) is way lower than that of the group effect, meaning that most of the clustering of data is between groups and not between cohorts (for more explanations see Annex 2 p. 248). Bray Curtis, Jaccard and Weighted Unifrac all revealed significant group effects both for S-A vs A and S-AG vs AG and in some cases, cohort effects, that were a lot weaker than the group effects except for weighted Unifrac (data not shown).

The differences in diversity were accompanied by differences in relative abundances of various bacterial taxa between groups (overview in Fig 14 E-F, detailed in Fig 15). At phylum level, the most prevalent phyla in each group were Firmicutes and Bacteroidota, and their relative abundance did not differ between groups (Fig 15 A, C). However, there were differences in relative abundance of families from the Firmicutes phylum. In the A group compared to S-A, there was an increased proportion of *Ruminococcaceae* ($p=0.001$) and a decreased proportion of *Lachnospiraceae* ($p=0.003$), whereas no differences were observed in families from this phylum between AG and S-AG groups (Fig 15 B). In the Bacteroidota phylum, there were decreased proportions of *Rikenellaceae* ($p=0.007$), *Marinifilaceae* ($p=0.02$) and *Barnesiellaceae* ($p<0.0001$), in A compared to S-A (Fig 15 D). In AG compared to S-AG, the proportion of *Prevotellaceae* was decreased

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($p=0.003$), and that of *Bacteroidaceae* ($p=0.03$) and *Marinifilaceae* ($p=0.0006$) were increased (Fig 15 D). We observed an increased relative abundance of the Actinobacteriota phylum in A compared to S-A ($p=0.0014$), probably driven by the increase in the *Bifidobacteriaceae* family ($p=0.0009$) (Fig 15 E-F). In this phylum, there was also a slightly decreased proportion of *Atopobiaceae* in AG compared to S-AG ($p=0.02$) (Fig 15 F). Plus, there was an increase in the relative abundance of Proteobacteria in AG compared to S-AG ($p=0.02$) which could be explained by an increase in relative abundance of *Suterellaceae* ($p=0.02$) (Fig 15 G-H). Finally, there was no difference between groups in relative abundance of Desulfobacterota (Fig 15 I) or *Desulfovibrionaceae* which was the only family from this phylum found in our samples (data not shown).

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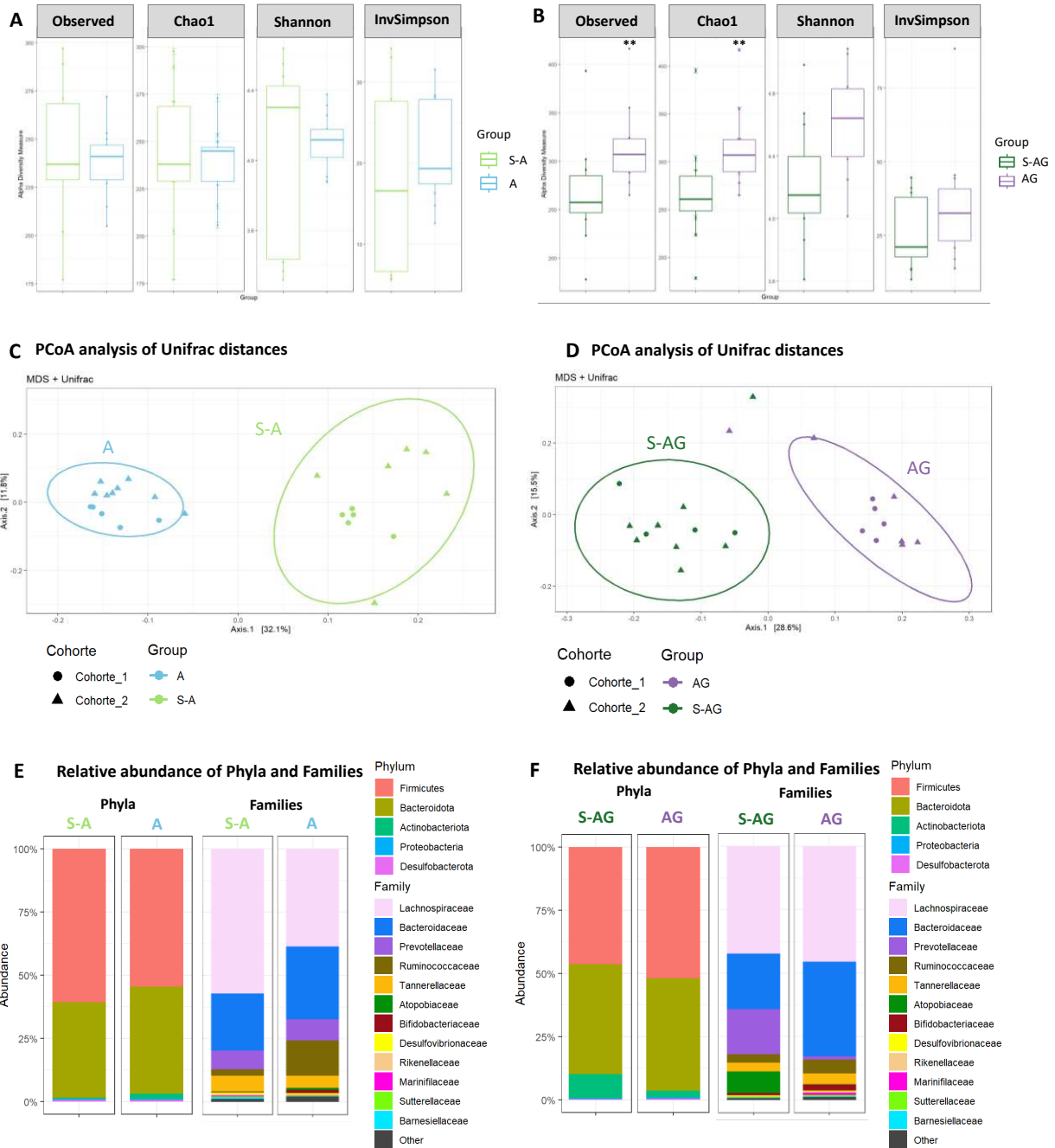


Figure 14 : Diversity and composition of fecal microbiota collected 6 weeks post FMT in BALB/c mice.

A-B) Indexes of α -diversity. Compared with Mann-Whitney test $**p < 0.01$ **C-D)** β -diversity (Unifrac distances) compared with Adonis test: SA-vs A: Group effect $p = 0,001$ $R^2 = 0,30$, and cohort effect $p = 0,007$ $R^2 = 0,087$; S-AG vs AG: Group effect $p = 0,001$ $R^2 = 0,23$; **E-F)** Overall view of relative abundance at phylum and family level

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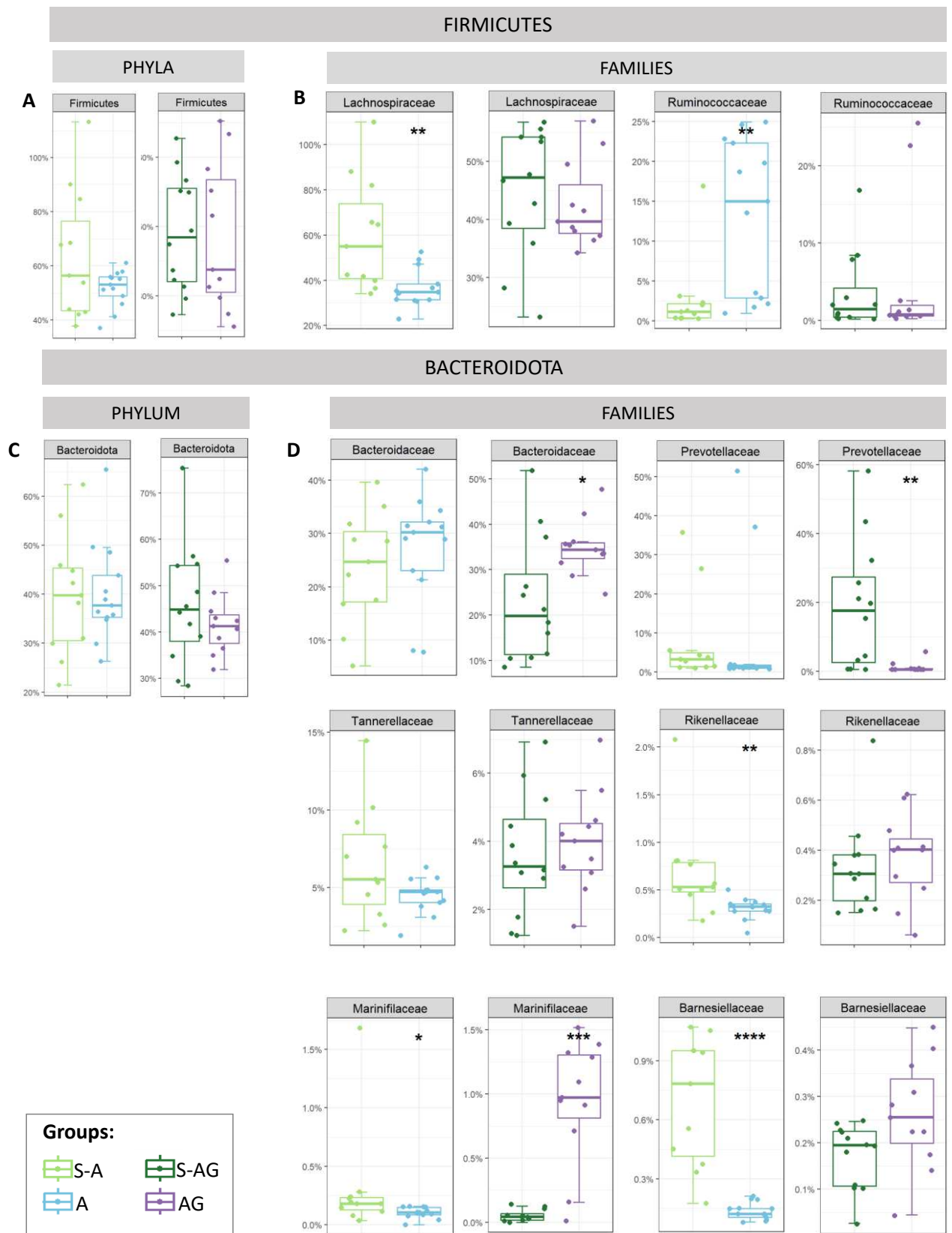


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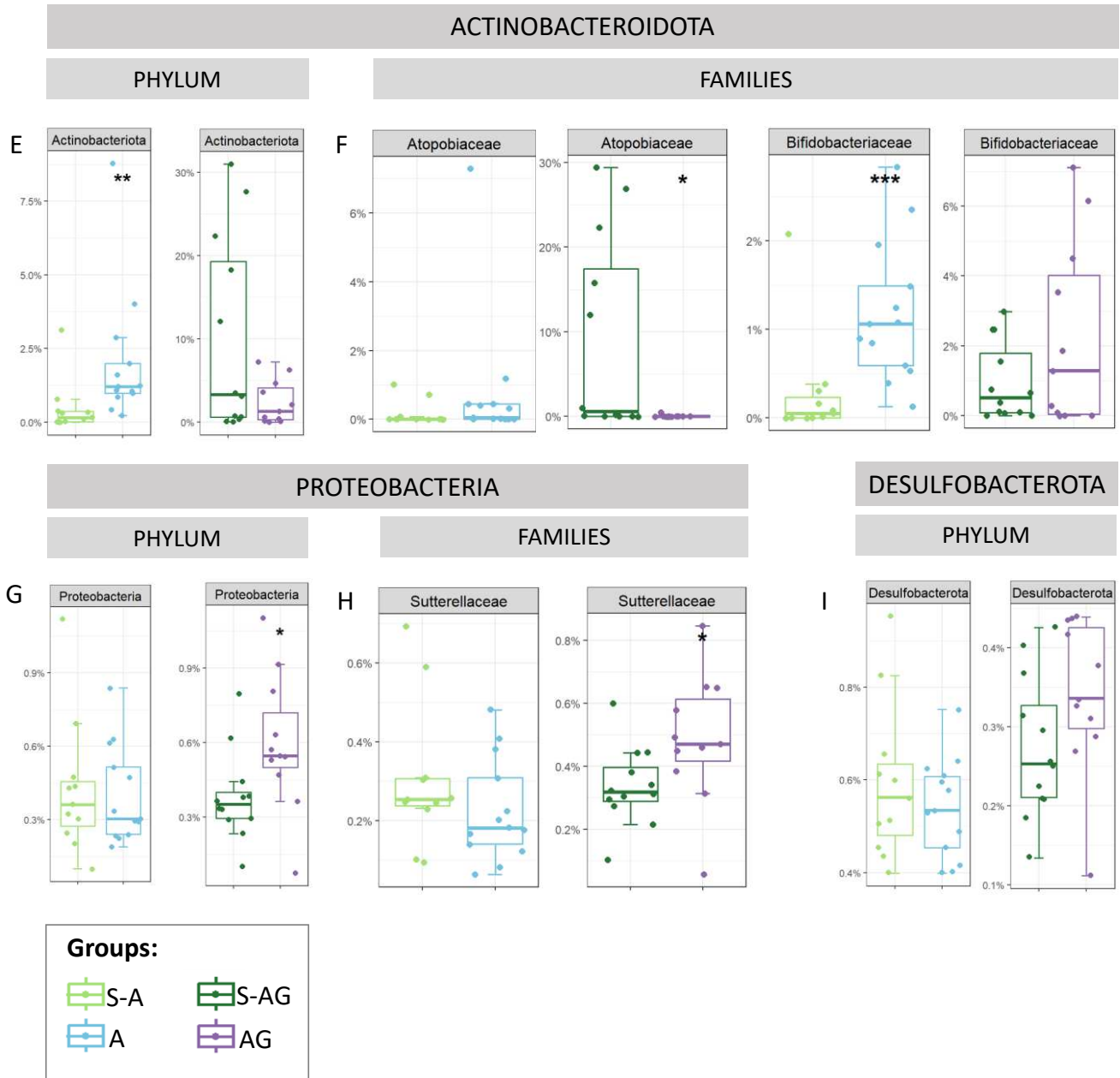


Figure 15 : Relative abundance of phyla and families in fecal microbiota collected 6 weeks post FMT in BALB/c mice.

A,C,E,G,I) Relative abundance of each phylum (% of total abundance) **B,D,F,H)** Relative abundance of families separated by phylum (% of total abundance). Only the families with a significantly different relative abundance between S-A and A, or S-AG and AG groups were plotted.

Groups were compared with Mann-Whitney test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

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2.3.1.1.2 Cecal microbiota

While analysis of microbiota from feces brings the advantage of choosing a specific timepoint, it is interesting to also assess the cecal microbiota composition, as it is a more accurate assessment of the actual microbial population in the large intestine. Plus, studies show quite important distinctions in the composition and functionality between fecal and cecal microbiota in mice [253, 254]. In cecal content, α -diversity was not found to be different between any of the groups (Fig 16 A-B). However, both A and AG groups clustered apart from their respective sibling groups in PCoA analysis according to Unifrac distances (S-A vs A Group effect $p=0.001$ $R^2=0.20$, Cohort effect $p=0.001$ $R^2=0.13$; S-AG vs AG Group effect $p=0.001$ $R^2=0.20$, Cohort effect $p=0.012$ $R^2=0.09$) (Fig 16 C-D). For S-AG and AG, the cohort effect was negligible compared to the group effect. However, for S-A and A, the cohort effect was almost as strong as the group effect, suggesting that the two cohorts in those groups had a distinct cecal microbiota. Bray Curtis, Jaccard and Weighted Unifrac all revealed significant group effects and sometimes cohort effects, both for S-A vs A and S-AG vs AG (data not shown).

The overall composition at phylum or family level is similar between cecal and fecal microbiota (Fig 14 E-F and Fig 16 E-F). However, the phyla and families that differed between groups in cecal content are in part different as the ones observed in fecal content (Fig 17). Firstly, as in fecal microbiota, there was no differences between groups in the relative abundance of Firmicutes and Bacteroidota Phyla (Fig 17 A, C). But at family level, in the Firmicutes phylum, there was an increase in *Ruminococcaceae* ($p=0.0006$) and a decrease in *Lachnospiraceae* ($p=0.002$) in A compared to S-A (Fig 17 B). In addition, the proportion of *Oscillospiraceae* was decreased in the AG group compared to S-AG ($p=0.02$) (Fig 17 B). In Bacteroidota, there was a decrease in *Tannerellaceae* in the A group compared to S-A ($p=0.03$) as well as decreased *Prevotellaceae* ($p=0.001$) and increased *Marinifilaceae* ($p=0.004$) in the AG group compared to S-AG (Fig 17 D). Finally, the relative abundance of Actinobacteriota was increased in A compared to S-A ($p=0.01$) (Fig 17 E), likely due to the increase in the proportion of *Bifidobacteriaceae* in this group ($p=0.002$) (Fig 17 F). Plus, in this phylum, the proportion of *Atopobiaceae* was decreased in AG compared to S-AG ($p=0.006$) (Fig 17 F). There were no differences between groups in the relative abundances of the Proteobacteria and Desulfobacterota phyla (Fig 17 G-H), nor in that of the *Desulfovibrionaceae* family (data not shown).

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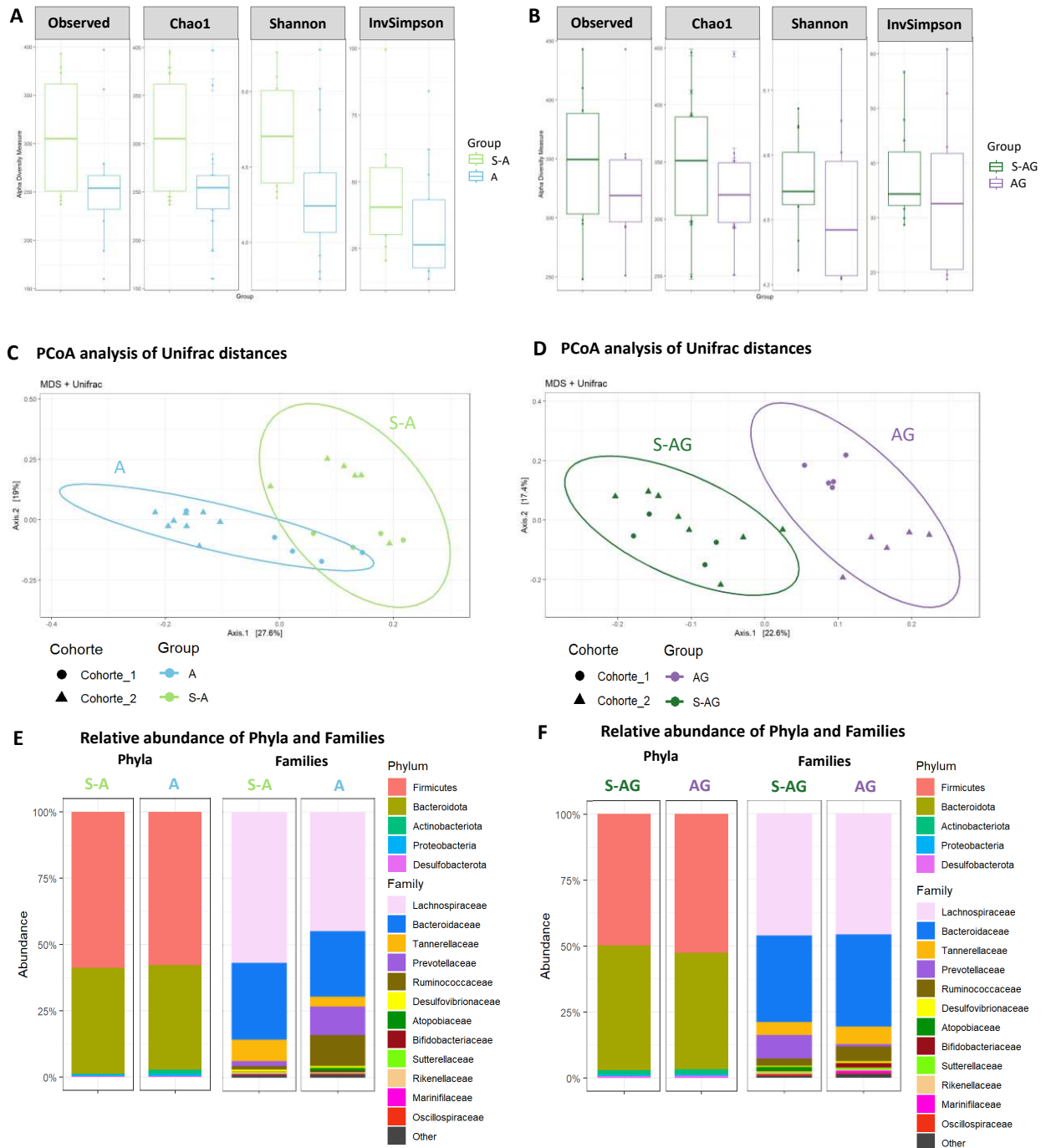


Figure 16 : Diversity and composition of cecal microbiota collected 9 weeks post FMT in BALB/c mice.

A-B) Indexes of α -diversity. Compared with Mann-Whitney test **C-D)** β -diversity (Unifrac distances) Adonis test: SA-vs A: Group effect $p=0,001$ $R^2=0,20$ and cohort effect $p=0,001$ $R^2=0,13$; S-AG vs AG: Group effect $p=0,001$ $R^2= 0,20$ and cohort effect : $p=0,012$ $R^2= 0,09$ **E-F)** Overall view of relative abundance at phylum and family level

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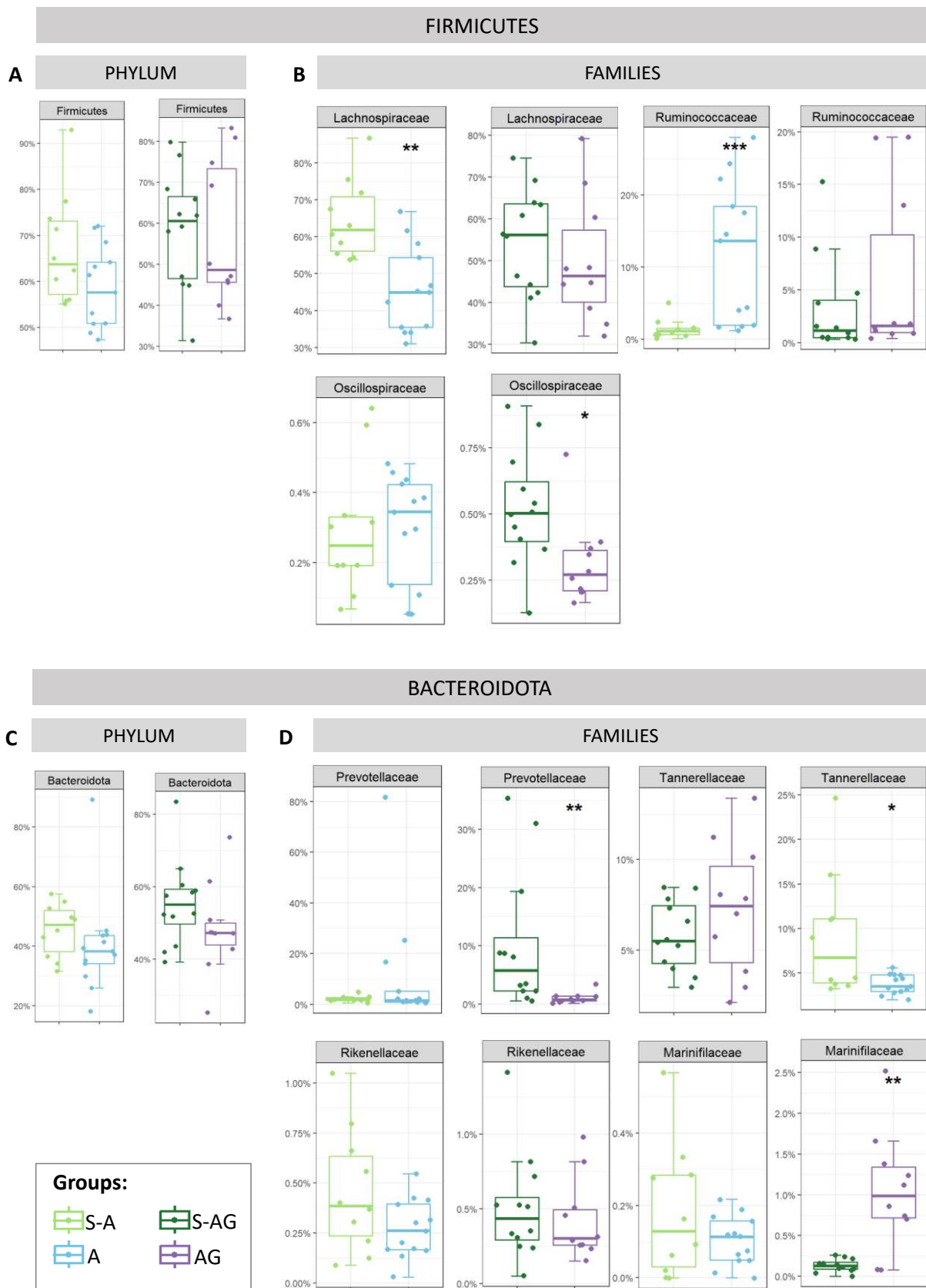


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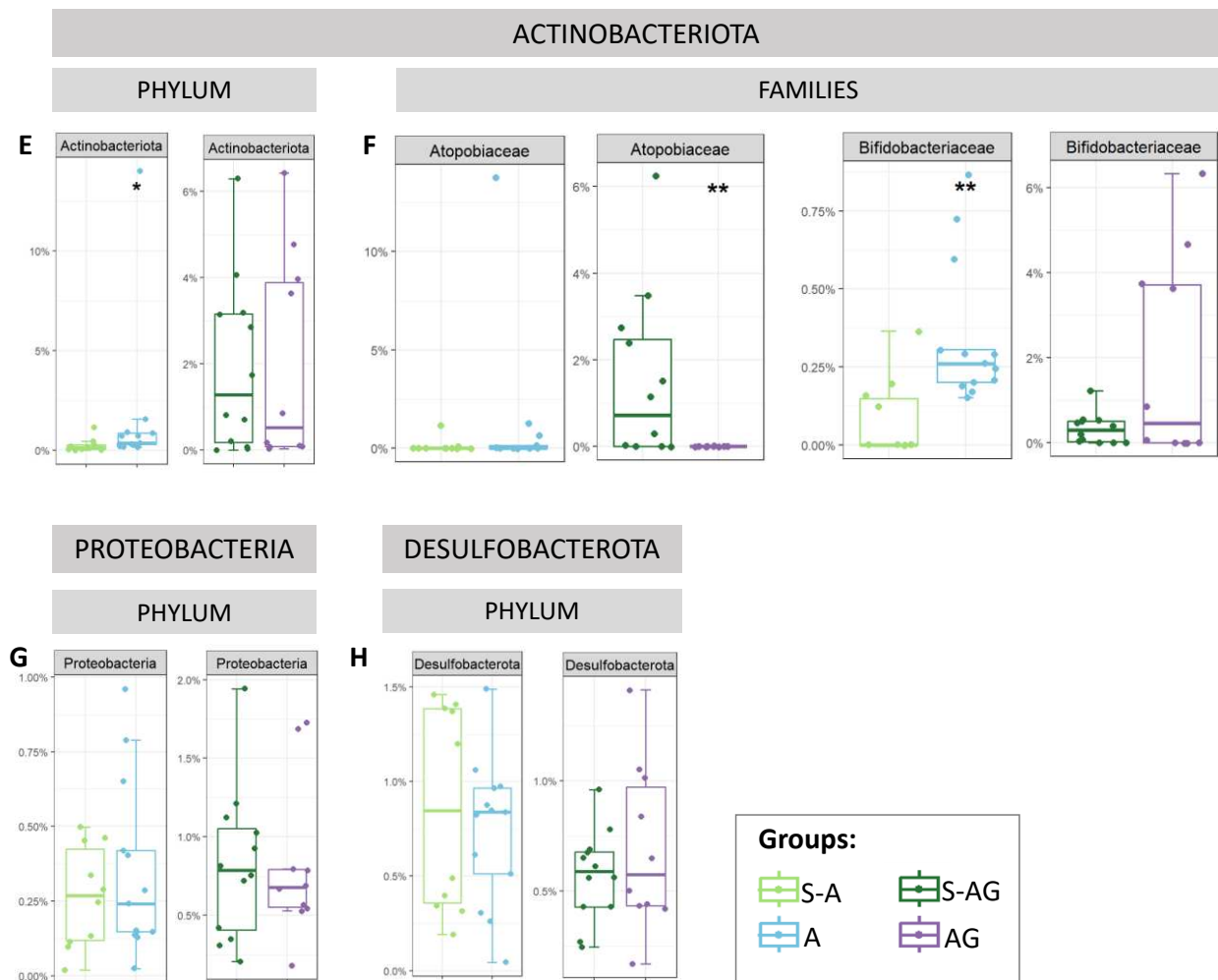


Figure 17: Relative abundance of bacterial phyla and families in cecal microbiota collected 9 weeks post FMT in BALB/c mice.

A,C,E,G,H) Relative abundance of each phylum (% of total abundance) **B,D,F)** Relative abundance of families separated by phylum (% of total abundance). Only the families with a significantly different relative abundance between S-A and A, or S-AG and AG groups were plotted. Groups were compared with Mann-Whitney test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

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2.3.1.2 Fermentation activity of the microbiota: SCFAs profile in the cecum

We next wondered if those differences in microbiota composition were accompanied by differences in the SCFAs profile, measured in the cecal content of the mice, 9 weeks after FMT. Indeed, SCFAs are highly absorbed along the large intestine, thus quantification in the cecal content reflects more accurately the actual functional production than quantification in the feces. Analyzing differences in SCFAs profile is interesting as they are known to be involved in gut-brain axis communication [86]. In BALB/c mice, there was a slightly increased cecal concentration of total SCFAs in group A compared to S-A ($p=0.04$), but no difference between S-AG and AG groups (Fig 18 A). The individual SCFAs were expressed as relative value (% of total SCFAs) to avoid bias from differences in total SCFAs concentration. We observed a slight increase in the proportion of acetate and butyrate in AG mice compared to S-AG ($p=0.02$), and a more significant increase in butyrate in the A group compared to S-A ($p=0.001$) (Fig 18 B and 18 D). The proportion of propionate was decreased in both A ($p=0.0008$) and AG ($p=0.0001$) groups compared to their respective sibling groups (Fig 18 C). Finally, the proportion of branched and long chain fatty acids was decreased in A compared to S-A ($p=0.007$) but increased in AG compared to S-AG ($p=0.03$) (Fig 5 E).

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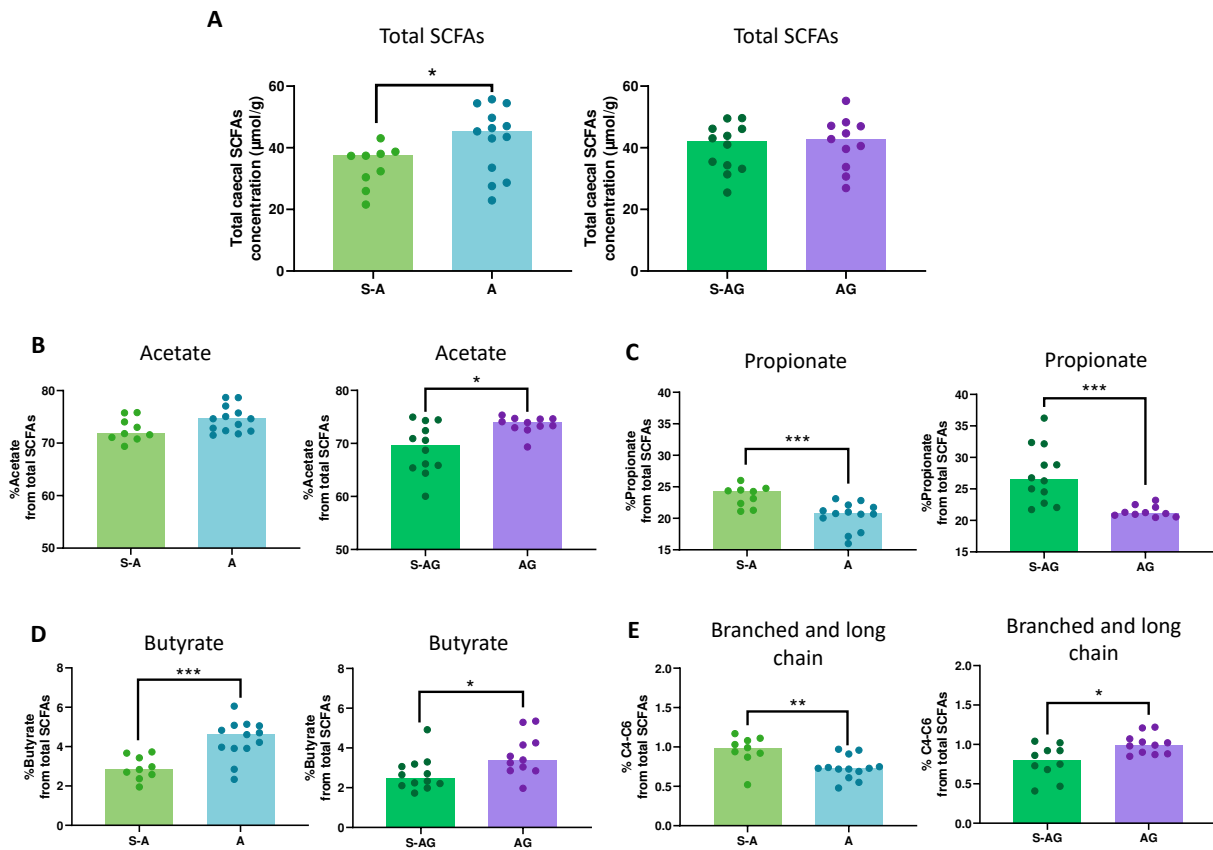


Figure 18 : SCFA analysis in cecal content collected at sacrifice (9 weeks post FMT) in BALB/c mice.

A) Total caecal concentration of SCFAs **B-E)** Relative proportion of acetate, propionate, butyrate and long and branched chain fatty acids. Groups were compared with Mann-Whitney test: *p < 0.05; **p < 0.01; ***p < 0.001.

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2.3.1.3 Spearman correlations between cecal microbiota composition and SCFAs

Since SCFAs are the major metabolites produced by bacteria, and as different bacterial taxa can produce different types of SCFAs, we wondered if there were correlations between bacterial diversity, or the relative abundance of bacterial phyla, and SCFAs proportions or total concentration in the caecum. We first carried out a Spearman correlation analysis on data from all BALB/c mice put together (detailed in table 6).

SCFAs	Microbiota	N	Correlation coefficient	p-value
Total SCFAs (in $\mu\text{mol/g}$)	Actinobacteriota	45	0,31	0,04
% Acetate (C2)	Firmicutes	45	0,34	0,02
	Actinobacteriota	45	0,32	0,03
% Acetate (C2)	Bacteroidota	45	-0,41	0,005
% Propionate (C3)	Chao1	45	0,35	0,02
	Shannon	45	0,40	0,01
	InvSimpson	45	0,36	0,02
	Bacteroidota	45	0,55	0,0001
% Propionate (C3)	Firmicutes	45	-0,50	0,0004
	Actinobacteriota	45	-0,33	0,03
% Butyrate (C4)	Firmicutes	45	0,38	0,01
% Butyrate (C4)	Shannon	45	-0,38	0,01
	Chao1	45	-0,36	0,02
	InvSimpson	45	-0,33	0,03
	Bacteroidota	45	-0,35	0,02

Table 6 : Correlation table of cecal SCFAs and microbiota α -diversity and phyla relative abundances in BALB/c mice when all mice were grouped together. Green: positive correlation, Red: negative correlation

There were many significant correlations, such as a positive correlation between total SCFAs concentration and the relative abundance of Actinobacteriota. The % of acetate was positively correlated with the relative abundance of Firmicutes and Actinobacteriota and negatively with that of Bacteroidota. Plus, the % of propionate correlated positively with α -diversity (Chao1, Shannon and InvSimpson Indexes) and with the relative abundance of Bacteroidota but negatively with the relative abundances of Firmicutes and Actinobacteriota. Finally, the % of butyrate was positively correlated with the relative abundance of Firmicutes, but negatively with the relative

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abundance of Bacteroidota and with α -diversity (Chao1, Shannon and InvSimpson indexes).

Next, we wondered if there could be more specific correlations in individual groups, as each group presents a distinct microbiota and SCFA profile (Table 7).

	SCFAs	Microbiota	N	Correlation coefficient	p-value
S-A	% Acetate (C2)	Chao1	9	0,73	0,02
	% Propionate (C3)	Chao1	9	-0,82	0,007
		Shannon	9	-0,77	0,02
A	% Branched and long chain	Proteobacteria	13	0,82	0,001
		Desulfobacterota	13	0,77	0,002
	% Butyrate (C4)	Actinobacteriota	13	-0,77	0,002
S-AG	Total SCFAs (μ mol/g)	Desulfobacterota	12	-0,63	0,03
AG	% Propionate (C3)	Shannon	11	0,81	0,003
	% Branched and long chain	Chao1	11	0,74	0,01

Table 7: Correlation table of cecal SCFAs and microbiota alpha-diversity and phyla relative abundances in BALB/c mice separated by groups. Green: positive correlation Red: negative correlation

When separating analysis by group, we found significant correlations with much higher coefficients, which is expected when n is reduced [255]. None of the correlations in a specific group were the same as the ones observed when all mice were grouped (Table 6). In group S-A, the % of acetate was positively correlated with α -diversity (Chao1 index), while the % of propionate was negatively correlated with it (Chao1 and Shannon indexes). In group A, the % of branched and long chain fatty acids was positively correlated with relative abundance of Proteobacteria and Desulfobacterota, while the % of butyrate was negatively correlated with the relative abundance of Actinobacteriota. In the S-AG group, the total SCFAs concentration was negatively correlated with the relative abundance of Desulfobacterota. Finally, in the AG group, both the % of propionate and branched and long chain fatty acids were positively correlated with α -diversity (Chao1 or Shannon indexes).

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2.3.2 C57BL/6J

2.3.2.1 Microbiota composition (fecal and cecal)

2.3.2.1.1 Fecal microbiota

In fecal microbiota from C57BL/6J mice, α -diversity was increased in mice from group A compared to S-A, both in number of observed ASVs ($p=0.04$) and Chao1 index ($p=0.04$) and decreased in AG compared to S-AG according to the InvSimpson index (Fig 19 A-B). As was observed in BALB/c mice, PCoA showed that there was a distinct microbiota composition between S-A and A ($p=0.001$ $R^2=0.12$), and S-AG and AG ($p=0.001$ $R^2=0.46$) groups according to Unifrac distances with a significant, but negligible, cohort effect for S-AG and AG ($p=0.009$ $R^2=0.07$) (Fig 19 C-D). Bray Curtis, Jaccard and Weighted Unifrac all revealed significant group effects both for S-A vs A and S-AG vs AG sometimes accompanied by cohort effects, always negligible in comparison (data not shown).

Again, there were differences between groups in relative abundances of various bacterial taxa (overview in Fig 19 E-F, detailed in Fig 20). At the phylum level, the most representative phyla in each group were Firmicutes and Bacteroidota, and the relative abundance of Firmicutes was higher in both A ($p=0.03$) and AG ($p=0.0002$) groups compared to their respective sibling group (Figure 20 A), which can be explained by the increase in *Ruminococcaceae* (A vs S-A $p=0.02$; AG vs S-AG $p=0.001$) and *Butyricoccaceae* (A vs S-A $p=0.002$; AG vs S-AG $p=0.03$) in those groups (Fig 20 B). In the same phylum, there was also a decrease of relative abundance of *Oscillospiraceae* in AG compared to S-AG ($p=0.006$) (Fig 20 B). The proportion of Bacteroidota was decreased in the AG group, compared to S-AG ($p=0.009$) (Fig 20 C), which can be explained by the strong decrease in *Prevotellaceae* in AG ($p<0.0001$), despite the increase of the most prevalent family in this phylum, *Bacteroidaceae* ($p=0.003$) in this group (Fig 20 D). The relative abundance of *Bacteroidaceae* was also increased in A compared to S-A ($p=0.05$) (Fig 20 D). As in the BALB/c strain, there was also a decreased proportion of *Barnesiellaceae* in A compared to S-A ($p=0.02$) (Fig 20 D). Again, as in BALB/c mice, we observed an increased proportion of Actinobacteriota in A compared to S-A ($p=0.04$) (Fig 20 E) although, in the C57BL/6J strain, it seems to be driven by an increase in the proportion of *Atopobiaceae* ($p=0.002$) (Fig 20 F) in this group. In the AG group, the proportion of this family was decreased compared to S-AG ($p=0.0001$) (Fig 20 F). There were no differences between groups in the relative abundance

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of Proteobacteria (Fig 20 G), or of the families in this phylum (data not shown). Finally, there was an increased proportion of Desulfobacterota and, consequently, of the only present family from this phylum, *Desulfovibrionaceae* (for both $p=0.02$) in A group compared to S-A (Fig 20 H-I).

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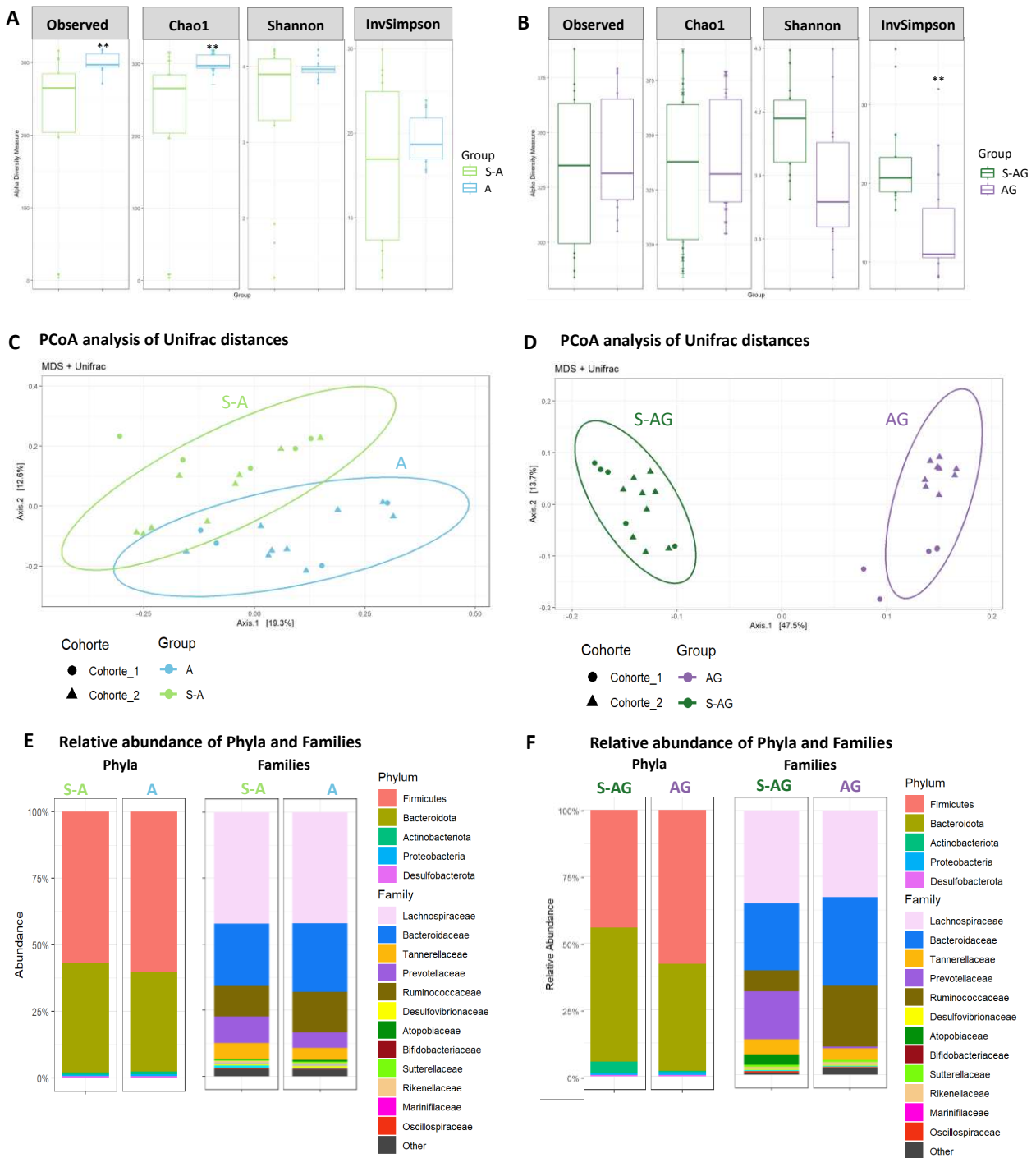
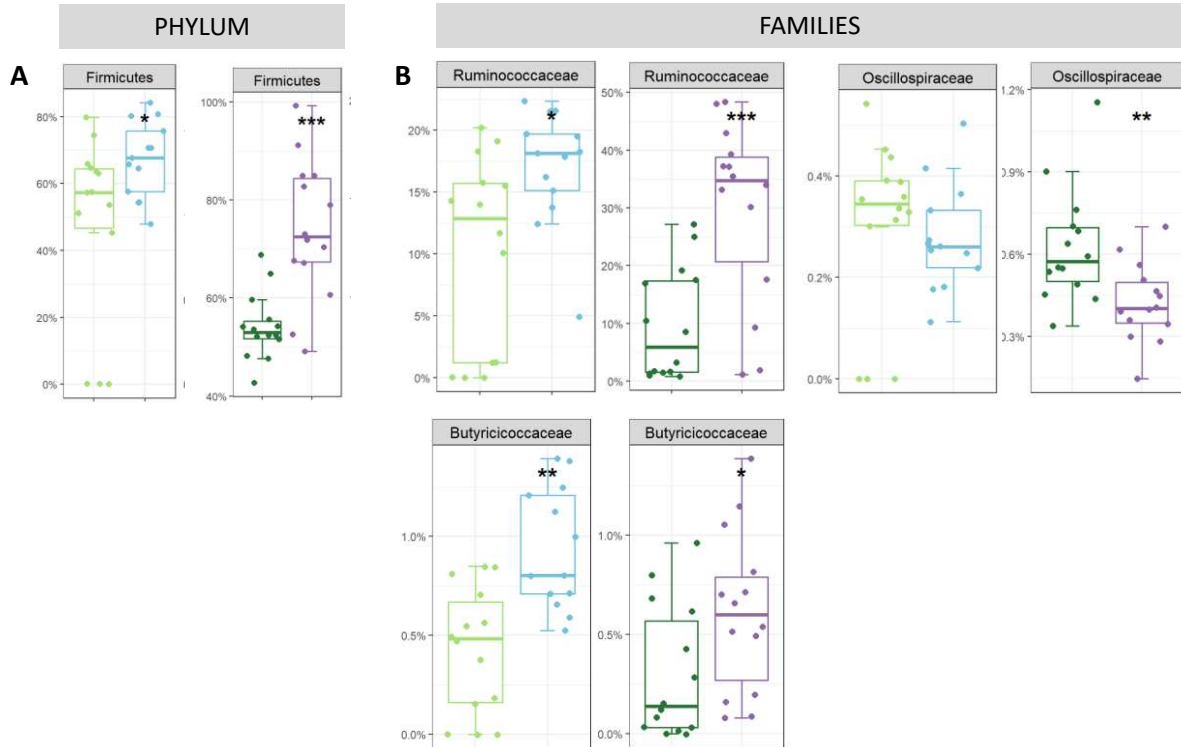


Figure 19 : Diversity and composition of fecal microbiota collected 6 weeks post FMT in C57BL/6J mice.

A-B Indexes of α -diversity. Compared with Mann-Whitney test $**p < 0.01$ **C-D** β -diversity (Unifrac distances) Adonis test. SA-vs A: Group effect $p = 0,001$ $R^2 = 0,12$; S-AG vs AG: Group effect $p = 0,001$ $R^2 = 0,46$ and cohort effect $p = 0,009$ $R^2 = 0,07$ **E-F** Overall view of relative abundance at phylum and family level

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FIRMICUTES



BACTEROIDOTA

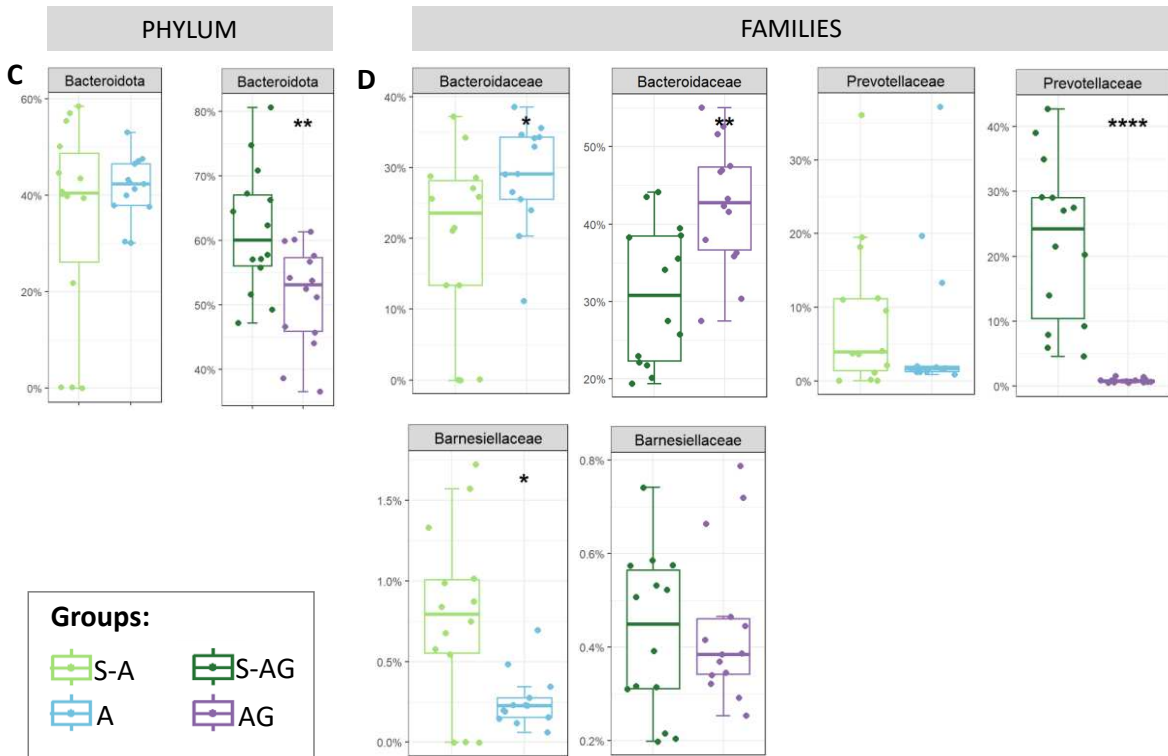


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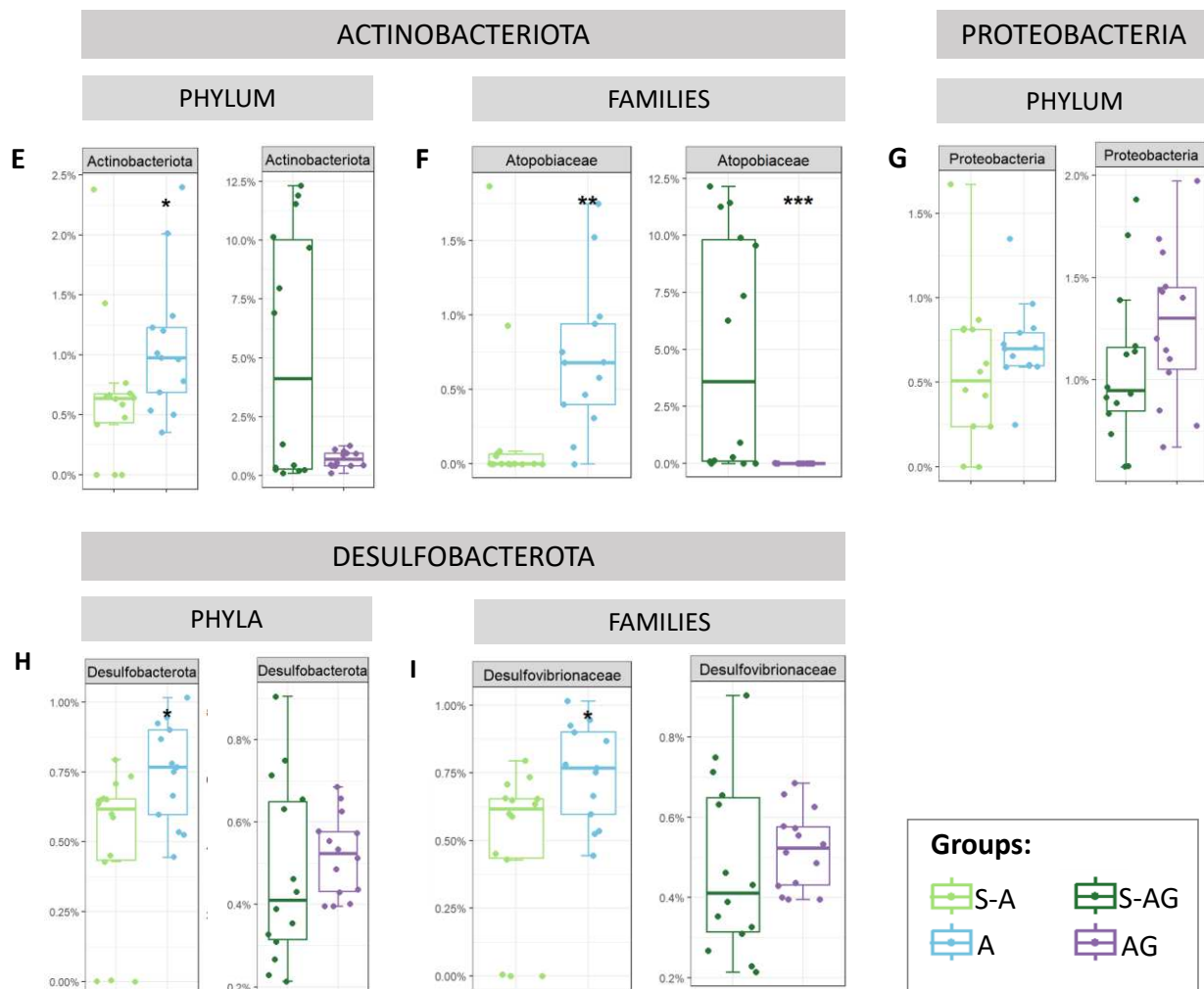


Figure 20 : Relative abundance of bacterial phyla and families in fecal microbiota collected 6 weeks post FMT in C57BL/6J mice.

A,C,E,G,H) Relative abundance of each phylum (% of total abundance) B,D,F,I) Relative abundance of families separated by phylum (% of total abundance). Only the families with a significantly different relative abundance between S-A and A, or S-AG and AG groups were plotted. Groups were compared with Mann-Whitney test : * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

2.3.2.1.2 Cecal microbiota

In cecal microbiota of C57BL/6J mice, there was no difference of α -diversity between groups (Fig 21 A-B). However, both A and AG groups clustered apart from their respective sibling groups in PCoA analysis according to Unifrac distances (S-A vs A: Group effect $p = 0.001$ $R^2 = 0.15$ Cohort effect $p = 0.01$ $R^2 = 0.08$; S-AG vs AG: Group effect $p = 0.001$ $R^2 = 0.15$), showing a distinct composition between them.

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Bray Curtis and Jaccard distances revealed significant group effects both for S-A vs A and S-AG vs AG (Fig 21 C-D), but Weighted Unifrac distance revealed a significant group effect only between S-AG and AG (data not shown). There were sometimes significant cohort effects in but, compared to the group effect, they were always negligible (data not shown).

The overall composition at phylum or family level was similar between cecal and fecal microbiota (Fig 19 E-F and Fig 21 E-F). There was an increase in the relative abundance of Firmicutes in the AG group compared to S-AG ($p=0.003$) (Fig 22 A), likely due to the increase in *Ruminococcaceae* ($p=0.0005$) and *Lactobacillaceae* ($p=0.005$), whereas relative abundance of *Lachnospiraceae* was decreased in this group ($p=0.02$) (Fig 22 B). The relative abundance of Bacteroidota, was decreased in the AG group compared to S-AG ($p=0.02$) (Fig 22 C), which can be explained by the strong decrease in relative abundance of *Prevotellaceae* ($p<0.0001$) (Fig 22 D). In addition, although this was not reflected by differences at the phylum level (Fig 22 C), the relative abundances of *Tannerellaceae* ($p=0.01$) and *Barnesiellaceae* ($p=0.03$) were decreased in the A group compared to S-A (Fig 22 D). There were no differences in relative abundance of Actinobacteriota between groups (Fig 22 E) but, in this phylum, proportions of *Atopobiaceae* ($p=0.04$) and *Eggerthellaceae* ($p=0.009$) were decreased in the A group compared to S-A, while the latter was increased in AG compared to S-AG ($p=0.02$) (Fig 22 F). The relative abundance of Proteobacteria was lower in the AG group compared to S-AG ($p=0.002$) (Fig 22 G) which can be explained by a decrease in *Sutterellaceae* ($p=0.002$) (Fig 21 H). This result is in opposition to the observation of increased relative abundance of Proteobacteria in the AG group compared to S-AG in BALB/c mice. Finally, the proportion of Desulfobacterota and, consequently, of the only present family of this phylum, *Desulfovibrionaceae*, was increased in group A compared to S-A ($p=0.02$) and decreased in group AG compared to S-AG ($p=0.05$) (Fig 22 I-J).

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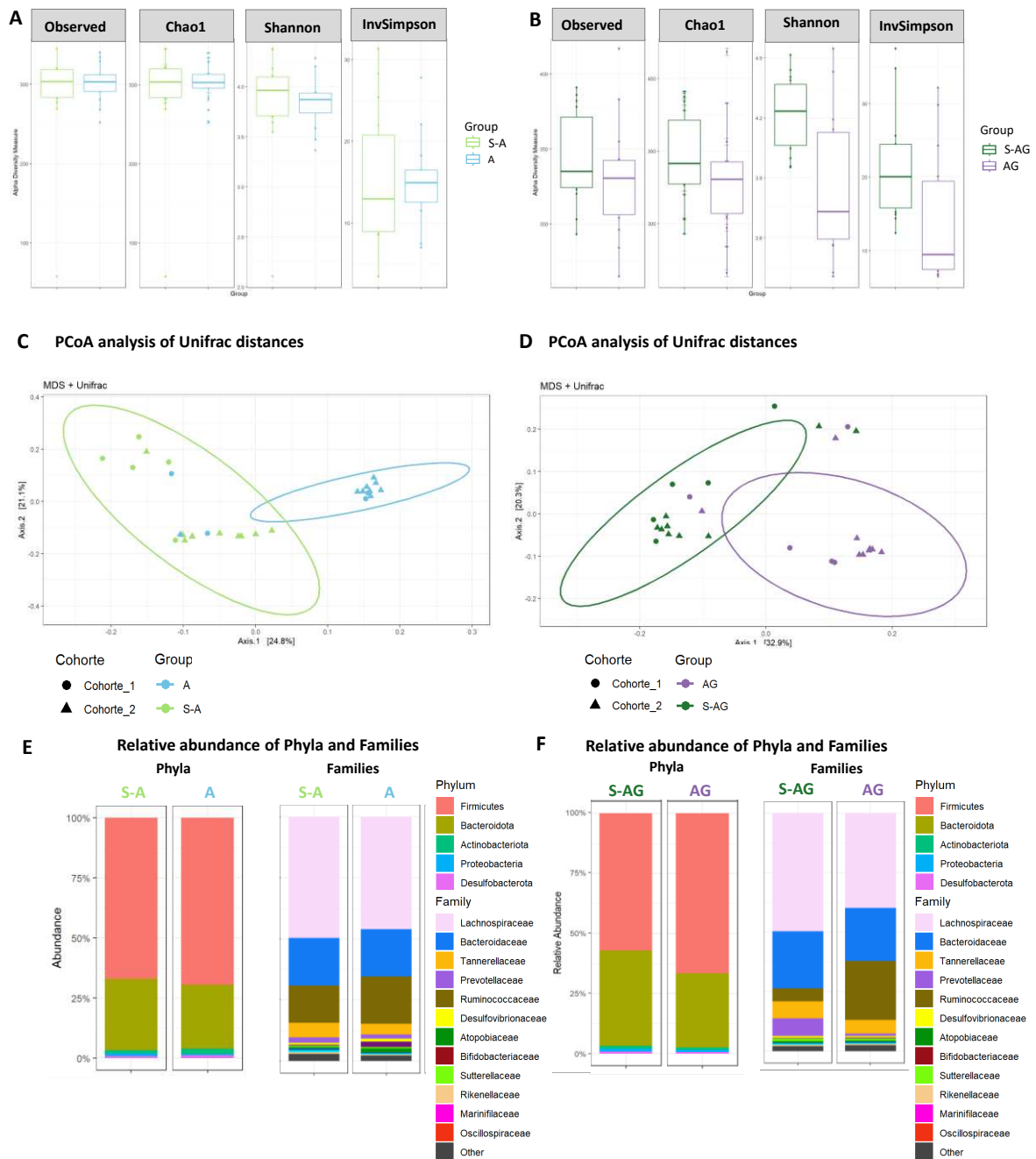


Figure 21 : Diversity and composition of cecal microbiota collected 9 weeks post FMT in C57BL/6J mice.

A-B) α -diversity indexes. Compared with Mann-Whitney test $**p < 0.01$ **C-D)** β -diversity (Unifrac distances) Adonis test. SA-vs A: Group effect $p = 0,001$ $R^2 = 0,15$ and cohort effect: $p = 0,01$ $R^2 = 0,08$; S-AG vs AG: Group effect $p = 0,001$ $R^2 = 0,19$; **E-F)** Overall view of relative abundance at phylum and family level

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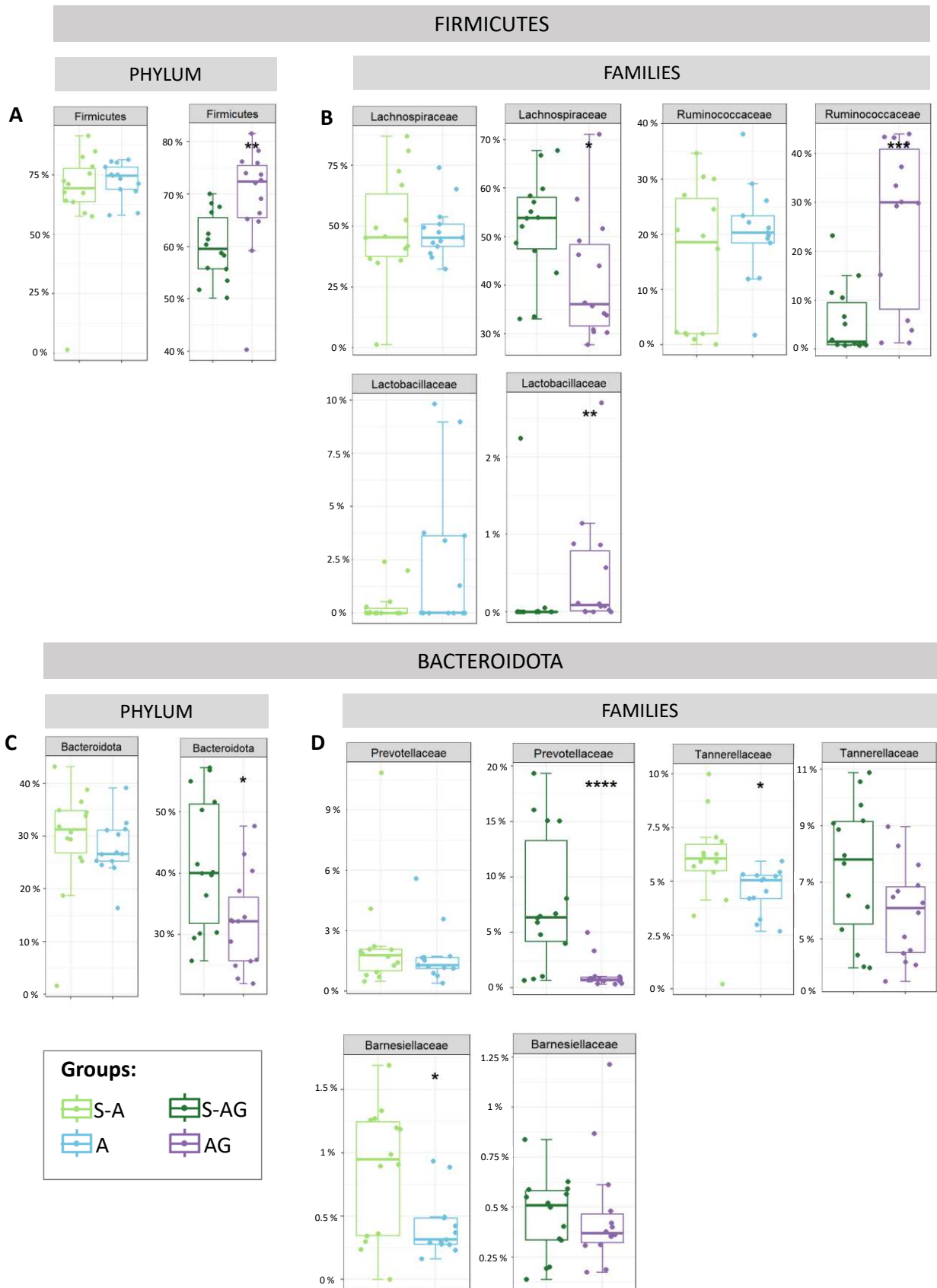


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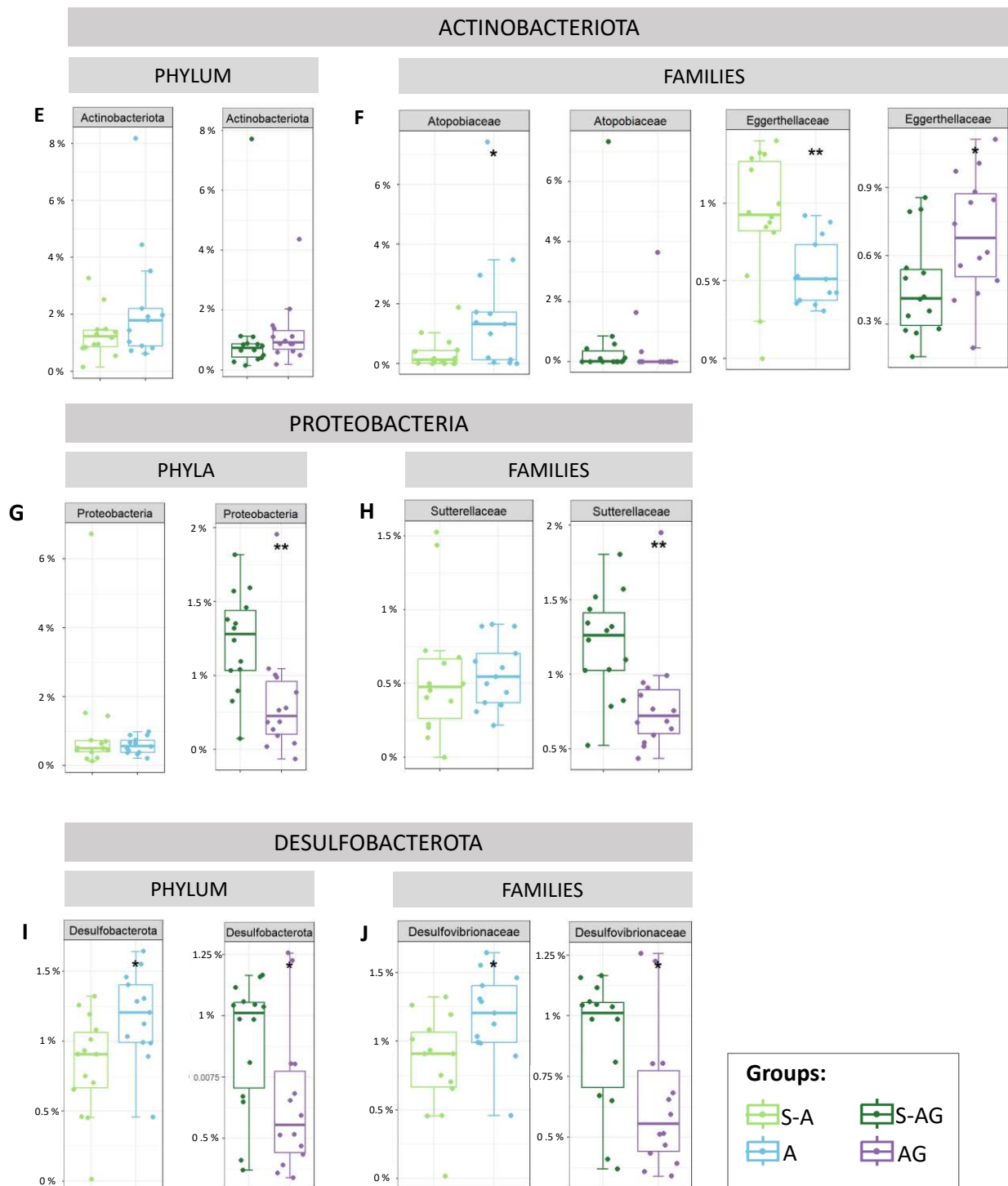


Figure 22 : Relative abundance of bacterial phyla and families in cecal microbiota collected 9 weeks post FMT in C57BL/6J mice.

A,C,E,G,I) Relative abundance of each phylum (% of total abundance) **B,D,F,H,J)** Relative abundance of families separated by phylum (% of total abundance). Only the families with a significantly different relative abundance between S-A and A, or S-AG and AG groups were plotted. Groups were compared with Mann-Whitney test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

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2.3.2.2 Fermentation activity of the microbiota: SCFAs profile in the caecum

Cecal total SCFAs concentrations and acetate proportions were not significantly different between groups (Fig 23 A and Fig 23 B).

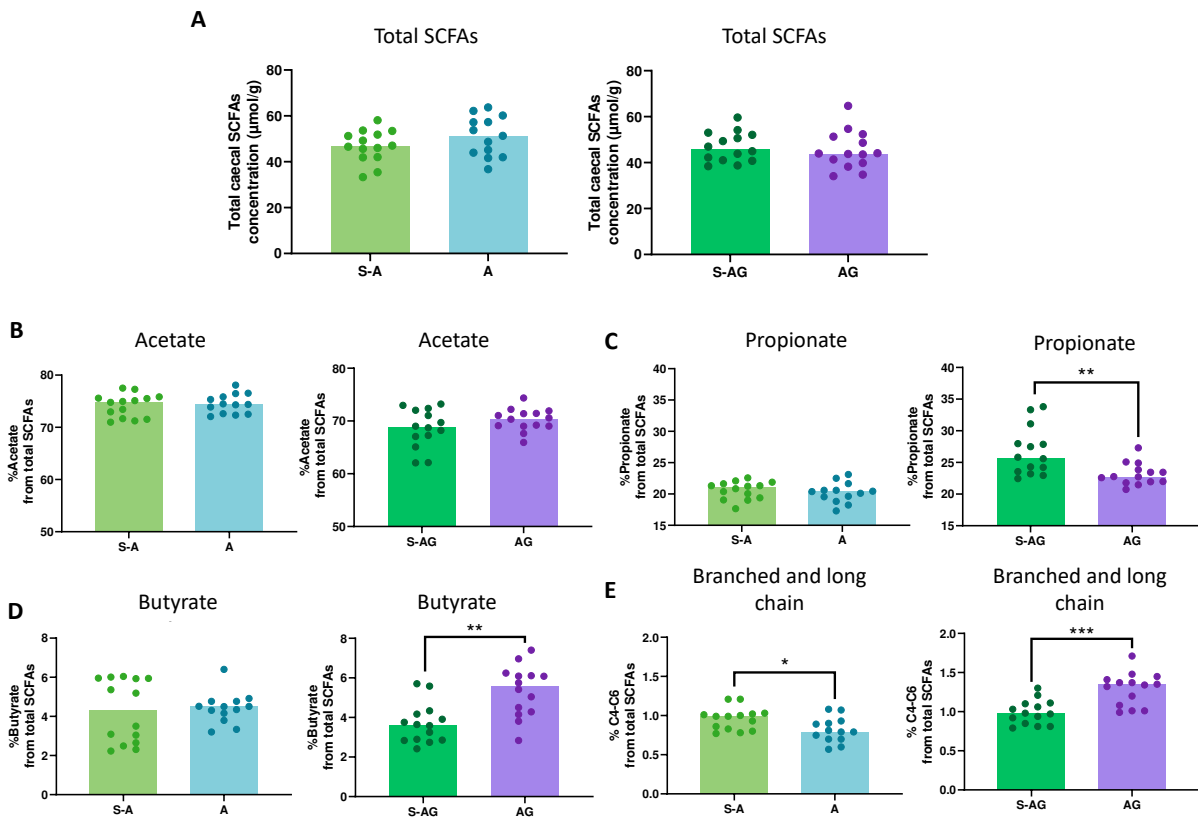


Figure 23: SCFA analysis in cecal content collected at sacrifice (9 weeks post FMT) in C57BL/6J mice.

A) Total cecal concentration of SCFAs **B-E)** Relative proportion of acetate, propionate (and long and branched chain fatty acids).

Groups were compared with Mann-Whitney test: **p<0.01

In the A group compared to S-A, the only difference was a slight decrease in the proportion of branched and long chain fatty acids ($p=0.03$) (Fig 23 E). There were more differences in the AG group compared to S-AG: the proportion of propionate was decreased ($p=0.02$) and proportions of butyrate ($p=0.001$) and branched and long chain fatty acids ($p=0.0003$) were increased (23 C D E).

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2.3.2.3 Spearman correlation between cecal microbiota composition and SCFAs

We found significant correlations between microbiota diversity and composition and SCFAs (Table 8-9).

SCFAs	Microbiota	N	Correlation coefficient	p-value
Total SCFAs ($\mu\text{mol/g}$)	Desulfobacterota	54	0,29	0,04
% Acetate (C2)	Firmicutes	54	0,30	0,03
	Actinobacteriota	54	0,34	0,01
% Acetate (C2)	Chao1	54	-0,39	0,003
	Proteobacteria	54	-0,39	0,004
% Propionate (C3)	Shannon	54	0,28	0,04
	Chao1	54	0,43	0,001
	Bacteroidota	54	0,48	0,0003
	Proteobacteria	54	0,57	0,00001
% Propionate (C3)	Firmicutes	54	-0,44	0,001
	Actinobacteriota	54	-0,47	0,0003
% Butyrate (C4)	Proteobacteria	54	-0,32	0,02
% Branched and long chain	Desulfobacterota	54	-0,37	0,01

Table 8 : Correlation table of cecal SCFAs and microbiota α -diversity and phyla relative abundances in C57BL/6J mice when all mice were grouped together. Green: positive correlation, Red: negative correlation.

When all the mice were grouped (Table 8), the total SCFAs concentration correlated positively with the relative abundance of Desulfobacterota while this phylum was negatively correlated with the % of branched and long chain fatty acids. The % of acetate was negatively correlated with α -diversity (Chao1 index) and positively with the relative abundance of Firmicutes and Actinobacteriota. In parallel, the % of propionate was correlated positively with α -diversity (Chao1 and Shannon indexes) and the relative abundances of Bacteroidota and Proteobacteria and negatively with the relative abundance of Firmicutes and Actinobacteria. Finally, the % of acetate and butyrate were both negatively correlated with the relative abundance of Proteobacteria (Table 8).

There were also a few correlations in specific groups (Table 9).

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	SCFAs	Microbiota	N	Correlation coefficient	p-value
S-A	Total SCFAs (μmol/g)	Chao1	13	0,75	0,003
A	% Butyrate (C4)	Desulfobacterota	13	0,56	0,05
S-AG	% Butyrate (C4)	Proteobacteria	14	-0,71	0,005

Table 9 : Correlation table of cecal SCFAs and microbiota α -diversity and phyla relative abundances in C57BL/6J mice separated by groups. Green: positive correlation Red: negative correlation

In S-A, the total SCFAs concentration was positively correlated with α -diversity (Chao1). In S-AG, % of butyrate was negatively correlated with the relative abundance of Proteobacteria (similarly to that observed when all groups were merged), while it was positively correlated with the relative abundance of Desulfobacterota in the A group.

2.3.3 Conclusion: Microbiota differences between groups in each strain

The composition and fermentation activity of the microbiota of mice transplanted with pooled microbiota from each donor group was distinct between groups. The differences observed between S-A and A groups are not necessarily the same as the ones observed between S-AG and AG groups. Plus, differences are quite heterogeneous depending on the type of sample (feces or cecal content) or the mouse strain. Firstly, α -diversity of fecal microbiota was increased in group AG compared to S-AG in BALB/c mice. However, in C57BL/6J mice it was increased in group A compared to S-A and decreased in group AG compared to S-AG (according to different indexes).

The following tables summarize the differences in relative abundances of phyla and families between groups in fecal or cecal microbiota in the two strains.

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		BALB/c	C57BL/6J
Fecal microbiota (6 weeks post FMT)	In A (compared to S-A)	↑ Actinobacteriota	↑ Firmicutes ↑ Actinobacteriota ↑ Desulfobacterota
	In AG (compared to S-AG)	↑ Proteobacteria	↑ Firmicutes ↓ Bacteroidota
Cecal microbiota (9 weeks post FMT)	In A (compared to S-A)	↑ Actinobacteriota	↑ Desulfobacterota
	In AG (compared to S-AG)	No difference	↑ Firmicutes ↓ Bacteroidota ↓ Proteobacteria ↓ Desulfobacterota

Table 10 : Summary table of microbiota differences at Phylum level

Bacterial phyla (sorted by relative abundance) with significantly different relative abundances between groups in feces collected 6 weeks after FMT and in cecal content collected 9 weeks after FMT ($p < 0.05$). Phyla in bold characters show the same variation in relative abundance between groups in fecal and cecal microbiota.

At the phylum level, the only common alteration between mouse strains is the increase in fecal Actinobacteriota in A group compared to S-A. Interestingly, in C57BL/6J mice, an increase in Firmicutes and decrease in Bacteroidota were observed in AG compared to S-AG, both in the fecal and cecal microbiota. Plus, the relative abundance of Desulfobacterota was also increased in A compared to S-A both in fecal and cecal microbiota (Table 10).

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		BALB/c	C57BL/6J
Fecal microbiota (6 weeks post FMT)	In A (compared to S-A)	↓ <i>Lachnospiraceae</i> ↑ <i>Ruminococcaceae</i> ↑ <i>Bifidobacteriaceae</i> ↓ <i>Rikenellaceae</i> ↑ <i>Marinifilaceae</i> ↓ <i>Barnesiellaceae</i>	↑ <i>Bacteroidaceae</i> ↑ <i>Ruminococcaceae</i> ↑ <i>Atopobiaceae</i> ↑ <i>Desulfovibrionaceae</i> ↓ <i>Barnesiellaceae</i> ↑ <i>Butyricoccaceae</i>
	In AG (compared to S-AG)	↑ <i>Bacteroidaceae</i> ↓ <i>Prevotellaceae</i> ↓ <i>Atopobiaceae</i> ↑ <i>Marinifilaceae</i> ↑ <i>Sutterellaceae</i>	↑ <i>Bacteroidaceae</i> ↑ <i>Ruminococcaceae</i> ↓ <i>Prevotellaceae</i> ↓ <i>Atopobiaceae</i> ↑ <i>Butyricoccaceae</i> ↓ <i>Oscillosporaceae</i>
Cecal microbiota (9 weeks post FMT)	In A (compared to S-A)	↓ <i>Lachnospiraceae</i> ↑ <i>Ruminococcaceae</i> ↑ <i>Bifidobacteriaceae</i> ↓ <i>Tannerellaceae</i>	↓ <i>Tannerellaceae</i> ↑ <i>Atopobiaceae</i> ↑ <i>Desulfovibrionaceae</i> ↓ <i>Eggerthellaceae</i>
	In AG (compared to S-AG)	↓ <i>Prevotellaceae</i> ↓ <i>Atopobiaceae</i> ↑ <i>Marinifilaceae</i> ↓ <i>Oscillosporaceae</i>	↓ <i>Lachnospiraceae</i> ↓ <i>Prevotellaceae</i> ↑ <i>Ruminococcaceae</i> ↓ <i>Sutterellaceae</i> ↑ <i>Lactobacillaceae</i> ↓ <i>Desulfovibrionaceae</i> ↑ <i>Eggerthellaceae</i>

Table 11 : Summary table of microbiota differences at family level

Bacterial families (sorted by relative abundance) with significantly different relative abundances between groups in feces collected 6 weeks after FMT and in cecal content collected 9 weeks after FMT ($p < 0.05$). Phyla in bold characters show the same variation in relative abundance between groups in fecal and cecal microbiota.

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In each mouse strain, some families were modulated in the same way in both A and AG groups compared to respective siblings. Indeed, in BALB/c mice, relative abundance of *Marinifilaceae* was increased in both groups. In the same way, in C57BL/6J mice, the relative abundances of both *Bacteroidaceae* and *Butyricoccaceae* were increased in both groups (Table 11).

In addition, some modulations were found both in cecal and fecal microbiota. In BALB/c mice, we observed in both types of samples an increase of *Bifidobacteriaceae* and a decrease of *Lachnospiraceae* in the A group compared to S-A, while the proportion of *Atopobiaceae* was decreased in AG compared to S-AG. In C57BL/6J mice there was an increase in *Atopobiaceae* and *Desulfovibrionaceae* in the A group compared to S-A present both in fecal and cecal microbiota.

Interestingly, in both mouse strains, a decrease of the relative abundance of *Prevotellaceae* was consistently observed in the fecal and cecal microbiota of the AG group, compared to the S-AG group. In fecal microbiota, an increased relative abundance of *Ruminococcaceae* in group A compared to S-A was also present in both strains.

	BALB/c	C57BL/6J
In A (compared to S-A)	↑Total SCFAs ↓%Propionate ↑%Butyrate ↓%Branched and long chain	↓%Branched and long chain
In AG (compared to S-AG)	↑%Acetate ↓%Propionate ↑%Butyrate ↑%Branched and long chain	↓%Propionate ↑%Butyrate ↑%Branched and long chain

Table 12 : SCFAs in cecal content 9 weeks after FMT with different proportions between groups in either strain. Common alterations between strains are in bold.

Several differences between groups in SCFAs profile in cecal microbiota were consistent in both mouse strains, namely, a decrease in branched and long chain fatty acids in A compared to S-A, and a decrease in propionate and an increase in butyrate and branched and long chain fatty acids in AG compared to S-AG. In BALB/c mice, the decrease in propionate and increase in butyrate were also present in A compared to S-A (Table 12).

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Finally, some of the correlations that we observed between SCFAs and microbiota composition or α -diversity when taking all mice together, regardless of the group, were common to BALB/c and C57BL/6J mice (Table 13).

SCFAs	Microbiota	BALB/c			C57BL/6J		
		N	Correlation coefficient	P-val	N	Correlation coefficient	P val
% Acetate (C2)	Firmicutes	45	0,34	0,02	54	0,30	0,03
	Actinobacteriota	45	0,32	0,03	54	0,34	0,01
% Acetate (C2)	Bacteroidota	45	-0,41	0,005	54	-0,33	0,01
% Propionate (C3)	Shannon	45	0,40	0,01	54	0,28	0,04
	Chao1	45	0,35	0,02	54	0,43	0,001
	Bacteroidota	45	0,55	0,0001	54	0,48	0,0003
% Propionate (C3)	Firmicutes	45	-0,50	0,0004	54	-0,44	0,001
	Actinobacteriota	45	-0,33	0,03	54	-0,47	0,0003

Table 13 : Common correlations in both strains between cecal SCFAs and microbiota composition and α -diversity. Green: positive correlation Red: negative correlation

2.4 RESULTS-SYSTEMIC AND INTESTINAL EFFECTS OF THE FMT

2.4.1 Effect of FMT on general parameters

At euthanasia, animals were weighed, as well as their cecal wall, spleen and adrenal glands. Decreased spleen weight or increased adrenal gland weight are considered markers of stress in mice [256–258]. Plus, animals were given an observational score of “constipation” depending on the number of fecal pellets in the colon during dissection, as well as their texture. There were no differences in any of those parameters for any strains (data not shown) except for an increased relative cecal wall weight in AG compared to S-AG in BALB/c mice ($p=0.04$) (Fig 24).

Part I- FMT from children with ASD on GF mice

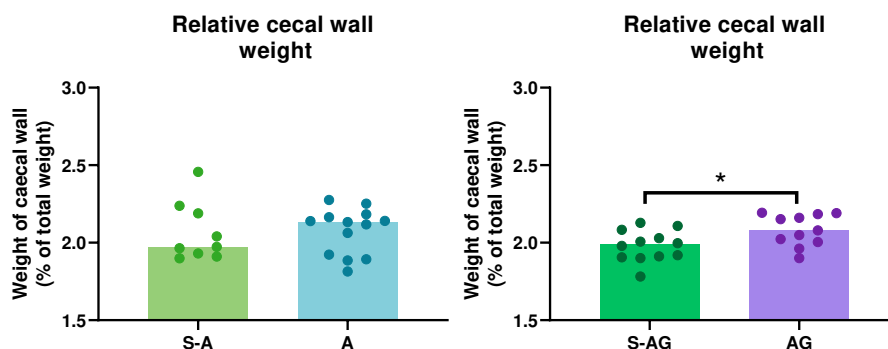


Figure 24: Relative cecal wall weight at euthanasia in BALB/c mice. Compared with Mann-Whitney test * $p < 0.05$

2.4.2 BALB/c

2.4.2.1 Effect of the FMT on general inflammation

2.4.2.1.1 T lymphocytes populations in the spleen

As mentioned in the introduction, differences in T lymphocytes populations, notably an elevated Th17 proportion or Th17/Treg ratio, has been found in individuals with ASD, and some ASD mice models [114, 158, 159, 171]. Plus, literature shows that the gut microbiota can influence T cell differentiation [154–157, 171]. Thus, we wondered if the transferred microbiota could have impacted those cell populations in the spleen of our animals. First, we analyzed the proportion of CD69+ cells among CD4+ cells in the spleen which is a marker of early immune activation [259], but found no difference between groups (Fig 25 A). We next measured the proportions of Th1, Th2, Th17 and Treg lymphocytes, to calculate Th1/Th2, Th17/Treg and Th1/Treg ratios, which are elevated in an inflammatory state and/or in individuals with ASD [147, 151]. None of those ratios were significantly different between groups (Fig 25 B-D), despite a trend to an increased proportion of Th1 lymphocytes in the A group compared to S-A ($p=0.05$) (Fig 25 E). Overall, we could not highlight a significant difference between groups in T lymphocytes populations in the spleen of our transplanted animals in the BALB/c strain.

Part I- FMT from children with ASD on GF mice

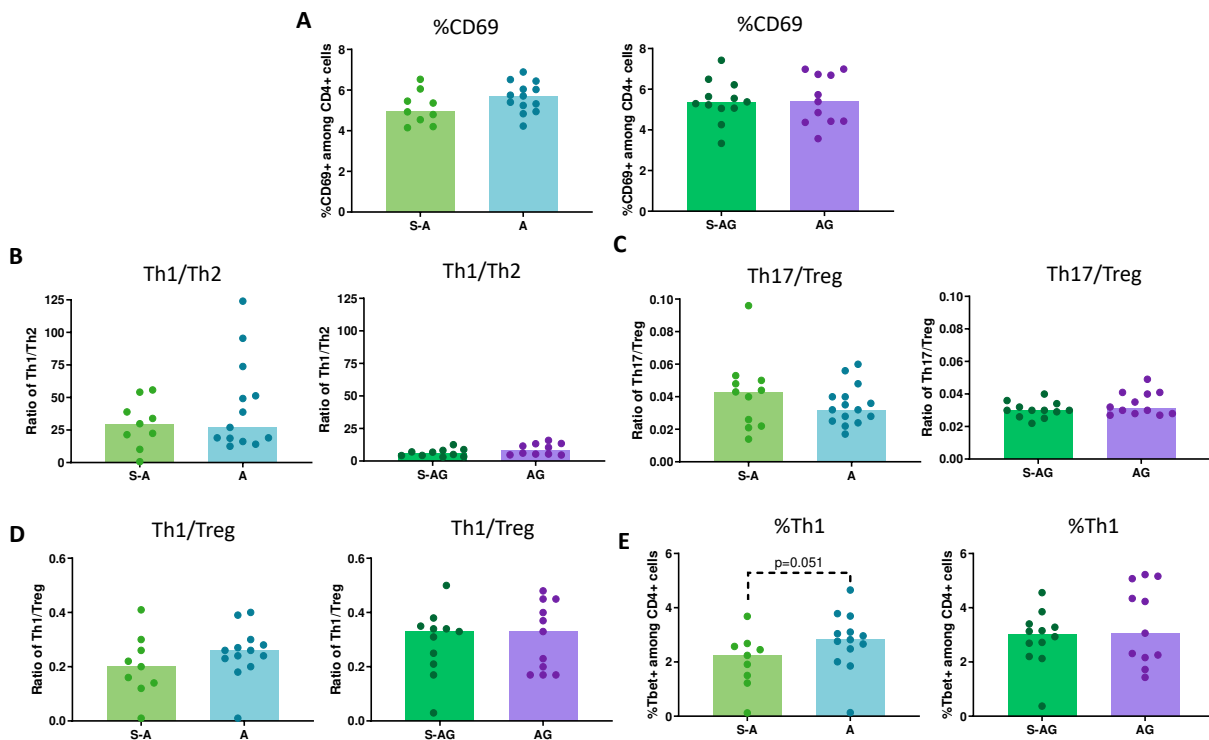


Figure 25 : Flow cytometry analysis of T cell populations in the spleen of BALB/c mice.

T cells populations were determined among CD4+ using surface and nuclear markers (Th1= %Tbet+; Th2= %Gata3+T1/ST2+; Th17: %FoxP3-RORγT+; Treg= %FoxP3+RORγT-) Compared with Mann-Whitney test. P-value of statistical trends are indicated on the graphs.

2.4.2.1.2 Seric KYN/Trp ratio

As detailed in the introduction, an elevated KYN/Trp ratio, which can indicate an inflammatory state, has been found in the serum of children with ASD [178]. Plus, the microbiota is involved in Trp metabolism regulation [260]. Thus, we wondered if this parameter could be modulated in our animals. However, no significant difference was found between groups for seric KYN/Trp ratio (Fig 26).

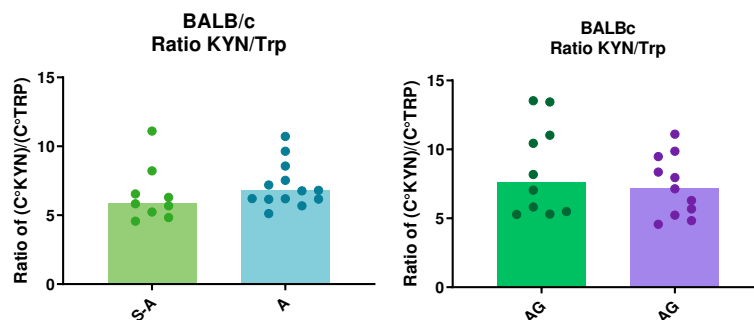


Figure 26 : Ratio of KYN/Trp in the serum of BALB/c mice
Compared with Mann-Whitney test.

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2.4.2.1.3 Spearman correlations between cecal microbiota and general inflammation

When taking all mice together for Spearman correlation there were a few significant correlations between general inflammation and cecal microbiota (Table 14).

General inflammation	Microbiota	N	Correlation coefficient	p-value
% CD69+ cells	Shannon	45	0,30	0,04
	InvSimpson	45	0,39	0,01
Th1/Th2	Firmicutes	45	0,34	0,02
Th1/Th2	Chao1	45	-0,34	0,02
	InvSimpson	45	-0,30	0,04
	Shannon	45	-0,33	0,03
	Bacteroidota	45	-0,32	0,03
Th17/Treg	Desulfobacterota	44	-0,35	0,02
Th1/Treg	Bacteroidota	44	0,32	0,03
	Proteobacteria	44	0,47	0,001
	Desulfobacterota	44	0,44	0,003
KYN/Trp ratio	Firmicutes	43	0,39	0,01
KYN/Trp ratio	Bacteroidota	43	-0,38	0,01
	Desulfobacterota	43	-0,45	0,002

Table 14: Correlation table of general inflammation markers and cecal microbiota in BALB/c mice when all mice were grouped together. Green: positive correlation, Red: negative correlation.

The % of CD69+ cells was positively correlated with α -diversity (Shannon and InvSimpson index). The Th1/Th2 ratio was positively correlated with the relative abundance of Firmicutes and negatively with α -diversity (Chao1, Shannon and InvSimpson indexes) and with the relative abundance of Bacteroidota. The Th17/Treg ratio was negatively correlated with the relative abundance of Desulfobacterota. Th1/Treg ratio was positively correlated with the relative abundances of Proteobacteria, Bacteroidota and Desulfobacterota. Finally, the ratio of KYN/Trp was positively correlated with the relative abundance of Firmicutes, but negatively with the relative abundance of Bacteroidota and Desulfobacterota.

When looking at the groups separately, we found some of the same correlations (Table 15).

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Group	General inflammation	Microbiota	N	Correlation coefficient	p-value
S-A	% CD69+ cells	Bacteroidota	9	-0,73	0,02
	Th1/Th2	InvSimpson	9	-0,92	0,001
		Shannon	9	-0,83	0,01
	Th1/Treg	InvSimpson	9	-0,83	0,01
		Shannon	9	-0,75	0,02
	KYN/Trp ratio	InvSimpson	9	0,87	0,002
Shannon		9	0,82	0,01	
A	% CD69+ cells	Firmicutes	13	-0,61	0,03
	Th1/Treg	Proteobacteria	13	0,91	0,000
AG	% CD69+ cells	Chao1	11	0,73	0,01
		Shannon	11	0,86	0,001
		InvSimpson	11	-0,72	0,01
		Shannon	11	-0,74	0,01
		Desulfobacterota	11	-0,72	0,01
	Th1/Treg	InvSimpson	11	0,75	0,01
		Shannon	11	0,82	0,002

Table 15 : Correlation table of general inflammation markers and cecal microbiota in BALB/c mice separated by groups. Green: positive correlation, Red: negative correlation

Indeed, in S-A and AG groups, Th1/Th2 ratio was negatively correlated with α -diversity (Shannon and InvSimpson indexes) like what was observed in the general analysis. Plus, in the AG group, like in the general analysis, the % of CD69+ cells was positively correlated with α -diversity, although not according to the same index. The analysis by group revealed other correlations between general inflammation and diversity: Th1/Treg was positively correlated with α -diversity (Shannon and InvSimpson indexes) in the AG group and KYN/Trp ratio was positively correlated with α -diversity (Shannon and InvSimpson indexes) in the S-A group. However, in this group, Th1/Treg was negatively correlated with α -diversity. In S-A the % of CD69+ cells correlated negatively with Bacteroidota, while in A group it was negatively correlated with the relative abundance of Firmicutes. In group A, the Th1/Treg ratio was positively correlated with the relative abundance of Proteobacteria. Finally, in group AG the ratio of Th1/Th2 was negatively correlated with the relative abundance of Desulfobacterota (Table 15).

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2.4.2.2 Effect of the FMT on Intestinal markers

2.4.2.2.1 Gut Inflammation

While there were no apparent differences in general inflammation between groups, we next wondered if the different microbiota populations could have had an impact on inflammation more locally, in the gut. Indeed, elevated levels of cytokines have been found in the gut of individuals with ASD [261] and ASD mice models, in which they could be reduced by modulation of the microbiota through a probiotic treatment or FMT [109, 111, 113]. For this reason, we have measured levels of transcripts of pro-inflammatory cytokines, IL-6 and TNF- α , as well as the anti-inflammatory cytokine IL-10, in the ileum and colon of the animals. There were no significant differences in cytokines transcripts levels in the ileum (Fig 14 A, C, E, G). However, colonic levels of IL-6 ($p=0.004$), TNF- α ($p=0.007$), and IL-10 ($p=0.05$) transcripts were significantly reduced in the A group compared to S-A (Fig 14 B, D, H). We also assessed the levels of GFAP in the gut, but this data is currently being re-analyzed due to issues in the original analysis.

Overall, those results tend to suggest that the gut microbiota of the A group led to reduced inflammation compared to S-A, specifically in the colon.

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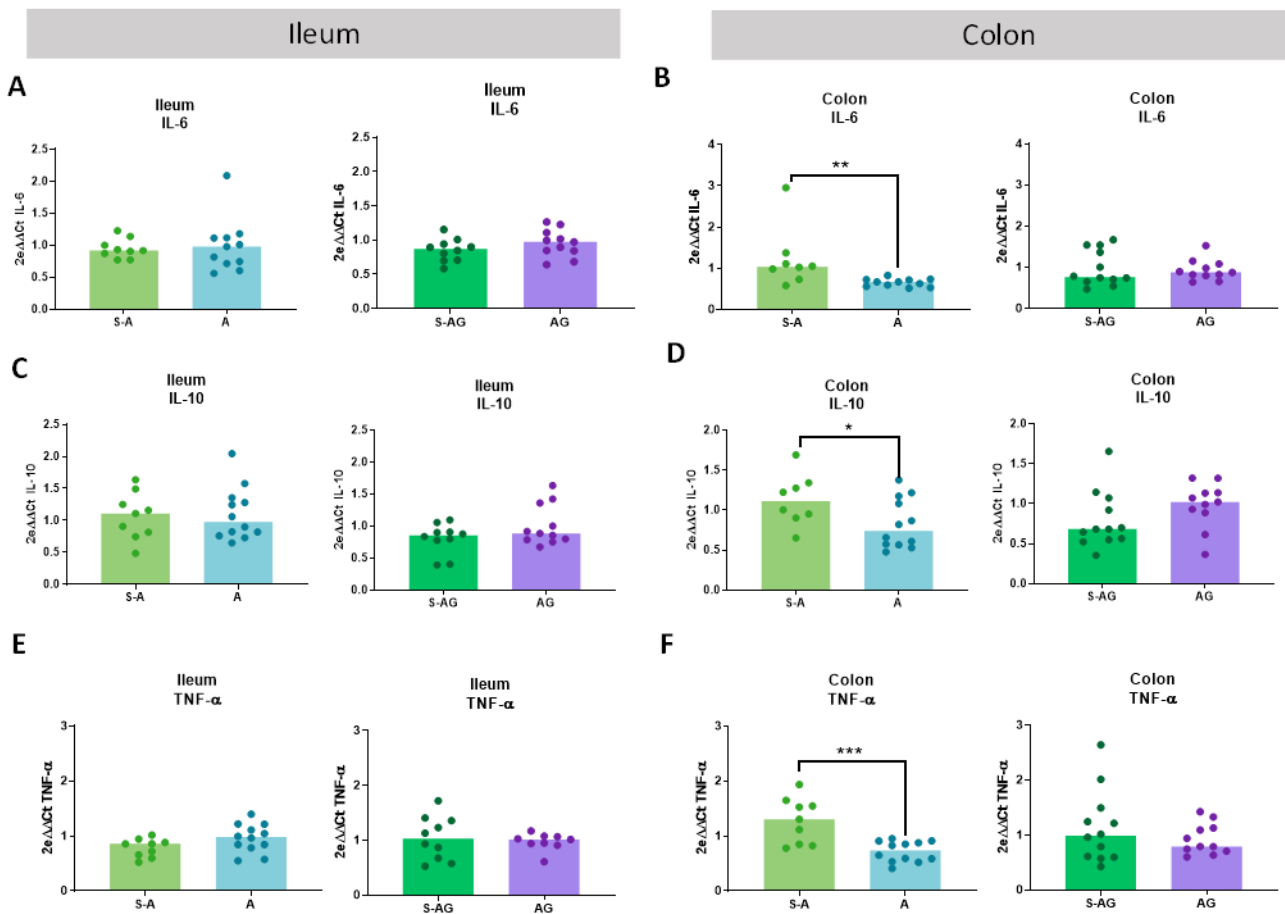


Figure 27 : Relative expression of cytokine genes in the gut of BALB/c mice

Relative expression as $2e\Delta\Delta CT$ of target gene – housekeeping gene (GAPDH). Compared with Mann-Whitney test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

2.4.2.2.2 Gut permeability

Increased gut permeability is present in many children with ASD [51, 52], as well as in ASD models [106–109]. To assess a potential impact of the transferred microbiota on gut permeability, we measured the levels of transcripts of genes coding for proteins involved in tight-junctions or their regulation (Claudin-2 (Cldn2), Occludin (Ocln1), Zona-occludens 1 or “ZO-1” (Tjp1), and Myosin light chain kinase (Mylk or MLCK)). Tight junctions are involved in paracellular permeability as they bind to cytoskeleton and form complex structures to regulate the passage of molecules between epithelial cells (Fig 28). Occludin and ZO-1 are necessary components of tight junctions thus their expression level is directly related to paracellular permeability. Proteins from the claudin family are also necessary

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components of tight junctions, but claudin-2 specifically is involved in the formation of pores that allow paracellular diffusion of small molecules. Plus, activated MLCK facilitates myosin binding to actin thus leading to reorganization of tight junctions to let bigger molecules pass through the epithelium in what is called the leak pathway [262]. Thus, increased claudin-2, and more importantly, increased MLCK expression could imply increased paracellular permeability (Fig 28). As another marker of permeability, we assessed the levels of e-cadherin in the ileum by immunohistochemistry. e-Cadherin is the main component of adherens junction and thus is involved in maintaining barrier function in the gut [263] (Fig 28). However, there were some technical difficulties with immunohistochemistry in BALB/c mice, which prevents us from including this data, as the analysis needs to be re-evaluated.

However, there were no differences between groups in the levels of transcript in any of those genes (Fig 29), thus, FMT from the different groups did not seem to impact gut permeability in BALB/c mice.

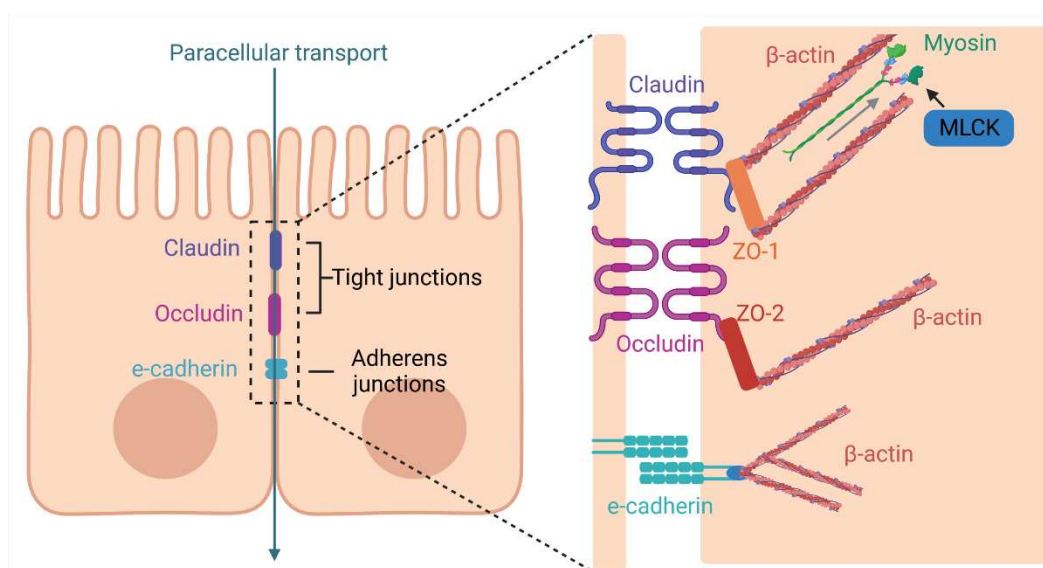


Figure 28 : Schematic representation of the ocludin and claudin in tight-junctions and e-cadherin in adherens junctions involved in paracellular permeability. Activated MLCK facilitates myosin binding to actin leading to tight junction structures rearrangement leading to increased paracellular permeability trough the leak pathway. Adherens junctions are tightly linked to the actin cytoskeleton and maintain cell to cell adhesion. Figure created on Biorender.com, inspired from Zhang et al. 2018 [262].

Part I- FMT from children with ASD on GF mice

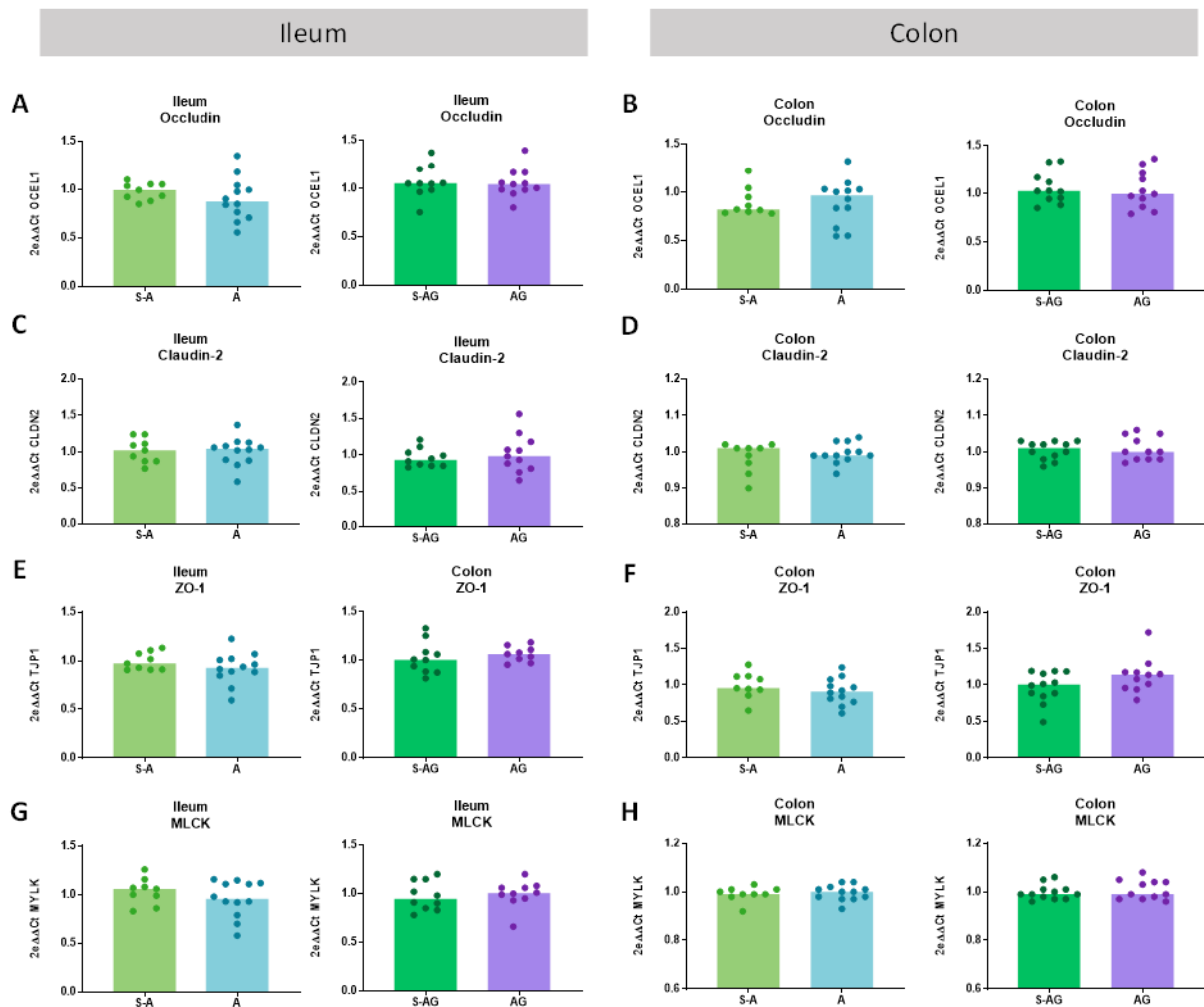


Figure 29 : Relative expression of tight-junction related genes in the gut of BALB/c mice
 Relative expression as $2e\Delta\Delta CT$ of target gene – housekeeping gene (GAPDH)
 Compared with Mann-Whitney test.

2.4.2.2.3 Gut serotonergic system

Alterations of the 5-HT metabolism in the gut has been described in individuals with ASD [183] and mice models of ASD [108, 109, 114, 124]. Plus, the gut microbiota can influence the release of 5-HT by enterochromaffin cells [195]. Thus, we decided to assess the level of TPH1 transcript in the gut, as it is the rate limiting enzyme for 5-HT production from Trp. The level of TPH1 transcript was not different between groups both in the colon and ileum of BALB/c mice (Fig 30).

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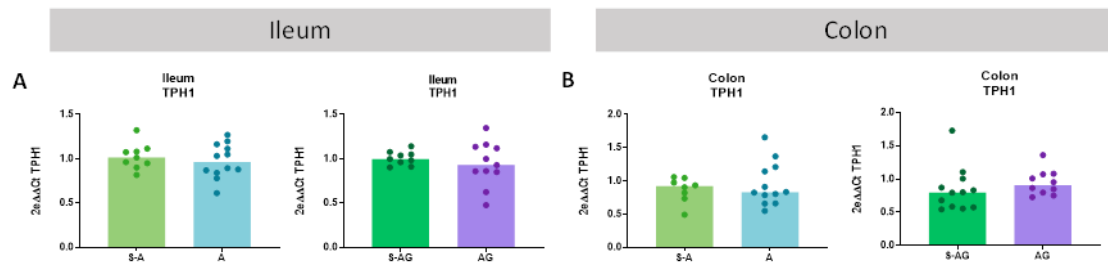


Figure 30 : Relative expression of TPH1 in the gut of BALB/c mice

A-B) Relative expression $2e\Delta\Delta CT$ of target gene – housekeeping gene (GAPDH) Compared with Mann-Whitney test.

Tissue from the ileum of those animals has been stained by immunohistochemistry to count the number of 5-HT positive cells, however, this data is also currently being re-analyzed.

From this data, we cannot conclude on an effect of the transferred microbiota from the different group on intestinal serotonin.

2.4.2.2.4 Spearman correlations between cecal microbiota and intestinal markers:

We next wondered if any of the intestinal markers were correlated with microbiota diversity or composition (Spearman's correlation) (Table 16-21)

Intestinal inflammation	Microbiota	N	Correlation coefficient	p-value
IL-6_colon	Shannon	44	0,34	0,02
	Chao1	44	0,30	0,05
IL-6_colon	Actinobacteriota	44	-0,37	0,01
IL-10_ileum	Shannon	42	-0,36	0,02
TNF- α _colon	Bacteroidota	44	0,30	0,05
TNF- α _ileum	Bacteroidota	42	0,37	0,02
TNF- α _ileum	Firmicutes	42	-0,42	0,01

Table 16 : Correlation table of intestinal inflammation markers and cecal microbiota in BALB/c mice when all mice were grouped together. Green: positive correlation, Red: negative correlation.

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Group	Intestinal inflammation	Microbiota	N	Correlation coefficient	p-value
S-AG	TNF- α _ileum	InvSimpson	10	0,76	0,01
AG	IL-6_ileum	Actinobacteriota	11	-0,66	0,03

Table 17 : Correlation table of intestinal inflammation markers and cecal microbiota in BALB/c mice separated by groups. Green: positive correlation, Red: negative correlation.

Firstly, for gut inflammation, when considering all the mice together (Table 16) we found a positive correlation between colonic expression of IL-6 and α -diversity (Chao1 and Shannon Indexes) while Shannon index was negatively correlated with the ileal expression of IL-10. Colonic expression of IL-6 was negatively correlated with the relative abundance of Actinobacteriota. Both ileal and colonic expression of TNF- α were positively correlated with relative abundance of Bacteroidota. Plus, ileal TNF- α expression correlated negatively with the relative abundance of Firmicutes. When separating the analysis by group, there was only a couple of significant correlations: Ileal TNF- α expression correlated positively with α -diversity (InvSimpson Index), and ileal IL-6 expression correlated negatively with the relative abundance of Actinobacteriota in S-AG and AG groups, respectively (Table 17).

Intestinal permeability	Microbiota	N	Correlation coefficient	p-value
Occludin_ileum	Chao1	42	0,41	0,01
	InvSimpson	42	0,39	0,01
	Shannon	42	0,37	0,01
Claudin-2_colon	Bacteroidota	44	0,41	0,01
Claudin-2_colon	Firmicutes	44	-0,37	0,01

Table 18 : Correlation table of permeability markers and cecal microbiota in BALB/c mice when all mice were grouped together. Green: positive correlation, Red: negative correlation.

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Group	Intestinal permeability	Microbiota	N	Correlation coefficient	p-value
S-A	Claudin-2_ileum	Actinobacteriota	9	-0,82	0,01
A	ZO-1_ileum	Shannon	12	0,62	0,03
		InvSimpson	12	0,63	0,03
	ZO-1_colon	Actinobacteriota	12	0,73	0,01
AG	ZO-1_colon	InvSimpson	11	0,76	0,01

Table 19 : Correlation table of permeability markers and cecal microbiota in BALB/c mice separated by group. Green: positive correlation, Red: negative correlation.

Then, for gut permeability, in the general analysis (Table 18), occludin expression in the ileum was positively correlated with α -diversity (Chao1, Shannon and InvSimpson Indexes). Colonic expression of claudin-2 was positively correlated with the relative abundance of Bacteroidota, and negatively with that of Firmicutes. When separating the analysis by group (Table 19), we found that, in the S-A group, claudin-2 expression was negatively correlated with the relative abundance of Actinobacteriota. The expression of ZO-1 in the ileum in group A and in the colon in group AG correlated positively with α -diversity (Shannon and InvSimpson indexes). In group A, ileal ZO-1 expression also correlated positively with the relative abundance of Actinobacteriota.

Intestinal serotonergic system	Microbiota	N	Correlation coefficient	p-value
TPH1_colon	Bacteroidota	44	-0,34	0,03
TPH1_colon	Firmicutes	44	0,34	0,02

Table 20: Correlation table of TPH1 expression and cecal microbiota in BALB/c mice when all mice were grouped together. Green: positive correlation, Red: negative correlation.

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Group	Intestinal serotonergic system	Microbiota	N	Correlation coefficient	p-value
A	TPH1_colon	Firmicutes	12	0,92	0,00003
	TPH1_colon	Bacteroidota	12	-0,86	0,0003
S-AG	TPH1_ileum	Bacteroidota	10	0,65	0,04
	TPH1_ileum	Firmicutes	10	-0,81	0,0049

Table 21: Correlation table of TPH1 expression and cecal microbiota in BALB/c mice separated by group. Green: positive correlation, Red: negative correlation.

Finally, we found that, when considering all BALB/c mice together, the colonic expression of TPH1 was positively correlated with the relative abundance of Firmicutes, and negatively with that of Bacteroidota (Table 20). The same correlations were observed in the A group specifically. Interestingly, in the S-AG group, the ileal expression of TPH1 showed an opposite correlation to those just described in group A and in the general analysis (Table 21).

2.4.3 C57BL/6J

2.4.3.1 Effect of FMT on general inflammation

2.4.3.1.1 T lymphocyte populations in the spleen

We assessed T lymphocytes populations in the spleen of C57BL/6J mice as well. The proportion of CD69+ cells among CD4+ cells was not significantly modulated in C57BL/6J, but there was a slight trend to a decreased proportion of those cells in the A group compared to S-A ($p=0.07$) (Fig 31 A). There was no significant difference between groups either in the ratio of Th1/Th2 lymphocytes, although there was a slight trend to a decrease of this ratio in AG mice compared to S-AG ($p=0.09$) (Fig 31 B). Plus, the proportion of Th1 lymphocytes was significantly decreased in this group compared to S-AG ($p=0.02$) (Fig 31 E). In addition, the ratio of Th17/Treg, was significantly decreased both in A ($p=0.006$) and AG ($p=0.0007$) groups compared to their respective sibling groups (Fig 31 C). Finally, the ratio of Th1/Treg, was slightly decreased in AG compared to S-AG ($p=0.05$) (Fig 31 H). Overall, this could highlight a reduced inflammatory state in the AG group, and to a lesser extent, in the A group, compared to respective sibling groups.

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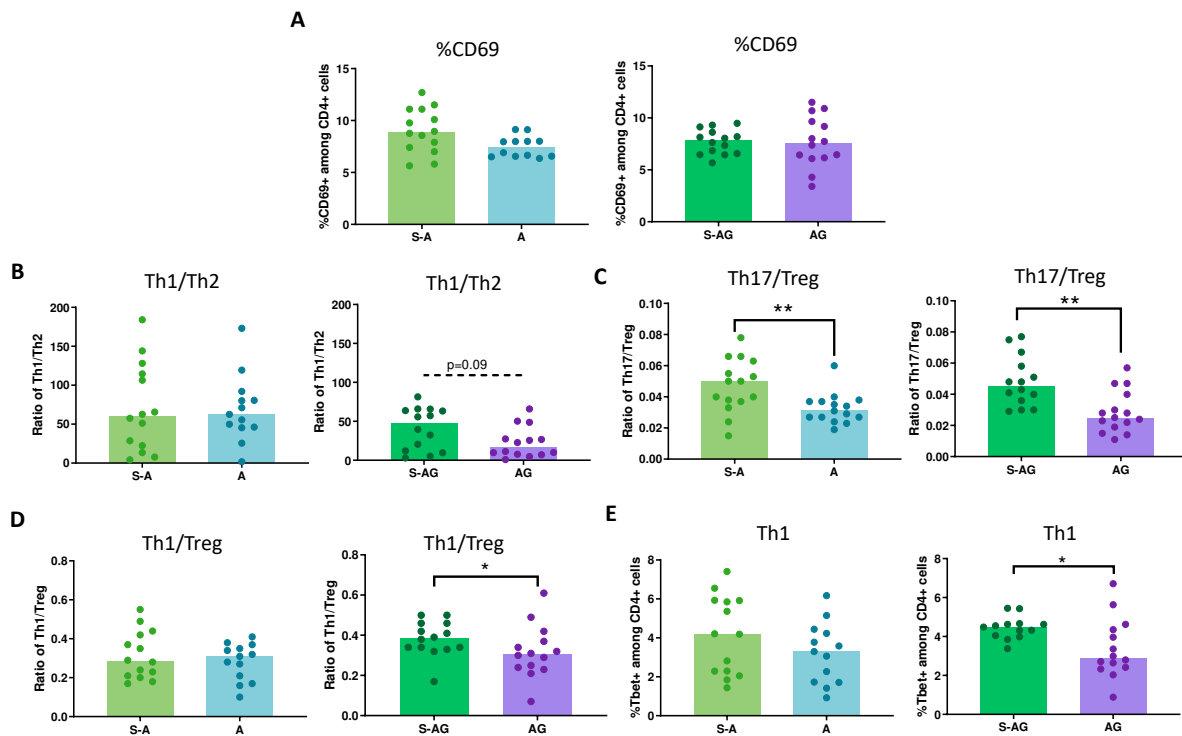


Figure 31: Flow cytometry analysis of T cell populations in the spleen of C57BL/6J mice
 T cells populations were determined among CD4+ using surface and nuclear markers (Th1= %Tbet+; Th2= %Gata3+T1/ST2+; Th17: %FoxP3-RORγT+; Treg= %FoxP3+RORγT-) Compared with Mann-Whitney test: *p<0.05; **p<0.01. P-value of statistical trends are indicated on the graphs.

2.4.3.1.2 Seric KYN/TRP ratio

As in BALB/c mice, the ratio of KYN/Trp in the serum was not different between groups in C57BL/6J mice although there was a trend to a decreased KYN/Trp ratio in group A compared to S-A (Fig 32).

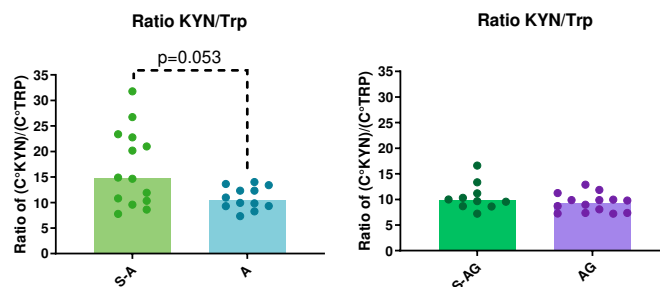


Figure 32: Ratio of KYN/Trp in the serum of C57BL/6J mice. Compared with Mann-Whitney test.

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2.4.3.1.3 Spearman correlations between cecal microbiota and general inflammation

We investigated Spearman's correlations between general inflammation and microbiota in all mice regardless of groups (Table 22).

General inflammation	Microbiota	N	Correlation coefficient	p-value
% CD69+	Shannon	54	0,28	0,04
	Actinobacteriota	54	0,45	0,001
Th1/Th2	Desulfobacterota	54	0,30	0,03
Th1/Treg	Shannon	54	0,28	0,04
	Proteobacteria	54	0,28	0,04
KYN/Trp ratio	Actinobacteriota	53	0,28	0,04
	Desulfobacterota	53	0,32	0,02
KYN/Trp ratio	Chao1	53	-0,34	0,01

Table 22: Correlation table of general inflammation markers and cecal microbiota in C57BL/6J mice when all mice were grouped together. Green: positive correlation, Red: negative correlation.

Both the % of CD69+ cells and the Th1/Treg ratio were positively correlated with α -diversity (Shannon Index), while Chao1 index was correlated negatively with KYN/Trp ratio. Both the % of CD69 cells and KYN/Trp ratio were positively correlated with the relative abundance of Actinobacteriota. Both Th1/Th2 and KYN/Trp ratios had a positive correlation with the relative abundance of Desulfobacterota. Finally, Th1/Treg ratio was positively correlated with the relative abundance of Proteobacteria.

Group	General inflammation	Microbiota	N	Correlation coefficient	p-value
S-A	KYN/Trp ratio	Actinobacteriota	13	0,81	0,001
S-AG	Th17/Treg	Shannon	14	-0,66	0,01

Table 23: Correlation table of general inflammation markers and cecal microbiota in C57BL/6J mice separated by group. Green: positive correlation, Red: negative correlation

When we analyzed each group separately, we found that KYN/Trp ratio correlated positively with the relative abundance of Actinobacteriota while Th17/Treg ratio correlated negatively with α -diversity (Shannon index) in S-A and S-AG groups, respectively (Table 23).

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2.4.3.2 Effect of FMT on Intestinal markers

2.4.3.2.1 Gut Inflammation

In the ileum of C57BL/6J mice, there were no differences between groups for the levels of IL-6 transcript (Fig 33 A). However, in the colon there was a slight trend for an increased in IL-6 transcript in group A compared to S-A (Fig 33 B). Levels of TNF- α transcript in the ileum were significantly reduced in both A ($p=0.04$) and AG groups ($p=0.04$) compared to their respective sibling groups (Fig 33 E-F). There was no difference between groups in the gene expression of IL-10 in either tissue (Fig 33 C-D).

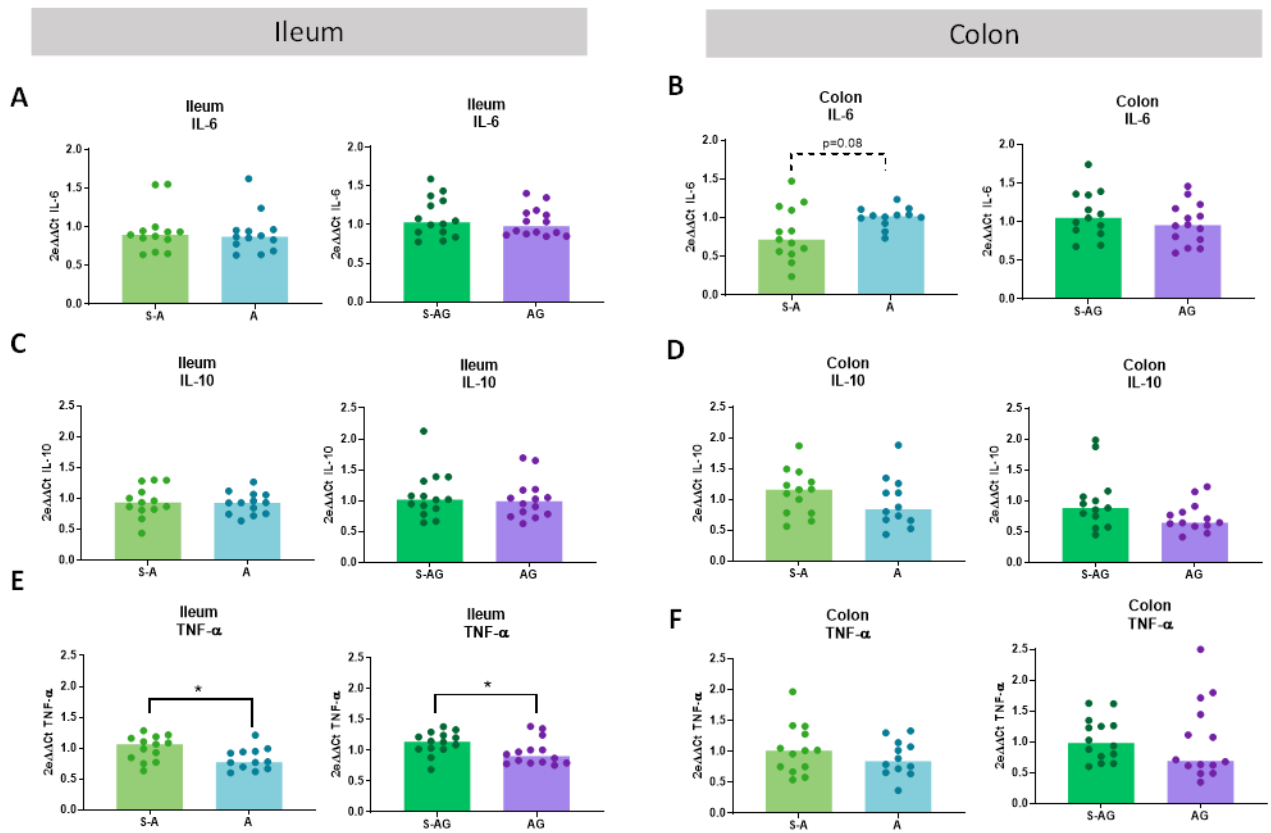


Figure 33: Relative expression of cytokine genes in the gut of C57BL/6J mice

A-F) Relative expression as 2e $\Delta\Delta$ CT of target gene – housekeeping gene (GAPDH)

Compared with Mann-Whitney test: * $p<0.05$. P-value of statistical trends are indicated on the graphs.

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In this strain, we assessed levels of GFAP in the gut by immunohistochemistry. This analysis revealed an increased quantity of GFAP, which has been found to be elevated in an inflammatory environment [264], in the ileum of AG mice compared to S-AG ($p=0.01$) (Fig 34 A-B). However, there was no difference between groups for GFAP in the colon (Fig 34 C-D).

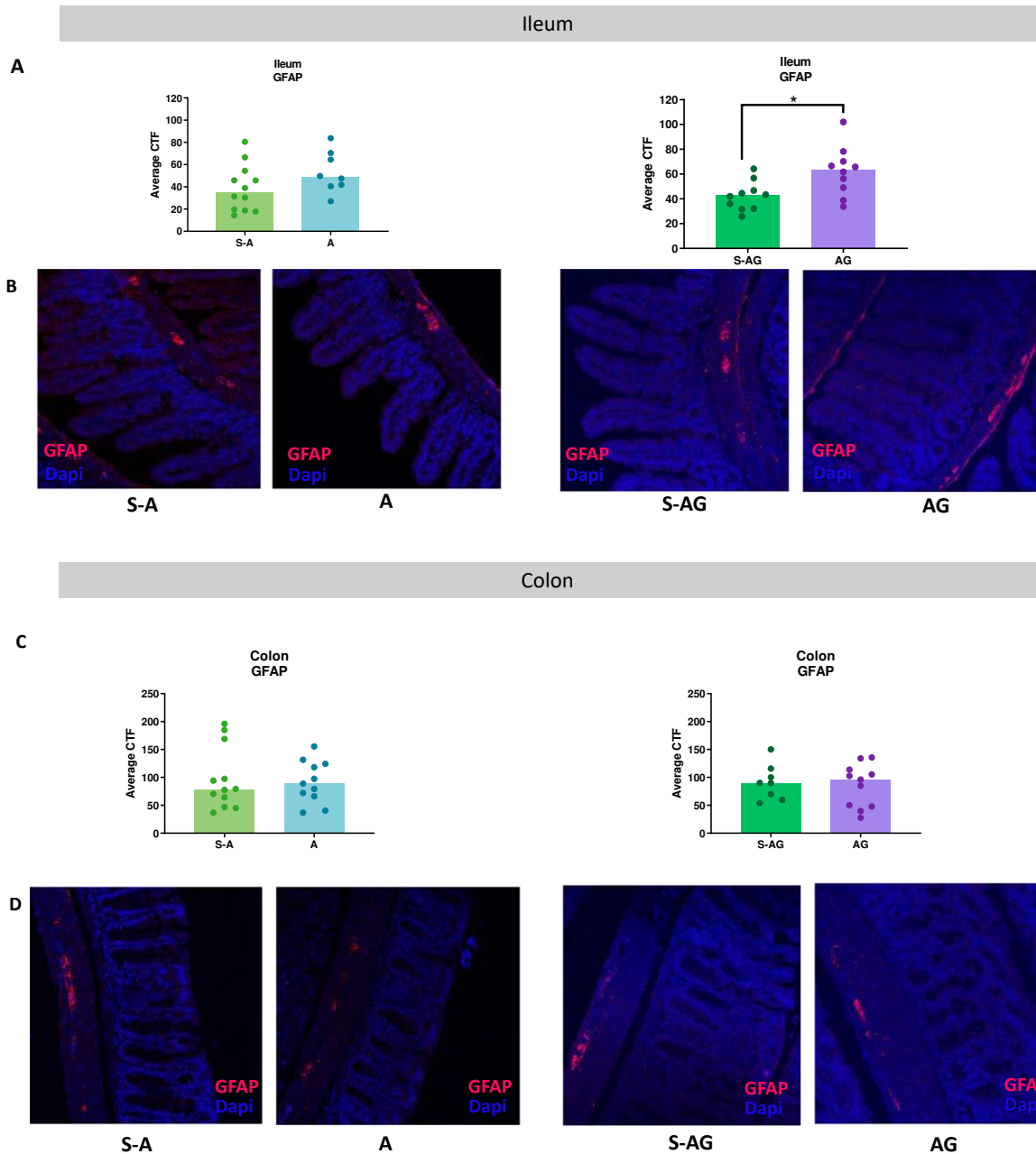


Figure 34 : Immunohistochemical quantification of GFAP in the gut of C57BL/6J mice
A-B) GFAP is expressed as average corrected total fluorescence (CTF)
C-D) Representative pictures (confocal microscope x200)
Compared with Mann-Whitney test: * $p < 0.05$

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This suggests that, in this strain, microbiota from group A and AG can either increase or reduce markers of gut inflammation compared to microbiota from the sibling groups, depending on the region and marker studied.

2.4.3.2.2 Gut permeability

Unlike in BALB/c mice, there were some significant differences between groups in the levels of transcripts from genes involved in tight junctions or their regulation in the gut. Like in BALB/c, there were no differences between groups in the levels of transcripts of occludin in ileum or colon (Fig 35 A, B). However, there was a trend to increased claudin-2 transcripts in the ileum of A ($p=0.07$) and AG ($p=0.08$) groups, compared to their respective sibling groups (Fig 35 C) but no difference in the colon (Fig 35 D). In addition, the level of transcripts of ZO-1 was significantly increased in the colon of group A compared to S-A ($p=0.02$) (Fig 35 F) while there was no difference between groups in the ileum (Fig 35 E). In AG compared to S-AG, there were increased levels of MLCK transcripts in the ileum ($p=0.006$) (Fig 35 G) and a trend to an increase in the colon ($p=0.07$) (Fig 35 H). Finally, immunohistochemistry analysis revealed no difference in the quantity of e-cadherin in the ileum or colon (Fig 36 A-D).

The increase of ZO-1 in the ileum of group A could suggest a better integrity of tight junctions in this group compared to S-A, thus, a decreased ileal permeability. The trend to an increase of claudin-2 expression in both A and AG group could suggest more pore formation, thus more passage of ions and small molecules. Meanwhile, the increase of MLCK expression in the ileum and trend to an increase in the colon of the AG group compared to S-AG, could reflect more MLCK activity, thus, disruption of tight-junctions increasing paracellular permeability [262].

2.4.3.2.3 Gut serotonin

In C57BL/6J mice, the level of TPH1 transcript was significantly increased in the colon of the A group compared to S-A ($p=0.004$) (Fig 37 A) but was not different between groups in the ileum (Fig 37 B). Immunohistochemistry analysis of 5-HT positive cells was completed in this strain but did not reveal any significant differences (Fig 37 C-F), although there was a trend to a decreased number of those cells in the A group compared to S-A ($p=0.07$) (Fig 37 C-D).

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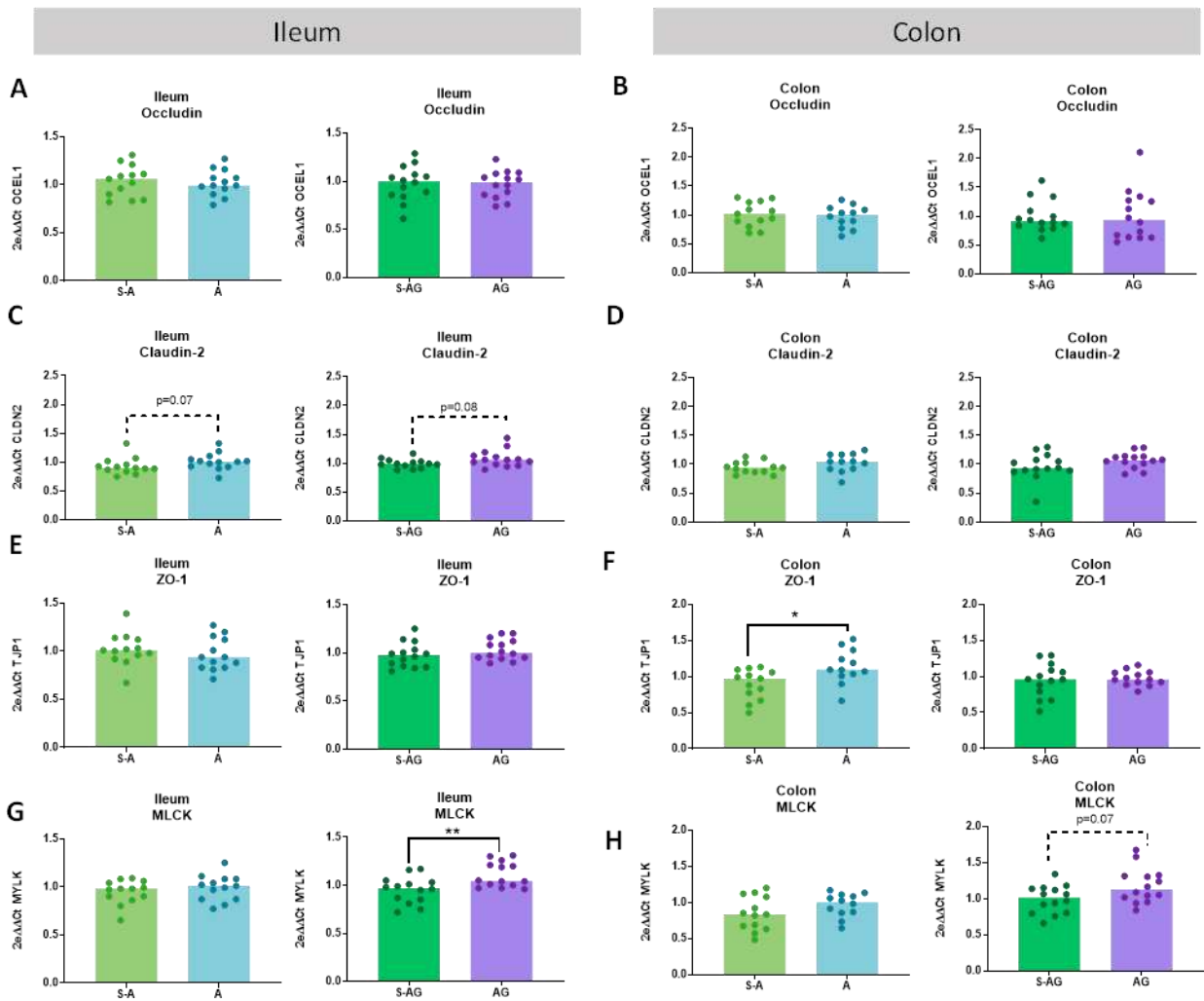


Figure 35 : Relative expression of tight junction related genes in the gut of C57BL/6J mice

A-G) Relative expression as $2^{-\Delta\Delta CT}$ of target gene – housekeeping gene (GAPDH)

Compared with Mann-Whitney test: * $p < 0.05$, p-value of statistical trends are indicated on the graphs.

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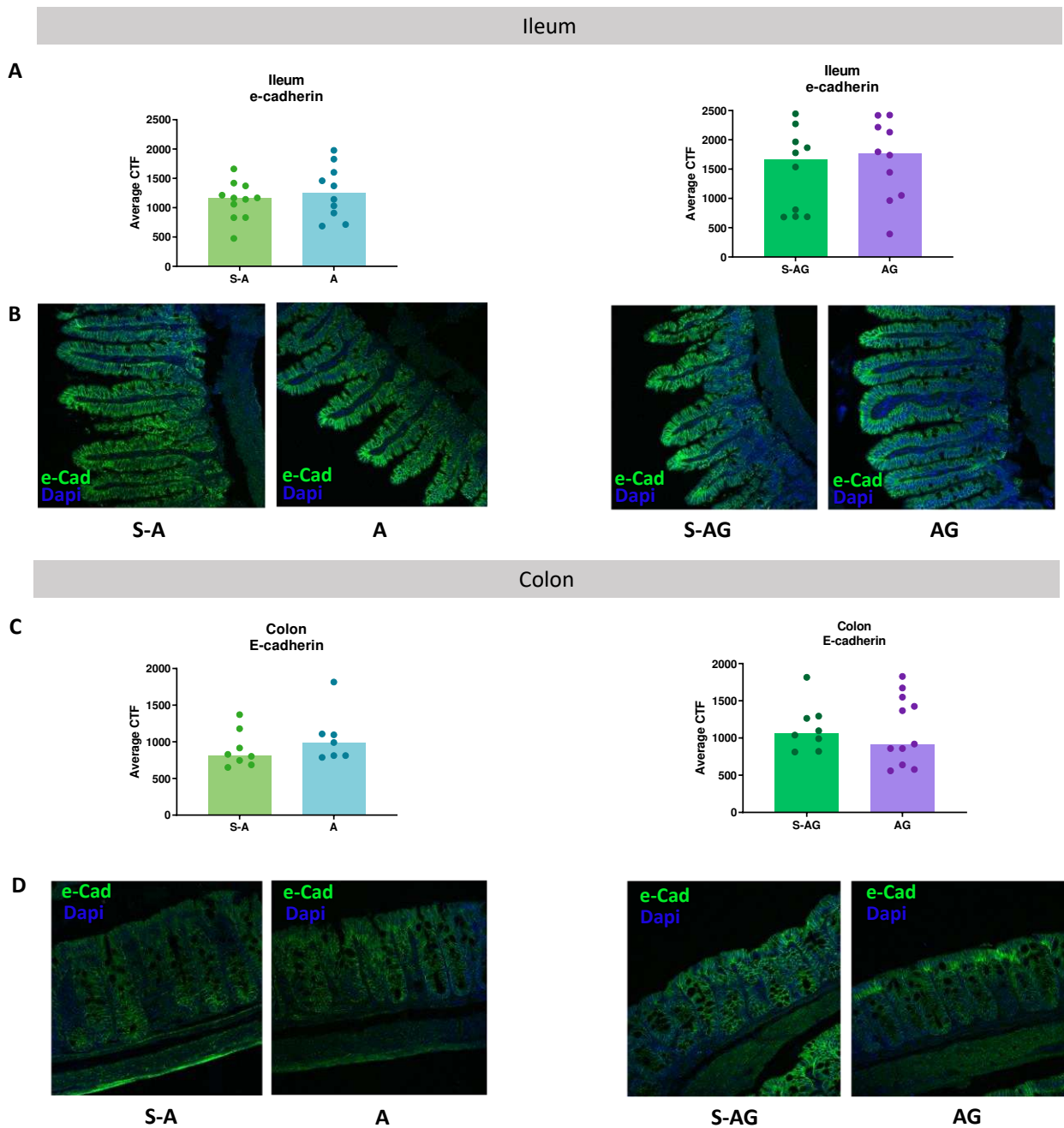


Figure 36 : Immunohistochemical quantification of e-cadherin in the gut of C57BL/6J mice

A-B) e-cadherin levels expressed as average corrected total fluorescence (CTF)

C-D) Representative pictures (confocal microscope x200)

Compared with Mann-Whitney test.

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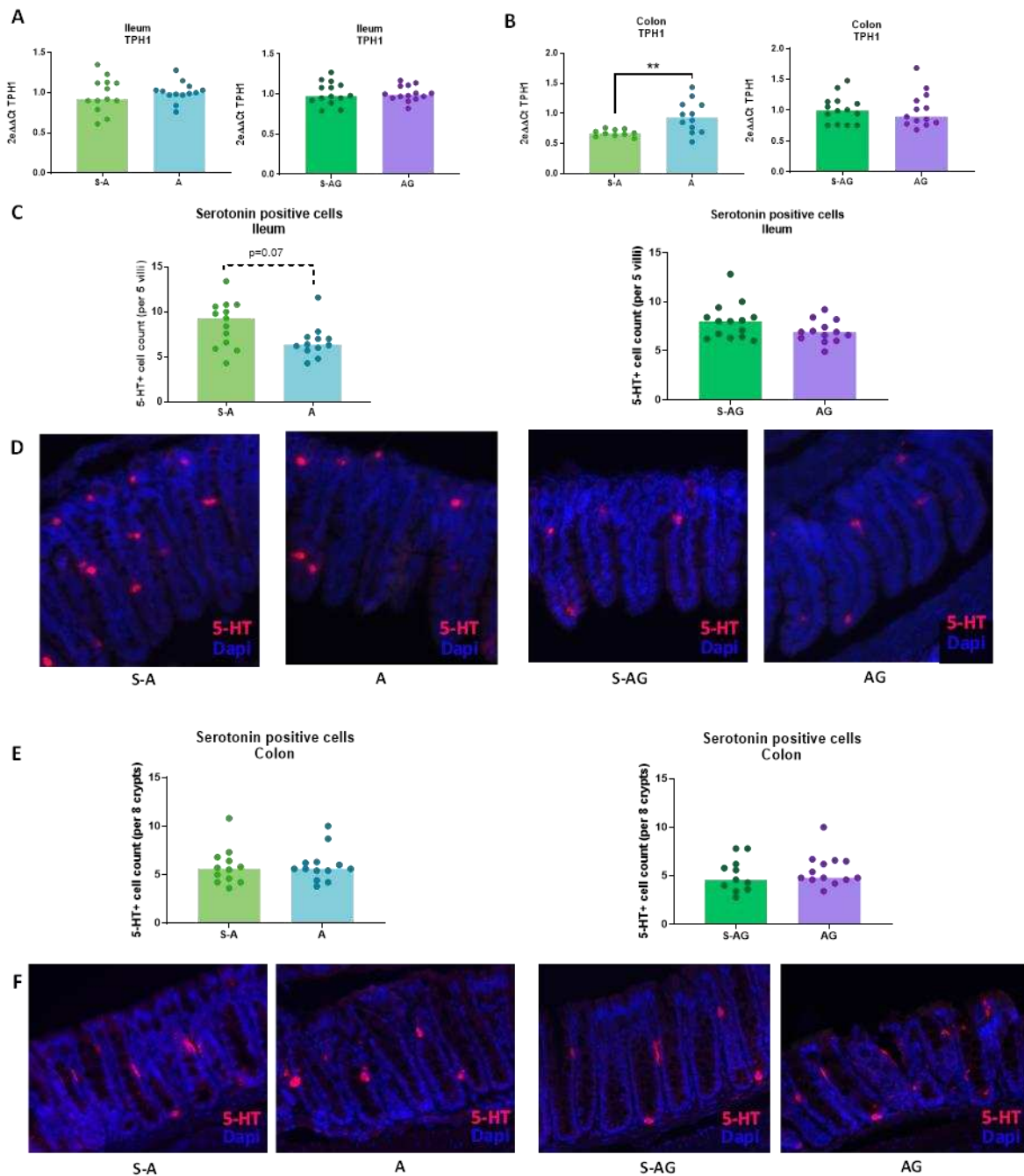


Figure 37 : Relative gene expression of TPH1 and immunohistochemical analysis of the number or serotonin positive cells in the gut of C57BL/6J mice

A-B) Relative TPH1 expression as $2e\Delta\Delta CT$ of target gene – housekeeping gene (GAPDH)

C-F) Count of 5-HT+ cells in the ileum and colon and representative pictures

Compared with Mann-Whitney test: ** $p < 0.01$. P-value of statistical trends are indicated on the graphs.

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2.4.3.2.4 Spearman correlation between cecal microbiota and intestinal markers

There were some correlations between intestinal markers and microbiota diversity or relative abundance of phyla (Table 24-28)

Intestinal inflammation	Microbiota	N	Correlation coefficient	p-value
TNF- α _ileum	Shannon	53	0,48	0,0002
	InvSimpson	53	0,43	0,001
	Proteobacteria	53	0,34	0,01
	Bacteroidota	53	0,52	0,00006
TNF- α _ileum	Firmicutes	53	-0,54	0,00003
IL-6_colon	Firmicutes	52	-0,34	0,02
IL-6_colon	Bacteroidota	52	0,30	0,03
	Proteobacteria	53	0,27	0,05

Table 24 : Correlation table of intestinal inflammation markers and cecal microbiota in C57BL/6J mice when all mice were grouped together. Green: positive correlation, Red: negative correlation.

Group	Intestinal inflammation	Microbiota	N	Correlation coefficient	p-value
A	IL-10_colon	Bacteroidota	12	-0,73	0,01

Table 25 : Correlation table of intestinal inflammation markers and cecal microbiota in C57BL/6J mice separated by group. Green: positive correlation, Red: negative correlation

When considering all the mice together (Table 24), ileal expression of TNF- α was positively correlated with α -diversity (Shannon and InvSimpson indexes). Plus, both ileal expression of TNF- α and colonic expression of IL-6 correlated positively with relative abundance of Proteobacteria and Bacteroidota, but negatively with relative abundance of Firmicutes. When separating mice by group, the only significant correlation between microbiota and gut inflammation was in group A, where colonic expression of IL-10 was negatively correlated with relative abundance of Bacteroidota (Table 25).

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Intestinal permeability	Microbiota	N	Correlation coefficient	p-value
ZO-1_colon	Shannon	52	-0,29	0,03
MLCK_ileum	Bacteroidota	53	-0,28	0,05
MLCK_colon	Actinobacteriota	52	-0,28	0,05

Table 26 : Correlation table of intestinal permeability markers and cecal microbiota in C57BL/6J mice when all mice were grouped together. Red: negative correlation

Group	Intestinal permeability	Microbiota	N	Correlation coefficient	p-value
A	MLCK_colon	Shannon	12	0,73	0,01
		InvSimpson	12	0,67	0,02
		Desulfobacterota	12	0,64	0,02
	ZO-1_colon	Desulfobacterota	12	0,78	0,003
	Occludin_ileum	Firmicutes	12	0,84	0,00
		Proteobacteria	13	0,68	0,01

Table 27 : Correlation table of intestinal permeability markers and cecal microbiota in C57BL/6J mice separated by group. Green: positive correlation

For intestinal permeability, when analyzing all the mice together (Table 26) we found negative correlations between colonic expression of ZO-1 and α -diversity (Shannon index). Ileal expression of MLCK was negatively correlated with relative abundance of Bacteroidota, while colonic expression of MLCK correlated negatively with relative abundance of Actinobacteriota. However, when the analysis was separated by group, there were some positive correlations, in group A specifically (Table 27). Colonic expression of MLCK was positively correlated with α -diversity (Shannon and InvSimpson indexes). Colonic expressions of both MLCK and ZO-1 were positively correlated with relative abundance of Desulfobacterota. Finally, the ileal expression of occludin was positively correlated with relative abundance of Firmicutes and Proteobacteria.

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	Intestinal serotonin	Microbiota	N	Correlation coefficient	p-value
All	TPH1_colon	Chao1	52	0,31	0,02
S-A	TPH1_colon	Desulfobacterota	12	-0,59	0,04

Table 28 : Correlation table of TPH1 expression and cecal microbiota in C57BL/6J mice when analyzed all together and separated by group. Green: positive correlation, Red: negative correlation

Finally, for intestinal serotonin, when analyzing all mice together, we found that colonic expression of TPH1 was positively correlated with α -diversity (Chao1 index). Specifically in the S-A group, we found a negative correlation between colonic TPH1 expression and the relative abundance of Desulfobacterota (Table 28).

2.4.4 Conclusion: Effect of the FMT on systemic and gut markers in each strain

Overall, FMT from the different groups did not induce differences in body and organ weights, at the exception of a heavier cecal wall in BALB/c mice in the AG group compared to S-AG. In both strains, transferred microbiota pools from the A and especially AG groups led mostly to reduced inflammation markers, systematically and/or in the gut, (except for the increase of GFAP in ileum in group AG of C57BL/6J). There was no difference in the expression of tight junction genes in BALB/c mice. However, in C57BL/6J mice, the increase in ZO-1 expression in the ileum in group A compared to S-A suggests a slightly decreased paracellular permeability in this group, while the increased levels of MLCK expression in the ileum in AG compared to S-AG could suggest an increased paracellular permeability in this group. However, measuring protein levels of activated MLCK would be necessary to conclude on a potential impact on permeability. In C57BL/6J we found increased TPH1 gene expression in the colon but a trend to a decreased number of 5-HT positive cells in the ileum in group A compared to S-A. This suggest that the transferred microbiota from this group can impact 5-HT metabolism in the gut in different ways depending on the marker studied and its localization along the GI tract.

There were a few correlations between microbiota composition and diversity

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and general or intestinal inflammation or other intestinal markers. Most of those were between general or gut inflammation and microbiota.

Interestingly, in both strains, gene expression of TNF- α in the ileum correlated positively with relative abundance of Proteobacteria and negatively with that of Firmicutes (Tables 16 and 24).

2.5 RESULTS-EFFECT OF FMT ON BRAIN AND BEHAVIOR

2.5.1 BALB/c

2.5.1.1 Effect of FMT on brain markers

2.5.1.1.1 *Neuroinflammation*

Individuals with ASD and mice models of ASD often show elevated markers of inflammation, and microgliosis [105, 164–166, 174–176]. Some studies show that microbial factors can influence microglial activation in the brain [16, 169]. Thus, we have assessed microglial structure by immunohistochemistry. By looking at distance between cell bodies (centroid distance), ramification index and branch length, we can have an idea of the morphology of the microglia. An activated microglial state is characterized by reduced centroid distance, increased occupied volume, and a less ramified morphology with shorter branches. We also looked at the gene expression levels of GFAP in the PFC and hippocampus, as it is a marker of glial cells, and its increase is a marker of neuroinflammation [265]. There were no significant differences for any of those parameters in BALB/c mice (Fig 38). There was a trend to a decreased level of GFAP transcripts in the PFC of mice from group A compared to S-A (Fig 38 E), which could suggest decreased brain inflammation in this region. However, as it is a trend, and not accompanied by morphological differences of the microglia, we cannot conclude on an effect of the microbiota on neuroinflammation.

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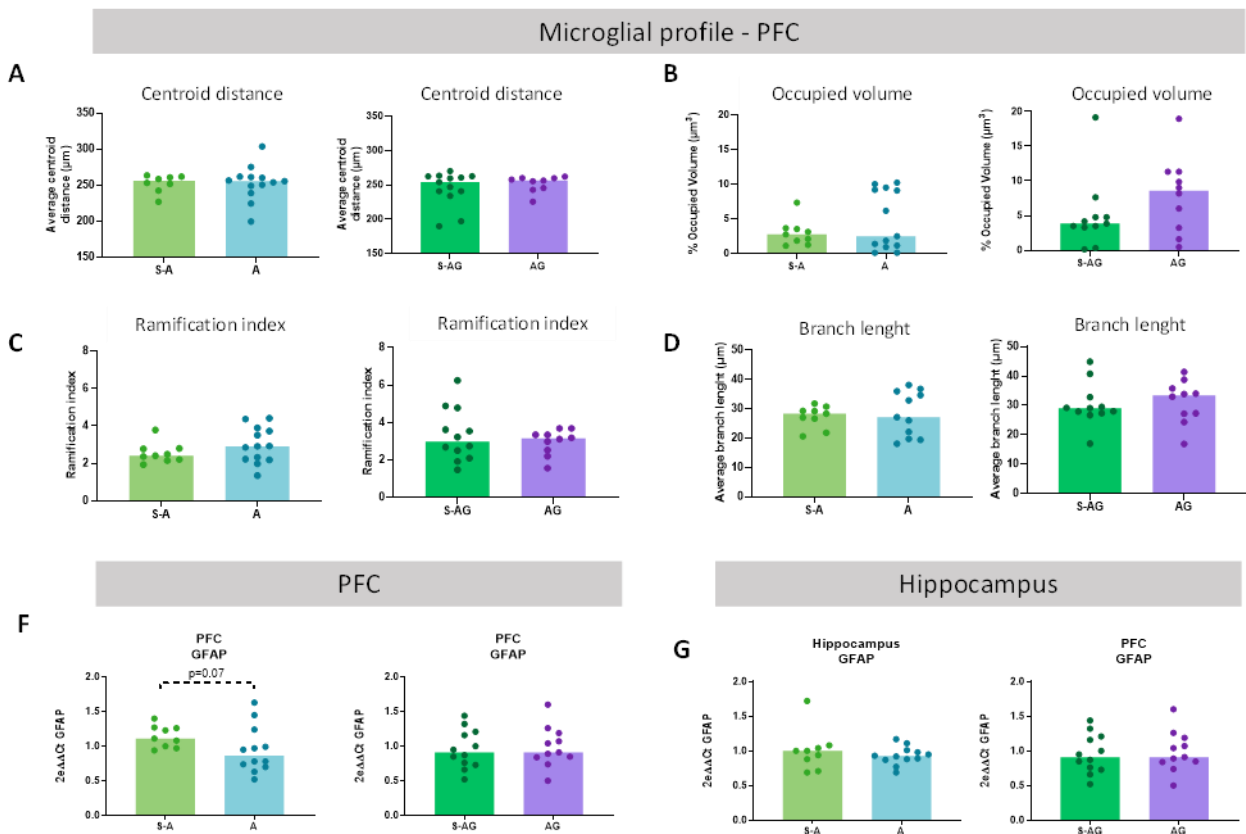


Figure 38: Microglial profile in the PFC and GFAP expression in the PFC and hippocampus of BALB/c mice

A-D) Microglial parameters were assessed using the 3DMorph MatLAB software on stacked pictures from whole brain imaging **E-F)** Relative gene expression of GFAP as $2e\Delta\Delta\text{CT}$ of target gene – housekeeping gene (β -actin). Compared with Mann-Whitney test, p-value of statistical trends are indicated on the graphs.

2.5.1.1.2 Brain serotonergic system

While most of the alterations in the serotonin system in children with ASD or ASD models are systemic, there are still a few reports of central alterations of 5-HT in individuals with ASD [184–186], and also in mice models [105, 170]. Plus, TPH2 polymorphism is associated with ASD [266]. Thus, we have analyzed the levels of transcripts of the serotonin transporter SERT (gene SLC6A4), and 5-HT1A (gene HTR1A) in the PFC and hippocampus of our animals. None of those markers were significantly different between groups (Fig 39 A-D), although there was a slight trend for a decrease in SERT transcript in the hippocampus of AG mice compared to S-AG ($p=0.09$) (Fig 39 D). We have also measured the number of serotonergic neurons in the raphe nuclei of the animals by counting TPH2 positive neurons in this region.

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As mentioned in the introduction, TPH2 is the enzyme responsible for the first step of serotonin synthesis in the brain and is mostly expressed in the raphe nuclei. The number of TPH2 positive neurons was significantly decreased both in A ($p=0.0005$) and AG ($p=0.03$) groups compared to respective sibling groups (Fig 39 E). Overall, these results indicate that, while the transferred microbiota from group A to an AG reduced the number of serotonergic neurons in the raphe nuclei, this was not accompanied by changes in some other markers of the serotonergic system in other brain regions.

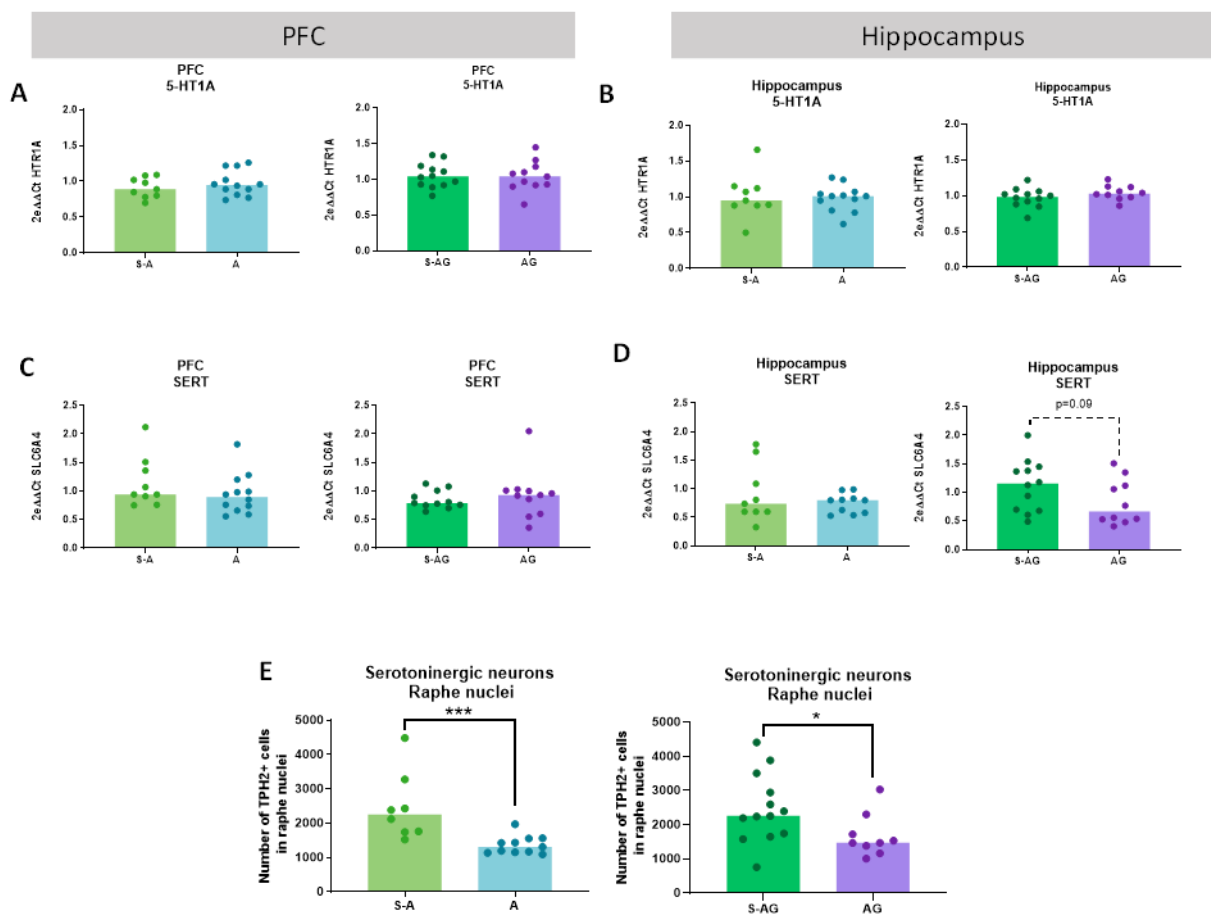


Figure 39: Relative gene expression of markers of the serotonergic system and immunohistochemical analysis of the number of TPH2 positive neurons in the brain of BALB/c mice.

A-D) Relative gene expression as $2^{-\Delta\Delta CT}$ of target gene – housekeeping gene (β -actin) **E)** Count of TPH2 positive neurons in the raphe nuclei (3D spot count in Imaris software) Compared with Mann-Whitney test: * $p < 0.05$; ** $p < 0.01$

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2.5.1.1.3 Brain BDNF expression

Individuals with ASD show alterations of neurotrophic factors in the brain [267]. Plus, microbiota can have an influence on BDNF levels in the brain [268]. Thus, we next wondered if this marker could also be modulated in our different groups. There was a significant increase in the expression of BDNF in the PFC of BALB/c mice in group AG compared to S-AG ($p=0.03$) (Fig 40 A), but no difference in the hippocampus (Fig 40 B).

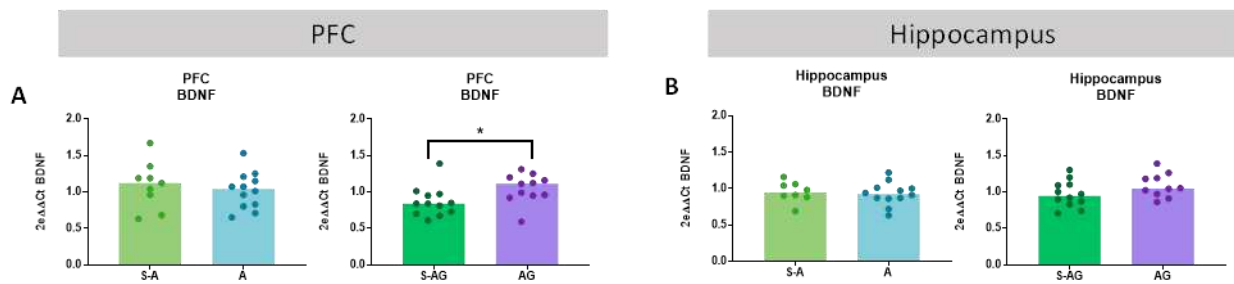


Figure 40 : Relative gene expression of BDNF in the brain of BALB/c mice

A-D) Relative BDNF gene expression as $2e\Delta\Delta$ CT of target gene – housekeeping gene (β -actin). Compared with Mann-Whitney test: $*=p<0.05$.

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2.5.1.1.4 Spearman correlations between cecal microbiota and brain markers

Spearman correlation analysis revealed some correlations between brain markers and microbiota diversity or relative abundance of phyla (Table 29-30)

Brain marker	Microbiota	N	Correlation coefficient	p-value
BDNF_PFC	Desulfobacterota	44	0,42	0,004
BDNF_PFC	Chao1	44	-0,52	0,0003
	Shannon	44	-0,35	0,02
BDNF_Hippocampus	Bacteroidota	43	0,30	0,05
BDNF_Hippocampus	Firmicutes	43	-0,32	0,04
GFAP_PFC	Actinobacteriota	44	-0,36	0,02
5-HT1A_PFC	Chao1	44	0,31	0,04
5-HT1A_Hippocampus	Desulfobacterota	43	0,32	0,03

Table 29 : Correlation table of brain markers and cecal microbiota in BALB/c mice when all mice were grouped together. Green: positive correlation, Red: negative correlation

When taking all the mice together, expression of BDNF in the PFC correlated negatively with α -diversity (Chao1 and Shannon indexes), while the expression of 5-HT1A in the same brain region correlated positively with α -diversity (Shannon index). Both the expression of BDNF in the PFC and of 5-HT1A in the hippocampus were positively correlated with the relative abundance of Desulfobacterota. The expression of GFAP in the PFC was negatively correlated with the relative abundance of Actinobacteriota. Finally, in the hippocampus, the expression level of BDNF correlated positively with the relative abundance of Bacteroidota, and negatively with that of Firmicutes (Table 29)

Group	Brain marker	Microbiota	N	Correlation coefficient	p-value
A	5-HT1A_Hippocampus	Actinobacteriota	12	0,6294	0,0283

Table 30 : Correlation table of brain markers and cecal microbiota in BALB/c mice separated by group. Green: positive correlation

When separating the mice by group, the only significant correlation was a positive one between the expression of 5-HT1A in the hippocampus and the relative abundance of Actinobacteriota only in the A group (Table 30).

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2.5.1.2 Effect of FMT on behavior

2.5.1.2.1 *Social and repetitive behaviors*

Impairment of social behavior, and sometimes increased stereotyped/repetitive behaviors are what characterize most ASD models [269]. Thus, we wondered if those behaviors could be altered in our animals. For this reason, we tested the mice in a version of the 3-chamber social interaction test and assessed their grooming behavior as a marker of stereotypy.

For the social test, we compared between groups the % of time where the animals interacted with the different cylinders. Plus, in a specific group, we considered that the mice had a preference for a cylinder if this % was different from 50% (according to a Wilcoxon signed rank test with 50 as a theoretical median). In the habituation phase, there were no differences between groups (Fig 41 A). However, mice from group A had a significant preference for the right cylinder ($p=0.02$) (Fig 41 B). This preference should not have impacted the results in the other phases, as the stranger mice were alternatively placed in either the right or left cylinder. In the social interaction phase, there were no differences between groups in the % of time spent interacting with the "Mouse cylinder" and mice from all groups showed a strong preference for the "Mouse cylinder" (Fig 41 C). In the social novelty phase, there was no difference between groups for time spent interacting with the "Unknown mouse cylinder". However, only mice from the S-AG group did not show a significant preference for the "Unknown mouse" cylinder (Fig 41 D), but there was a trend to a preference ($p=0.08$).

In the self-grooming test, there were no differences between groups in number of grooming bouts, mean duration of each grooming bout, latency to first grooming, or proportion of "incomplete" grooming bouts (Fig 42). Thus, the differences in microbiota between groups in BALB/c mice did not have an impact on repetitive behavior measured in this test.

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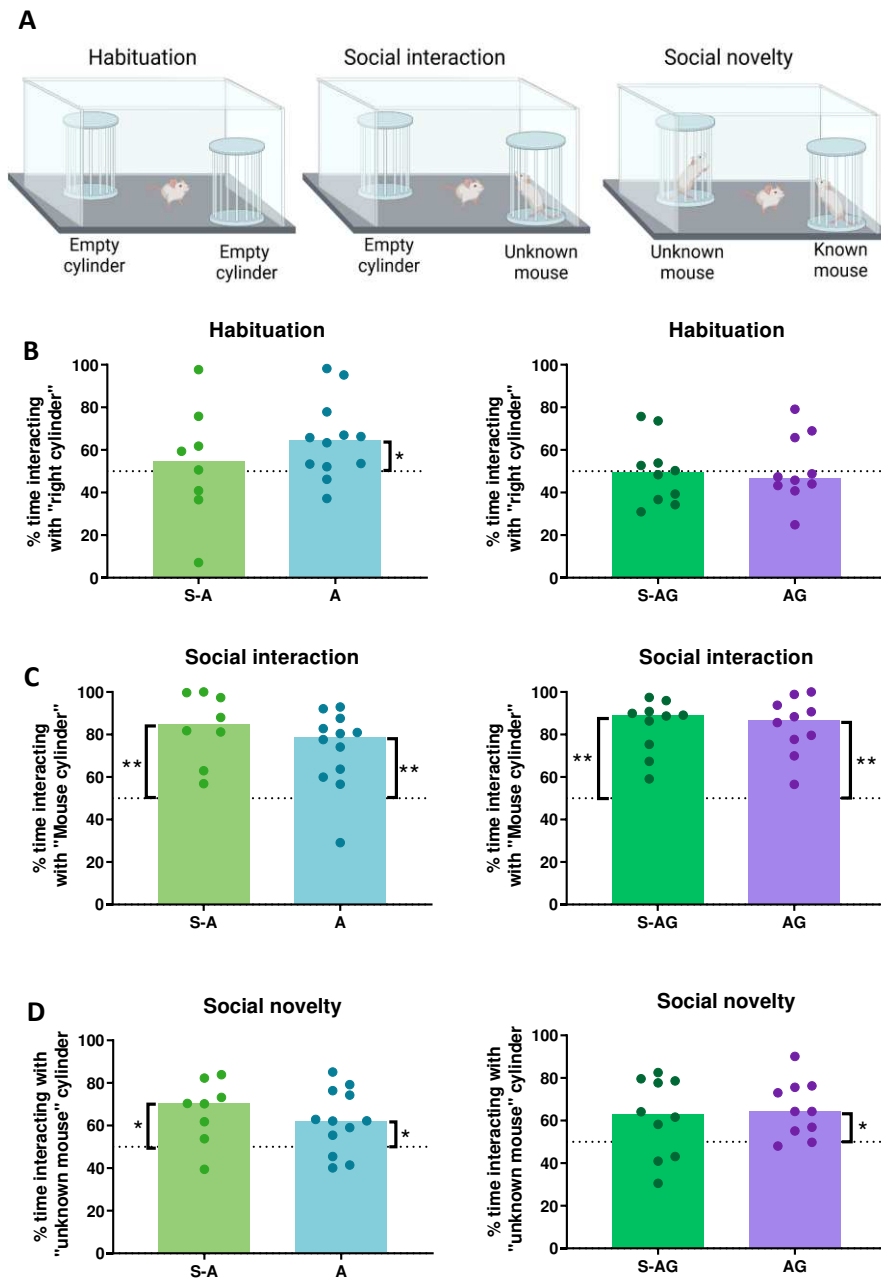


Figure 41 : Results of social interaction and novelty tests in BALB/c mice.

A) Schematic representation of the test (created with Biorender.com)

B-D) % of interaction time with cylinders in each phase of the test

Groups compared with Mann-Whitney test, and in each group, the median was compared to a theoretical value of 50 with the Wilcoxon signed rank test. * $p < 0.05$; ** $p < 0.01$

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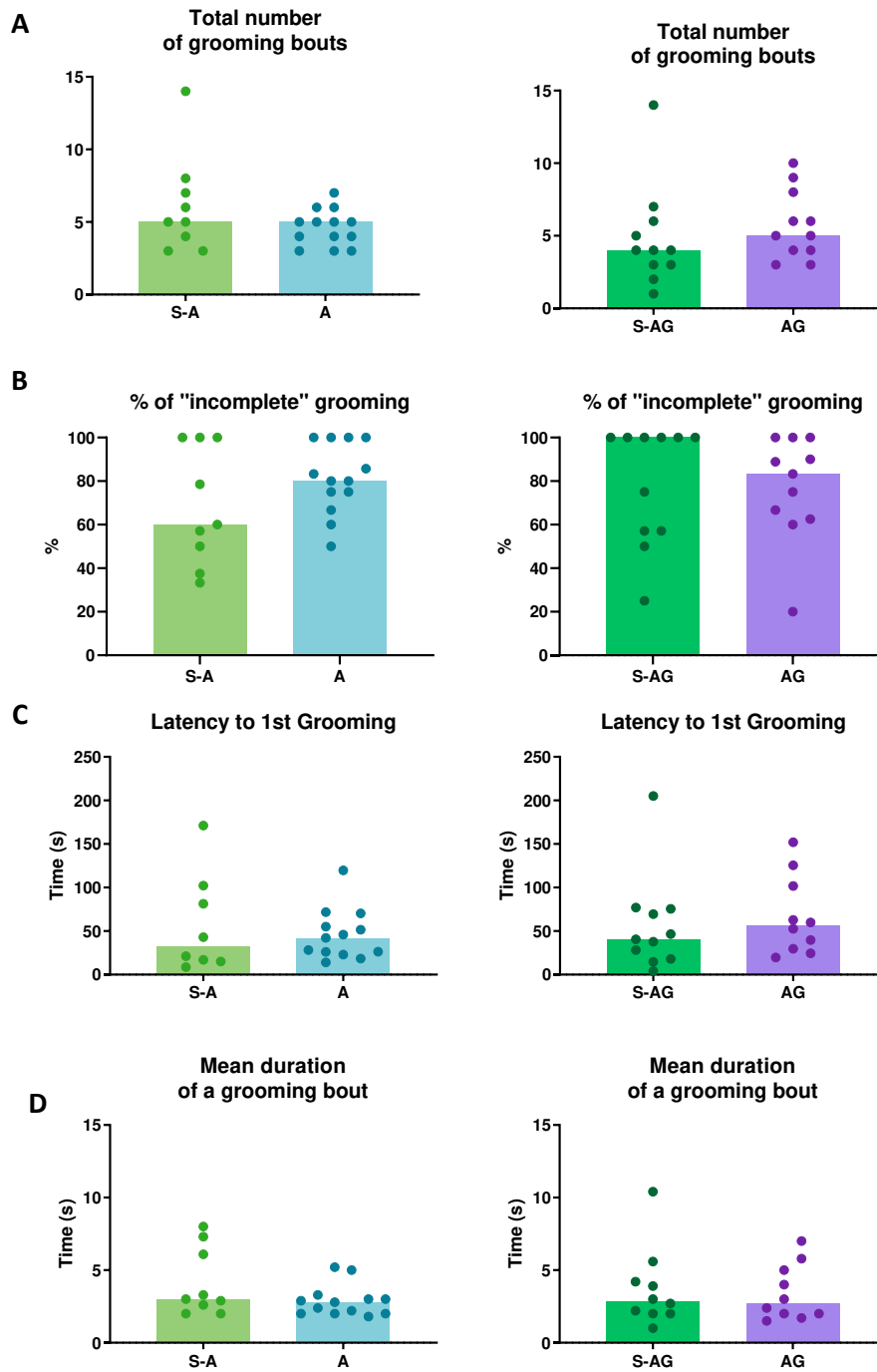


Figure 42 : Results of the self-grooming test of BALB/c mice
 Compared with Mann-Whitney test.

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2.5.1.2.2 Anxiety-like behavior

While anxiety is not part of the core symptoms of ASD, it is a very common occurrence in individuals with ASD and is observed in some ASD models [269]. Thus, we tested the mice in the OF test (Fig 43). To check for potential locomotion differences between groups, we measured total distance travelled during the test, and there were no significant differences between groups (Fig 43 A). Of note, distance travelled was also measured during social behavior tests and spatial memory tests, and there were no differences between groups (data not shown). When looking at anxiety-related parameters, we found no difference between groups in the % of time spent in center and % of time spent in corners (Fig 43 B-C). Thus, the differences in microbiota in BALB/c mice did not seem to impact anxiety-like behavior in this test.

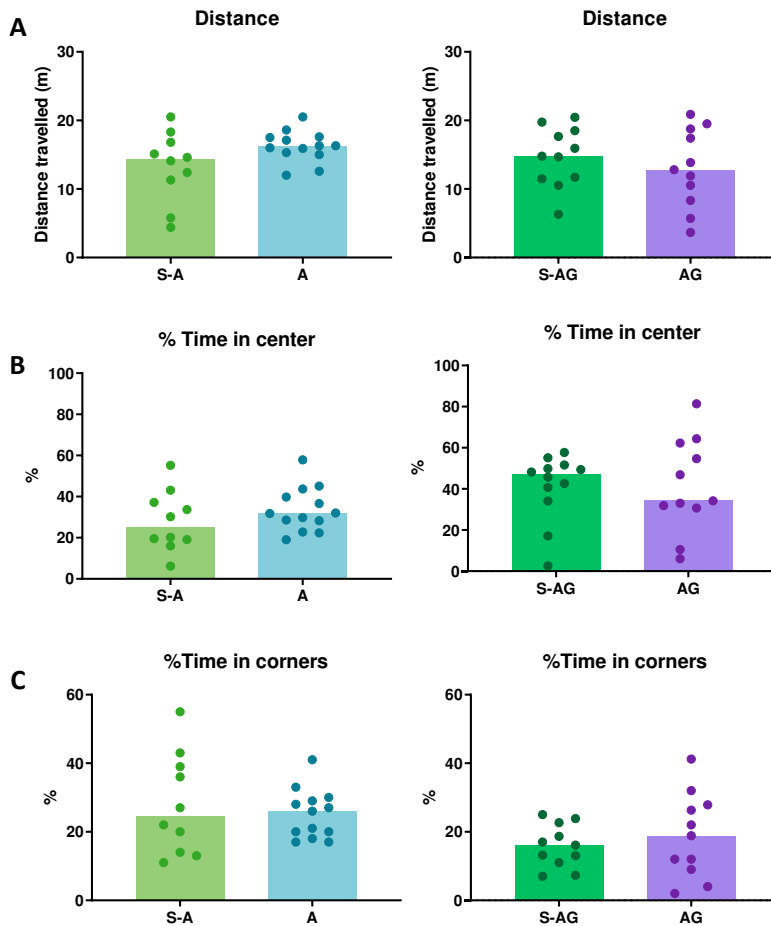


Figure 43 : Results of the Open-field test of BALB/c mice
A) Distance travelled during the test **B-C)** Anxiety related parameters. Groups compared with Mann-Whitney test.

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2.5.1.2.3 Spatial memory

Finally, as some individuals with ASD, present cognitive impairments, behavioral tests that assess memory are sometimes used in ASD models [270, 271]. Plus, SCFAs treatment can improve memory and brain plasticity in mice, suggesting that the gut microbiota could influence cognitive function [272]. For these reasons, we wondered if spatial memory could be modulated in our mice. Thus, we have tested our animals in the 5-object spatial recognition test. We measured the recognition index, as explained in the material and methods (p.72) but found no difference in this parameter between groups (Fig 44 B), indicating that the microbiota did not lead to differences in spatial memory in this test.

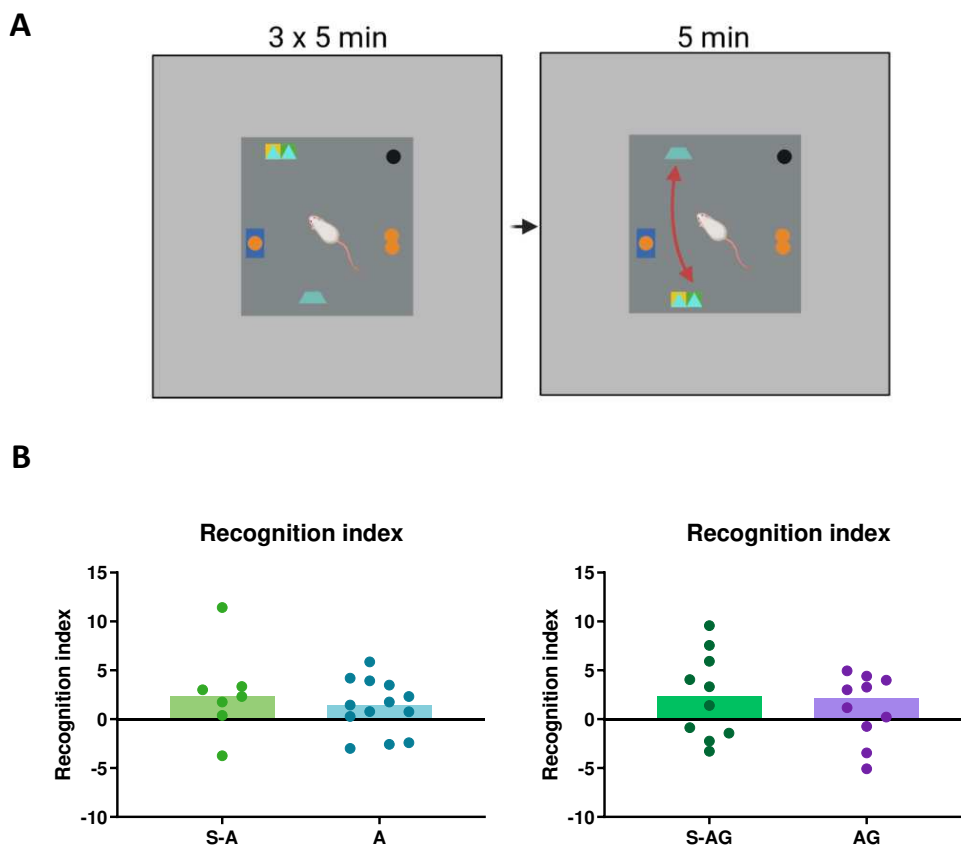


Figure 44: Results of the spatial recognition test in BALB/c mice
A) Schematic representation of the test (created with Biorender.com)
B) Recognition index. Compared with Mann-Whitney test.

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2.5.1.3 Spearman correlations between microbiota and behavior

2.5.1.3.1.1 Correlations between fecal microbiota and behavior

Firstly, we wondered if there could be correlations between behavior and the composition of fecal microbiota collected just before the start of the behavioral testing period (Table 31-32).

Behavior	Fecal microbiota	N	Correlation coefficient	p-value
%Time in center	Actinobacteriota	44	0,42	0,005
% Unknown mouse cylinder	Proteobacteria	40	0,38	0,02

Table 31 : Correlation table of behavior and fecal microbiota in BALB/c mice when all mice were grouped together. Green: positive correlation

When considering all mice together, the % of time spent in the center of the OF correlated positively with the relative abundance of Actinobacteriota, while the % of time spent on the “Unknown mouse cylinder” in the social novelty phase correlated positively with the relative abundance of Proteobacteria (Table 31).

Group	Behavior	Fecal microbiota	N	Correlation coefficient	p-value
S-A	Latency to 1st grooming	Shannon	9	-0,82	0,007
		Bacteroidota	9	-0,82	0,007
	Latency to 1st grooming	Firmicutes	9	0,82	0,007
	Total number of groomings	Proteobacteria	9	0,67	0,05
	% Unknown mouse cylinder	InvSimpson	8	0,74	0,04
Desulfobacterota		8	0,76	0,03	
A	% Unknown mouse cylinder	Chao1	12	0,66	0,02
S-AG	% Time in center	InvSimpson	12	-0,664	0,02
		Shannon	12	-0,594	0,04
	% Time in center	Actinobacteriota	12	0,706	0,01
	% Incomplete grooming	InvSimpson	11	-0,616	0,04
AG	% Mouse cylinder	Desulfobacterota	10	0,661	0,04
	% Mouse cylinder	Actinobacteriota	10	-0,830	0,003
	Total number of groomings	Proteobacteria	11	0,647	0,03

Table 32 : Correlation table of behavior and fecal microbiota in BALB/c mice separated by group. Green: positive correlation Red: negative correlation.

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When separating the analysis by group, there were more significant correlations (Table 32). In S-A, latency to first grooming was negatively correlated with α -diversity (Shannon index) and relative abundance of Bacteroidota but negatively with that of Firmicutes. Interestingly, for that second correlation, the adjusted p-value of comparison between these correlations in S-A and A group was significant ($p=0.04$). This is the only correlation we found where this was the case, showing that this association is strongly specific to group S-A. Plus, total number of grooming was positively correlated with relative abundance of Proteobacteria both in S-A and AG groups. Still in S-A, % of time interacting with "Unknown mouse cylinder" was positively correlated with α -diversity (InvSimpson). Interestingly, in the A group, the correlation between % of time interacting with "Unknown mouse cylinder" and α -diversity (Chao1) was negative. In the S-AG group, the % of time spent in the center of the OF and the proportion of incomplete grooming bouts correlated negatively with α -diversity (Shannon and/or InvSimpson indexes). The % of time in the center of the OF correlated positively with the relative abundance of Actinobacteriota. Finally in the AG group, the % of time interacting with the "Mouse Cylinder" in the social interaction test was positively correlated with the relative abundance of Desulfobacterota, and negatively with that of Actinobacteriota.

2.5.1.3.1.2 Correlations between cecal microbiota and behavior

Cecal microbiota was sampled after behavior, and thus its composition could have shifted due to the stress of behavioral tests. Nonetheless, we still wondered if there could be any correlations between its composition and the behavior, since the differences between groups in fecal and cecal microbiota were not the same (Table 33-34).

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Behavior	Cecal microbiota	N	Correlation coefficient	p-value
Latency to 1st grooming	Chao1	44	0,35	0,02
%Time in center	Proteobacteria	44	0,41	0,01

Table 33 : Correlation table of behavior and cecal microbiota in BALB/c mice when all mice were grouped together. Green: positive correlation

When taking all the mice together, there was a positive correlation between latency to first grooming and α -diversity (Chao1 index). Plus, % of time spent in center of the OF correlated positively with the relative abundance of Proteobacteria (Table 33).

Group	Behavior	Cecal microbiota	N	Correlation coefficient	p-value
S-A	Latency to 1st grooming	Shannon	9	0,83	0,01
		InvSimpson	9	0,83	0,01
		Firmicutes	9	0,80	0,01
	Latency to 1st grooming	Bacteroidota	9	-0,70	0,04
S-AG	% Incomplete groomings	Chao1	11	0,67	0,03

Table 34 : Correlation table of behavior and cecal microbiota in BALB/c mice separated by group. Green: positive correlation Red: negative correlation.

In the analysis separated by group, we found in S-A, like in the general analysis, a positive correlation between latency to first grooming and α -diversity although not according to the same indexes. Plus, in this group, this behavioral parameter was also positively correlated with the relative abundance of Firmicutes and negatively with that of Bacteroidota. In S-AG, the number of incomplete grooming was positively correlated with α -diversity (Chao1 index). There were no significant correlations in A and AG groups in this strain (Table 34).

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2.5.2 C57BL/6J

2.5.2.1 Effect of FMT on brain markers

2.5.2.1.1 Neuroinflammation

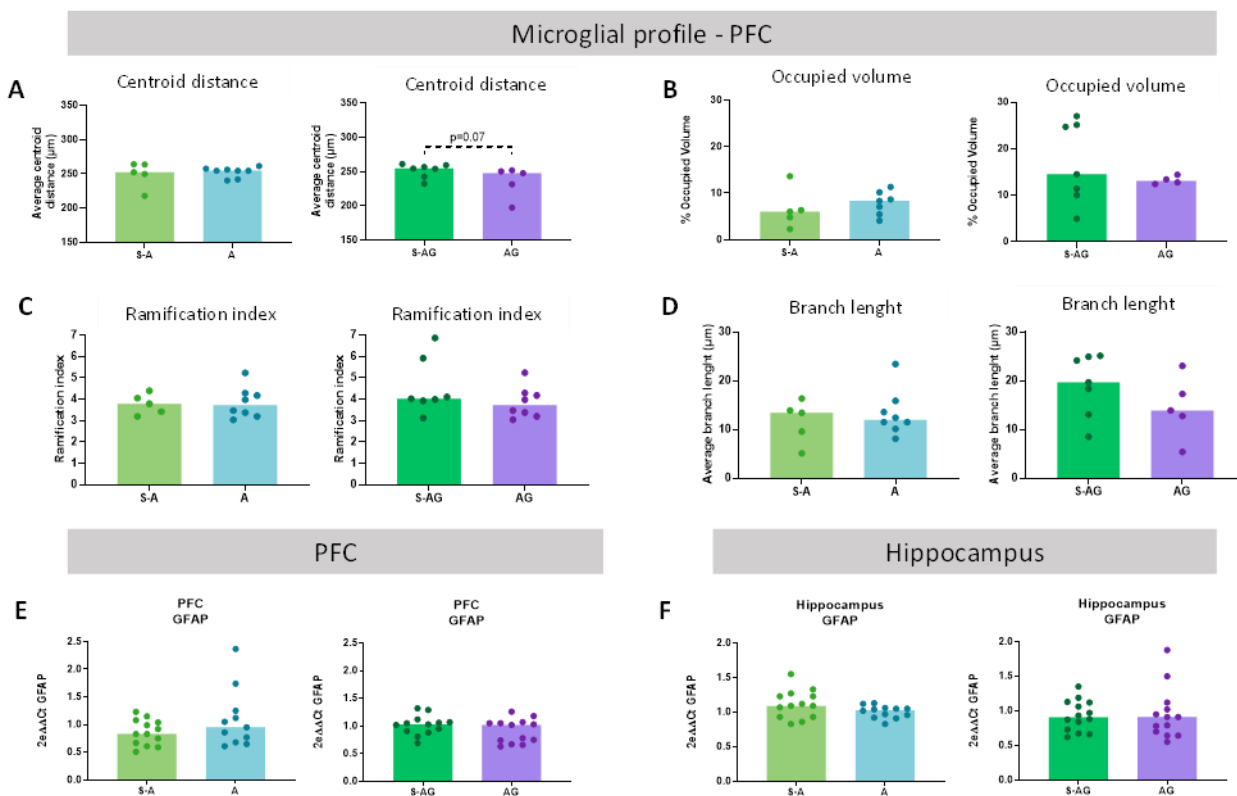


Figure 45 : Microglial profile in the PFC and GFAP expression in the PFC and hippocampus of C57BL/6J mice

A-D) Microglial parameters were assessed using the 3DMorph MatLAB software on stacked pictures from whole brain imaging **E-F)** Relative gene expression of GFAP as $2e\Delta\Delta CT$ of target gene – housekeeping gene (β -actin). Compared with Mann-Whitney test, p-value of statistical trends are indicated on the graphs.

Like in BALB/c mice, there was no significant difference between groups in C57BL/6J for any of the assessed parameters for microglial morphology (Fig 45 A-D) or expression of GFAP in the PFC and hippocampus (Fig 45 E-F). There was a trend to a decreased centroid distance (Fig 45 A), which is a marker of microglial activation, but as it was not significant, and not accompanied by any difference in microglial morphology, we cannot conclude on an effect of the microbiota on this parameter. To note, the results on C57BL/6J mice are only those of cohort 2, as cohort 1 could not

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be analyzed due to technical difficulties.

2.5.2.1.2 Brain serotonergic system

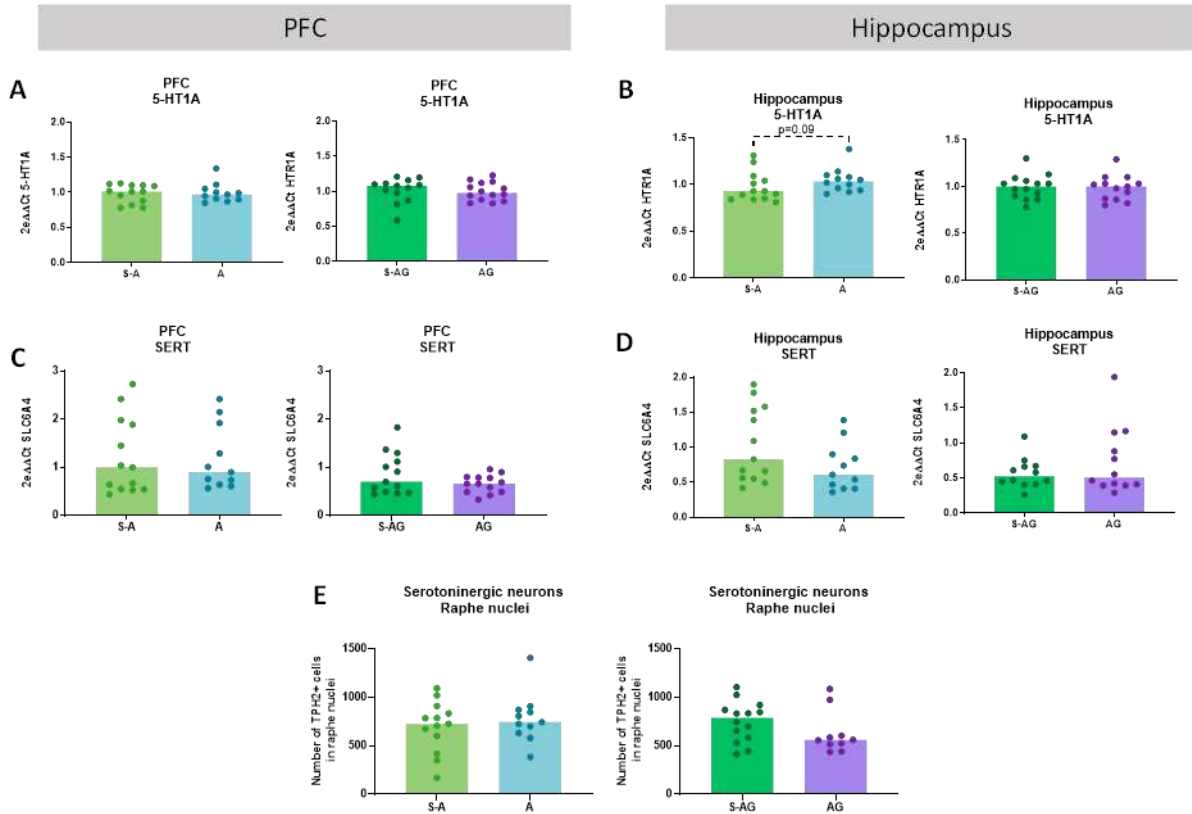


Figure 46: Relative gene expression of markers of the serotonergic system and immunohistochemical analysis of the number of TPH2 positive neurons in the brain of C57BL/6J mice.

A-D) Relative gene expression as $2\Delta\Delta CT$ of target gene – housekeeping gene (β -actin)
E) Count of TPH2 positive neurons in the raphe nuclei (3D spot count in Imaris software) Compared with Mann-Whitney test.

In C57BL/6J mice, there were no significant differences between groups for the levels of transcripts of SERT (SLC6A4) and 5-HT1A (Fig 46), although there was a slight trend to an increase in 5-HT1A expression in the hippocampus of A mice compared to S-A ($p=0.09$) (Fig 46 A). When we analyzed the number of TPH2 positive neurons in the raphe nuclei, we did not observe any significant difference between groups, unlike what was observed in BALB/c mice (Fig 46 E).

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2.5.2.1.3 Brain BDNF expression

In C57BL/6J mice, there was no significant differences between groups in the expression of BDNF, either in the PFC or in the hippocampus (Fig 47 A-B).

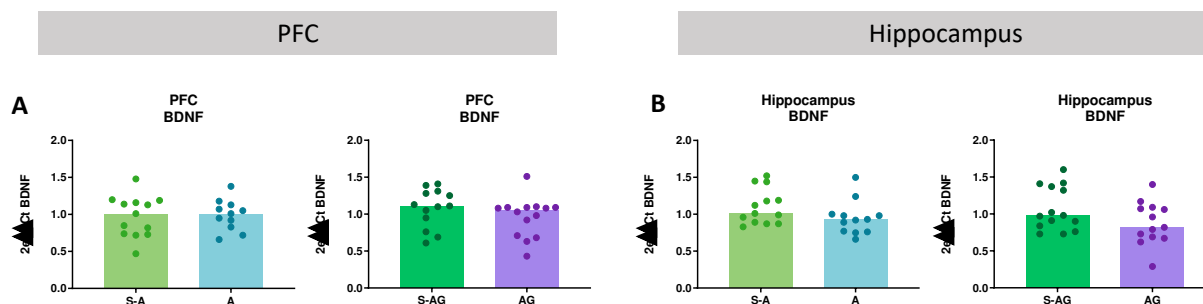


Figure 47: Relative gene expression of BDNF in the brain of C57BL/6J mice.

A-D) Relative BDNF gene expression as $2e\Delta\Delta CT$ of target gene – housekeeping gene (β -actin). Compared with Mann-Whitney test.

2.5.2.1.4 Spearman correlations between cecal microbiota and brain markers

There were a few significant correlations between brain markers and cecal microbiota composition and diversity in this strain (Table 35-36).

Brain marker	Microbiota	N	Correlation coefficient	p-value
GFAP_Hippocampus	Desulfobacterota	51	0,48	0,0003
GFAP_PFC	Actinobacteriota	50	-0,34	0,02
5-HT1A_PFC	Firmicutes	50	-0,34	0,01
5-HT1A_PFC	Shannon	50	0,40	0,004
	InvSimpson	50	0,40	0,004
	Bacteroidota	50	0,34	0,01
5-HT1A_Hippocampus	Desulfobacterota	51	0,40	0,003

Table 35 : Correlation table of brain markers and cecal microbiota in C57BL/6J mice when all mice were grouped together. Green: positive correlation, Red: negative correlation

When considering all the mice together, we found a positive correlation between 5-HT1A expression in the PFC and α -diversity (Shannon and InvSimpson indexes) (Table 35).

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Both the expression of GFAP and 5-HT1A in the hippocampus were positively correlated with the relative abundance of Desulfobacterota. There was a negative correlation between expression of GFAP in the PFC and relative abundance of Actinobacteria. Finally, 5-HT1A expression in the PFC was positively correlated with relative abundance of Bacteroidota and negatively with that of Firmicutes (Table 35).

Group	Brain marker	Microbiota	N	Correlation coefficient	p-value
S-A	GFAP_Hippocampus	Actinobacteriota	12	0,73	0,01
A	BDNF_Hippocampus	Actinobacteriota	12	-0,69	0,01
AG	5-HT1A_PFC	Shannon	14	0,75	0,002
		InvSimpson	14	0,67	0,01
	GFAP_Hippocampus	InvSimpson	13	0,64	0,02
	GFAP_PFC	Actinobacteriota	14	-0,61	0,02

Table 36 : Correlation table of brain markers and cecal microbiota in C57BL/6J mice separated by groups. Green: positive correlation, Red: negative correlation

When considering groups separately (Table 36), the positive correlation between 5-HT1A in the PFC and α -diversity and the negative correlation between GFAP expression in the PFC and relative abundance of Actinobacteria found in the general analysis were also present specifically in the AG group. Both those correlations were stronger than in the general analysis, which is to be expected since n is greatly reduced. Still in the AG group, the expression of GFAP in the hippocampus was also positively correlated with α -diversity (InvSimpson index) while in group S-A it correlated positively with the relative abundance of Actinobacteriota. Finally, BDNF expression in the hippocampus in the A group correlated negatively with relative abundance of Actinobacteriota (Table 36).

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2.5.2.2 Behavior

2.5.2.2.1 Social and repetitive behaviors

In the habituation phase of the 3-chamber test, mice from group S-AG showed a significant preference for the right cylinder ($p=0.02$) (Fig 48 B). However, again, this should not have impacted the results in the other phases as position of the stranger cylinder was alternated. There were no differences between groups in % of time spent interacting with "Mouse cylinder" or "Unknown mouse cylinder" in the social interaction and social novelty phase (Fig 48 C-D). However, in the social novelty phase, mice from group A and S-AG did not show a significant preference for the "Unknown mouse" cylinder (although there was a trend for the A group ($p=0.08$) (Fig. 48 D).

In the self-grooming test, mice from the AG group had a significantly increased total number of self-grooming bouts compared to the S-AG group ($p=0.023$) (Fig 49 A). None of the other parameters measured in this test were different between groups.

Overall, this suggests that the transferred microbiota impacted social novelty in slightly different ways depending on the group, and the microbiota from group AG increased repetitive behaviors compared to S-AG.

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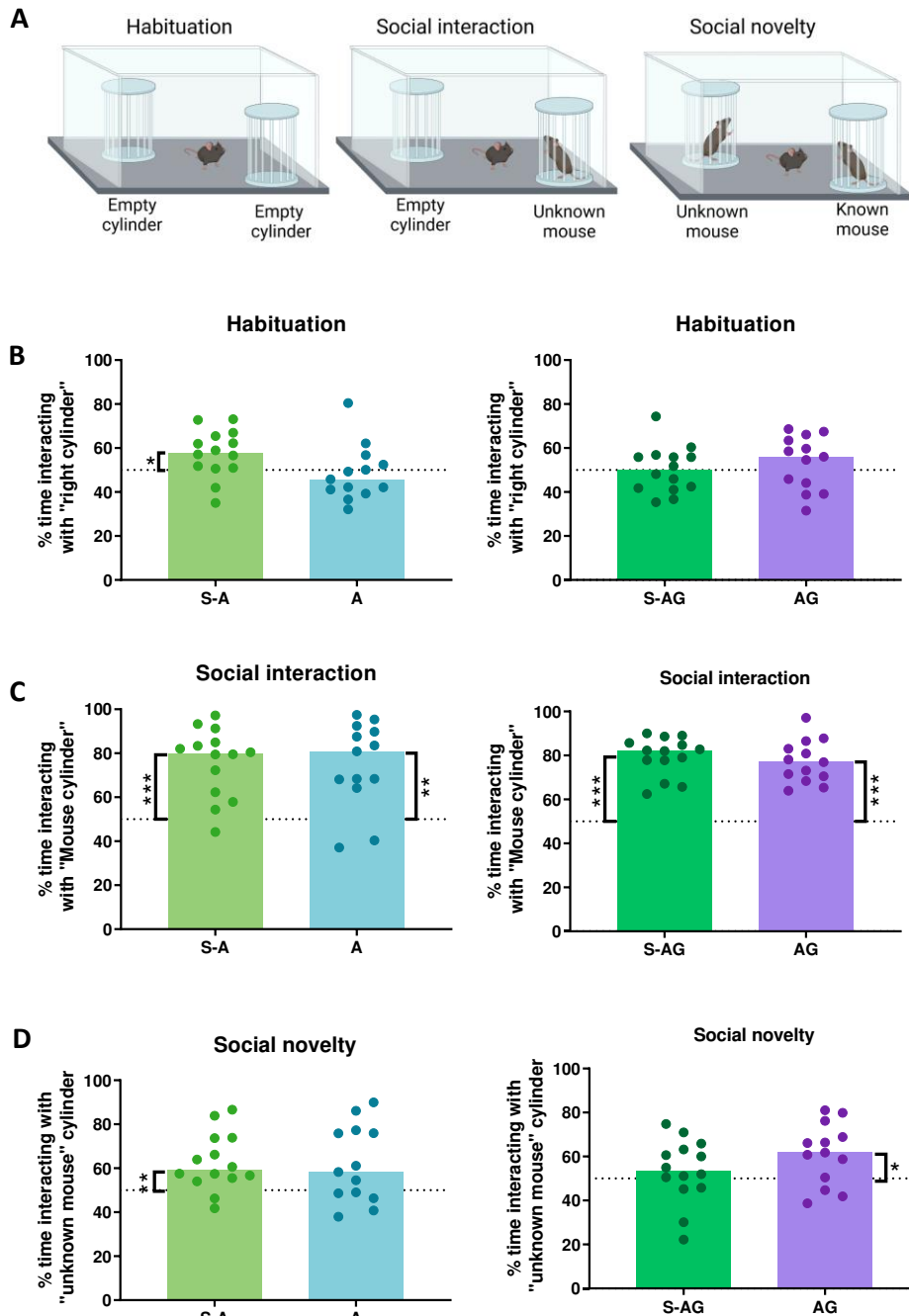


Figure 48 : Results of social interaction and novelty tests in C57BL/6J mice

A) Schematic representation of the test (created with Biorender.com)

B-D) % of interaction time with cylinders in each phase of the test. Groups compared with Mann-Whitney test, and in each group, the median was compared to a theoretical value of 50 with the Wilcoxon signed rank test.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

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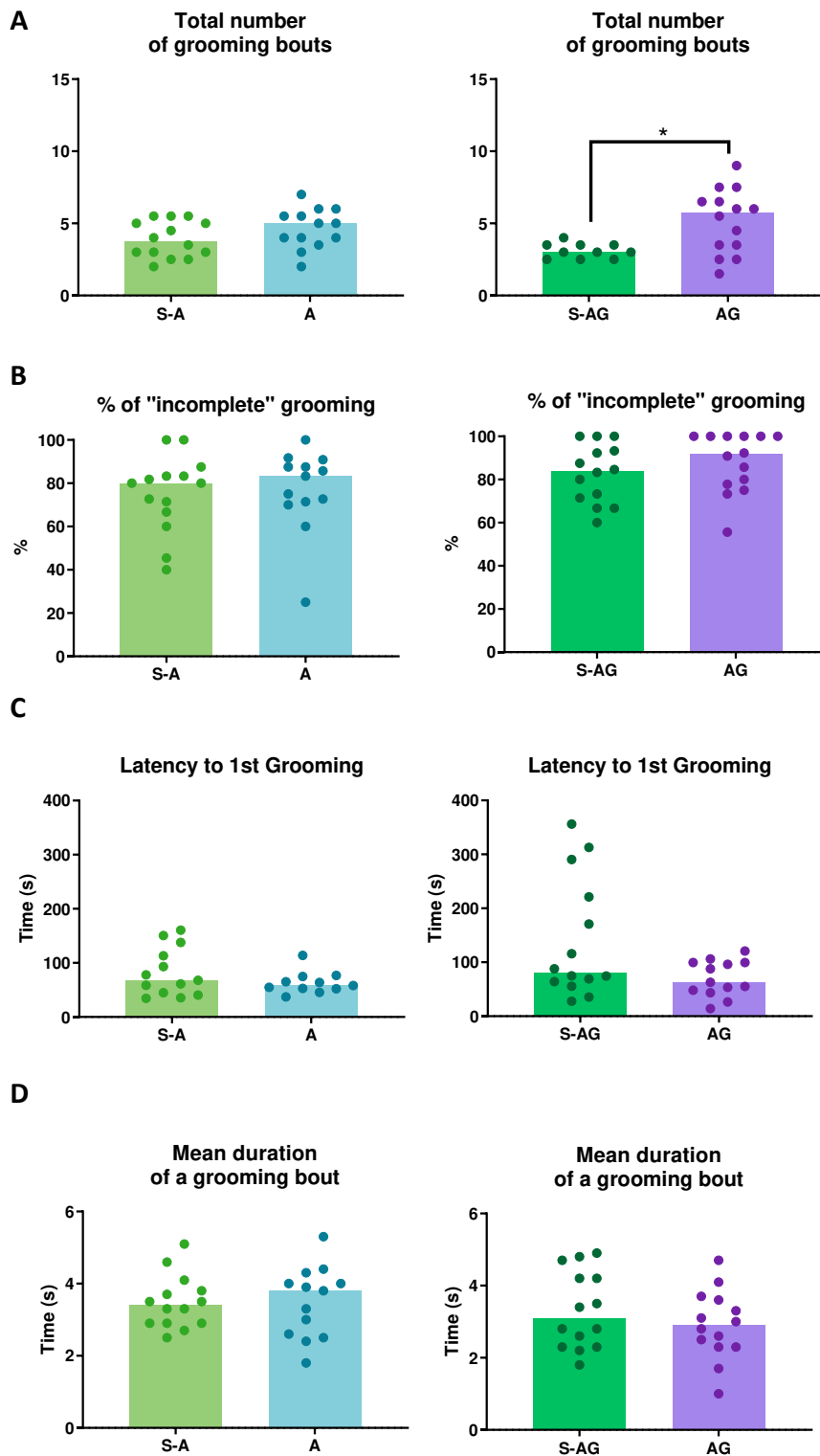


Figure 49 : Results of the self-grooming test of C57BL/6J mice
 Compared with Mann-Whitney test: * $p < 0.05$

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2.5.2.2.2 Anxiety-like behavior

Like in BALB/c mice, there were no differences between groups in the % of time in center or corners of the OF (Fig 50 A, B). However, the total distance travelled by mice from group A during the test was significantly lower than that of mice from group S-A, showing that the transferred microbiota had an impact on locomotor activity in this strain (Fig 50 A).

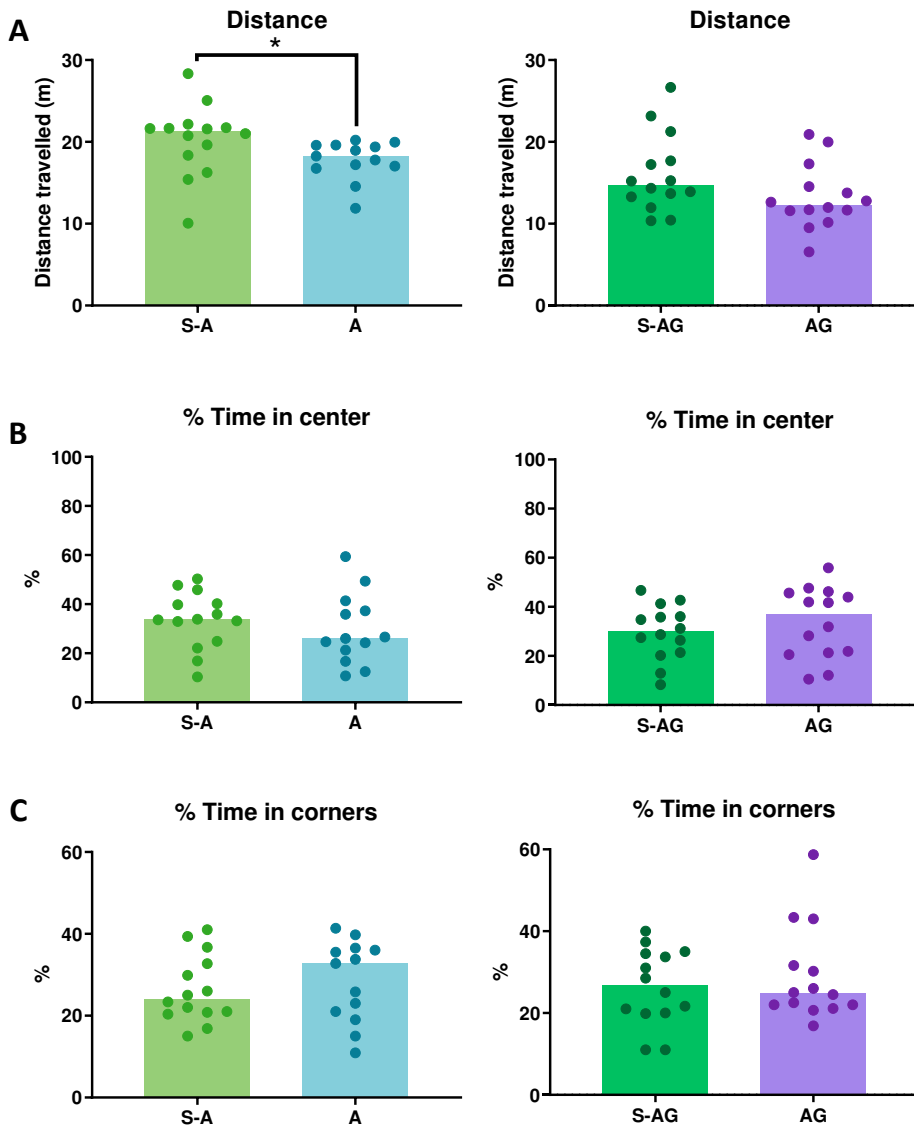


Figure 50 : Results of the OF test in C57BL/6J mice

A) Distance travelled during the test **B-C)** Anxiety related parameters. Compared with Mann-Whitney test: * $p < 0.05$

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2.5.2.2.3 Spatial memory

In the spatial recognition test, mice from group A had a higher recognition index than the S-A mice ($p=0.002$), while the AG mice showed a significantly reduced recognition index compared to the S-AG group ($p=0.003$) (Fig 51 B). This shows that the microbiota from the different donor groups had an impact on spatial memory in this strain.

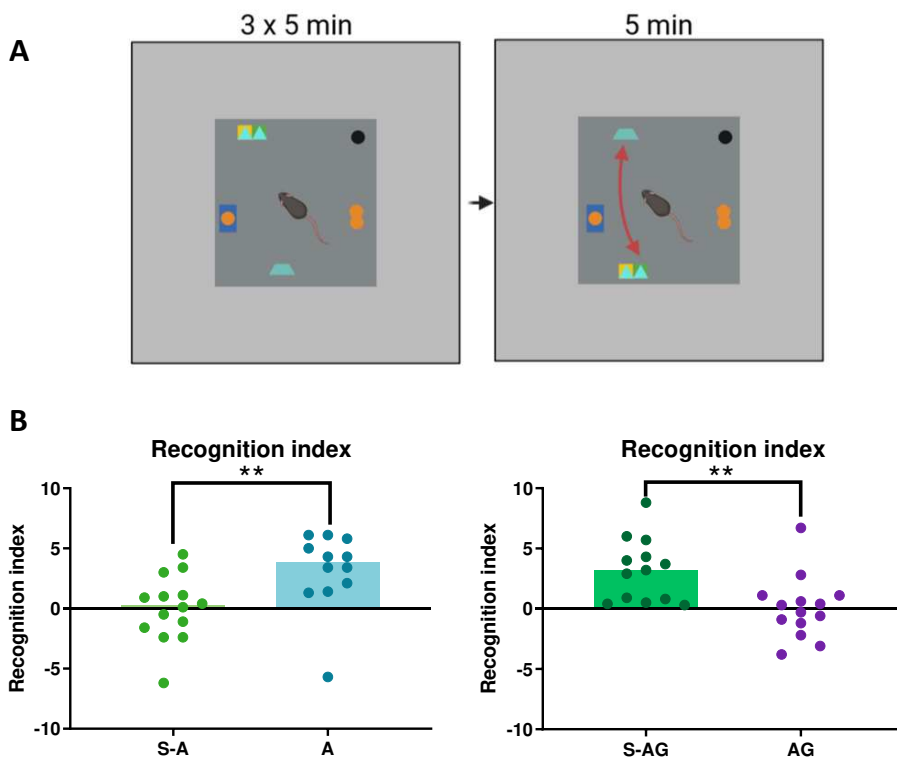


Figure 51: Results of the Spatial recognition test in C57BL/6J mice

A) Schematic representation of the test (created with Biorender.com)

B) Recognition index Compared with Mann-Whitney test: ** $p < 0.01$

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2.5.2.2.4 Correlation fecal and cecal microbiota –Behavior

2.5.2.2.4.1 Fecal microbiota-Behavior

We investigated Spearman correlations between behavioral parameters and fecal microbiota diversity and phyla relative abundance (Table 37)

Behavior	Fecal microbiota	N	Correlation coefficient	p-value
% Incomplete groomings	Chao1	52	0,27	0,05
Recognition index	InvSimpson	51	0,34	0,01
	Shannon	51	0,34	0,02

Table 37 : Correlation table of behavior and fecal microbiota in C57BL/6J mice when all mice were grouped together. Green: positive correlation.

When analyzing all mice together for correlations between fecal microbiota and behavior, we found that both the proportion of incomplete grooming and the recognition index were positively correlated with α -diversity (Chao1 or Shannon and InvSimpson indexes) (Table 37).

Group	Behavior	Fecal microbiota	N	Correlation coefficient	p-value
S-A	% Incomplete groomings	Chao1	11	0,65	0,03
	Recognition index	Proteobacteria	11	0,73	0,01
A	% Mouse cylinder	InvSimpson	13	0,59	0,03
		Shannon	13	0,66	0,01
AG	% Mouse cylinder	Actinobacteriota	13	-0,66	0,01

Table 38 : Correlation table of behavior and fecal microbiota in C57BL/6J mice separated by group. Green: positive correlation, Red: negative correlation

When separating the analysis by group, in S-A, the positive correlation between proportion of incomplete grooming and Chao1 index was also significant. In this group the recognition index correlated positively with the relative abundance of Proteobacteria. In group A, the % of time interacting with “Mouse cylinder” was positively correlated with α -diversity (Shannon and InvSimpson indexes) and, in AG, it was correlated negatively with the relative abundance of Actinobacteriota (Table 38).

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2.5.2.2.4.2 Cecal microbiota-Behavior

Behavior	Microbiota	N	Correlation coefficient	p-value
% Mouse cylinder	Shannon	53	0,34	0,01
	InvSimpson	53	0,35	0,01
% Mouse cylinder	Actinobacteriota	53	-0,32	0,02
% Unknown mouse cylinder	InvSimpson	53	-0,28	0,04

Table 39 : Correlation table of behavior and cecal microbiota in C57BL/6J mice when all mice were grouped together. Green: positive correlation, Red: negative correlation

In this strain, the % of time spent interacting with the “Mouse cylinder” was positively correlated with α -diversity of the cecal microbiota (Shannon and InvSimpson indexes) (Table 39) like what was observed in fecal microbiota in group A (Table 33). This behavioral parameter was also correlated negatively with relative abundance of Actinobacteriota in cecal microbiota. However, the “% Unknown mouse cylinder” was negatively correlated with α -diversity (InvSimpson index) (Table 39).

Group	Behavior	Microbiota (ce-cal)	N	Correlation coefficient	p-value
S-A	% Time in center	Shannon	13	0,57	0,04
	Latency to 1st grooming	Bacteroidota	13	-0,66	0,01
	Latency to 1st grooming	Firmicutes	13	0,64	0,02
A	% Time in center	InvSimpson	13	-0,58	0,04
	% Mouse cylinder	Actinobacteriota	13	-0,55	0,05
AG	% Mouse cylinder	Desulfobacterota	13	0,60	0,03

Table 40: Correlation table of behavior and cecal microbiota in C57BL/6J mice separated by group. Green: positive correlation, Red: negative correlation

In the analysis separated by groups (Table 40), both in S-A and A groups, the % of time spent in the center of the OF was positively correlated with α -diversity but not according to the same indexes (Chao1 or InvSimpson). In S-A, latency before first grooming was positively correlated with relative abundance of Firmicutes and negatively with that of Bacteroidota. Finally, the % of interaction with “Mouse cylinder” was negatively correlated with Actinobacteriota in the A group, and positively with the relative abundance of Desulfobacterota in the AG group.

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2.5.3 Conclusion: Effect of FMT on brain and behavior in each strain

There were no differences between groups in microglial profile in both strains. The decrease in TPH2+ positive neurons in the raphe nuclei that was observed in BALB/c mice in A and AG groups, compared to their respective sibling groups was not present in C57BL/6J mice. The impact of the transferred microbiota on other brain markers was limited, as the only difference observed between groups was an increased expression of BDNF in the PFC of BALB/c mice from group AG compared to S-AG. Finally, while there were no differences between groups for behavior in BALB/c mice, C57BL/6J mice from group AG showed increased repetitive behaviors and impaired spatial memory compared to mice from S-AG. However, mice from group S-A showed decreased spatial memory compared to A, and mice from group S-AG showed no significant preference for the “Unknown mouse” cylinder in the social novelty test. This implies that microbiota from donors without ASD can have a negative impact on behavior in this strain.

Some of the correlations between brain markers and cecal microbiota composition were common between strains. Indeed, in both strains, expression of GFAP in the PFC correlated negatively with Actinobacteriota and 5-HT1A expression in the hippocampus positively with the relative abundance of Desulfobacterota. Also, 5-HT1A expression in the PFC, was positively correlated with α -diversity in both strains, but not always with the same indexes (Tables 29 and 35).

Some of the correlations observed between microbiota and behavior were also common between strains. Indeed, in the S-A group, latency to first grooming was positively correlated with relative abundance of Firmicutes and negatively with that of Bacteroidota, both in fecal and cecal microbiota in BALB/c and in cecal microbiota in C57BL/6J mice (Tables 32, 34 and 40).

2.6 : SUMMARY OF RESULTS AND DISCUSSION

This study brings new evidence on the effects of FMT from individuals with ASD compared to FMT from TD individuals to mice. As mentioned in the introduction, a few studies with similar approaches were recently published. Those have been summarized in table 41 at the end of this part.

In our study, the implanted gut microbiota was distinct between groups in both strains, both in composition and metabolic activity. However, the differences between groups were not all the same between strains, suggesting that the genetic background of the animals may play a role in how microbiota is implanted.

In BALB/c mice, α -diversity of the fecal microbiota, assessed by the number of ASVs or Chao1 index, was higher in group AG than in group S-AG, while in C57BL/6J mice it was higher in group A compared to S-A. Meanwhile, in this strain, InvSimpson index, was lower in group AG than S-AG both in fecal and cecal microbiota, suggesting a less diverse microbiota in this group. We also found distinct β -diversity between groups, for both strains and both in cecal and fecal microbiota. Unlike us, none of the studies included in table 41 saw differences in α -diversity, but, like us, they saw distinct β -diversity between ASD and control mice [237, 239].

We also found differences in relative abundance of some phyla and families, either specific to one strain, found in both, or opposite between strains. None of the phylum differences were specific to BALB/c mice. In C57BL/6J mice specifically, we found an increased proportion of Firmicutes in both A and AG groups compared to sibling groups and a decreased proportion of Bacteroidota in the AG group compared to S-AG, both in fecal and cecal microbiota. This is contrary to the commonly observed decrease of Firmicutes/Bacteroidota ratio in individuals with ASD [62], which is also found in patients with inflammatory bowel disease [273]. Nonetheless, an increase of Firmicutes and decrease of Bacteroidota, and/or an increase of their ratio has been found in Shank3 KO [106], MIA [123, 124] and VPA models of ASD [121–123]. In C57BL/6J mice, this increase in Firmicutes in A and AG groups is mostly due to the increased proportion of *Ruminococcaceae*. In BALB/c mice, although there were no difference at phylum level for Firmicutes, relative abundance *Ruminococcaceae* was also increased, but only in the A group compared to S-A. Interestingly, an increased proportion of this family has also been observed in MIA mice compared to WT mice [170]. Plus, the *Ruminococcus* genus, which is part of

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the *Ruminococcaceae* family, was also found to be increased in individuals with ASD [61] and in MIA and VPA models [122, 123].

The decreased proportion of Bacteroidota in the AG group of C57BL/6J mice compared to S-AG seems to be driven by a strong decrease in the relative abundance of *Prevotellaceae*. While, in BALB/c mice, there was no difference in abundance of Bacteroidota, the proportion of *Prevotellaceae* was strongly decreased in the AG group compared to S-AG, as in C57BL/6J mice. Interestingly, decreased proportion of *Prevotella*, which, in our samples, was the most abundant representant of the *Prevotellaceae* family, was reported in the microbiota of individuals with ASD, some of which having GI symptoms [126]. *Prevotella* was also reported to be decreased in Shank3 KO, MIA, and VPA animal models of ASD [117, 122, 123]. The fact that this modification is present in both strains and found in individuals with ASD and ASD models suggests that it could be an ASD related microbial signature. In addition, decreased *Prevotellaceae* have also been reported in constipated patients [274], which is interesting as children from the AG group were all constipated. Still in this phylum, we found an increased relative abundance of *Bacteroidaceae* in AG compared to S-AG in fecal microbiota of both strains. In BALB/c mice, this increase was also observed in A compared to S-A. The most prevalent genus in this family in our samples was *Bacteroides*, which was reported to be decreased in individuals with ASD [61] and some ASD models [117, 119]. However, many other studies reported increased abundance of *Bacteroidaceae* and/or *Bacteroides* in MIA, BTBR and VPA animals compared to WT [107, 108, 122–124].

Another common alteration between strains was the increased relative abundance of Actinobacteria in the A group compared to S-A, likely due to the increase of *Atopobiaceae*. Interestingly, an increased proportion of Actinobacteria has been reported in individuals with ASD [65] and in the Shank3 KO and VPA models [106, 120].

Finally, relative abundance of the Proteobacteria phylum, and its most prevalent family in our samples, *Sutterellaceae*, was increased in group AG compared to S-AG in fecal microbiota of BALB/c mice, while the opposite was observed in C57BL/6J mice. In children with ASD, the relative abundance of Proteobacteria tends to be increased [62]. In ASD models, a study found an increase of this phylum in Shank3 KO mice [106], while it was decreased in BTBR mice in another study [107]. Overall, those discrepancies between strains and models in the literature suggest that the modulations of this phylum and family are not as robust and may be influenced by the genetic

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background.

We also found some alterations in cecal SCFAs profiles. Interestingly, in both strains, the cecal proportion of propionate and branched and long chain fatty acids was decreased in the AG group compared to S-AG, while the proportion of butyrate was increased. Plus, in both strains, the proportion of branched and long chain fatty acids was increased in group A compared to S-A. The Firmicutes phylum is known to produce butyrate while the Bacteroidota phylum produces primarily acetate and propionate [275]. The changes in butyrate and propionate proportions in the AG group reflect the changes in relative abundance of those phyla. Interestingly we found a very significant positive Spearman correlation between the % of propionate and the relative abundance of Bacteroidota in both strains, while a less strong positive correlation was found between proportion of butyrate and relative abundance of Firmicutes in BALB/c mice only. In individuals with ASD, studies also reported increased levels of fecal butyrate [76] and decreased levels of fecal propionate [57] compared to TD individuals, although different studies made opposite observations [76, 87, 88]. The % of valerate, which is part of branched and long chain fatty acids, whose overall proportion was increased in group A, but decreased in group AG, was also reported to be increased in individuals with ASD [76, 88]. In addition, increased levels of cecal butyrate were found in the VPA and BTBR mouse models of ASD [108, 121].

Those differences in microbiota composition and fermentation activity led to differences between groups in terms of immune function, gut inflammation and permeability markers, the serotonin system in the gut and the brain, and behavior.

No significant difference between groups in T cell populations in the spleen was observed in BALB/c mice. However, in C57BL/6J mice, T lymphocytes populations in the spleen exhibited a more regulatory profile, characterized by a decreased Th17/Treg ratio in both A and AG groups compared to sibling groups accompanied by decreased Th1/Treg ratio and % of Th1 in AG compared to S-AG. These differences suggest reduced general inflammation in A and AG groups and contribute to the evidence of a role of the microbiota in regulation of T cell populations. Plus, in the Spearman correlation analysis grouping all mice, Th1/Treg ratio was positively correlated with α -diversity and relative abundance of Bacteroidota.

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In both strains, mice from the different groups exhibited differences in markers of inflammation in the gut. The decreased expression of TNF- α and IL-6 in the colon of BALB/c mice of group A compared to S-A suggests decreased colon inflammation. In C57BL/6J the results are more mitigated. There was a decreased expression of TNF- α in the ileum of groups A and AG compared to respective siblings, which would suggest decreased inflammation in this gut section. However, GFAP which has been found to be elevated in an inflammatory environment [264], was increased in the ileum of group AG compared to S-AG. Interestingly, in both strains, the expression of TNF- α in the ileum was positively correlated with the relative abundance of Bacteroidota and negatively with the relative abundance of Firmicutes. In BALB/c this was also the case in the colon. Interestingly, in MIA mice, TNF- α expression in the brain was also positively correlated with the relative Bacteroidota and negatively with Firmicutes [123].

Overall, while some results are contradictory, it seems like the microbiota from both ASD groups compared to sibling groups led to reduced inflammation in the colon of BALB/c mice, and systematically in C57BL/6J mice. This is consistent with the observation of increased butyrate in the caecum of A and AG groups compared to sibling groups, as this SCFA is known to have anti-inflammatory properties [276]. Interestingly, decreased markers of inflammation in the colon have also been found by Gonzales et al. (2021) in SPF mice after FMT from ASD donors compared to FMT from unrelated TD donors [238].

Aside from inflammation, the microbiota had other impacts on the gut. In BALB/c mice the caecum was enlarged in AG compared to S-AG. The elevated proportion of cecal butyrate in the AG group could be related to this enlargement, since a study in piglets reported that butyrate could induce cell proliferation in the caecum [277]. In C57BL/6J mice, there was an increase of the gene expression of TPH1 in the colon of group A compared to S-A. Interestingly, Xiao et al. (2021) also found increased TPH1 at the protein level in the colon of GF mice after FMT from ASD donors compared to FMT from unrelated TD donors [239].

In C57BL/6J mice, there was an increased expression of the gene coding for MLCK, a protein involved in tight junction reorganization, in the ileum of AG mice compared to S-AG suggesting there could be increased ileum permeability in this group. This hypothesis will be tested in a follow-up experiment using Ussing chambers to measure paracellular permeability. Interestingly, increased markers of permeability have also been found in the

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colon of SPF mice that received microbiota from ASD donors [238]. However, in the A group, the increased expression of ZO-1 in the colon compared to S-A could suggest decreased colon permeability as ZO-1 is a necessary component of tight junctions. Interestingly, in the colon, there was a positive Spearman correlation between Desulfobacterota and ZO-1, specifically in group A. Members of this phylum have been associated with increased gut inflammation [278], but to our knowledge, their implication in gut permeability is unknown.

In the brain, there was also an impact of the microbiota, but only in BALB/c mice. There was a decreased number of serotonergic neurons in the raphe nuclei, in A and AG groups compared to their respective sibling groups. This is highly interesting as TPH2 polymorphism is associated with ASD, and KO of the TPH2 gene leads to ASD-like phenotype in mice [279, 280]. In Xiao et al. (2021), protein levels of TPH2 in the PFC were increased in BALB/c GF mice that received FMT from children with ASD compared to mice that received FMT from unrelated TD donors [239]. In our study, there was also an increased gene expression of BDNF in the PFC of group AG compared to S-AG. Interestingly, expression of BDNF in the PFC was positively correlated with abundance of Desulfobacterota.

Despite the lack of effect of FMT from different groups on brain markers in C57BL/6J mice, most behavioral differences between groups were seen in this strain. FMT from group A led to decreased locomotion during the OF test. Interestingly, decreased locomotion has also been found by Sharon et al. (2019), in the offspring of C57BL/6J GF mice that received FMT from children with ASD and GI symptoms compared to FMT from unrelated TD donors [237]. In our study, FMT from group AG led to increased repetitive behaviors in C57BL/6J mice. Increased repetitive behavior has also been observed in mice or offspring of mice that received microbiota from children with ASD [237, 239]. Interestingly, repetitive behaviors were positively correlated with α -diversity, which is opposite to what has been found in BTBR mice [108]. For spatial memory, we found surprising results. Indeed, while spatial memory seemed to be impaired in the AG group compared to S-AG, mice from group A had better performances in the spatial memory test than mice from group S-A, that did not have a preference for the displaced objects. None of the studies included in table 41 analyzed this behavior, but spatial memory is impaired in some ASD models [270, 271].

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In the social novelty test, the AG groups of both strains, had a significant preference for the cylinder containing the unknown mouse, while this was not the case for mice from the S-AG groups. Only in C57BL/6J, mice from group A, had no preference for the “unknown mouse” cylinder, while the group S-A did. While the % of preference was never significantly different between groups, these findings could suggest a slightly impaired social novelty behavior in mice from group A compared to S-A in the C57BL/6J strain and, in both strains, a slightly improved social novelty behavior in mice from group AG compared to S-AG. Two of the studies presented in table 41, reported impaired social interaction behavior in mice that received microbiota from individuals with ASD compared to microbiota from TD donors [239, 241], but none of them tested social novelty behavior. Interestingly, in group A, performance in the social interaction phase was positively correlated with α -diversity, which has also been found in BTBR mice [108].

Overall, our results are not completely consistent with what has been observed in literature in similar studies. Some of the discrepancies could be due to methodological differences. Firstly, in all studies, TD donors were unrelated to the individuals with ASD [237–239]. Thus, the differences in microbiota could have been influenced by genetic and environmental differences, and not necessarily by ASD specific differences. By using siblings as controls in our study, we limited this issue as they are genetically close and live in the same environment, with similar diet. Although, regarding diet, we cannot exclude the possibility that the children with ASD displayed specific dietary preferences compared to their siblings. In addition, some of our donors with ASD received antipsychotic or anticonvulsant medication that could have altered composition of their gut microbiota [282]. Another difference is related to GI comorbidities. Xiao et al. (2021) did not mention if the donors displayed GI symptoms or not [239], which, in our opinion, should be taken in account, considering the differences in results of A and AG groups. Finally, only Xiao et al. (2021) have, like us, studied the effect of FMT directly in GF mice [239]. Sharon et al. (2019) also performed FMT in GF mice, but they studied behavior and other parameters in their offspring. In consequence, those mice did not have GF mothers and were thus exposed to bacterial products from the microbiota from the ASD donors during gestation, and then to the microbiota from birth and not later in life, which is quite different from our protocol [237]. The other study included in table 41 performed FMT on SPF animals after antibiotic depletion [238].

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To conclude, this study confirms that microbiota from individuals with ASD can impact ASD-related systemic GI brain and behavioral markers in a different way than microbiota from their siblings. Our study also brings new insights into the importance of the genetic background in the effects of FMT as it was very different depending on the strain. In addition, the fact that the microbiota and its effect on behavior and other ASD-related markers, were not the same between A and AG groups, compared to sibling groups, is interesting as none of the previous studies investigated this difference. Interestingly, microbiota from AG groups leads to markers of increased ileum permeability, although we cannot be sure of this without a functional analysis. Interestingly, the children from group AG have severe constipation and it has been shown that gut permeability was increased in constipated patients [283].

The microbiota from the A and AG group led to a less inflammatory phenotype overall, which is not what we expected, as children with ASD tend to display more inflammatory T cell profiles [151, 152] and increased gut inflammation [51] but it could mean that, in this case, their microbiota actually plays more of a regulatory role to reduce this inflammation.

In terms of behavior, the increase in repetitive behavior and decrease in spatial memory in the AG group in C57BL/6J mice shows that microbiota from this group could transfer some of the behavior commonly altered in ASD models. However, the S-A group has impaired spatial memory compared to the A group. For social behavior, the results were mitigated, as mice from group A had a worst performance than mice from group S-A in C57BL/6J mice, while the opposite was observed in both strains in mice from group AG compared to S-AG although those were minimal differences and there was no significant difference when comparing between groups.

Considering the heterogeneity due to the different strains and donor groups, we cannot conclude that the ASD microbiota in general transferred a complete "ASD-like" phenotype in mice. However, C57BL/6J mice that received AG microbiota did show some ASD-like behavioral symptoms, as well as markers of increased ileal permeability.

The use of GF mice in FMT experiments can bring some issues, as effects of the lack of microbiota in the mother during gestation and in the offspring in the first 3 weeks of life might not be reversible by recolonization at weaning. Thus, the "healthy" microbiota from S-A and S-AG group may have failed to improve those alterations. In order to draw more accurate

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conclusions from these behavioral differences, we assessed behavioral characteristics of GF C57BL/6J mice in the same experimental conditions in part 2 of this thesis.

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	Study	Sharon et al. (2019) [237]	Gonzales et al. (2021) [238]	Xiao et al. (2021)[239]	Our study			
Protocol	Species, strain n per group	GF Mice (C57BL/6J) n=85-121	SPF Mice (C57BL/6J) ABX depletion n=12	GF Mice (BALB/c) n=16	GF Mice (BALB/c) n=9-13		GF Mice (C57BL/6J) n=13-14	
	Pooled microbiota or not	No	No	Yes	Yes			
	GI symptoms in donors	Yes	No	?	No	Yes	No	Yes
	Control group	Unrelated TD donors	Unrelated TD donors	Unrelated TD donors	TD siblings			
α -diversity		No \neq	---	No \neq	No \neq	\nearrow	\nearrow	\searrow
β -diversity		\neq	---	\neq	\neq	\neq	\neq	\neq
Behavior	Locomotion	\searrow	---	---	No \neq	No \neq	\searrow	No \neq
	Anxiety	No \neq	---	\nearrow	No \neq	No \neq	No \neq	No \neq
	Stereotypy	\nearrow	---	\nearrow	No \neq	No \neq	No \neq	\nearrow
	Sociality	\searrow	---	\searrow	No \neq	\nearrow^*	\searrow^*	\nearrow^*
	Memory	---	---	---	No \neq	No \neq	\nearrow	\searrow
Other parameters	General inflammation	---	---	---	No \neq	No \neq	\searrow	\searrow
	Gut inflammation	No \neq	\searrow	---	\searrow	No \neq	\searrow \nearrow	\searrow
	Gut permeability	No \neq	\searrow	---	No \neq		\nearrow	\searrow
	Trp metabolism	---	---	\nearrow Cecal Trp and KA	No \neq	No \neq	No \neq	No \neq
	Gut 5-HT system	---	---	\nearrow TPH1, \searrow SERT and 5-HT1A in colon	No \neq	No \neq	\nearrow TPH1 in colon	No \neq
	Brain 5-HT system	---	---	\nearrow TPH2 and SERT	\searrow TPH2+ neurons	\searrow TPH2+ neurons	No \neq	No \neq
	Neuroinflammation	---	---	---	No \neq	No \neq	No \neq	No \neq
	Brain BDNF mRNA	---	---	---	No \neq	\nearrow PFC	No \neq	No \neq

Table 41 : Table detailing methods and results of studies of FMT from human individuals with ASD to mice (GF or SPF, with or without depletion). Arrows represent parameters increased/improved (\nearrow) or decreased/impaired (\searrow) in ASD group compared to TD group. \neq : Difference between groups, No \neq : Parameter measured but no difference was found, ---=parameter not measured in this study. \nearrow^* or \searrow^* = group comparison not significant but one group and not the other shows preference in social novelty

3 PART 2: COMPARISON OF GF AND CONVENTIONAL MICE BEHAVIOR AND GUT PERMEABILITY

3.1 INTRODUCTION-CHARACTERISTICS OF GF ANIMALS

3.1.1 Use of GF mice in microbiota research

The concept of GF animals has first been introduced by Louis Pasteur in 1885 who hypothesized that GF life was impossible. However, in following years, researchers have started trying to produce GF animals starting with guinea pigs and chickens and, in the 1940s and 1950s, the first GF mice and rats were successfully developed [284].

Since then, GF mice have been used to study the implication of the gut microbiota in various physiological functions. Indeed, comparing GF and SPF mice of the same strains allows to test whether a physiological parameter or pathological symptom is impacted by the presence of micro-organisms in the body.

Those studies have found that GF mice have morphological differences of the GI tract, an immature immune system and reduced fat and skeletal muscle volume [17, 285–292]. In addition, a few studies reported altered behaviors and brain function in GF animals compared to SPF [3, 4, 6–17, 20–22]. This proves that the microbiota plays an active role in those functions throughout life.

In this short introduction I will provide a brief overview of the GI and systemic alterations observed in GF mice and a more exhaustive list of behavioral and brain alterations in those animals.

3.1.2 Brief overview of intestinal and systemic characteristics of GF mice/rats

One of the early observations made on GF rodents is the morphological and functional differences of their GI tract. The most obvious difference is the hypertrophied caecum of GF mice or rats, containing a large volume of almost liquid cecal content. Early reports also found reduced gut motility and impaired bile acid secretion in GF animals compared to conventional

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ones [285]. More recent studies report other morphological alterations of the GI tract, such as altered morphology of villi and crypts and shorter small intestine [284, 293]. Functional alterations of the endocannabinoid system has also been observed in the gut of GF mice compared to SPF which can lead to differences in regulation of gut motility, inflammation, or neuropeptide release [286, 287]. Plus, two studies found decreased colonic 5-HT in GF vs SPF mice, accompanied by decreased gene expression of TPH1 and increased expression of SERT [195, 196]. Finally, paracellular permeability of the colon epithelium was found to be decreased in GF mice compared to SPF [293].

Many studies report immune alterations in GF mice, such as impaired cytokines profile (basal or following immune activation), imbalance of T cell profiles (TH1/TH2) and morphological alterations of lymphatic organs. Plus, specifically in the gut, GF mice lack Th17 cells and have a thinner mucosal layer [288, 289, 291].

In addition, GF mice have reduced adiposity compared to SPF mice. This is accompanied by lower circulating levels of insulin and leptin (hormones that decrease appetite) and hypersensitivity to those hormones. Plus, in the hypothalamus and brainstem, GF mice show dysregulation of neuropeptides involved in appetite and adiposity regulation [290, 292].

Finally, a recent study found that GF mice displayed reduced skeletal muscle mass, and alteration of gene expression in skeletal muscles that indicate atrophy and reduced muscle strength compared to SPF. In addition, the glucose metabolism was impaired in the liver and muscle and oxidative metabolism was also impaired in muscle cells of GF mice. This was accompanied by reduced spontaneous locomotor activity and grip strength in GF mice compared to SPF [17].

In many cases, those changes are reversible when mice are recolonized with SPF microbiota or specific beneficial bacterial species [17, 194, 287, 292, 293], which definitively proves that the microbiota plays a direct and active role in those functions.

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3.1.3 Brain and behavior of GF mice/rats

Remark: All the brain and behavioral alterations presented in this part are regrouped in tables 42 and 43 (p 181 and 184).

3.1.3.1 Brain alterations

Many studies report differences in markers of different brain functions between GF and SPF rodents.

Firstly, GF mice display alterations in general brain functionality markers. Three studies [3, 6, 21], found decreased BDNF gene expression or protein levels in the hippocampus of GF mice (BALB/c, Swiss Webster, NMRI) . Decreased gene expression of BDNF gene was also found in the amygdala of GF NMRI [6] and Swiss Webster mice [15] compared to SPF. Decreased hippocampal BDNF and other neurotrophic factor levels have been observed following chronic stress [294], thus confirming an altered stress regulation in GF mice. However a different study found increased BDNF gene expression in the dentate gyrus of Swiss Webster GF mice (not subjected to any stress) [4]. In NMRI mice, the levels of neural growth factor in anterior olfactory region and orbital frontal cortex were decreased in GF compared to SPF, suggesting impaired plasticity in those regions [6]. Differences of neuronal activation characterized by cFOS expression in the brain of GF vs SPF mice have also been found. Compared to SPF, GF C57BL/6J mice have increased cFOS positive neurons in the basolateral amygdala and decreased cFOS positive neurons in infralimbic cortex, which are regions involved in fear regulation [12].

As briefly mentioned in the general introduction, the pioneer paper that described altered brain function in GF mice is Sudo et al in 2004 [3]. This study described increased blood levels of adrenocorticotrophic hormone and corticosterone following an acute psychological stress (environment change). Increased corticosterone after acute stress in GF rodents compared to SPF was found in four other studies in Swiss Webster, NMRI and C57BL/6J mice and in F344 rats [4, 7, 13, 21]. Interestingly, this was reversed by recolonization with SPF microbiota in two of those papers [3, 13]. In addition, increased levels of corticotropin releasing hormone in the hypothalamus was found both in BALB/c mice and F344 rats compared to SPF animals [3, 7]. Plus, decreased levels of glucocorticoid receptor were found in the cortex

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of GF BALB/c mice [3], and in two regions of the hippocampus (dentate gyrus and CA1) in GF F344 rats compared to SPF [7]. All those results show that the lack of microbiota leads to an overactivation of the HPA axis in response to stress.

NMDA receptors are glutamatergic receptors and are thus involved in neuronal activation. Decreased levels of NMDA receptor subunits, NR2A in hippocampus and NR-1 in hippocampus and cortex, were found in GF BALB/c mice compared to SPF [3]. In a different study, NR2B expression was decreased in the central amygdala of GF Swiss Webster mice compared to SPF [4]. A decreased proportion of those receptors suggests impaired function of those brain regions.

The 5-HT system in the brain of GF rodents is also different to that of SPF ones. 5-HT_{1A} protein levels were decreased in the dentate gyrus of GF Swiss Webster mice [4]. Decreased 5-HT was found in the hippocampus of GF F344 rats compared to SPF [7]. However, concentration of 5-HT and its main metabolite 5-HIAA was increased in the hippocampus of GF Swiss Webster mice compared to SPF. Plus in those GF mice, serotonin transporter (SERT) expression was decreased in the hippocampus [21]. In BALB/c mice, the ratio of 5-HIAA/5-HT, which reflects 5-HT turnover, was decreased in the striatum of GF mice compared to descendants of GF mice recolonized with SPF microbiota, suggesting less 5-HT use in this region, while in the brainstem, levels of 5-HIAA were increased in GF mice [14]. In GF NMRI mice, 5-HT turnover in the striatum was increased compared to SPF mice [6]. Overall, this indicates that the microbiota has an impact on brain 5-HT metabolism, but this impact is different depending on the strain and brain region studied.

There are also differences in levels of other monoamines in the brain of GF rodents. For context, DA is derived from tyrosine or phenylalanine and can be metabolized into 3,4-dihydroxyphenylacetic acid (DOPAC) which, in turn, can be metabolized into homovanillic acid (HVA). But DA can also be metabolized into HVA through another pathway (Fig 52). Norepinephrine (NE) is derived from dopamine and can be metabolized into 3-methoxy-4-hydroxyphenylglycol (MHPG) by most of the same enzymes involved in DA degradation (Fig 52).

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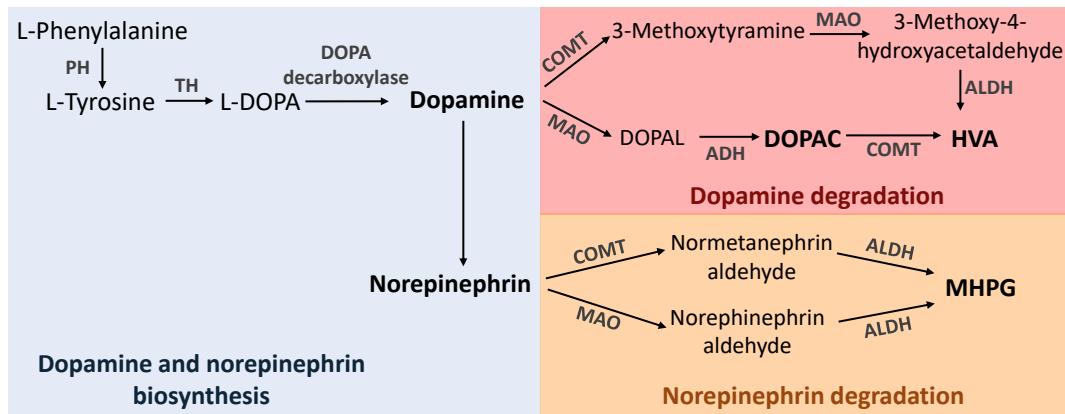


Figure 52 : Biosynthesis and degradation pathways of dopamine and norepinephrin. Figure inspired from Zahoor et al. (2018) Chap.7 Fig 1 [295] and Chen et al (2015) Fig 1 [296]. ADH=Alcohol deshydrogenase; ALDH= Aldehyde deshydrogenase; COMT=Catechol-O-methyltransferase; DOPAL=3,4-Dihydroxyphenylacetaldehyde; DOPAC=3,4-Dihydroxyphenylacetic acid; HVA=Homovanillic acid; L-DOPA = L-Dihydroxyphenylalanine; MAO=Monoamine oxidase; MHPG=3-methoxy-4-hydroxyphenylglycol; PH=Phenylalanine hydroxylase ; TH=Tyrosine hydroxylase.

In GF NMRI mice, DA and NE turnovers (DOPAC/DA and MHPG/NE ratios) were increased in the striatum [6]. Plus, the expression of dopaminergic receptor 1 was increased in the hippocampus [6]. However, somewhat contradictory observations were made in GF BALB/c mice and F344 rats. In BALB/c mice, DA turnover (DOPAC/DA and/or HVA/DA) was decreased in the PFC and the striatum of GF mice compared to the descendant of GF mice recolonized with SPF microbiota. GF BALB/c mice also displayed decreased NE turnover (MHPG/NE) in the brainstem [14]. Similarly, in GF F344 rats, DA turnover (HVA/DA) was decreased in the frontal cortex, hippocampus, and striatum compared to SPF rats. In addition, DOPAC and HVA concentrations were decreased in the frontal cortex of GF stressed rats compared to SPF stressed rats [7]. This shows that microbiota can impact the dopaminergic and norepinephrinergic systems, in different ways depending on the strain.

In addition, two recent studies reported changes in brain oxytocin of GF rodents compared to SPF. In Wu et al. (2021) the number of oxytocin positive cells was decreased in the paraventricular nucleus and bed nucleus of stria terminalis of GF C57BL/6J mice compared to SPF [13]. Plus, oxytocin receptor binding was increased in many regions of the olfactory cortex in

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GF F344 rats compared to SPF [19]. Interestingly, in the olfactory cortex and the PVN, oxytocin signaling can promote social interaction [297, 298], while in the bed nucleus of stria terminalis, it is involved in cued fear learning [299]. Both those behaviors have been found to be altered in GF mice (detailed in the next part).

Two studies reported increased microglial density and differences in microglial profile in the brain of GF C57BL/6 mice. In adult mice, Erny et al. (2015) found an increased number of microglial cells, whose gene expression pattern suggested immature microglia that tend to stay in a steady state. Plus, when triggering immune activation with LPS or a virus, microglia of GF mice did not display the morphological changes or gene upregulations that microglia of SPF mice display in those conditions [16]. Thion et al. (2018) found sex and age specific differences. They observed an increase in microglial cells in embryonic brains (in both male and females) and adult female brains. Plus, the microglia had more ramifications in GF embryonic brains compared to SPF. Some differences in microglial gene expression were also found between GF and SPF in embryonic brain of males and adult brains of females, suggesting that the microbiota impacts microglia at different stages depending on the sex of the animal [11].

Finally, Braniste et al. (2014) reported increased BBB permeability in the adult brain and decreased expression of tight junction proteins in the embryonic and adult brain of GF mice compared to SPF [8].

Overall, these findings show that the brain of GF rodents present general functional differences, an overactivated HPA axis, alteration of various neurotransmitter systems, immature and under reactive microglia, and increased BBB permeability. Nonetheless, the precise nature of those alterations is highly dependent on the strain of the studied model.

3.1.3.2 Behavior

Note: The principles of the behavioral tests used in these studies and not otherwise described in the material and methods of part 1 are described in Annex 3 p. 254)

While the pioneer paper by Sudo et al. (2004) [3] did not investigate behavior, many of the following studies did, and five of them found decreased anxiety in GF mice compared to SPF (Swiss-Webster, NMRI and BALB/c) assessed by the light/dark box (L/D box), elevated plus maze (EPM)

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and/or OF test [4, 6, 10, 13, 21] . Interestingly, in Clarke et al. (2012), this behavior alteration was reversed by colonization with SPF microbiota [21]. Plus, Luk et al. (2018) found decreased anxiety in the EPM in Swiss Webster GF mice compared to ex-GF mice of the same strain that had been colonized at birth with SPF microbiota or with *Bifidobacterium* species. This shows that a complex microbiota is not necessary to restore this behavior [18]. However, increased anxiety in the OF test was observed in GF BALB/c and C57BL/6J mice and F344 rats compared to SPF [7, 9, 14].

Impaired social behavior in GF animals has been described in Swiss Webster and C57BL/6J mice as well as F344 rats in either the three-chamber test or the reciprocal social interaction test compared to SPF and/or to ex-GF mice colonized with SPF microbiota [7, 9, 13, 18, 20, 22]. However, Arentsen et al. (2015) reported better performance in the three-chamber social novelty test in GF Swiss Webster mice compared to SPF [15].

GF C57BL/6J and BALB/c mice had impaired fear extinction learning (see Lexicon in Annex 3 p.254) characterized by increased % of time spent freezing in the cued extinction phase (mice in the shock chamber with the cued sound) compared to SPF in two studies [9, 12]. In Lu et al. (2018), GF C57BL/6J mice spent less time freezing in a contextual extinction phase (mice in the shock chamber but without the shocks or associated sound) suggesting that the learning impairment of GF mice in this test is specific to cued memory. In addition, GF mice in this study also showed impaired spatial memory in the Morris water maze test [9]. Interestingly, in Chu et al. (2021) the fear extinction learning impairment was reversed by fostering of GF pups by SPF mothers, but not by recolonization with SPF microbiota after weaning [12]. Memory was also impaired in GF Swiss Webster mice in the novel object test in comparison to ex-GF mice colonized at birth with microbiota from SPF mice. Gnotobiotic colonization with *Bifidobacterium* species also improved memory but only in female mice [18].

Luo et al. (2018) reported decreased resignation behaviors in GF BALB/c mice characterized by a decreased latency to feed in novelty suppressed feeding test (NSFT) and by decreased immobility in forced swimming test (FST) compared to SPF. The behavioral alteration in the FST was reversed by administration of LPS to GF mice [10].

Two studies have investigated repetitive behaviors in GF or SPF BALB/c and Swiss Webster mice but reported no differences [14, 15].

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Finally, decreased mobility of C57BL/6J GF mice has been observed in three studies [9, 13, 17], while other studies have found increased mobility in NMRI, BALB/c and Swiss Webster GF mice compared to SPF or GF mice colonized with SPF microbiota [10, 14, 15, 18, 21]. Other studies assessed locomotor activity [7, 20] but saw no differences between GF and SPF animals (C57BL/6J mice or F344 rats).

Because of these mobility differences, the decreased anxiety observed in Swiss Webster mice [15] and increased anxiety observed in C57BL/6J mice [9] can be debatable. Indeed, it is possible that the difference in time spent in the center of the OF are due to the mobility differences, and not to a difference in anxiety.

3.1.4 Conclusion

GF rodents have an altered immune system, gut function, and metabolism but also display several brain alterations, namely in the HPA axis, monoaminergic and glutamatergic systems, neuroimmune system and BBB permeability. However, the precise nature of those dysregulations varies according to the genetic background and, sometimes, in the same strain depending on the study. GF rodents also display multiple behavioral differences compared to SPF, in locomotor activity, anxiety, social behavior, fear regulation and memory. Again, those alterations vary depending on the strain. This shows that the microbiota is at interplay with the genetic background in regulation of brain function and, in turn, of behavior.

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Table 42 : Brain alterations observed in GF mice and rats compared to their SPF counterparts. #=Alteration or behavior restored, at least partially, by colonization with SPF microbiota, specific bacterial strains or LPS Abbreviations: ACTH= Adrenocorticotrophic hormone, AO= Anterior olfactory region, BNST= Bed nucleus of stria terminalis, BLA= Basolateral amygdala, CRF= Corticotropin-releasing factor, DG=Dentate gyrus, DRD1A= Dopamine D1 receptor 1 A ,GR=Glucocorticoid receptor, IL=Infralimbic cortex, Prefrontal cortex, PVN=Paraventricular nucleus, SERT=Serotonin transporter. NGF=Nerve growth factor, NR-1 or NR2A or NR2B=NMDA receptor subunits, OFC=orbital frontal cortex OR=oxytocin receptor, PFC= Prefrontal cortex

Study	Species	Strain	Sex	Age	HPA axis	Neurotrophic factors	Serotonin	Other monoamines	Others
Sudo et al. 2004 [3]	Mice	BALB/c	M	9 weeks	↑blood ACTH and corticosterone after acute stress* ↑CRF in hypothalamus (PVN) and ↓GR in PFC	↓BDNF concentration in hippocampus			↓NR1 and NR2-a concentration in cortex and/or hippocampus
Neufeld et al. 2011 [4]	Mice	Swiss Webster	F	8 weeks	↑blood corticosterone after acute stress	↑BDNF expression in hippocampus (DG)	↓5-HT1A expression in hippocampus (DG)		↓NR2B in central amygdala
Heijtz et al. 2011 [6]	Mice	NMRI	M	8-10 weeks		↓BDNF expression in BLA and hippocampus (CA1) ↓ NGF expression in OFC and AO	↑5-HT turnover in striatum	↑NE and DA turnover in striatum, ↑DRD1A expression in hippocampus (DG)	↑ Synaptophysin and PSD-95 expression in striatum
Clarke et al. 2013 [21]	Mice	Swiss Webster	F and M	6-9 weeks	↑blood corticosterone after acute stress	↓BDNF expression in hippocampus	↑5-HT and 5-HIAA, and ↓SERT expression in hippocampus		

Table continues on the next page.

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Study	Species	Strain	Sex	Age	HPA axis	Neurotrophic factors	Serotonin	Other monoamines	Others
Nishino et al. 2013 [14]	Mice	BALB/c	M	16 weeks			↓5-HT turnover in striatum ↑5-HIAA in brainstem	↓NE turnover in brainstem, ↓DA turnover in PFC and striatum	
Crumeyroille-Arias et al. 2014 [7]	Rats	F344	M	11 weeks	↑blood corticosterone after acute stress, ↑CRF in hypothalamus (PVN) and ↓GR in hippocampus (CA1 and DG)		↓5-HT in hippocampus	↓DA turnover in PFC, hippocampus and striatum. ↓ DOPAC and HVA in PFC after acute stress	
Braniste et al. 2014 [8]	Mice	NMRI C57BL/6J	F and M	Embryon and 8-10 weeks					↓occludin, claudin-5 in embryonic and adult PFC, and adult striatum and hippocampus ↑ BBB permeability in adult#
Arentsen et al. 2015 [15]	Mice	Swiss Webster	M	12 weeks		↓BDNF expression in amygdala			
Erny et al. 2015 [16]	Mice	C57BL/6	F and M	6-10 weeks					↑Nb of microglial cells in adult brain. Gene expression profile suggesting immature microglia. Blunted immune response of microglia after immune activation

Table continues on the next page.

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Study	Species	Strain	Sex	Age	HPA axis	Neurotrophic factors	Serotonin	Other monoamines	Others
Lu et al. 2018 [9]	Mice	C57BL/6J	F and M	4 and 12 weeks					Structural brain differences, \searrow cell number in corpus callosum
Luo et al. 2018 [10]	Mice	BALB/c	M	8 weeks					\nearrow genes of the GR pathway in hippocampus
Thion et al. 2018 [11]	Mice	C57BL/6	F and M	Embryo and 8-9 weeks					\nearrow microglial density in embryonic and adult brain, excessive ramifications in embryonic brains. Different expression profile of microglial genes in embryonic and adult brains (sex dependent)
Chu et al. 2019 [12]	Mice	C57BL/6J BALB/c	M	7-16 weeks					\nearrow cFOS+ neurons in BLA, \searrow CFOS + neurons in IL \searrow PSD-95 in PFC \searrow Phenolic metabolites in cerebro-spinal fluid #
Effah et al. 2020 [19]	Rats	Fischer	M	1 and 4 days					\searrow OR binding in the eyes of newborn male and female rats and \nearrow OR binding in various regions of the olfactory nuclei
Wu et al. 2021 [13]	Mice	C57BL/6J	M	11-15 weeks	\nearrow blood corticosterone after social encounter and acute stress#				\searrow oxytocin positive cells in PVN, BNST and hippocampus (DG)

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Table 43 : Behavioral alterations observed in GF mice and rats compared to their SPF counterparts. ⁽¹⁾ = in this study, GF mice were compared to ex-GF mice that had been recolonized at birth by SPF microbiota; #=Alteration or behavior restored, at least partially, by colonization with SPF microbiota, specific bacterial strains or LPS; ~ =Potentially biased because of the reduced or increased mobility by itself could explain the reduced or increased distance or time in center of OF. Abbreviations: EPM=Elevated plus maze, FST=Forced swim test, L/D box= Light/dark box, NSFT=Novelty suppress feeding test, OF=Open field.

Study	Species	Strain	Sex	Age (week)	Mobility	Anxiety	Resignation	Social behavior	Memory	Fear conditioning
Neufeld et al. 2011 [4]	Mice	Swiss Webster	F	8		↘ EPM				
Heijtz et al. 2011 [6]	Mice	NMRI	M	8-10	↗ OF	↘ L/D box, EPM				
Clarke et al. 2013 [21]	Mice	Swiss Webster	F and M	6-9		↘# L/D box				
Nishino et al. 2013 [14]	Mice	BALB/c	M	7, 10 and 16	↗ OF	↗ OF				
Arentsen et al. 2015 [15]	Mice	Swiss Webster	M	12	↗ OF	↘~ OF		↗ 3-chamber interaction test		
Crumeyrolle-Arias et al. 2014 [7]	Rats	F344	M	11	→ OF	↗ OF		↘ (Slightly) Reciprocal social interaction		
Desbonnet et al. 2014 [22]	Mice	Swiss Webster	M	8				↘ # 3-chamber interaction and novelty test		

Table continues on the next page.

Part 2- Comparison of GF and CV mice

Study	Species	Strain	Sex	Age (week)	Mobility	Anxiety	Resignation	Social behavior	Memory	Fear conditioning
Buffington et al. 2016 [20]	Mice	C57BL/6J	F and M	7-12	→ 3-chamber test			↘# 3-chamber interaction and novelty test		
Lu et al. 2018 [9]	Mice	C57BL/6J	F and M	4 and 12	↘ OF (at 4 weeks only) Morris water maze (in males at 12 weeks only)	↗ ~ OF (at 4 weeks only)		↘ 3-chamber social novelty (at 12 weeks only)	↘ Morris water maze (at 12 weeks only)	Impaired (at 12 weeks only)
Luo et al. 2018 [10]	Mice	BALB/c	M	8	↗ OF	↘ OF	↘ FST#, NSFT			
Luk et al. 2018⁽¹⁾ [18]	Mice	Swiss Webster	F and M	6-7	↗ # OF (in females only)	↘ # EPM		↘ # 3-chamber social novelty (females only)	↘ # Novel-Object	
Chu et al. 2019 [12]	Mice	C57BL/6J BALB/c	M	7-16						Impaired #
Lahiri et al. 2020 [17]	Mice	C57BL/6	M	6-8	↘# OF					
Wu et al. 2021 [13]	Mice	C57BL/6J	M	11-15	↘ 3-chamber test	↘ OF EPM L/D box		↘ # Reciprocal social interaction		

Part 2- Comparison of GF and CV mice

3.2 STUDY DESIGN AND OBJECTIVE

Objective:

A few studies in the past decade investigated behavior and other parameters in GF rodents, but there are contradicting results between strains, and even among studies on the same strains. Plus, there is only little evidence on the impact of GF status on cognition and repetitive behaviors. Finally, we cannot be sure that those alterations would be the same in our experimental conditions.

As we used originally GF mice in the FMT experiment, we aimed to study ASD-related behavior and other ASD-related parameters in both GF strains used in the FMT experiment, in the same experimental conditions. This could allow better interpretation of our results from the previous experiment.

The experiment was carried out on both strains, to characterize potential differences between them. Unfortunately, the isolators of GF BALB/c mice became contaminated due to a leak during the first week of the behavioral experiments. For this reason, those mice could no longer be considered GF and thus only C57BL/6J mice were considered in the study.

Study design:

We characterized the behavior of C57BL/6J mice in the same tests of anxiety, social behavior, stereotyped behaviors, and spatial memory, as the ones used in the FMT study as well as in two additional tests measuring anxiety and repetitive behaviors. We also aimed to study some of the ASD related intestinal, systemic and brain markers that were investigated in the FMT study. However, those analyses are still ongoing. Thus, the results presented in this thesis are only those of intestinal permeability and behavior.

The GF mice used in this experiment came from a local colony of the Anaxem GF animal facility. We decided to create a conventional (CV) colony by recolonizing GF progenitors from this same animal facility with microbiota from SPF mice and breeding them for 2 generations. This prevents potential genetic differences between the SPF and GF groups, which might have occurred if we had bought SPF mice from a conventional rodent provider. Indeed, we cannot rule out a genetic drift in the Anaxem GF colony, as it has been established several years ago.

Part 2- Comparison of GF and CV mice

GF mice, and the second generation of conventional mice coming from recolonized GF progenitors (called CV mice in the rest of the manuscript), were transferred to identical isolators at 4 weeks of age to avoid any environmental differences that could impact behavior. Mice were raised in those isolators until adult age, when behavioral tests were performed in the behavioral compartment of the isolators. This ensured that the GF mice stayed GF for the entirety of the tests and prevented the potential impact of a drastic environmental change on behavior.

3.3 MATERIALS AND METHODS

3.3.1 Animals

Each group was composed of 15 C57BL/6JCrI mice either GF or conventional (CV) (Anaxem facility, Micalis Institute, Jouy-en-Josas, France). To obtain CV mice with a similar genetic background as the GF one, a F0 generation of GF mice was colonized with fecal microbiota from sex- and age- matched SPF mice from the same strain (Charles River, Ecully, France) first by putting fecal pellets in their cages every 2 days for 3 weeks, then through one oral administration by having the mice suck on a pipette containing a suspension of 5 pellets in 1.5 mL of PBS 1X. These F0 conventionalized mice were bred to obtain successively F1 and F2 generations. The F2 generation was used in the study as the CV group. GF mice were born in breeding isolators, and F1 and F2 CV mice in a conventional breeding room where there were no other mice. All mice were transferred to identical (but separate) experimental isolators at around 4-5 weeks of age to avoid environmental bias. As was done for the FMT experiment, 4 or 5 additional male mice of the same strain and bacterial status were also brought into the isolators at the beginning of the experiment to serve as unknown mice for the social interaction test. The living conditions of the mice were the same as previously described.

To ensure that the isolator of the GF mice was sterile at the beginning of the experiment, and stayed sterile for the duration of it, drinking water and fecal pellets were collected weekly, starting two weeks prior to the start of the experiment (first from sentinel mice and then from the experimental mice). These samples were observed under an optical microscope and cultured in various media at different temperatures (see table 44 for details) to screen for the presence of any contaminant microorganisms (bacterial or fungal).

Part 2- Comparison of GF and CV mice

Type of culture medium	Temperature	Media	Type of sample
Liquid	37°C	LB, LCY	Feces
	30°C	LB, LCY, Sabouraud	Feces and drinking water
	22°C	LB, LCY, Sabouraud	Drinking water
Agar	37°C	BHI, GVF	Feces

Table 44 : Details of the media and temperature of incubation to check GF status. LB=Luria Bertani, BHI=Brain heart infusion GVF=Gélose Viande Foie. Sabouraud liquid medium was bought ready-made from Biomerieux (Craponne, France). The other media were prepared locally in the Micalis Institute. Detailed compositions of the media are in Annex 4 p. 257.

At the end of the experiment, animals (13 weeks old) were weighed and euthanized by decapitation. Their caecum, spleen and adrenal glands were dissected and weighed. Those tissues, as well as sections of ileum and colon, brain and blood, were collected and stored for future analyses. Another portion of the colon was also collected and used for measure of permeability using Ussing chambers.

3.3.2 Intestinal permeability (Ussing chambers):

At euthanasia, a fresh 2 cm segment of colon was collected from 8 mice chosen at random in each group. It was put in cold 1X Dulbecco/Vogt modified Eagle's minimal essential medium (DMEM) containing L-Glutamine and devoid of L-glucose and sodium pyruvate (Gibco, Fisher Scientific). It was then put on a glass plate covering a box filled with ice and opened along the mesenteric border. The sample was then mounted in Ussing chambers (Physiologic Instruments, San Diego, USA) in a 0.1 cm² slider (Fig 53).

Part 2- Comparison of GF and CV mice

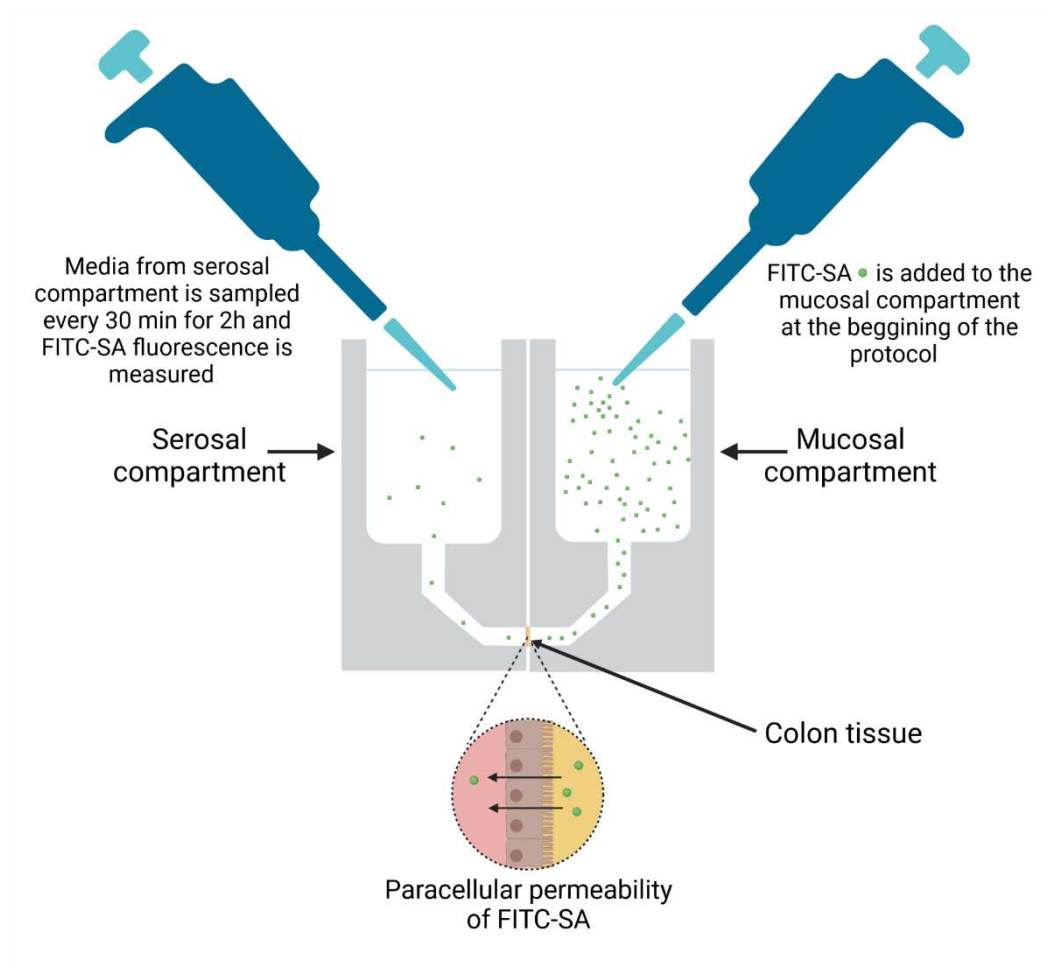


Figure 53: Schematic representation of measurement of paracellular permeability of colon tissue using Ussing Chambers.

The mucosal side was exposed to oxygenated Krebs-mannitol buffer (10 mM) and the serosal side to oxygenated Krebs-glucose buffer (10 mM) maintained at 37°C. Fluorescein isothiocyanate-sulfuric acid (FITC-SA) (Thermo Fisher Scientific, France) that has a molecular weight of 400 Da was used as a marker of paracellular permeability of tight junctions. 40 µg/mL of FITC-SA was added to the mucosal chamber, and 100 µL of buffer from the serosal chamber was collected every 30 min, during 2 h. FITC-SA concentration was measured by fluorescence (485 nm excitation, 538 nm emission). Paracellular permeability was defined as the flux of FITC-SA expressed as ng/cm²/h. The FITC-SA flux at each timepoint was used for statistical analysis.

Part 2- Comparison of GF and CV mice

3.3.3 Behavioral tests

All behavioral tests were performed in the behavioral compartment of the isolator in the morning (after 9 am) up to early afternoon (no later than 3 pm). The tests were performed by experimenters who had been regularly involved in handling of the mice throughout the protocol so that the animals were used to them. To avoid experimental bias, behavioral tests were conducted simultaneously in the GF and CV isolators by two experimenters. The animals from each group were evenly distributed between the two experimenters. Figure 54 details the calendar of the behavioral experiments.

OF, spatial object recognition, social interaction and novelty tests were performed in the same way as described previously. The self-grooming test was also performed in the same way as before, adding the assessment of the number of rearings (i.e mouse standing on its hindlegs to look at its environment) as an additional indicator of anxiety-like behavior [300]. It was easier to count this behavior in this test, as the camera was filming the animals from the side, and closer than in the other tests (filmed from the top). Plus, two additional tests were performed, which are detailed below.

GF vs CV experiment

Week 1							Week 2						
Mon	Tue	Wed	Thu	Fri	Sat	Sun	Mon	Tue	Wed	Thu	Fri	Sat	Sun
Open-field and Spatial object recognition							Social interaction and novelty			Self-grooming			

Week 3						
Mon	Tue	Wed	Thu	Fri	Sat	Sun
Step-down test		Marble burying				

Figure 54 : Calendar of the behavioral tests- CV vs GF experiment

3.3.3.1 Anxiety-like behavior: Step-down test

Mice were placed on a platform 12.5 x 9.5 cm wide and 4.0 cm high, located in the center of the behavior isolator. The latency for the mouse to step-down from the platform was measured. Once the mouse stepped down, or after a maximum duration of 5 min without stepping down, it was returned to its cage (after being left to explore on the floor around the platform for 5 s). The test was repeated three times with a 1-min delay between each run on the platform during which the mouse was back in its home cage. A longer

Part 2- Comparison of GF and CV mice

time spent on the platform reflects higher anxiety. The average latency to get off between the 3 trials was used for statistical analysis.

Reference: Anisman et al. (2001) [301]

3.3.3.2 Stereotyped behavior: Marble burying test

Mice were placed in an empty clean cage with 4 cm of clean litter mixed with some litter from the home cage, on the surface of which were placed 3 rows of 4 marbles (1.5 cm in diameter) (Fig 55). Mice were left in this cage for 30 min, during which the number of marbles buried (defined as two thirds of the marble not visible) was manually recorded every 5 min during the test by two experimenters that each analyzed half of the animals in each group. Two mice were performing the test at the same time in each isolator, separated by an opaque wall so that they could not see each other (Fig 55). A picture was taken before and after each test. The number of marbles buried at each timepoint over 30 min was used for statistical analysis. It is considered that mice that show repetitive behaviors will bury more marbles during the test.

Reference: Fournet et al. (2012) [302]

Part 2- Comparison of GF and CV mice



Figure 55 : Representation of the marble burying test A) Schematic of the marble burying test (created on Biorender.com) B) Picture of the test cages before and after 30 min of test. Circled in yellow are the marbles not buried after 30 min. Here, animal 1 buried 3 out of 12 marbles and animal 2 buried 8 out of 12 marbles.

3.3.4 Statistical analysis

Some of the data did not follow normal distribution (Shapiro-Wilk test) or had unequal variances between groups (Fischer test). Therefore, for consistency in the statistical analysis, we decided to compare all data using non-parametric tests and to represent them on graphs as individual values with median or just medians for analysis through time (marble burying test and Ussing chamber). Comparisons between the two groups (CV and GF) were performed using a Mann-Whitney test, for analysis through time each timepoint was analyzed separately. Comparison to a theoretical value (for social interaction test) were performed using the Wilcoxon signed rank test. The level of significance was set at $p < 0.05$. Calculations were performed with the GraphPad Prism software (version 7.03, La Jolla, CA, USA). Outliers were identified using the "Identify outliers" function in the GraphPad Prism software with a ROUT value of 1%. All values identified as outliers were removed from the statistical analysis.

Part 2- Comparison of GF and CV mice

3.4 RESULTS : COMPARISON OF GF AND CV C57BL/6J MICE

3.4.1 Body and organ weight and intestinal permeability:

As GF mice are known to have some physiological differences compared to CV mice, the most striking being the enlarged caecum [285], we have measured mouse weight and weight of full and empty caecum. As expected, the GF mice had very significantly enlarged caeca, characterized by an increased weight of cecal content ($p < 0.0001$) (Fig 56 B) and cecal wall ($p < 0.0001$) (Fig 56 C) compared to CV. The total mouse weight was increased in GF mice compared to CV ($p = 0.005$) (Fig 56 A), but this difference was due to the extra weight of the cecal content, as it was no longer observed when cecal content weight was removed from total weight (Fig 56 D).

As previously mentioned, GF mice have an overactive HPA axis and disturbed anxiety like behavior. Plus, as mentioned in part 1 of this thesis, increased weight of spleen and adrenal glands could be a marker of increased stress. Thus, we have weighed those organs, but found no significant difference between GF and SPF mice (Fig 56 E-F).

We measured the flux of FITC-SA during 2 hours through fresh colonic tissue in Ussing chambers to assess intestinal paracellular permeability. This flux was significantly higher in GF mice after 60 min ($p = 0.004$) 90 min ($p = 0.0006$) and 120 min ($p = 0.0003$) (Fig 56 G). This shows that C57BL/6J GF mice have increased colonic paracellular permeability compared to CV mice.

Part 2- Comparison of GF and CV mice

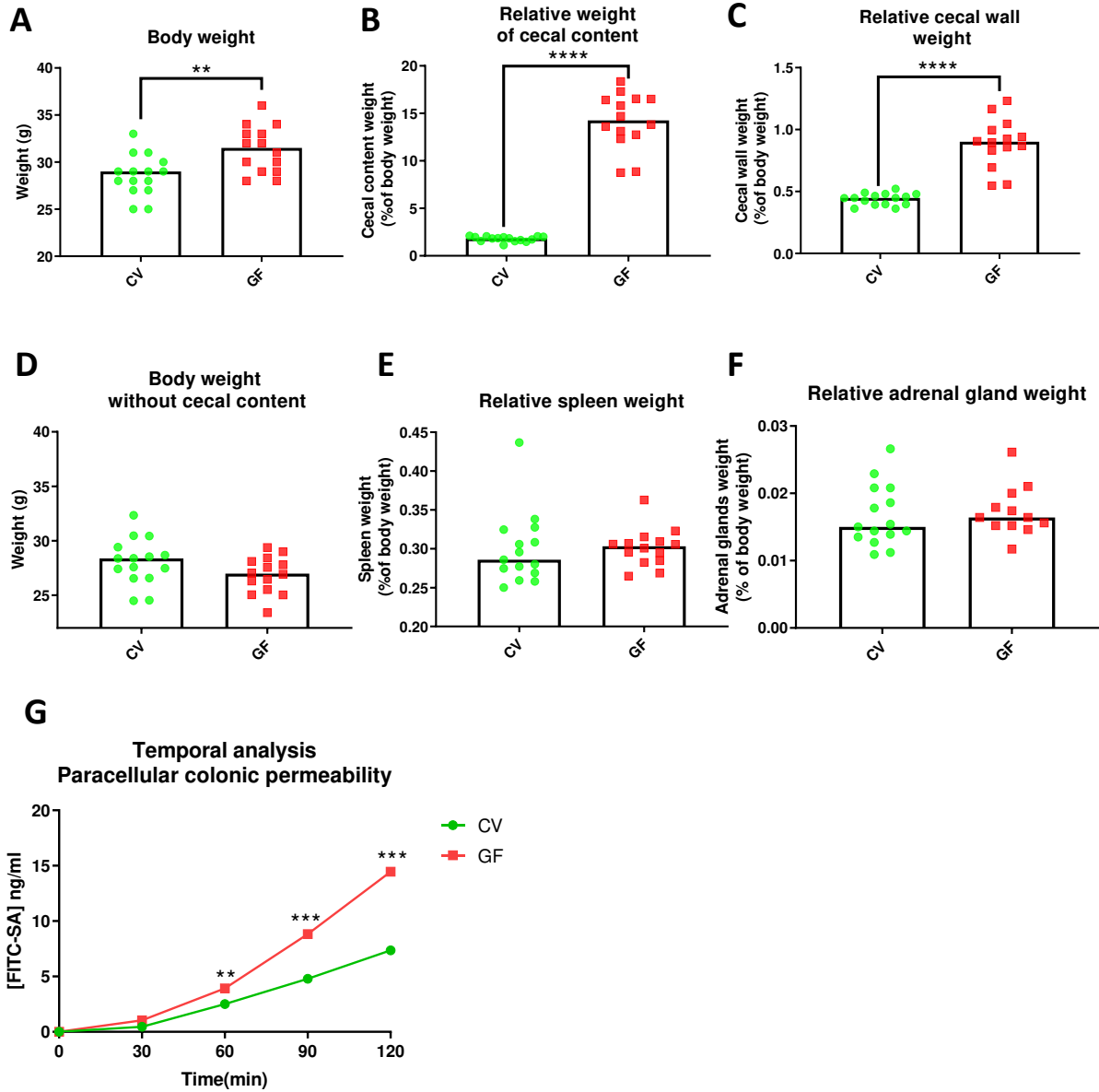


Figure 56: Body and organ weight at sacrifice (A-F) and ex-vivo analysis of paracellular permeability in the colon (G) in CV and GF mice. Groups were compared with Mann-Whitney tests. **p < 0.01; ***p < 0.001; ****p < 0.0001.

Part 2- Comparison of GF and CV mice

3.4.2 Behavior

3.4.2.1 Locomotor activity

Most behavioral tests in mice are dependent on mobility of the animals, thus it is important to assess mobility in every test where it is relevant. Plus, studies report differences in mobility in GF mice compared to SPF [6, 9, 10, 13–15, 17]. To assess potential locomotor activity differences between CV and GF mice, we measured distance travelled and speed, during the OF test. Total distance travelled ($p < 0.0001$) and average speed ($p < 0.0001$) were very significantly decreased in GF mice compared to CV mice (Fig 57). While this is an interesting result, this difference in locomotor activity could impact the interpretation of some of the behavioral tests in this study, and thus should be taken into consideration.

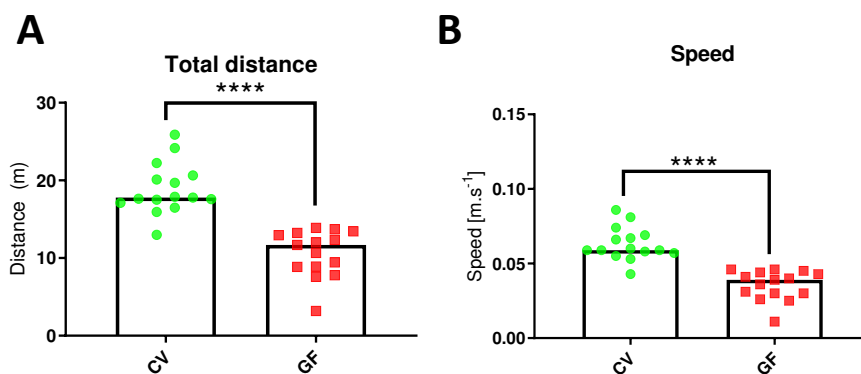


Figure 57: Locomotor activity of CV and GF mice during the OF test

Groups were compared with Mann-Whitney test **** $p < 0.0001$.

3.4.2.2 Anxiety-like behavior

In the OF test, there were no difference in the % of time spent or distance travelled in center of the OF or in the % of time spent in corners of the OF (Fig 58 A C D). However, GF mice had a significantly reduced number of entries in the center compared to CV mice ($p = 0.005$) (Fig 58 B). Because of the decreased locomotion of GF mice, we cannot interpret this as increased anxiety, as the GF mice may have entered the center zone less often solely because they were slower to move around the OF. There was no significant difference between groups in latency to step-down from the platform in the step-down test (Fig 58 E). However, it is interesting to point out that, in the GF group, 1/3 of the mice took more than 30s on average to step down,

Part 2- Comparison of GF and CV mice

while no mice from the CV group took this long. This could suggest that the GF mice have increased anxiety. However, this result could be also impacted by the decreased mobility of GF mice as the 4 mice with the highest latencies to step-down are those who travelled the smallest distances on average in the tests where distance was measured.

Finally, GF mice showed a very significantly reduced total number of rearings ($p < 0.0001$) during the self-grooming test (Fig 58 F). Reduced number of rearings can be associated with decreased anxiety [300] but again, this decrease is likely to be due, at least in part, to the decreased locomotor activity of the animals.

Overall, in those tests, GF mice show behaviors that can be associated with increased anxiety, but we cannot draw this conclusion, as all those parameters could be highly biased by the difference in locomotion.

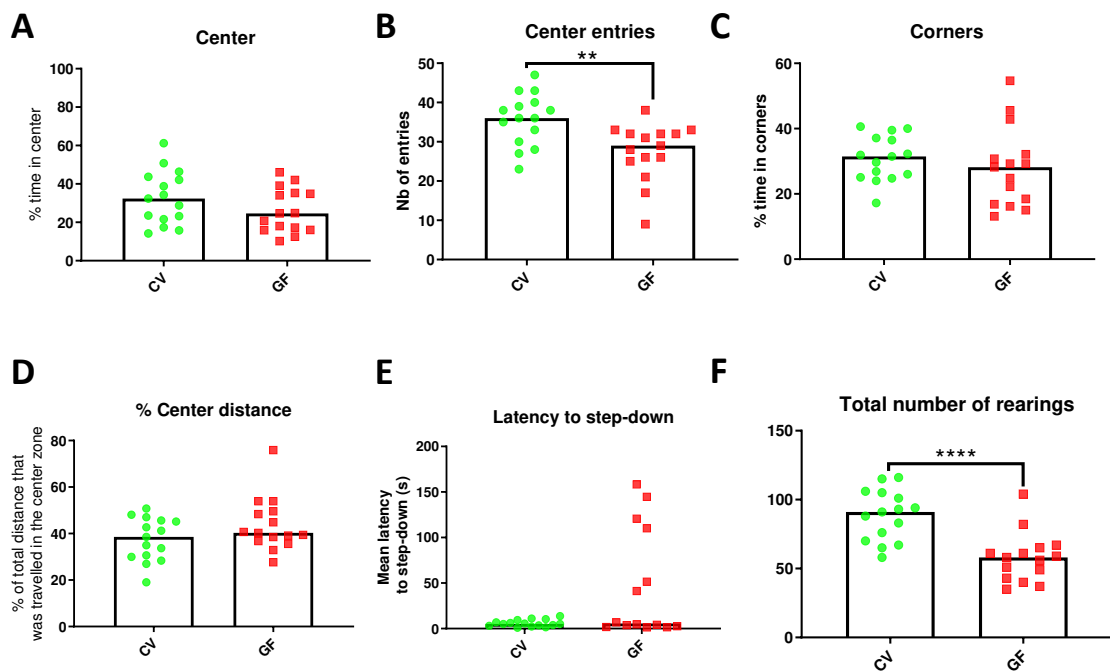


Figure 58 : Anxiety related behaviors in the different tests in CV and GF mice

A-D) OF test E) Step-down test F) Number of rearings during the self-grooming test
Groups were compared with Mann-Whitney test ** $p < 0.01$; **** $p < 0.0001$.

Part 2- Comparison of GF and CV mice

3.4.2.3 Social behavior

We tested the mice in the three-chamber social interaction test. GF mice also presented reduced mobility in this test as indicated by a reduced distance travelled in all 3 phases of the test ($p=0.0007$) (Fig 59 A). In the habituation phase, there were no differences between groups for % of time interacting with right cylinder and both groups showed no preference for any of the cylinders (Fig 59 B). In the social interaction phase, there was a trend for a higher % of time spent interacting with the "Mouse cylinder" in GF compared to CV even though both groups showed a significant preference for the "Mouse cylinder" (Wilcoxon signed rank test for a difference to a theoretical median of 50: CV $p=0.0002$ GF $p=0.0001$) (Fig 59 C). In the social novelty test, there was no difference between groups for the % of time interacting with the "Unknown mouse" cylinder (Fig 59 D). However, this % was significantly different from a theoretical median of 50 only in the GF group ($p=0.02$) (Fig 3 E).

Overall, these results suggest slightly improved social behavior in GF mice, which is different from what has been observed in three papers in this strain [9, 13, 20].

Part 2- Comparison of GF and CV mice

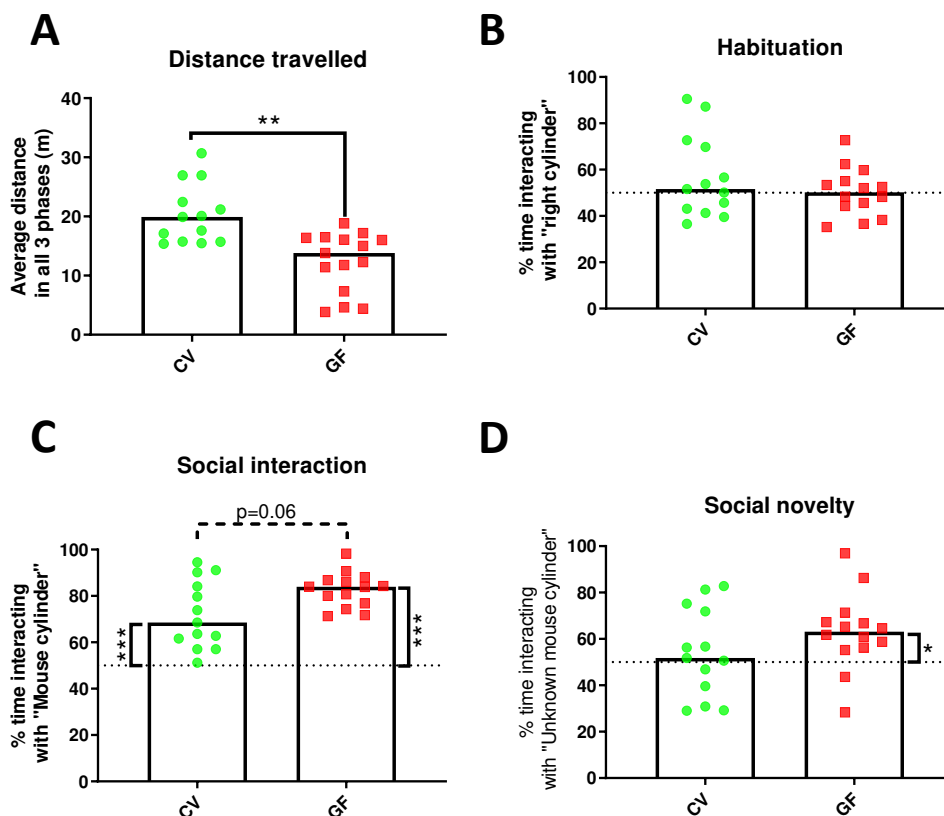


Figure 59 : 3-chamber social interaction test in CV and GF mice

A) Average distance travelled in the 3 phases of the test **B-D)** % of interaction with cylinders during the 3 phases of the test. Groups compared with Mann-Whitney test, and in B-D, in each group, the median % was compared to a theoretical value of 50 with the Wilcoxon signed rank test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. P-value of statistical trends was indicated on the graphs.

3.4.2.4 Repetitive behavior

Increased repetitive behavior is often observed in ASD models, but it has not been found to be modulated in GF mice, although it was rarely tested. We have tested the repetitive behaviors of the mice in the self-grooming test and the marble burying test.

In the self-grooming test, GF mice showed a trend towards an increased total grooming time ($p = 0.051$) and decreased latency to first grooming compared to CV mice ($p = 0.0003$) (Fig 60 A, D). However, there was no difference in total number of grooming, % of incomplete grooming and mean duration of a grooming bouts (Fig 60 B, C, E), which are stronger indicators of repetitive behaviors.

Part 2- Comparison of GF and CV mice

In the marble burying test, an increased number of marbles buried is considered a marker of increased repetitive behavior. We analyzed the number of marbles buried every 5 minutes during the test. The GF mice buried significantly less marbles than the CV mice at the 10 min time point ($p=0.0003$) but there was no difference between groups at the other time points, and both groups buried the same median number of marbles at the end of the test (Fig 60 F). Overall, it seems that the GF mice are simply less efficient at initiating the burying of the marbles, which may be due to decreased mobility in this group.

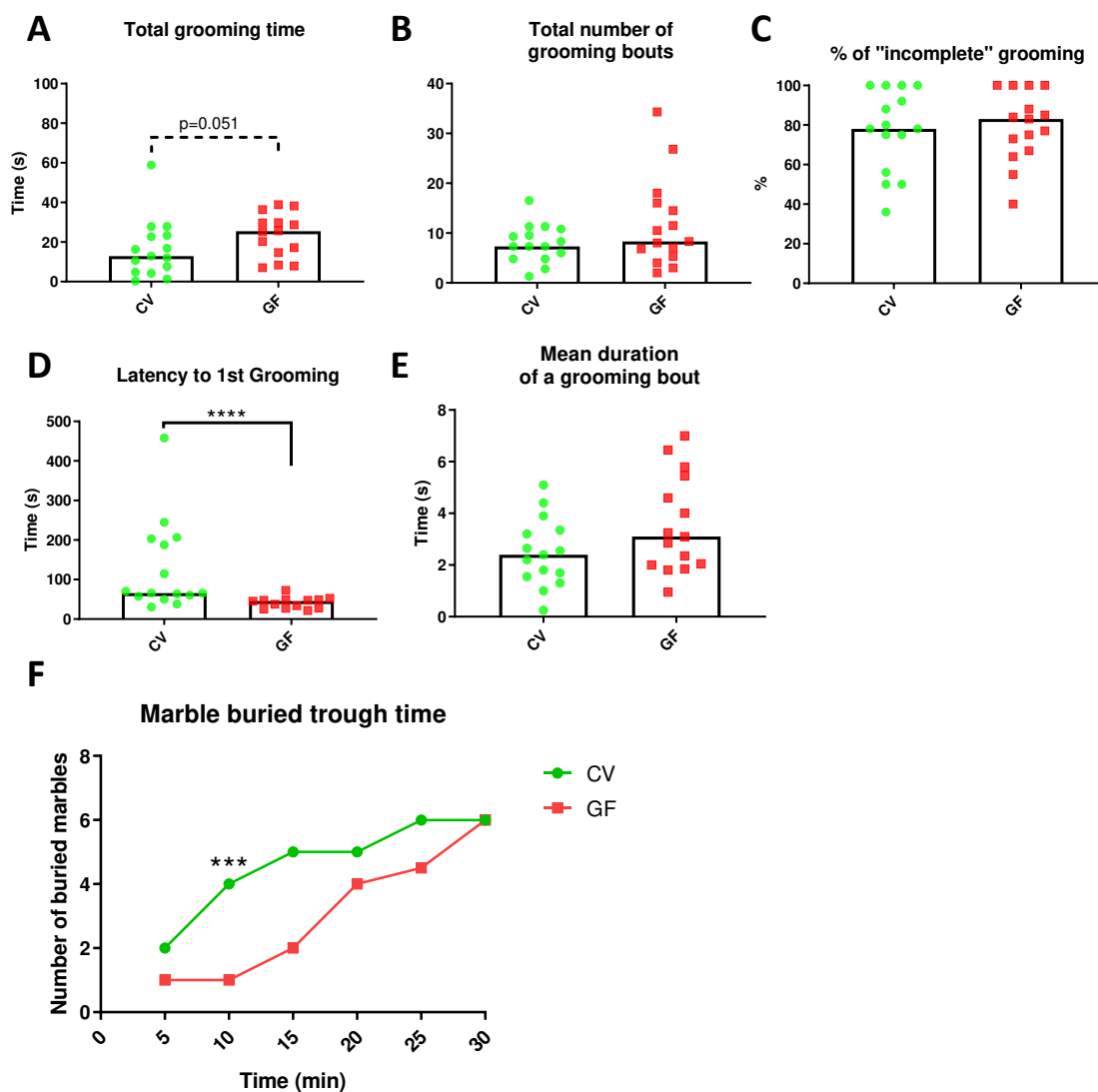


Figure 60 : Repetitive behaviors of CV and GF mice A-E) Self grooming test F) Marble burying test. Compared with Mann-Whitney test. $****p<0.0001$. P-value of statistical trends was indicated on the graphs.

Part 2- Comparison of GF and CV mice

Overall, GF mice groom sooner and tend to groom for a longer total time, but not more often or in a more “repetitive” way (incomplete grooming bouts). In addition, they do not bury more marbles. Thus, they do not seem to show more repetitive behaviors than the CV mice. Nonetheless, the increased grooming time and latency to first grooming could indicate an increased anxiety-like behavior in the GF mice [303].

3.4.2.5 Spatial memory

We decided to assess spatial memory with the 5-object spatial recognition test. The average of the total distance travelled in phase 4 and 5 of the spatial recognition test was decreased in GF mice (Fig 61 A). The recognition index (RI= (DO.P5-All.P4)-(NDO.P5-All.P4), detailed in material and methods of part 1 p. 72) was not significantly different between groups, although there was a trend for a decreased RI in GF ($p=0.09$) (Fig 61 B). This trend suggests a slightly impaired spatial memory in GF C57BL/6J mice similar to what was observed in Lu et al. (2018) [9].

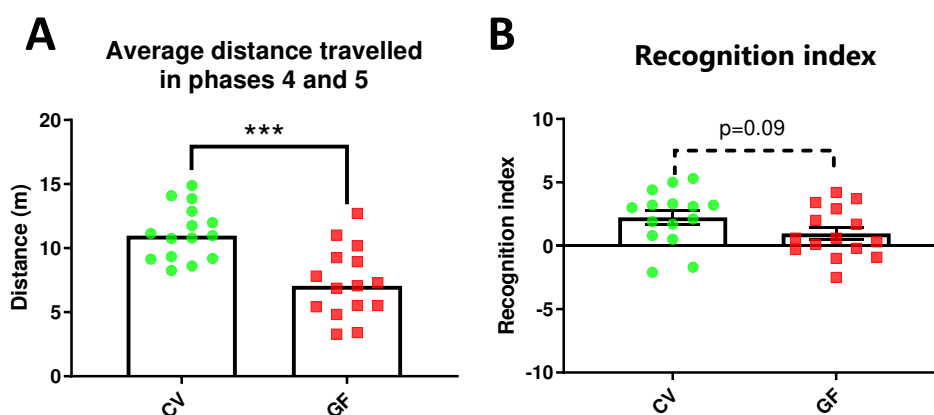


Figure 61: Spatial recognition test in CV and GF mice

A) Average distance travelled in P4 and P5 B) recognition index Groups were compared with Mann-Whitney test *** $p < 0.001$.

P-value of statistical trends was indicated on the graphs.

Part 2- Comparison of GF and CV mice

3.5 SUMMARY OF RESULTS AND DISCUSSION

Overall, we observed various behavioral alterations in GF C57BL/6J mice that do not really reflect an ASD-like phenotype. In the literature, the behavior of GF mice has been studied in various mouse strains and one rat strain. Some of our observations are similar to what has been described in GF C57BL/6J mice or other strains, but there are also differences which are detailed and summarized in table 45 below.

Study	Animals	SPF in isolators?	Same supplier?	Mobility	Anxiety	Repetitive behavior	Social behavior	Memory
[7]	F344 rat	Yes	No	→	↗	N/A	↘	N/A
[6]	NMRI mice	Yes	Yes	↗	↘	N/A	N/A	N/A
[4]	Swiss Webster mice	No	Yes	N/A	↘	N/A	N/A	N/A
[21]		No	Yes	N/A	↘	N/A	N/A	N/A
[15]		Yes	Yes	↗	↘	→	↗	N/A
[22]		No	Yes	N/A	N/A	N/A	↘	N/A
[18]		Yes	Yes	↗	↘	N/A	↘	↘
[14]	BALB/c mice	Yes	Yes	↗	↗	→	N/A	N/A
[10]		No	Yes	↗	↘	N/A	N/A	N/A
[20]	C57BL/6J mice	No	No	→	N/A	N/A	↘	N/A
[9]		No	No	↘	↗	N/A	↘	↘
[17]		No	Yes	↘	N/A	N/A	N/A	N/A
[13]		No	No	↘	↘	N/A	↘	N/A
Our study		Yes	Yes	↘	(↗)	→	(↗)	(↘)

Table 45 : Summary of the findings in literature and our study on mobility anxiety, repetitive behavior, social behavior, and spatial memory in GF vs SPF mice. Some experimental differences are detailed (SPF mice raised in isolators or not and use of SPF and GF mice from the same supplier or not). Legend: → =No change in behavior; ↗=Behavior increased or improved in GF mice compared to SPF; ↘=Behavior decreased or impaired in GF compared to SPF; (↘) or (↗)=statistical trend to a change in behavior in GF ; N/A= behavior not tested in this study.

Part 2- Comparison of GF and CV mice

Our observation of reduced locomotor activity in C57BL/6J GF mice confirms what has been observed in literature on this strain [9, 13, 17] and may be linked to the reduction of skeletal muscle volume and metabolism observed in this strain in one study in GF mice compared to SPF [17]. Since other strains of GF mice (BALB/c, NMRI and Swiss Webster) display increased mobility [6, 10, 14, 15], it could be interesting to compare skeletal muscle in GF and SPF mice of those strains, which, to our knowledge, has not yet been done.

While this impaired mobility is an interesting result, it limits the interpretation of the seemingly increased anxiety in GF mice compared to CV mice in the OF and step-down tests, as the reduced number of entries in center or increased latency to step down, could be due to the reduced locomotion of GF animals. However, the trend to an increased grooming time, and decreased latency to grooming are not influenced by mobility differences and suggest that the GF C57BL/6J mice are more anxious than CV mice. However, in the same strain, Wu et al. (2021), observed decreased anxiety in GF mice compared to SPF mice in the OF, EPM, and L/D box test [13]. This difference, and, in general, some of the discrepancies between studies in the same strains, could be due to differences in experimental procedures.

Indeed, behavioral experiments can be highly influenced by environmental factors and in some studies, SPF mice were not raised in isolators [4, 9, 10, 12, 13, 20–22]. The isolator is a closed environment with different environmental exposure (sounds, smells) compared to a regular animal facility, so raising SPF and GF mice in such different environments may lead to behavioral differences that are not linked to microbial status. Plus, as behavior took place outside of the isolators in those studies, even if the mice were given time to acclimate in the testing room, the GF mice experienced a more extreme change in environment when moving from isolator to regular facility. By raising the SPF mice from birth or early age in isolators, as was done in a few studies [6, 7, 15] this issue is limited as the SPF mice experienced a similar environmental change as the GF mice before the tests. However, performing the tests in isolators, as was done by Nishino et al. (2013) [14] and in our study, avoids other issues that may arise from the sudden environmental change. Indeed, when taken out of the isolator, GF mice are suddenly exposed to environmental microbes. Wu et al. (2021) [13] mentioned that GF mice were kept out of the isolators (in normal cages in an SPF facility) for up to a week (supposedly the duration of behavioral

Part 2- Comparison of GF and CV mice

tests). Thus, those mice cannot really be considered GF and the colonization could have impacted behavior even in the short term. Indeed, Nishino et al. (2014), showed that the sole action of taking a GF mouse out of an isolator for 24 h led to decreased anxiety in the OF [14].

We also found differences between GF and CV mice in social behavior and spatial memory. The expression of the results by the recognition index or % of time interacting with a cylinder compared to total time allows to interpret the results regardless of mobility differences.

The trend to a better performance of GF mice compared to CV mice in the social interaction test and significant preference for the “unknown mouse” cylinder only in GF mice implies that they show slightly improved sociability. This is opposite to what has been observed in three studies in this strain, who reported decreased performance in GF compared to SPF mice in the 3-chamber social test [9, 20] or the reciprocal social interaction test [13]. However, increased social interaction has been found in GF Swiss Webster mice compared to SPF [15].

It is possible that the deficits in social behavior observed in GF mice especially for social novelty, are in part influenced by the fact that the SPF mice were not housed in isolators in some of those studies [9, 20, 13], and that the tests were performed outside of the isolators. This means that the GF mice were exposed to a lot more environmental smells during the test than they were used to in the isolator, and this may have interfered with their ability to recognize the smell of an unknown mouse. Discerning between smells in a complex environment could be more difficult for GF mice, as they present functional differences in the olfactory epithelium [304]. In addition, in our study the unknown mice used for 3-chamber social interaction test were of the same microbial status as the tested animals. This was not the case in any of the studies included in table 45 that reported differences in the 3-chamber test, who all used SPF mice as unknown mice for both GF and SPF groups [9, 15, 18, 20, 22]. It is possible that GF mice have no issues in recognizing the smell or discerning between smells of other GF mouse but cannot as efficiently recognize or discern between the smells of SPF mice, explaining the deficiency in social behavior observed in those studies, particularly for social novelty. However, this does not explain why the SPF mice had no preference for the SPF unknown mouse in our study.

Part 2- Comparison of GF and CV mice

In the spatial object recognition test, we found a trend to a lower RI in C57BL/6J GF mice compared to CV. This would suggest a slightly impaired spatial memory in GF mice, which is also what had been observed by Lu et al. (2018) [9]. In addition, impaired memory has also been observed in GF Swiss Webster mice [18].

Finally, GF mice do not display more repetitive or stereotyped grooming compared to SPF and did not bury more marbles in the marble burying test. While repetitive behavior has not been studied in GF mice from this strain, previous studies on BALB/c and Swiss Webster GF mice also reported no differences in repetitive behaviors in those tests [14, 15].

Overall, aside from the environmental living conditions of the animals, other experimental differences could explain some dissimilarities in results.

Firstly, in some of the studies, animals originally came from 2 different facilities (SPF mice directly from a private supplier and GF mice from a local gnotobiotic colony) [9, 12, 13]. This is not recommended for behavioral studies as handling and living conditions of the mice can greatly differ between facilities and thus influence behavior. In addition, because of genetic drift, it is possible that there are genetic differences between mice of a given strain from a commercial supplier and a colony of the same strain from a local facility. In Luk et al. (2018), the authors did not compare the animals to SPF mice, but to GF mice colonized at birth with SPF microbiota [18]. While this allows both groups to have identical genetic backgrounds, it is possible that having been developed in the womb of a GF dam could lead to developmental differences that could not be reversed by colonization at birth. Thus, those animals are not totally comparable to SPF animals. We have tried to overcome this issue by creating a CV line in our local facility, from breeding colonized GF mice with SPF microbiota and breeding them for two generations. With this approach, the CV mice have CV parents and grandparents, so any developmental issue due to coming from originally GF mice was likely restored. A similar approach has been used by Nishino et al. (2013) for one generation [14]. However, we cannot exclude the possibility that the microbiota composition of those recolonized animals and their descendancy is different from that of the original SPF donors, which could also lead to behavioral differences. For this reason, we are currently analyzing microbiota composition of SPF donors, F0 recipients and F1 and F2 CV mice.

Part 2- Comparison of GF and CV mice

For future GF studies, it would be ideal to use of SPF and GF mice from the same supplier or facility, raised in the same environmental conditions, and, when possible, tested for behavior inside the isolators. This would allow to limit the influence of environmental and genetic factors and thus, may lead to more reproducibility among studies.

Aside from behavioral differences, we have found that GF C57BL/6J mice had an enlarged caecum typical of all GF rodents. Interestingly, we found increased intestinal paracellular permeability in the colon of GF mice compared to SPF, which contradicts what was observed by Hayes et al. (2018) [293]. This difference could be due to the fact that their study used a different substrain of mice, C57BL/6N. Interestingly, previous unpublished work from our team also found increased paracellular permeability in GF F344 rats compared to SPF (Véronique Douard, personal communication).

Ongoing analyses will investigate potential differences between GF and SPF mice in gene expression in the gut and brain of some of the markers that we looked at in the FMT experiment, and/or that have been found to be altered in GF mice in literature.

This experiment brought us insight into the behavioral characteristics of GF C57BL/6J mice in our experimental conditions. While we cannot directly compare the behavior of GF mice to that of our FMT groups, as they are different experiments, we can still formulate hypotheses on the effect that the FMT from the different donor groups could have had on those alterations observed in C57BL/6J GF mice.

The decreased locomotion of C57BL/6J GF mice has seemingly been restored by FMT from group S-A and A, but maybe to a lesser extent in the A group as locomotion was lower in compared to S-A. In the other two groups, especially AG, the values of total travelled distance are closer to what was observed in GF mice. While we cannot directly compare, this suggests that microbiota from those groups had less of an impact on the locomotion of GF mice.

Social novelty behavior of GF mice, tended to be better than that of CV mice. In the FMT experiment, only mice from group S-A and AG had a preference for the novel mouse. This could reflect a negative impact of FMT from the S-AG and A groups on social novelty performance, while it was not the case with FMT from AG and S-A donor groups.

Part 2- Comparison of GF and CV mice

While GF mice did not display changes in number of grooming bouts in the self-grooming test compared to CV, in the FMT experiment, this parameter was increased in AG group compared to S-AG group. The values of this parameter in the S-AG group are closer to that of GF mice, thus, we can hypothesize that microbiota from the AG group increased this behavior.

Finally, the trend to an impaired spatial memory observed in GF mice is interesting, considering the difference in spatial memory between mice that received ASD or sibling microbiota in C57BL/6J mice. We can make the hypothesis that microbiota from A and S-AG groups ameliorated spatial memory in originally GF mice, while microbiota of S-A and AG groups did not impact it or had less of an impact.

It would be interesting to also study GF BALB/c mice in the same conditions, as was originally planned, in order to compare those results to the ones observed in the FMT study. Indeed, even if there were no difference in behavior for FMT in BALB/c mice, it would be interesting to know whether GF mice of this strain showed pre-existing behavioral alterations. Plus, it would be interesting to investigate in GF mice the number of TPH2 positive cells in the raphe nuclei that was decreased in both A and AG groups compared to siblings in BALB/c. To our knowledge, this has not been investigated in GF mice, but multiple studies saw alterations in the 5-HT system in other brain regions in GF mice or rats [4, 6, 7, 14, 194].

4 GENERAL DISCUSSION AND PERSPECTIVES

Overall, our results in the FMT experiment were quite strain specific. Nonetheless, our results partially validated our principal hypothesis. Indeed, C57BL/6J mice that received AG microbiota did show some ASD-like behavioral symptoms. Interestingly, however, FMT from sibling groups seemed to also negatively impact, or not restore some of the behavioral alterations of GF mice. This suggests that FMT from humans could negatively impact, or fail to improve, altered behaviors in originally GF mice, regardless of if the donors have ASD or not. The other two studies in literature that performed FMT from human donors to GF mice saw impairments in social behavior in mice that received ASD microbiota compared to TD microbiota, which was not the case in our study, except for slightly impaired performance in social novelty in group A in C57BL/6J mice. As we used siblings of the donors as controls, their microbiota might have been closer to the one of the ASD groups, explaining the more mitigated results. However, this does not seem to be the case, as we found strong differences between groups in microbiota composition, and when looking at differences in β -diversity we saw very significant differences, like what was observed in the other studies. Another possibility is that, by pooling microbiota, we altered the complex ecosystem of the microbiota from each donor, which could have implanted in a way that is not representative of the original population. Nonetheless, in some studies, pooled microbiota from humans has efficiently transferred phenotypic traits of ASD or depression to mice [27, 30, 239]. To gain more insight into the fidelity of microbiota engraftment from donors to the mice, we plan to compare the composition of the individual donors and pooled microbiota to the microbiota implanted in each mice group.

It is also possible that testing the mice in other behavioral tests might have revealed more behavioral alterations. Although we did not see differences in anxiety using the OF test, the step-down might have revealed some differences. For repetitive behavior, using the marble burying test, as was used by Sharon et al. (2019) [237], could have strengthened the result observed in the AG group. Plus, for social behavior, investigating the performance of the animals in reciprocal social interaction, a test which studies social behavior more precisely, differentiating between different

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types of interaction (for details see annex 3 p 254), could have reinforced our observation on social novelty. However, there is a limit to the number of behavioral tests that can be done in one experiment, as it may lead to increased stress in the animals, if they are subjected to many different tests within a few weeks, thus possibly impacting the results.

The Spearman correlation analysis highlighted some interesting correlations that were sometimes found in both strains and consistent with previous literature. It could be interesting to continue this analysis in lower phylogenetic levels of the microbiota, looking specifically at the bacterial families, or even genera, that are most modulated between groups. In addition, while in this thesis we focused only on correlations between microbiota and other factors, the analysis also encompassed correlations between different markers. We could further investigate parameters that correlate with the same microbial phyla to see if they also correlate together and, in consequence, emit hypotheses on how those different factors may be linked.

In addition, looking into microbiota modulations in more details could be interesting. This thesis focused on phylum and family level, as there were already important differences at those levels, reflecting global microbiota differences between groups. In the families that are modulated between groups, we could focus on specific genera that might contain species that have been studied in the context of ASD, for example, *p*-Cresol producing species. For a reminder, *p*-Cresol is a bacterial metabolite produced by some bacterial species that was found to be increased in individuals with ASD and to be correlated with stereotyped behavior [77, 78]. In two studies on mice, treatment with *p*-Cresol induced increased repetitive behavior and /or social interaction deficits, which were mediated by the microbiota, as FMT from *p*-Cresol treated animals transferred those behaviors to WT mice. It may therefore be relevant to measure fecal or urinary *p*-Cresol in the animals that received FMT from individuals with ASD. There could be a different abundance of *p*-Cresol producing species in the different groups, which could play a role in the differences in social and repetitive behaviors. For example, differences between groups in *Clostridioides*, *Olsenella*, *Blautia* or *Rombustia* genera might be interesting to investigate, as they all contain one of the major *p*-Cresol producing species [128]. If they are modulated, we could try to design primers to amplify DNA from specific *p*-Cresol producing species they contain by qPCR and see if they are differentially represented between groups. Additionally, we could use a KEGG (Kyoto

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Encyclopedia of Genes and Genomes) based analysis to identify differences in microbial genes involved in *p*-Cresol biosynthesis pathway in the different groups [305, 306].

Overall, despite the lack of impaired social behavior, the AG group in C57BL/6J mice recapitulates some ASD-related characteristics, with increased repetitive behaviors and impaired spatial memory. Plus, this group presents markers of increased ileal permeability. Thus, furthering the investigation in this group is interesting. To pursue this experiment, our team is currently testing the effects of a synbiotic formula (*Lactobacillus paracasei* L411+ Galacto-oligosaccharide + inulin) on the same ASD-related systemic, intestinal, central, and behavioral markers in GF C57BL/6J mice that have been colonized with microbiota from the S-AG or AG groups. Considering the previous remarks, the marble burying test will be added to the panel of behavioral tests in this study.

In addition, in the GEMMA project, FMT from the S-AG and AG donors is being investigated in SPF WT mice and SPF BTBR and CMA mice models of ASD by our collaborators in Utrecht University. The hypothesis is that the microbiota from AG group would worsen pre-existing ASD like behavior in the animal models of ASD, while microbiota from S-AG groups may ameliorate it. In the two models, the synbiotic intervention is also being tested to investigate whether it could reduce ASD-related symptoms in those animals. The study in the CMA model will also bring more insight in the effect of the FMT in a model that shows increased inflammation. One possibility is that the microbiota from the AG group, in a potential inflammatory environment, will have more deleterious effects on systemic, gut, brain or behavioral markers in the animals. Another possibility is that the microbiota from the AG group will limit the inflammation in the CMA model, as we observed reduced gut or systemic inflammation in our GF mice that received this microbiota compared to S-AG. Overall, those studies on CMA and BTBR models will bring more insight into the effect of ASD microbiota on mice, having preexisting inflammation and/or ASD-like behavior.

Aside from those ongoing experiments, it could be interesting to investigate the effect of the microbiota on spatial memory in more detail. In the S-AG group, the proportion of *Prevotellaceae* was much higher than in AG group. This is interesting, as a recent study found that increased gut *Prevotellaceae* due to resistant starch supplementation improved spatial memory, in a model of memory impairment due to high-fat diet [307]. This study found

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that this effect was dependent on the activation of GABAergic neurons in the medial septum area, a region that can regulate hippocampal CA1 excitability. As the medial septum area receives projections from the brain stem, it can be modulated by vagal signals, making this region a good target for an involvement of the gut-brain axis in memory regulation [307]. Thus, it could be interesting to study activation of this region in the S-AG and AG animals.

In addition, we could investigate brain circuits that have been shown to be implicated in an effect of the microbiota on social behavior. In Pascucci et al. (2020) and Sgritta et al. (2019), the effect of *p-Cresol* or *L. reuteri* MM4 probiotic treatment on social behavior implicated DA or oxytocin signaling in the ventral tegmental area. This region is part of the mesolimbic social reward system and thus involved in regulation of social interaction [25, 84].

Whole brain immunostaining after transparisation could be used to study those networks, as this method could allow to mark neuronal bodies and axonal projections towards other brain regions.

To conclude, there is still little literature on FMT in ASD, whether it is as a therapeutic endeavor, or in a mechanistic investigation. Our results add to this literature, showing that FMT from individuals with ASD can induce some immune, GI, brain, and behavioral alterations in originally GF mice, although not fully representative of an "ASD-like" phenotype. In addition, we highlight the importance of choice of control groups, presence of GI symptoms in the donors and genetic background of the model used in the effect of FMT.

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6 ANNEXES

6.1 ANNEX 1: PRINCIPLE OF 16S rRNA SEQUENCING ANALYSIS FOR MICROBIOTA COMPOSITION

6.1.1 General principle

Note: This part was written using my notes and some figures from the course “Analyses de données de séquençage en metabarcoding” dispensed by the MIGALE platform in INRAE that I attended in June of 2022 [308]. Other materials used to write this part have been cited.

To analyze microbiota composition of a sample, one option is to sequence some regions from a gene that is well referenced and contains conserved regions among all bacteria, surrounded by variable regions to distinguish between bacterial groups. One popular candidate in prokaryotes that suits those characteristics is the 16S subunit of ribosomal RNA. Thus, it is most often used for analysis of gut microbiota composition. In this project the regions used for sequencing were V3-V4 variable regions, but other alternatives are possible (Fig 62).

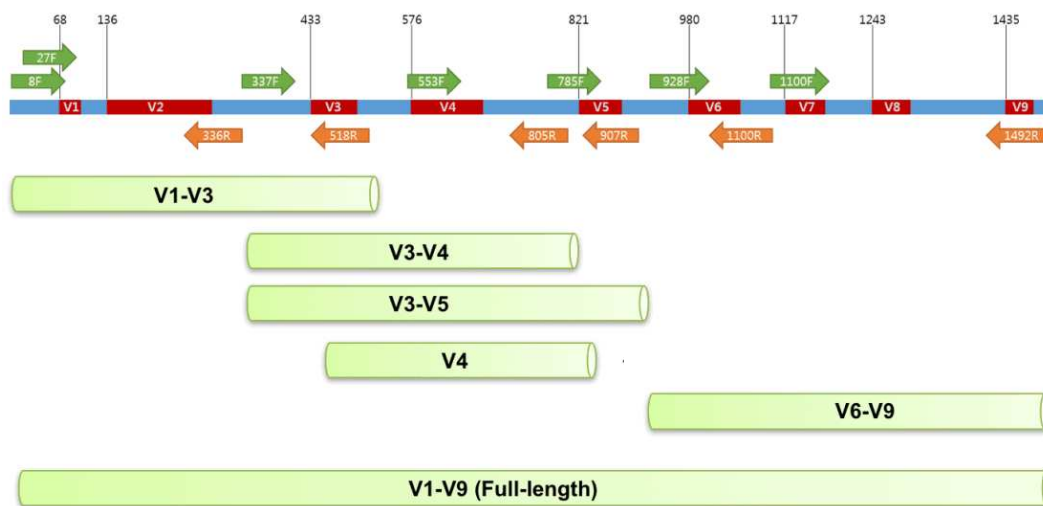


Figure 62 : Regions of bacterial 16S rRNA that are often targeted for microbiota analysis.

Green and orange arrows represent forward and reverse primers respectively, that allow to amplify selected regions. Variable regions are in red, conserved regions in blue. Image modified from <https://help.ezbiocloud.net> [309]

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Those regions are amplified by PCR in DNA samples (in our case extracted from feces or cecal content) and sent for sequencing. The sequencing reads are then filtered, and similar sequences are grouped together (as there are many copies of a same sequence). One option is to regroup the sequences that are very close (more than 97% homology) into operating taxonomic units (OTUs). Another option is the use of Amplicon Sequence Variant (ASVs). ASVs groups together the sequences that are identical and identifies differences in sequences that are likely due to sequencing errors. For example, a sequence that is different from another by only one nucleotide, and that is found in very few copies, is likely to be a sequencing error so it will be grouped in the ASV of the closest sequence. This method is more reproducible than OTUs and can lead to better resolution in certain cases [310]; thus this is the one we chose in this project for microbiota analysis. After being grouped into OTUs or ASVs, the sequences are aligned to a phylogenetic database of 16S genes in order to classify them into taxa (in order Domain, Kingdom, Phylum, Class, Order, Family, Genus, Species). However, because the amplified region is often quite small, this analysis is not specific down to the species level, and sometimes not even up to genus level. Once this analysis is complete, a bioinformatics process allows to determine various parameters described in the following sections.

6.1.2 α -diversity

α -diversity represents the diversity of ASVs (or OTUs) in a sample. There are different indexes of α -diversity:

Richness Observed: Number of different ASVs observed in the sample.

Chao1: Number of different ASVs with an added estimation of non-observable species.

Shannon index: Measures heterogeneity of the sample as it takes into account the relative abundance of ASVs.

Simpson index: Based on probability that two bacteria picked at random in the community belong to the same ASVs (so it decreases when diversity increases, thus the inverse of this index, "InvSimpson", is most commonly used).

Faith's phylogenetic diversity index: Takes into account phylogenetic

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information without considering relative abundance of each ASV. Defined as the number of phylogenetic branches covered by ASVs of the sample. A higher number of branches means higher diversity [311].

6.1.3 β -diversity

β -Diversity represents how different two samples are from each other. It considers two datapoints and focuses on shared or specific features between them to calculate a “distance” between those two samples in terms of composition. Those distances can be calculated in different ways:

Jaccard: represents the fraction of ASVs specific to one or the other sample without taking abundance of those ASVs into account (Fig 63).

Bray-Curtis: fraction of ASV abundance that is specific to one or the other sample (Fig 63).

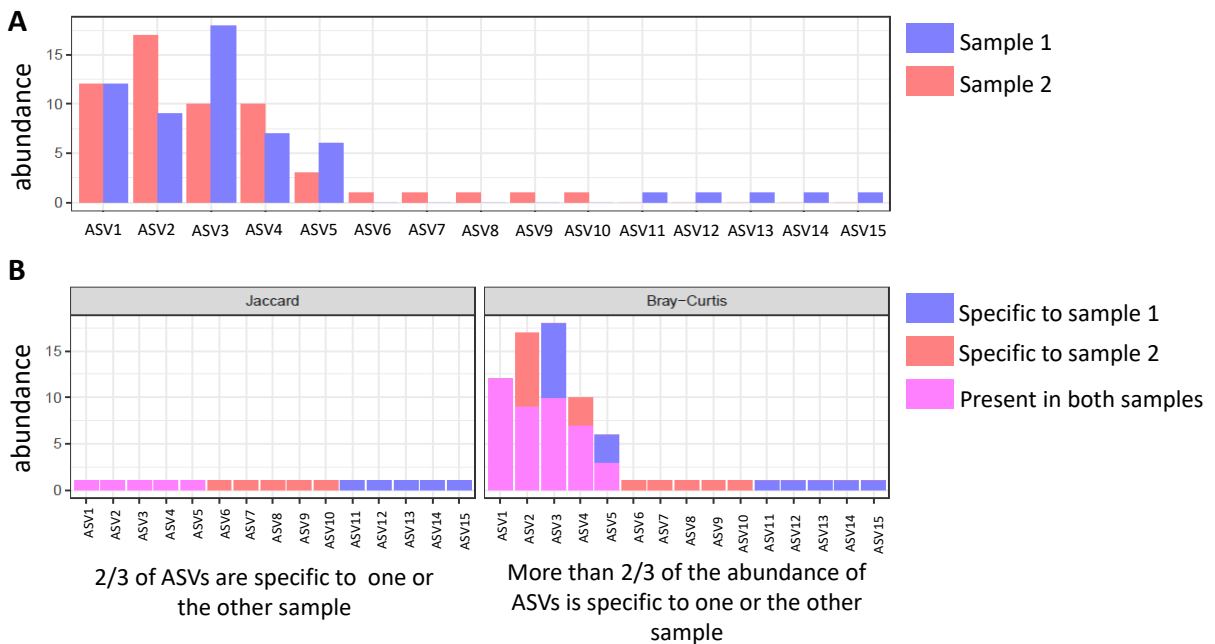


Figure 63 : Principle of Jaccard and Bray-Curtis distances.

A) An example of the repartition of ASVs in two samples **B)** Representation of those communities according to Jaccard and Bray-Curtis indexes. In this example, Jaccard distance will be lower than Bray-Curtis distance. Figure modified from materials provided in the course: “Analyses de données de séquençage en metabarcoding” [340].

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There are also indexes that consider the phylogenetic distance between ASVs:

Unifrac: Considers the phylogenetic tree branches that are specific to one or the other sample (Fig 64).

Weighted Unifrac: Same principle as Unifrac but takes into account abundance of each ASV in the community, expression as a fraction of phylogenetic tree branches (Fig 64).

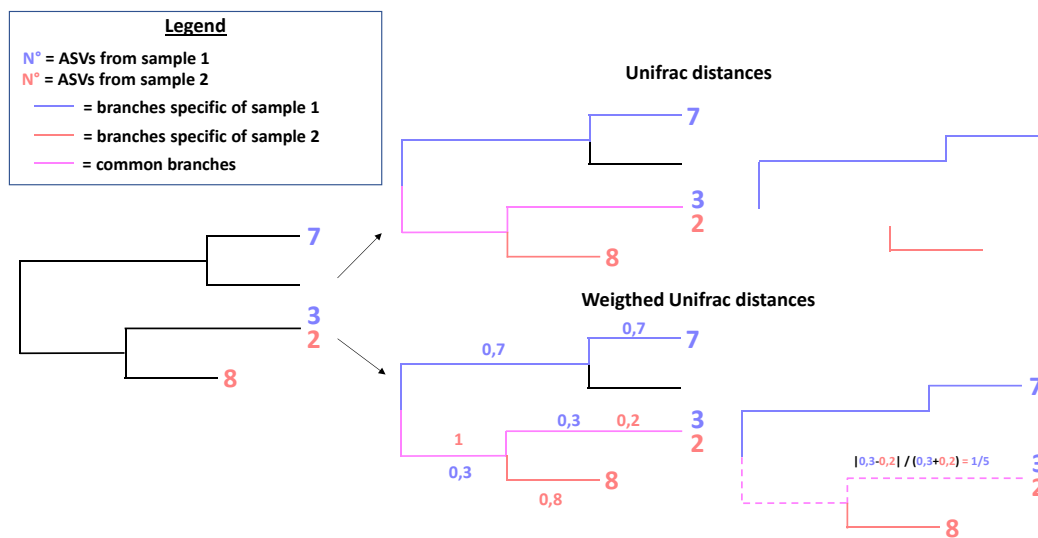


Figure 64 : Representation of Unifrac and Weighted Unifrac distances on a phylogenetic tree.

Unifrac distance=branch length that is specific to one or the other sample. Weighted Unifrac=fractions of total ASVs on a branch specific to one or the other sample. Here 1/5 of the « common » branch is specific to sample 1. Figure modified from materials provided in the course: "Analyses de données de séquençage en metabarcoding" [308].

Those distances can be used to perform Principal Component Analysis (PCoA) and plot the samples on a map to visually represent heterogeneity between samples. An Adonis test (also called PERMANOVA) can be used to test if data from two distinct groups significantly cluster from each other. Adonis test results in a R^2 value, representing the effect size (i.e fraction of clustering explained by the group or treatment) and a p-value of statistical significance. For example, a R^2 of 0.5 for the "treatment" factor accompanied by a significant p-value indicates that 50% of the clustering is explained by the "treatment" factor and that the treatment groups clusters significantly apart, suggesting they have distinct β -diversity. Multiple factors can be

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tested at a time, for example, a “treatment” factor and an “sex” factor. Even if Adonis reveals significant effects for both, the R^2 brings more information to see if one or the other factor is responsible for most of the clusterin of data.

6.1.4 Relative abundance of taxa:

After ASVs have been assigned to different taxa, we can determine their relative abundance (i.e. proportion of ASVs that belong to each taxa). It is important to note that 16S analysis is only quantitative in a comparative way among samples that were processed at the same time, so 16S data should always be described in relative abundance or proportion (not in absolute abundance).

6.2 ANNEX 2: CLINICAL EVALUATIONS OF GI SYMPTOMS AND ASD

There are many scales that can be used in clinical research to evaluate gastro-intestinal (GI) or behavioral symptoms. Here, I will describe the ones that are mentioned in the introduction of this thesis.

6.2.1 GI symptoms scales

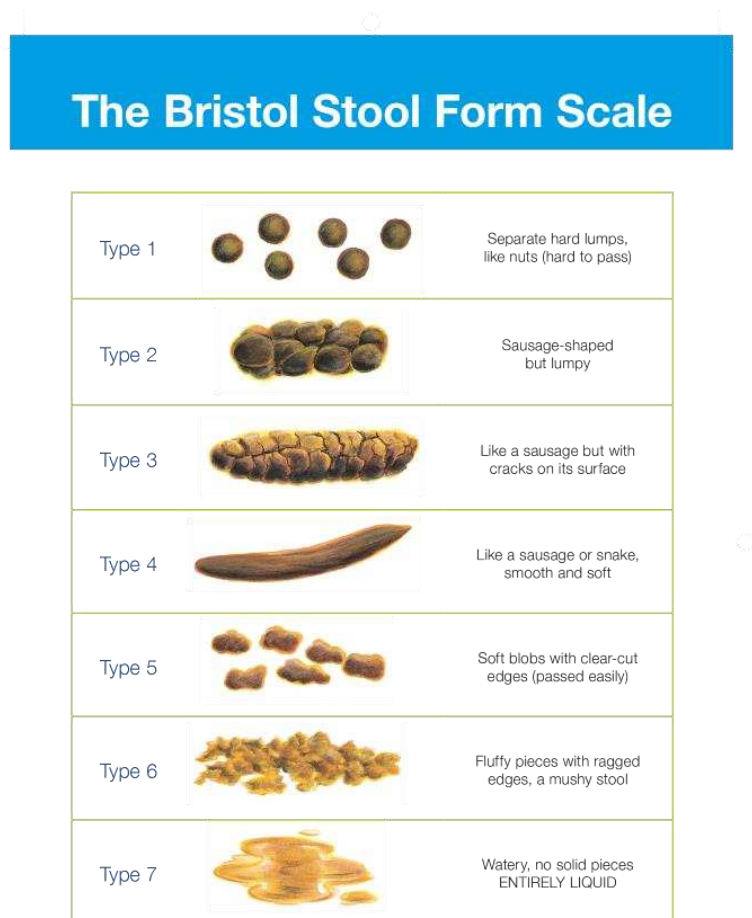


Figure 65: Bristol Stool chart.

Source: Continence foundation of Australia [344]

Bristol score: The Bristol scale is used to determine constipation by rating the aspect and texture of stools over a varying time period. There are 7 categories rated from 1 (very hard stool) to 7 (liquid stool) (Fig 65).

Gastro-intestinal symptoms rating scale (GSRS): Questionnaire used to rate abdominal pain, indigestion symptoms, constipation, and diarrhea over

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the prior week [312].

GI severity index (GSI): This rating scale has been developed for studies on individuals with ASD, and rates symptoms of gastrointestinal distress most commonly reported by parents of children with ASD [313]. Some studies use a simplified version of this index with only 6 items (6-items GI severity index).

6.2.2 Behavioral scales for ASD diagnosis:

DSM-5 scores: The 5th edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) is used by clinicians and repertoires the diagnostic criteria of all mental disorders, including ASD. For ASD, the DSM-5 considers deficits in social communication and social interaction, restricted, repetitive patterns of behavior, interests, or activities. It rates severity of those behaviors as follows: Level 1: Requiring support; Level 2: Requiring substantial support; Level 3: Requiring very substantial support. The clinical report also should specify if there is accompanying intellectual and language impairment [314].

Autistic Behavior Checklist (ABC): Questionnaire used by parents and clinicians to characterize ASD behavioral symptoms. It contains 57 questions grouped into 4 categories (sensory relating, body and object use, language, and social and self-help skills) and scored from 1 to 4 according to the impairment degree [315].

Autism Diagnostic Observation Schedule (ADOS): Standardized assessment used by clinicians to diagnose ASD by observing and interacting with children in specific situations and scenarios during which the following behaviors are assessed: stereotyped/idiosyncratic words or phrases, facial expressions directed to others, imagination/fictional play, repetitive behaviors or interests, negative behavior, speech abnormalities. There are 4 different modules used depending on the level of communication of the child [316].

Autism Treatment Evaluation Checklist (ATEC): Free questionnaire used by parents and clinicians to characterize ASD behavioral symptoms which contains 77 questions that are classified into four subscales: Speech/language/communication, Sociability, Sensory/Cognitive awareness, and Physical/Health/Behavior. It is often used to quantify potential improvement of ASD symptoms following therapeutic interventions [317].

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Vineland Adaptive Behavior Scales (VABS): Standardized test used in the assessment of intellectual and neurodevelopmental disorders (including ASD). It includes forms filled by parents/caregivers and teachers and a standardized interview to rate behaviors divided in 5 subscales: communication, daily living skills, socialization, motor skills and maladaptive behaviors [318].

Psycho-educational profile: Assessment to evaluate behavior and skills of young children (2-7 years old) suspected to have ASD and measure developmental strengths, weaknesses, and learning style to help caregiver, teachers and clinicians to offer adapted solutions for the children [319].

Social Responsiveness Scale (SRS): Used by parents/caregivers or teacher and measures social ability through a questionnaire evaluating social skills by rating them on a scale of severity. It exists in different versions for different ages and even a self-report questionnaire for adults [320].

6.3 ANNEX 3: LEXICON-BEHAVIOR

This lexicon describes the principle of all behavioral tests mentioned in this thesis that have not otherwise been described in materials and methods of either part.

Anxiety:

Many tests of anxiety in mice or rats are based on the fact that they tend to explore new environments but are also cautious by instinct and tend to hide away from open areas (anxiogenic areas) where they could be easily spotted by potential predators. They have been developed as tests of anxiety as it was shown that treatment of mice or rats with anxiolytics increased time spent in the anxiogenic areas of those tests.

-Elevated-plus-maze (EPM): The test takes place in a maze that is high from the ground in the shape of a cross with two open-arms and two closed arms (outside walls) (Fig 66 A). The animal is placed in the center of the cross and left to explore. The open arms are a more anxiogenic zone as they are open, elevated, and sometimes brightly lit. Thus the % of time spent by the animal or rat in the open arms is inversely proportional to its anxiety [321].

-Light-Dark-box (L/D box): The test takes place in a box which is divided in two compartments. One is enclosed (with a roof) and dark and one is open and brightly lit (Fig 66 B). The animal is placed in the bright compartment and left to explore. The time spent in each compartment and the transition between the dark and bright compartments during the test are measured. The time spent in the dark compartment is proportional to anxiety [322].

Resignation/motivation:

-Forced swim test (FST): In this test, mice are put in a high cylinder filled with water 22-24° C for 6 min. This is a stressful situation from which they cannot escape, and the time immobile (when the animal has given up trying to escape) is measured [323].

-Novelty suppressed feeding test (NSFT): In this test, mice or rats that have been deprived from food overnight are placed in a brightly lit OF with a food pellet in the center. The latency to start eating the pellet is measured, to assess motivation to eat despite the stressful environment [324].

Immobility time in the FST and latency to feed in the NSFT are decreased in

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rodents treated with antidepressant molecules, so those tests are used to assess anti-depressant properties of treatments.

Social behavior:

-Reciprocal social interaction: Two mice or two rats from the same sex, age and bacterial status are placed in a neutral environment and different behaviors or interactions between them are measured (sniffing, crawling under or over and following) [325].

Learning/memory:

-Novel object recognition: Mice or rats are habituated to the testing arena in a first habituation phase. Then in the learning phase, they are presented with two identical objects. Then animals are returned to their cage for a fixed duration of time that varies depending on the protocol. They are placed back in the testing arena and presented to one of the same objects and one new one for a retention phase. The amount of interaction with each object during this phase is calculated as an indicator of memory, as the animals should have a preference for the novel object [326].

-Morris Water-maze (spatial memory): Mice or rats are placed in a large pool with opacified water so that they cannot see under the surface. A platform is placed in one specific place in the pool (under the water but high enough for the animal to stand on it and have its head out) and visual cues are placed around the pool (Fig 66 C). The animal is put in the pool at different localizations for multiple sessions, to measure the delay to find or not the platform and the evolution of it over time. After a retention period, the animal is put back in the pool, but the platform is removed. Successful learning is considered when the animal goes directly in the right quadrant of the pool. The latency to reach the platform and time spent in this quadrant are measured as indicators of spatial memory [327].

-Fear-conditioning/extinction learning: Mice or rats are placed in a chamber with an electrified grid at the bottom that can deliver small electrical shocks which are stressful for the animals (Fig 66 D). A sound is played for a short duration of time, at the end of which the animals receive a shock. This is repeated a fixed number of times (learning period), then animals are returned to their cage (retention period). After this period, animals are put back in the same cage, and the sound is played without associated shocks (extinction period). Freezing behavior is measured during the extinction

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period as a marker that the animal is still scared to receive a shock and thus has good memory. Another option is to test contextual freezing, (same environment but no sound) or cued freezing (different environment but same sound). Contextual and cued freezing reflect contextual and cued memory [328].

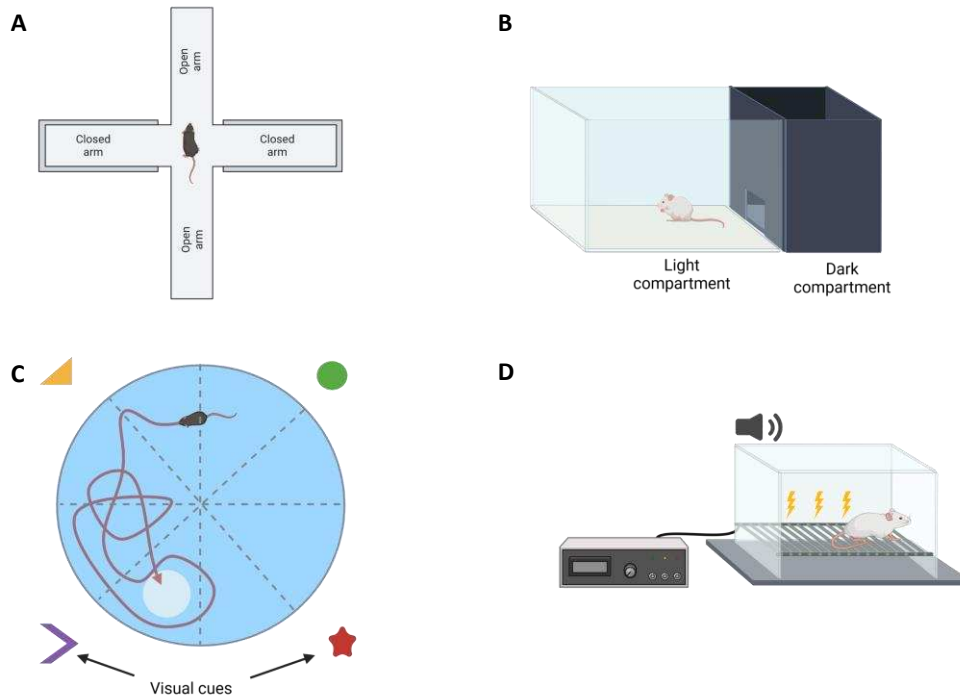


Figure 66 : Schematic representation of behavioral tests.

A) Elevated plus maze (EPM) B) Light/dark box (L/D box) C) Morris water maze D) Fear conditioning. Figure created on Biorender.com

6.4 ANNEX 4 : COMPOSITION OF CULTURE MEDIA USED TO CHECK GF STATUS

Media	Composition (for 1L total volume)
LB (Luria Bertani©) broth	Luria Broth powder (BD Biosciences) 25g Osmosed water
LCY	2 g N-Z amine A, 5 g sodium chloride, 1g monobasic potassium phosphate (Sigma-Aldrich) 2 g yeast extract (BD Biosciences) Osmosed water
Sabouraud	Liquid Sabouraud media (Biomérieux) (undisclosed composition)
BHI (Brain Heart Infusion)	BHI powder (7.7 g veal brain infusion, 9.8 g beef heart, 10 g pepton proteose, 2 g dextrose, 5 g sodium chloride, 2.5 g disodium phosphate (Invitrogen)) 15 g agar (Invitrogen) Osmosed water
VFA (Viande Foie© Agar)	38g Meat liver 0.6‰ (Biorad) 8g agar (Invitrogen)

Table 46 : Detail of the composition of the media used for culture of feces and water from GF isolator to check GF status.

6.5 ANNEX 5: PUBLISHED LITTERATURE REVIEW (MICROORGANISMS, SEPTEMBER 2020)

The published version of the review is provided below.



Review

Role of the Gut Microbiota in the Pathophysiology of Autism Spectrum Disorder: Clinical and Preclinical Evidence

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Received: 10 July 2020; Accepted: 3 September 2020; Published: 7 September 2020



Abstract: Autism spectrum disorder (ASD) is a neurodevelopmental disorder affecting 1 in 160 people in the world. Although there is a strong genetic heritability to ASD, it is now accepted that environmental factors can play a role in its onset. As the prevalence of gastrointestinal (GI) symptoms is four-times higher in ASD patients, the potential implication of the gut microbiota in this disorder is being increasingly studied. A disturbed microbiota composition has been demonstrated in ASD patients, accompanied by altered production of bacterial metabolites. Clinical studies as well as preclinical studies conducted in rodents have started to investigate the physiological functions that gut microbiota might disturb and thus underlie the pathophysiology of ASD. The first data support an involvement of the immune system and tryptophan metabolism, both in the gut and central nervous system. In addition, a few clinical studies and a larger number of preclinical studies found that modulation of the microbiota through antibiotic and probiotic treatments, or fecal microbiota transplantation, could improve behavior. Although the understanding of the role of the gut microbiota in the physiopathology of ASD is only in its early stages, the data gathered in this review highlight that this role should be taken in consideration.

Keywords: autism spectrum disorder; microbiota-gut-brain axis; immune system; tryptophan metabolism; animal models of autism spectrum disorder

1. Introduction

Autism spectrum disorder (ASD) is one of the most prevalent neurodevelopmental disorders, characterized by impairment in social behavior, communication, prevalence of repetitive and stereotyped behavior and lack of adaptation to change. It can sometimes also involve cognitive impairments and anxiety disorders. Although the behavioral diagnosis of ASD has improved in the past decade, it is still very hard to characterize, especially for high-functioning ASD. In addition, the diagnosis is only possible after 18 months of age and cannot be confirmed until a later age, which compromises any preventive measures [1,2].

In the last 50 years, the prevalence of ASD has tremendously increased, more than 35-fold since the 1970s, and is now estimated at 1 in 160 children worldwide, 1 in 54 in the United States and 1 in 89 in the European Union [3–5].

In the early 1990s, ASD was believed to be due at 90% to genetic factors and highly heritable due to the high risk for siblings of ASD patients. More recent studies described a genetic heritability of around 50%. However, these data are difficult to assess accurately, as the genetic variants responsible for ASD are also associated with other neurodevelopmental disorders. The same studies have proven that environmental factors also play an important role in this disorder and can in part explain such an increase in the prevalence of ASD [6–10].

It is important to point out that the prevalence of gastrointestinal (GI) symptoms is four-times higher in ASD children than in typically developing (TD) children and that many studies report a specific GI phenotype in ASD, characterized by increased gut permeability and abnormal immune function in the gut [11]. Interestingly, a recent study reports that ASD children with functional GI disorders show a distinct gut microbiota and immune signature compared to TD children with the same GI symptoms. This study also found a correlation between the ASD-specific dysbiosis (more specifically the increase in species from the *Clostridiales* order) and GI symptoms (inflammation and abdominal pain) [12]. This suggests that these GI symptoms are an integral part of ASD pathophysiology, and are in interaction with the gut microbiota and the immune system.

The involvement of the microbiota-gut-brain axis in ASD has been the focus of numerous studies over the past decade. The purpose of this review is to summarize key findings from clinical and preclinical studies and to describe how ASD-related symptoms can be affected by the gut microbiota. This review begins by describing the changes in microbiota composition and activity that have been first observed in ASD patients but have then also been found in various murine models of ASD over the past decade. It then documents the clinical and preclinical evidence of the implication of the gut microbiota and its metabolites in some important ASD biomarkers such as inflammation and immune impairments, as well as alterations in tryptophan (Trp). Finally, the second part of this review examines the effect of intervention studies targeting the gut microbiota on behavior in ASD patients and animal models of ASD.

2. Clinical and Preclinical Evidence for Involvement of the Gut Microbiota in Various Aspects of ASD

2.1. Dysbiosis and Changes in Bacterial Metabolites in ASD

2.1.1. Clinical Evidence

In 2000, Sandler et al. [13] hypothesized that dysbiosis due to antibiotic treatment in young children was involved in the apparition of regressive autism observed in some of those children. They postulated that this was due to colonization of neurotoxin-producing bacteria, and started a clinical trial on 18 children with a vancomycin treatment with the aim of eliminating these bacteria. They observed improvement in the behavior of those children during the treatment, but it did not last after stopping the treatment. Although the association between early-life antibiotic treatment and ASD has not been confirmed since then [14], this princeps study proved the existence of a causative link between modification of the gut microbiota composition and behavior, in a subset of children with ASD. In 2012, the same team published a summary of their research on the subject, highlighting a dysbiosis in ASD children. One genus, *Desulfovibrio* was present in 50% of ASD children, some of their siblings, but never in unrelated controls. The proportion of *Desulfovibrio* correlated with severity of ASD symptoms [15].

Since then, many research teams have been investigating the gut microbiota of ASD children, and most of them have observed a dysbiosis. Recently, two meta-analyses compared these data in an attempt to identify specific genera or species with a consistent pattern of change across studies. The first one [16] analyzed 9 papers and the second [17] studied 18 papers including 8 from the 9 papers reviewed in the first meta-analysis. Both meta-analyses reported a decrease in *Bifidobacterium* and increase in *Faecalibacterium* and *Clostridium* in ASD children despite high interstudy heterogeneity. Only Iglesias-Vasquez et al. (2020) [17] reported differences at the phylum level, such as higher

Bacteroidetes/Firmicutes ratio in ASD children, or elevated relative abundance of *Proteobacteria*. The two meta-analyses present discrepancies, as Xu et al. (2019) [16] reported a decrease of *Bacteroides* and *Parabacteroides* in ASD children when Iglesias-Vázquez et al. (2020) [17] reported an increase of both those genera. Furthermore, Xu et al. (2020) [16] observed a lower abundance of *Akkermansia* in ASD children when Iglesias-Vázquez et al. (2019) [17] reported no difference. Around the same time, two systematic reviews were also published by Ho et al. (2020) [18] and Bezawada et al. (2020) [19] who compared 26 and 28 studies, respectively, including 14 and 16 of the studies included in any of the meta-analyses. Both underlined the heterogeneity of results among studies and reported a few consistent results. They pointed out that many studies observed an increase in some *Clostridium* species, and a lower proportion of *Bifidobacterium*. Bezawada et al. (2020) [19] also reported that the *Sutterella* genus was found to be more abundant in ASD children in many studies.

Overall, those meta-analyses and reviews confirm the presence of dysbiosis in ASD, despite heterogeneous results among studies. These could be due to methodological differences, but also to the fact that the different cohorts come from multiple countries with different lifestyles and dietary habits. In addition, the age groups of the children recruited in the different studies vary significantly, with some including children as young as 2 years old, an age at which the gut microbiota is not completely stabilized [20]. Despite this, there seems to be a rather consistent increase of *Clostridium*, which is considered to be a putative harmful genus, and a decrease in *Bifidobacterium* which is considered beneficial. Surprisingly, however, both meta-analyses report an increase in *Faecalibacterium* in ASD patients, when the only known species from this genus, *Faecalibacterium prausnitzii*, is considered a beneficial bacterium with anti-inflammatory properties [21].

It is interesting to point out that among the different studies; the control groups differed in their constitution. They were of three types, either composed only of siblings of ASD children, or only of unrelated individuals or of both siblings and unrelated individuals. In the studies with both types of control groups, the sibling group seemed to have a different microbiota profile compared to unrelated individuals, and was sometimes closer to the “ASD profile” [22–26]. This is not surprising considering the influence of genetics and environment on gut microbiota composition. Recent unpublished work from Luna et al. presented at the 74th Annual Meeting Society of Biological Psychiatry Chicago (May 2019) [27] reported a significant difference between the microbiota of TD siblings of ASD children and unrelated controls. This team also reported an influence of GI symptoms on the gut microbiota composition. This heterogeneity in the composition of the ASD and control groups might explain in part the variability between studies. Only an increase in the number of studies and a better standardization of the composition of groups could address this issue.

The existence of a dysbiosis in the gut of many ASD children is now well-accepted but its precise nature is still not completely understood. In order to understand the impact of this dysbiosis on health, researchers have been focusing on bacterial metabolites that are differentially modulated in ASD children. Different teams have found an increase in urinary p-cresol, a bacterial metabolite derived from tyrosine, in young children with ASD [28–31]. These teams hypothesized that this increase could be due to a higher level of p-cresol producing bacteria such as *Clostridium difficile*. However, a study by Gabriele et al. (2016) [32] reported that levels of p-cresol did not correlate with elevated proportions of *Clostridium* species, but correlated with slow intestinal transit. Although the increase of urinary p-cresol in young children with ASD has been observed several times, there is little evidence so far explaining the mechanisms underlying this increase.

Short chain fatty acids (SCFAs) are considered to be key actors of the microbiota-gut-brain axis, and their involvement in multiple neurological disorders has been increasingly described [33]. In the context of ASD, some studies have reported altered fecal levels of SCFAs in ASD children, but with great diversity in results [34–36]. Adams et al. (2011) [34] reported a decrease in total SCFAs in stool of ASD patients while Wang et al. (2012) [35] reported an increase. In Liu et al. (2019) [36] acetate and butyrate levels were decreased in stools of ASD patients while valerate levels were increased. Only Wang et al. (2012) [35] reported significant alteration in propionate levels, which they found to be

elevated in stools of ASD patients. Finally, Averina et al. (2020) [37] found decreased expression of genes related to production of butyrate in the metagenome of ASD children.

As of now, there is still little clinical evidence of the impact of those SCFAs in ASD pathophysiology, most of the evidence comes from animal studies and will be discussed later in this review. Interestingly, patients suffering from propionic acidosis, a genetic disorder characterized by an accumulation of propionate, present neurodevelopmental delay, and a recent publication reported a very high prevalence of ASD (21%) in patients with this disease [38]. Propionate can increase oxidative stress, thus influencing mitochondrial activity. Mitochondrial dysfunction has been reported in many ASD patients and believed to play a role in its pathophysiology [39].

Frye et al. (2016) [40] showed that lymphoblastoid cell lines (LCL) derived from ASD patients had a different response to propionate than LCL from control subjects, especially in an oxidative environment, where propionate induced an overproduction of ATP and mitochondrial dysfunction. In another similar in vitro study, Rose et al. (2018) [41] found that LCL derived from ASD patients also responded differently to butyrate than LCL from control subjects. In LCL from controls, butyrate decreased mitochondrial respiration when it did not significantly alter it in LCL from ASD patients and increased it in LCL from ASD patients with mitochondrial dysfunction.

2.1.2. Preclinical Evidence

Because of the multifactorial aspect of ASD, a number of murine models have been developed. Some are genetic models, like the Shank3 KO, NL3^{R451C} or PCDH9 KO models, based on extinction or mutation of genes known to be involved in some cases of ASD [42–45], or the BTBR mouse strain, considered an idiopathic ASD model, based on its behavioral phenotype [46]. There are also many environmental models either based on a challenge during gestation (maternal high-fat diet (MHFD)), maternal immune activation (MIA), maternal exposure to valproic acid (VPA) or during life (cow's milk allergy (CMA)) [47–50]. All those ASD models have been classified as such based on the fact that they present altered behaviors related to ASD symptoms (social interaction and communication deficits, stereotyped behaviors). However, a growing number of studies report GI symptoms in some of these models similar to those observed in ASD patients. More precisely, increased intestinal permeability was found in Shank3 KO, BTBR and MIA mice [51–54]. Abnormal cytokine profiles have been found in the gut of BTBR, MIA and MHFD mice [52,54,55] and an increase of myeloperoxidase (MPO) expression (marker of inflammation) was found in the ileum of VPA mice [56]. Finally, Hosie et al. (2018) [57] reported a faster transit associated with an increase of inhibitory signaling in the GI epithelium in NL3^{R451C} mice, when the opposite was observed in BTBR mice [53]. Interestingly, many studies have also found dysbiosis in those models, detailed in Tables A1 and A2 (Appendix A). Although the nature of the dysbiosis is very different among the different models, there are a few similarities. Firstly, a decrease in α -diversity has been described in Shank3 KO, BTBR, MIA and MHFD mice and VPA rats [53,55,58–61]. However, other studies in Shank3B KO, NL3^{R451C}, BTBR, MIA and VPA mice did not observe any change in α -diversity [52,54,57,62,63]. All of the studies included in Tables A1 and A2 that assessed β -diversity observed a difference between controls and model animals, except for Hsiao et al. (2013) [54]. At the phylum level, an increase in *Bacteroidetes* and a decrease in *Firmicutes* was observed in BTBR and MIA mice, and in male VPA rats [53,59–61]. This is in agreement with the elevated *Bacteroidetes/Firmicutes* ratio in ASD patients described in a meta-analysis previously mentioned [17]. However, other studies reported an increase of *Firmicutes* in Shank3 KO, VPA and MHFD mice [51,55,63,64] as well as a decrease in *Bacteroidetes* in VPA and MHFD mice [55,63]. The increase in *Proteobacteria* reported in ASD patients was not seen in ASD mice models except in Shank3 KO mice by Sauer et al. (2019) [51]. Plus, a decrease in this phylum was observed in BTBR mice by Coretti et al. (2017) [52]. At lower taxonomic levels, as in ASD patients, a decrease in *Lactobacillus* has been observed in MIA and Shank3 KO mice [54,58] as well as a decrease in *L. reuteri* in Shank3 KO, Shank3B^{-/-} and BTBR mice [58,62] and a decrease in *L. brevis* and *L. ruminis* in Shank3 KO mice [58]. However, Coretti et al. (2017) [52] found increased *Lactobacillus* in male BTBR mice. The *Prevotella*

genus, which has been found to be decreased in ASD patients by Kang et al. (2013) [65], was also decreased in Shank3 KO mice [58] but was increased in MIA and BTBR mice [52,59,60]. Although changes in proportion of *Clostridium* species seem to be recurrent in ASD patients, they were only observed in BTBR mice by Newell et al. (2016) [59], who found decreased and increased levels of various *Clostridium* species in cecal contents and feces, respectively. This study underlies important differences between cecal and fecal composition, which has to be taken in consideration, as most of the studies cited only assessed microbial composition of feces. Plus, most studies used only male mice; however, among the few that used both male and female mice, most observed strong sex-related differences in microbiota composition [52,58,61,63]. Both of those criteria should be considered in future studies.

Overall, the bacterial alterations observed in ASD models vary considerably between studies and models, and do not necessarily reflect the changes observed in ASD patients. However, the occurrence of those alterations in multiple genetic and environmental rodent models of ASD is a strong indicator of the implication of the microbiota-gut-brain axis in ASD pathophysiology. Another observation reinforces this assumption: germ-free (GF) mice, which are devoid of microbiota, present some altered behaviors related to ASD, such as reduced social interaction, and increased stereotyped behavior [49,66] and thus have been proposed as an environmental ASD model.

Interestingly, there are also reports of altered bacterial metabolites levels in different ASD models, similar to what is observed in ASD. In the MIA model, alterations in several serum metabolites have been observed, in particular, 4-ethylphenylsulfate (4-EPS), a metabolite that is derived from the bacterial metabolite 4-ethylphenol, was found to be drastically increased. 4-EPS is derived from tyrosine, and is structurally close to p-cresol. Interestingly, a probiotic treatment with *B. fragilis* NCTC 9343 restored normal serum levels of 4-EPS and ameliorated anxiety-like behavior, but neither social nor repetitive behaviors in the MIA model [54]. A whole range of intestinal bacteria such as *Coriobacteriaceae*, *Enterobacteriaceae*, *Fusobacteriaceae* and *Clostridium* clusters I and XIVa, can catabolize tyrosine into aromatic derivatives, including p-cresol [67]. However, the reasons behind such an increase in this model are currently not known. Interestingly, in a recent report from Bermudez-Martin et al. (2020) [68] a 4-week administration of p-cresol in the drinking water changed microbiota composition and induced impaired social behavior and increased repetitive behavior in wild-type (WT) mice. P-cresol treatment also impaired excitability of dopaminergic neurons in the ventral tegmental area of those mice, a circuit involved in the social reward system [69]. Plus, the authors showed that the effect of p-cresol on behavior was dependent on microbiota composition, as fecal microbiota transplantation (FMT) from p-cresol treated mice to WT mice induced the similar behavioral impairments, and, in contrast, FMT from WT mice to p-cresol-treated mice restored normal social behaviors [68].

The implication of SCFAs has also been investigated in ASD models. In the BTBR model, Golubeva et al. (2017) [53] reports decreased levels of acetate and isobutyrate, but increased levels of butyrate. In addition, increased levels of butyrate have been observed in male VPA mice [54]. Neither of those studies reported on a difference in the levels of propionate. However, there has been reports of an effect of propionate on ASD-related behavior, as its administration to rats alters social behavior, increases repetitive behaviors and alters cognitive functions [70–72]. In addition, similarly to what is observed in the VPA model, mother exposure to propionate induces social deficits in the offspring [73]. On the contrary, butyrate improves social deficits in the BTBR mouse model [74], which is surprising considering that butyrate levels are increased in this model [53].

2.2. Influence of the Gut Microbiota on Immune System Dysregulation in ASD

2.2.1. Clinical Evidence

Immune system impairments such as higher blood levels of pro-inflammatory cytokines, dysfunctional immune cells or presence of antibodies targeting brain proteins, have been observed in many ASD children, and in their mothers during pregnancy and post-partum. Interestingly, studies

have showed that those increases in pro-inflammatory cytokines can correlate with the severity of some behavioral symptoms [75–77].

Many clinical studies report higher prevalence of ASD following bacterial or viral infection during pregnancy, which could lead to an inflammatory environment in the placenta and amniotic fluid. It has been hypothesized that those infections, whether they occur before or after birth, could play a crucial role in ASD pathogenesis, as they can influence important neurodevelopmental mechanisms, like microglial maturation and synaptic pruning [77].

The gut microbiota and the immune system are intrinsically linked. It is accepted that a major constitutive function of the immune system is to control the microbiota and reinforce the intestinal barrier. In turn, the microbiota also has a direct effect on the immune system, as bacterial metabolites or compounds can influence differentiation of immune cells, or regulate their activity, not only in early postnatal development but throughout the lifespan. The mammalian immune system has co-evolved with the establishment of the microbiota, to reach a symbiotic relationship. However, this relationship can become more deleterious depending on genetic background, environmental challenges or changes in nutrition [78,79].

Because of those observations, it has been hypothesized that one way of action of the gut microbiota in ASD was through its action on the immune system, more specifically on the balance between T regulatory cells (Treg) and effector T cells, such as T helper (Th) cells in the gut. Those Th cells are the results of naïve CD4+ T cells differentiation. One subtype of Th cells, Th17, is pro-inflammatory and can be involved in autoimmunity. On the contrary, Treg cells are anti-inflammatory and play a protective role against autoimmunity. Disruption of Treg/Th17 balance has been linked to the pathophysiology of many autoimmune diseases, and could also be involved in ASD [80,81]. Although differentiation is mostly driven by immune signals such as chemokines or cytokines, the Treg/Th17 balance could be influenced by an altered microbiota. Indeed, differentiation into Treg can be induced by some species of *Clostridiales* and by *Bacteroides fragilis*, whereas the differentiation into Th17 cell can be induced by some segmented filamentous bacteria (SFB) [82].

Interestingly, in Rose et al. (2018) [83] pro-inflammatory cytokines were elevated in the serum and gut of ASD patients, and this elevation was higher in children with ASD and GI symptoms than in children with ASD and no GI symptoms. In addition, a more recent study by the same team characterized circulating effector T cell populations in ASD patients with or without GI symptoms in comparison to TD controls. They found increased levels of IL-17 positive CD4+ and CD8+ T cells in ASD patients compared to controls, and this increase was even stronger in ASD patients with GI symptoms. The levels of IFN γ were also increased in ASD patients with GI symptoms compared to ASD patients without. Furthermore, they found decreased regulatory T cells in both ASD groups compared to TD, and a decrease in Treg/Th17 ratio in ASD patients with GI symptoms [84]. These observations have been completed by the fact that some of the bacterial species altered in ASD patients appear to be associated with overproduction of interferons (IFN) and pro-inflammatory cytokines in the gut. Indeed, a correlation was found between fecal levels of *Faecalibacterium* and increased levels of genes involved in type I IFN and IFN- γ signaling in immune cells of ASD children compared to TD-unrelated controls [85]. Type I IFN signaling induces antimicrobial programs and is involved in regulation of innate and adaptive immunity, but also in autoimmune diseases [86]. In addition, Luna et al. (2017) [12] reported the existence of a correlation between levels of multiple bacterial species in children with ASD and GI symptoms, and elevated levels of various cytokines in their blood.

Clinical studies also report a neuroinflammatory state in ASD, characterized by proliferation and morphological modification of microglia and astrocytes into a reactive state in the brain. Indeed, post mortem observation of the brains of ASD patients revealed increased glial fibrillary acidic protein (GFAP)-positive cells and GFAP protein levels (marker of astrogliosis). Plus, increased markers of reactive microglia and astrocytes were found in various brain regions, but most notably in the cerebellum. Similarly to what has been observed in the blood of ASD patients; increased levels of many pro-inflammatory cytokines were found in the brain and cerebrospinal fluid of ASD patients post

mortem [87,88]. Suzuki et al. (2013) [89] used positron emission tomography (PET) and observed more reactive microglia in various brain regions of ASD patients compared to controls, most strikingly in the cerebellum. Those neuroglial alterations are believed to play a role in ASD pathophysiology, as microglia and astrocytes are involved in neurodevelopment, in part via synaptic pruning. In physiological conditions, synaptic pruning consists in reinforcement of important connections and removal of redundant connections by phagocytosis. This process plays a crucial role in wiring the brain during development and is involved in plasticity during life, but could be deleterious if overly activated. Thus, a reactive state of microglia and astrocytes in development and throughout life in ASD may result in changes in neuronal morphology and connectivity which could contribute to behavioral and cognitive alterations [90]. To our knowledge there are no clinical studies that link those neuroinflammatory defects to the impaired microbiota in ASD. Only animal studies, as described below, provide evidence for a crucial role of a complex microbiota in microglial maturation and function.

2.2.2. Preclinical Evidence

First, in studies in the VPA-induced murine model of ASD, markers for neuroinflammation were found to be increased in the dorsal hippocampus associated with marked changes in microbiota composition in the intestinal tract [56,63]. In addition, a study by Erny et al. (2015) [91] showed that absence of microbiota from birth (GF mice) or depletion during life (SPF mice treated with antibiotics) led to immature microglia exhibiting a blunted response to LPS challenge. This was reversed by co-housing with SPF mice with a complex microbiota. Interestingly, a normal microglial phenotype was also restored by the administration of a cocktail of SCFAs in the drinking water. Finally, another more recent study demonstrated therapeutic effects of a Trp-derived bacterial metabolite, Indoxyl-3-sulfate (I3S), on microglia- and astrocyte-related neuroinflammation in a mouse model of multiple sclerosis [92]. Those results proved that a complex microbiota and its metabolites are necessary for microglia maturation and influence microglia and astrocyte function both during development and throughout life.

Animal studies also provide most of the evidence on the implication of the microbiota in the other immune alterations observed in ASD. First, studies on germ-free animals prove that the microbiota is important for maturation of the immune system and helps maintain immune homeostasis [93]. Plus, as previously mentioned, an immune challenge during pregnancy in the MIA model results in a dysbiosis in the offspring along with altered communication, social and repetitive behaviors and cortical defects similar to ASD [48,60,94]. Immune alterations similar to those seen in ASD patients were observed in MIA offspring, such as an increase in IL-6 and IL-17 pro-inflammatory cytokines, and higher proportion of Th17 cells [54,95]. Interestingly, in Hsiao et al. (2013) [54], a probiotic intervention with *B. fragilis* NCTC 9343 was sufficient to restore normal IL-6 levels. More recently, a study by Kim et al. (2017) [95] demonstrated that a vancomycin treatment in MIA mothers during the whole gestational period prevented Th17 dysregulation in mothers and the appearance of behavioral and cortical alterations in offspring. The authors suspected that the vancomycin treatment induced a depletion of SFB which can induce T cell differentiation into Th17. Plus, they observed no MIA-induced behavioral phenotypes in mouse strains lacking SFB, and in consequence producing less Th17. Gavage of those mice with SFB was sufficient to restore MIA-induced phenotypes in the offspring. These data demonstrated that the presence of SFB in the gut, and consequent Th17 differentiation, were necessary to induce behavioral and cortical abnormalities in MIA offspring.

It is interesting to note that immune dysregulations can also be observed in genetic or environmental models of ASD that are not related to an immune challenge. Plus, these dysregulations are often associated with bacterial modifications. In BTBR mice, an enhanced inflammatory response to LPS challenge has been observed, and basal colonic levels of TNF- α and IL-6 are elevated which correlates with some of the alterations in bacterial composition [52,96]. In models based on deletion or mutation of the Shank3 gene, systemic increase of IL-6 and IL-17 have been observed, as well as more GFAP-positive cells, which is a marker for astrogliosis. Interestingly, treatment with *L. reuteri* MM4-1A could lower

IL-17 levels in this model [51,58]. In the MHFD model, an increase of IL-1 β , IL-6 and TNF- α has been observed [55] as well as an increase in intestinal levels of IL-17 due to a higher proportion of innate lymphoid cells 3 (ILC3) cells in the intestinal lamina propria of the offspring. The authors treated pregnant mice with antibiotics to obtain offspring with depleted microbiota. These offspring were then transplanted with gut microbiota from either MHFD or control mice of the same age. They observed a higher proportion of ILC3 in offspring colonized with MHFD microbiota compared to offspring colonized with microbiota from controls. This result proved that the effect on ILC3 cells was dependent on the microbiota [64]. Finally, in the VPA model, various studies reported an increase of microglial density in various brain regions, and a LPS challenge induced overproduction of IL-6, IL-1 β and TNF- α in the brain and in the spleen [56,97–99]. As previously mentioned, VPA mice and rats also have a disturbed microbiota with elevated butyrate production [61,63]. Butyrate is often considered a beneficial SCFA in gut-brain axis regulation, and has been found to enhance intestinal and blood-brain barrier (BBB) functions and promote anti-inflammatory responses [33]. However, de Theije et al. (2014) [56,63] proposed that the microbiota changes and the elevated butyrate levels they observed in the caecum of VPA mice could be associated to increased intestinal inflammation through modulation of the mucus composition. This lead would be interesting to pursue in order to gain a better understanding of the link between elevated butyrate and inflammation in this model.

Overall, those preclinical results show that immune challenges either during pregnancy or throughout life lead to ASD-like behaviors, and that this effect can be microbiota-dependent. Those observations and the fact that immune dysregulations are present in many ASD models, and often correlate with microbiota changes and altered behaviors, implies the existence of a microbiota-immune-brain relationship that could be part of the pathophysiology of ASD. However, most of the evidence of a gut microbiota-immune-brain axis in ASD is still based on preclinical research and there is a need for more clinical research on the subject.

2.3. Influence of the Gut Microbiota on Dysregulation of Tryptophan Metabolism in ASD

2.3.1. Clinical Evidence

Trp cannot be produced by the body and only comes from dietary consumption. Dysregulations of the Trp metabolism in ASD have been described; however, their implication in the disorder is still unclear. Dietary Trp is the precursor for serotonin (5-HT) and kynurenine (KYN), whose pathways have been shown to be dysregulated in ASD [100]. KYN is derived from Trp via the indoleamine-2,3-dioxygenase (IDO), activated in presence of pro-inflammatory cytokines (IL-6 and IL-1) and TNF. KYN can then cross the BBB and be transformed into two derivatives, kynurenic acid (KA) or quinolinic acid (QA). KA is neuroprotective and reduces excitotoxicity via inhibition of the N-methyl-D-aspartate (NMDA) receptors, whereas QA is an agonist of those receptors and is thus neurotoxic [100,101]. Serum of ASD patients presents lower KA concentration [101,102], higher KYN/KA ratio and higher QA concentration [101]. Decrease of KA and increase of QA in the serum might reflect similar changes at the central level, thus leading to increased excitotoxicity, which may be involved in ASD pathophysiology.

The other main derivative of Trp is 5-HT. Ninety-five percent of 5-HT circulating in the body is produced by the gut enterochromaffin cells (ECs) through the action of the rate-limiting enzyme tryptophan hydroxylase 1 (TPH1) and of the aromatic acid decarboxylase (AADC). It plays a crucial role in regulation of GI functions. In the brain, 5-HT is produced via TPH2 and plays an important role in various brain functions such as mood, sleep or appetite regulation. Furthermore, both central and peripheral 5-HT play a role in pre- and postnatal neurodevelopment, thus their dysregulation has been hypothesized to be involved in ASD pathophysiology [103,104]. A few studies report central alterations of 5-HT in ASD patients. However, most of the evidence towards dysregulation of the 5-HT metabolism in ASD comes from the fact that increased blood levels of 5-HT have been widely observed in ASD patients since the 1960s, being found in more than 25% of them [104,105]. While this increase could be due to increased uptake by platelets or decreased breakdown, it could also be due to

increased 5-HT release by ECs in the gut [104,106]. One study has found a small positive correlation between severity of GI symptoms and whole blood 5-HT levels in ASD patients [107]. Interestingly, Luna et al. (2017) [12] found decreased levels of Trp, and elevated levels of the 5-HT metabolite 5-HIAA in rectal tissue of ASD patients with GI dysfunction, and those modulations correlated with the increase or decrease of some bacterial species in the gut microbiota of those patients. To our knowledge, the effect of a probiotic intervention on the Trp pathway has not been investigated in ASD patients. However, it has been investigated in healthy subjects or patients with other pathologies. In a study by Kato-Kataoka et al. (2016) [108], daily intake of a fermented drink containing *L. casei* Shirota for 8 weeks prevented the elevation of plasmatic Trp of healthy subjects before a stressful examination period. In another study, after a long-term administration (105 days) of *L. reuteri* DSM-17938, adults suffering from functional constipation had lower plasmatic levels of 5-HT [109]. Finally, daily administration of probiotics for 8 weeks, either the *L. helveticus* R0052/*B. longum* R0175 mix or *L. plantarum* 299v, resulted in a decrease in seric KYN/Trp ratio and a decrease in seric KYN levels, respectively, in patients suffering from depressive disorders [110,111]. While these studies provide evidence that probiotic treatments can influence the Trp pathway, it is still unclear if the changes of microbiota in ASD are involved or not in the Trp alterations observed in ASD patients.

2.3.2. Preclinical Evidence

Multiple preclinical studies have proved that the microbiota can influence Trp metabolism. Clarke et al. (2012) [112] have observed increased plasmatic Trp and a decreased plasmatic KYN/Trp ratio in male and female GF mice, the latter being restored by gut colonization with SPF microbiota. Plus, two separate studies found that GF mice had lower colonic levels of 5-HT and lower colonic expression of TPH1 mRNA, compared to SPF mice or mice colonized with microbiota from healthy human donors [113,114]. The study from Yano et al. (2015) [114] found an increased expression of the 5-HT transporter gene, SLC6A4, which they hypothesize to be a compensatory response to the deficit in 5-HT synthesis. Interestingly, colonization of GF mice at postnatal day 42 with spore-forming bacteria from either SPF mice or healthy human donors restored colonic and seric levels of 5-HT and normal TPH1 and SLC6A4 gene expression in the colon. The other study, from Reigstad et al. (2015) [113] found that in vitro stimulation of human-derived ECs with acetate or butyrate could induce TPH1 expression. Overall, those results show that certain types of bacteria from the gut microbiota and their metabolites can influence Trp metabolism along the 5-HT and KYN pathways.

Interestingly, BTBR, MIA, CMA and VPA mouse models of ASD all show impaired 5-HT metabolism. MIA mice present increased serum 5-HT [54], and CMA mice have increased 5-HT but decreased 5-HIAA in the ileum [50]. A decrease of intestinal 5-HT was also found in BTBR mice, as well as an increase in 5-HT/5-HIAA ratio [53]. In the VPA model, de Theije et al. (2014) [56] observed decreased 5-HT levels in the ileum associated with fewer ECs. The authors also observed alterations in 5-HT metabolism in the brain, such as a decrease of 5-HT and increase in 5-HIAA/5-HT ratio in the prefrontal cortex and amygdala. Plus, a recent study found that MIA mice presented increased expression of the 5-HT_{2A} receptor in the frontal cortex [94]. However, it is still unclear if those central alterations are influenced by intestinal 5-HT.

As previously mentioned, microbiota alterations were described in all those models. Interestingly, in VPA, BTBR and MIA mice, the 5-HT alterations correlated with some of the observed microbiota changes [53,63,94].

Although most evidence towards an influence of the gut microbiota on alteration of neurotransmitter systems in ASD is focused around 5-HT, there has been sporadic evidence on involvement of the gut microbiota in other neurotransmitter systems related to ASD, particularly the GABAergic (gamma-aminobutyric acid) and glutamatergic systems. Clinical studies have found alterations in central or peripheral levels of GABA or glutamate, or altered expression of their receptors in the brain of ASD patients [31,115–117]. Some bacteria of the human gut microbiota are capable of producing GABA, which could be one of the ways that the microbiota impacts the gut-brain axis [118].

Interestingly, Kang et al. (2018) [30] found lower levels of GABA, as well as lower GABA/glutamate ratio in the feces of children with ASD. However, the authors found no correlation between these changes and the microbiota modulations observed in these patients. Another recent study looked at gene expression in the metagenome of ASD patients and found a decrease in genes related to GABA production [37]. More evidence comes from animal studies, as decreased expression of GABA receptors has been observed in the hippocampus of Shank3 KO mice. Interestingly, this alteration correlated with *L. reuteri* levels in the microbiota of those mice, and *L. reuteri* MM4-1A treatment partly restored those expression levels [58]. There is still little evidence of the implication of the microbiota in those GABA and glutamate alterations in ASD, but those first results provide a promising avenue to pursue.

In conclusion to this first part, it is now well-accepted that ASD patients have a disturbed microbiota, with altered metabolic activity. Increasing evidence shows that those disruptions can influence the immune system and Trp metabolism, both in the periphery and in the brain. Thus, the gut microbiota may have an influence on neurodevelopment and brain function during the life of ASD patients. These new findings have prompted many teams to test whether interventions on the gut microbiota could have beneficial effects on GI symptoms, brain function and behavior in ASD.

3. Clinical and Preclinical Interventions Targeting the Gut Microbiota

3.1. Probiotic Intervention Studies for ASD Symptoms

Recently, a few interventional clinical studies and more interventional preclinical studies have been published, bringing evidence that modulation of the gut microbiota can influence ASD-related behaviors, as well as some elements explaining the underlying mechanisms of this effect.

3.1.1. Clinical Studies

As previously mentioned, one of the first studies to establish a link between gut microbiota dysbiosis and ASD was published in 2000 by Sandler et al. [13], who observed behavioral improvement of children with ASD during a vancomycin treatment, showing that modification of gut microbiota can induce changes in behavioral symptoms. However, those effects did not persist after the treatment, and a long-term antibiotic treatment is not feasible, thus, researchers have started to investigate the potential role of probiotic treatments in ASD. Multiple studies have reported effects of probiotic treatments on microbiota composition and GI symptoms in ASD children. Although many of these studies did not analyze the behavior of the children, or did not see any improvement after probiotic administration [26,119–121], other studies did report behavioral improvement [122–125]. In Shaaban et al. (2017) [123], 30 children with ASD were given a 3-month, daily treatment with a patented probiotic mixture (composed of strains of the species *L. acidophilus*, *L. rhamnosus* and *B. longum*) which induced an improvement in communication, sociability and cognitive awareness, characterized by a decrease in the ATEC (autism treatment evaluation checklist) score. In Liu et al. (2019) [124], *L. plantarum* PS128 was given to 36 children for 4 weeks in a placebo-controlled trial. The authors did not observe an improvement in behavioral scores using different diagnosis scales, but saw a decrease in anxiety behavior, hyperactivity and opposition/defiance behaviors. They propose that the effects of the treatment could have been stronger if it had been administered for a longer period of time.

Overall, considering the variations in the probiotic choice, group size, duration of treatment and behavioral assessment tools, these results are not yet sufficient to establish a beneficial effect of probiotic interventions on behavior in ASD. However, the use of probiotics could be an interesting lead of treatment or preventive measures as suggested by numerous preclinical studies showing an effect of probiotics on behaviors related to ASD, which will be detailed later. Since each strain or even species could have a different influence on ASD symptoms, only an increased number of studies could allow to identify specific beneficial probiotic strains.

3.1.2. Preclinical Studies

As previously mentioned, there are multiple murine models of ASD, genetic or environmental, that present altered behaviors relative to ASD. As many of those models also have impaired GI function and gut microbiota, as previously described, many research teams have wondered if modulating this microbiota composition using a probiotic treatment could improve the altered behaviors of those models. One of the first groups to publish such a study was Sarkis Mazmanian's group, who tested the effect of *Bacteroides fragilis* NCTC 9343 in the MIA mouse model [54]. They observed an improvement in anxiety-like behavior, stereotyped behavior, communication and cognitive function. However, the probiotic treatment had no impact on social behavior. Interestingly, the authors identified one metabolite, 4-EPS, whose increased serum levels in MIA mice was restored to control values by the probiotic treatment. In parallel, a chronic systemic administration of 4-EPS to naïve mice induced an anxiety-like behavior. Since then, some other studies have tested probiotics in other mouse models of ASD. Buffington et al. (2016) [49], showed that *Lactobacillus reuteri* gut concentration was decreased in the MHFD mouse model, and that treatment with *L. reuteri* MM4-1A restored social behavior in those mice. Based on this observation, another team showed improvement of social and repetitive behavior in the Shank3 KO genetic model of ASD, following treatment with *L. reuteri* MM4-1A [58]. This has been later further explored by Sgritta et al. (2019) [62] who found that *L. reuteri* MM4-1A treatment improved social behavior in the VPA environmental model, the BTBR idiopathic model and the Shank3B KO genetic model. They also reported that administration of *L. reuteri* MM4-1A improved social behavior of GF mice, proving that this bacterium could act on its own. Interestingly, the same study also demonstrated that the effect of *L. reuteri* MM4-1A in Shank3B^{-/-} mice was dependent on the vagus nerve, as treatment with this bacterium was inefficient in vagotomized Shank3B^{-/-} mice. This work also brought a very thorough mechanistic explanation of the probiotic effect, showing that it was dependent on the presence of oxytocin receptors in the ventral tegmental area, which is involved in social interaction-induced neuronal plasticity [62].

These recent data provide good arguments on the potential effect of specific probiotic treatments on behavior in ASD patients, and on the mechanistic functioning of the microbiota-gut-brain axis in the context of this disorder. All these results obtained in rodents are a first step that will have to be confirmed in studies on ASD patients. In the coming years we should see the first studies published, owing to large-scale projects involving longitudinal surveys of children at risk for ASD and intervention trials with probiotics, e.g., the European-funded GEMMA project [126] or an American study testing the effects of an *L. reuteri* treatment, associated or not with an oxytocin nasal spray, on social behaviors in ASD patients [127].

3.2. FMT Studies

3.2.1. Clinical Studies

To our knowledge, there are very few clinical studies exploring the impact of fecal microbiota transplantation (FMT), on ASD symptoms in patients. FMT is most commonly used as treatment of *C. difficile* infections, where it seems very efficient [128]. Besides, some studies have proven that FMT could have a therapeutic effect in patients with irritable bowel syndrome [129,130] and promising effects on insulin resistance and metabolic parameters in patients with metabolic syndrome, though this needs further investigation [131,132]. Some studies have also started to investigate the impact of FMT in various neurological disorders [133]. While those results are promising, it is worth noting that this procedure is not yet completely mastered and there is a need for more large-scale longitudinal studies. In 2017, Kang et al. published an open label study in which they performed FMT in 18 patients with ASD (7–16 years old) and comorbid GI symptoms [134]. Eighteen weeks after FMT, the team observed in all patients an amelioration of GI symptoms, an increase of microbiota diversity, assessed using Faith's phylogenetic diversity index, and an amelioration of ASD-related behavioral symptoms, assessed according to clinical and parental based scales. The same team published a follow up study

2 years later, and the previously observed ameliorations had remained the same or even improved [135]. Those results offer a promising lead on the efficacy of FMT for amelioration of behavior and GI symptoms in ASD, but it needs to be further investigated in controlled studies with larger cohorts.

3.2.2. Preclinical Studies

The use of ASD mice models in preclinical studies is necessary for a more mechanistic understanding of the impact of FMT on microbiota, GI symptoms, other ASD-related markers and ASD-like behavior. Preclinical research has brought evidence of a potential therapeutic role of FMT in many neurological disorders, including ASD [133]. As previously mentioned, GF mice present impaired social interaction [49,66]. Interestingly, in Buffington et al. (2016) [49] colonization of GF mice at weaning with microbiota from normal mice normalized anxiety-like and social behaviors, while colonization with microbiota from MHFD mice did not. It is interesting to point out that the effects of the FMT did not appear when it was done at 8 weeks of age, highlighting the existence of a critical time window during which FMT in initially GF mice can impact behavior. In a recent study, Saunders et al. (2020) [94] transplanted gut microbiota from adult MIA mice or from control mice into 28-days-old control mice whose microbiota had been depleted by antibiotic treatment. The mice that received MIA microbiota showed impaired performances in the object recognition test compared to mice that received microbiota from controls. Overall, those results show that FMT from healthy mice can improve behavior in ASD models, whereas FMT from an ASD mouse model can induce behavioral deficits in healthy mice.

Recently, Sharon et al. (2019) [136] reported that transferring microbiota of children with ASD to GF mice could influence ASD-like behavior. GF mice were colonized at weaning with microbiota from ASD patients and from their typically developing (TD) siblings as control (humanization of mice). Their offspring (named ASD and TD, respectively) were tested for social interaction (three chamber sociability test), stereotyped behavior (marble burying test) and anxiety-like behavior (open-field). ASD mice presented decreased social interaction and increased stereotyped behavior compared to TD mice. Following this observation, the team observed a correlation between those behavioral differences and the higher or lower quantity of specific bacteria in ASD mice microbiota. They also observed alternate splicing for many genes in the prefrontal cortex, including a few that are known to be involved in some human cases of ASD or other neurodevelopmental syndromes. Finally, the team highlighted some metabolomic differences in colon content of ASD mice compared to TD mice, particularly a decrease in 5-aminovaleric acid and taurine, which could rescue some behavioral defects when administered to mice.

Another recent study performed FMT in the MIA mouse model, using pooled stools from three healthy human donors [60]. Mice received microbiota either directly after collection from the donors or after an *in vitro* culture step. Both FMT procedures reduced repetitive behavior assessed with the marble burying test and self-grooming analysis. Only the FMT with microbiota coming directly from the donors had an effect on anxiety-like behavior and none of the two FMT procedures had a significant impact on social behavior in the three-chamber social interaction test.

FMT is a promising approach to improve behavior and GI symptoms in ASD patients. However, more clinical studies need to be done in order to reinforce this hypothesis. More preclinical studies are also necessary in order to gain more insight into the mechanisms by which FMT can induce systemic and neuronal changes leading to behavioral improvement.

4. Conclusions

Despite discrepancies between studies, the data presented in this review converge to conclude that ASD patients exhibit an abnormal microbiota composition, with altered activity. Whether these alterations are involved in the onset of ASD, or occur during the development of the disease, a growing body of research suggests that they may aggravate the behavioral symptoms and biological signs of ASD.

This led to the use ASD animal models to try to elucidate the mechanisms underlying the involvement of the gut microbiota in this disorder. To date, these preclinical studies conducted in rodents have particularly shown alterations in the immune system and in the metabolism of Trp (summarized in Figure 1). In addition, recent studies bring evidence of a role of the gut microbiota, through its metabolites, in other neurological and physiological aspects that are disturbed in ASD patients, such as the GABAergic and glutamatergic transmission in the brain or respiratory mitochondrial activity [31,40,41].

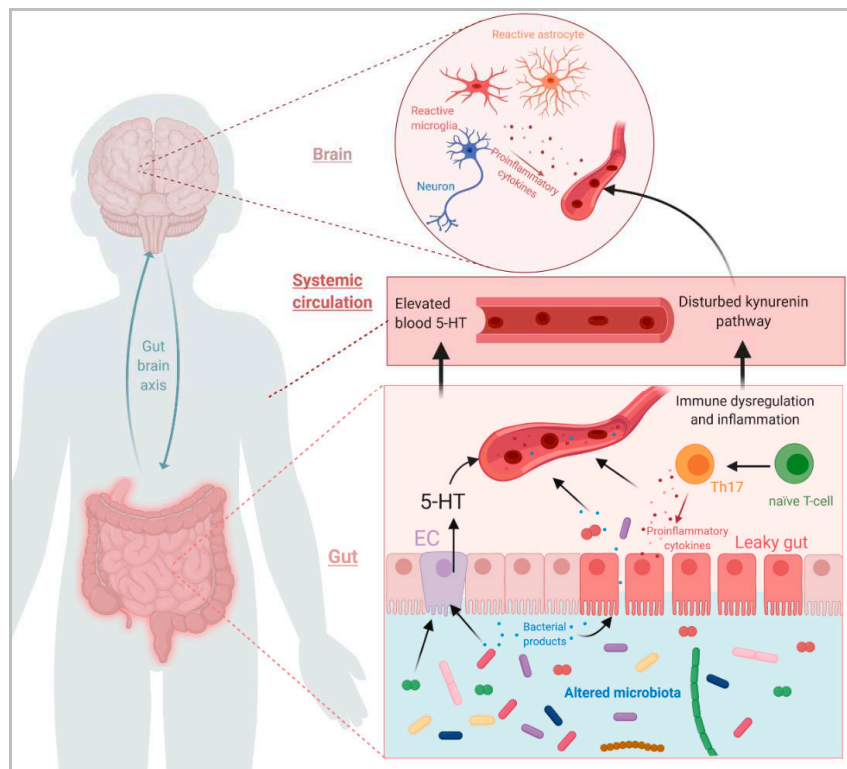


Figure 1. Summary schematic showing the potential impacts of a disturbed gut microbiota on various parameters in the gut, systemic circulation and brain in ASD, and how those parameters can be linked. (Figure created with [BioRender.com](https://www.biorender.com)).

Other factors could be associated with ASD and influenced by the gut microbiota. For example, it has been suggested that heavy metal imbalance could be involved in ASD, and studies show that heavy metals can influence gut-microbiota composition, that heavy metal imbalance is correlated with abundance of some bacterial genera, and that some bacterial species are capable of transforming heavy metals into more or less-toxic derivatives [137–139]. In a similar way, deficiencies in vitamin B levels, which have been found in ASD patients [140], could be linked to dysbiosis, considering the role of the gut microbiota in vitamin synthesis [141]. However, to our knowledge, there are still very few reports as of now of those interactions in the context of ASD, and this would be interesting to deepen the research on these subjects.

Recent clinical interventional studies, using probiotic treatments or FMT, have produced some promising results, supported by data from preclinical studies. Hopefully, by pursuing this back and forth between clinical and preclinical work, new evidence of the involvement of the intestinal microbiota in ASD are expected to be found, as well as new mechanisms underlying the action of the gut microbiota. Furthermore, since it is still unclear whether microbiota alterations appear as a consequence of ASD or are involved in its onset—there is also a need for longitudinal studies, in order to characterize when the microbiota comes into play in this disorder, and if it can be used as

an early biomarker. This type of research will be implemented, in the coming years, for example by the European GEMMA project [126].

Author Contributions: Writing—original draft preparation: L.R., L.N.; writing—review and editing: L.R., N.P., P.P.-P., A.D.K., S.R., L.N. All authors have read and agreed to the published version of the manuscript.

Funding: This review was written in the context of the GEMMA project, funded by the European Commission by means of the Horizon 2020 program (call H2020-SC1-BHC-03-2018) with the project ID 825033.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

ASD	Autism spectrum disorder
BBB	Blood brain barrier
CMA	Cow's milk allergy
EC	Enterochromaffin cells
4-EPS	4-Ethylphenylsulfate
FMT	Fecal microbiota transplantation
GABAergic	gamma-aminobutyric acid
GF	Germ free
GFAP	Glial fibrillary acidic protein
GI	Gastrointestinal
5-HT	Serotonin
IFN	Interferon
ILC3	Innate lymphoid cells
KA	Kynurenic acid
KYN	Kynurenin
LCL	Lymphoblastoid cell lines
LPS	Lipopolysaccharide
MHFD	Maternal high fat diet
MIA	Maternal immune activation
MPO	Myeloperoxidase
NMDA	N-methyl-D-aspartate
QA	Quinolinic acid
SCFAs	Short chain fatty acids
SFB	Segmented filamentous bacteria
SPF	Specific pathogen free
TD	Typically developing
Trp	Tryptophan
VPA	Valproic acid
WT	Wild-type

Appendix A

Table A1. Microbiota modulations in genetic models of ASD.

Model	Sex	Sample	Method	Difference in Microbiota Compared to Controls	Ref.
Shank3 ^{-/-}	F/M	Feces	16S rRNA seq RT-PCR	<p>α-diversity: ↓ β-diversity: Modulated Phylum level: N.S Class level: ↓<i>Bacilli</i> Order level: ↓<i>Lactobacillales</i>, <i>Rhodospirillales</i>, <i>Rickettsiales</i> and <i>Turicibacteriales</i> Family level: ↑<i>Veillonellaceae</i>; ↓<i>Lactobacillaceae</i>, <i>Bacteroidaceae</i>, <i>Acetobacteriaceae</i>, <i>mitochondria</i> and <i>Turicibacteriaceae</i>; ↑<i>Veillonellaceae</i> Genus level: ↓<i>Lactobacillus</i>, <i>Coprococcus</i>, <i>Bacteroides</i>, <i>Acetobacter</i>, <i>Turicibacter</i> and <i>Prevotella</i>; ↑<i>Veillonella</i> in males ↓ in females Species level: ↓<i>L. reuteri</i>, <i>L. brevis</i>, <i>L. ruminis</i> in both male and female; ↓<i>V. parvula</i> and <i>V. dispar</i> in females, ↑<i>V. dispar</i> in males</p>	[58]
	N.S	Feces	16S rRNA seq	<p>No assessment of diversity Phylum level: ↑<i>Actinobacteria</i> and <i>Firmicutes</i>; ↓ <i>Proteobacteria</i> Absence of <i>Verrucomicrobia</i>; Presence of <i>Deferribacteres</i>, <i>Chlamydiae</i> and <i>Tenericutes</i> Class level: N.S Order level: ↑ <i>Bifidobacteriales</i> and <i>Eggerthellales</i> Family level: N.S Genus level: ↑ <i>Asaccharobacter</i>, <i>Eggerthella</i>, <i>Enterorhabdus</i> and <i>Paraeggerthella</i> Species level: ↑ <i>B. pseudolongum</i>, <i>Assacharobacter</i> WCA-131-CoC-2, <i>Eggerthella</i> YY7918 and <i>Enterorhabdus caecimuris</i>.</p>	[51]
Shank3B ^{-/-}	M	Feces	16S rRNA seq	<p>α-diversity: No changes β-diversity: Modulated Bacterial modulation were not detailed except for: Species level: ↓ <i>L. reuteri</i></p>	[62]
NL3 ^{R451C}	M	Feces	ARISA * 16S rRNA seq	<p>α-diversity: No changes β-diversity: Modulated at 3 weeks of age (not at 9 weeks) Bacterial modulations were only detailed at OTU level: Species level (OTUs): ↑ OTUs from <i>Lachnospiraceae</i> family, ↓ OTUs from <i>Candidate</i> phylum</p>	[57]

Table A1. Cont.

Model	Sex	Sample	Method	Difference in Microbiota Compared to Controls	Ref.
BTBR	M	Feces and cecal content	16S rRNA seq	<p>α-diversity: ↓ β-diversity: Modulated in cecal content only Phylum level: ↑ <i>Bacteroidetes</i> in cecal content Class level: N.S Order level: N.S Family level: ↓ <i>Enterobacteriaceae</i> both cecal and fecal Genus level: N.S Species level: ↑ <i>A. Muciniphila</i>, <i>Lactobacillus</i> spp., <i>Roseburia</i> spp., <i>C. leptum</i>, <i>Prevotella</i> spp.; ↓ <i>Clostridium</i> cluster XI both cecal and fecal In cecal content only, ↑ <i>Methanobrevibacter</i> spp.; ↓ <i>C. coccoides</i> and <i>Clostridium</i> cluster I In feces only, ↑ <i>C. coccoides</i> and <i>Clostridium</i> cluster I; ↓ <i>Methanobrevibacter</i> spp.</p>	[59]
	F/M	Feces	16S rRNA seq	<p>α-diversity: No changes β-diversity: Modulated Phylum level: ↑ <i>Proteobacteria</i> and TM7 in female Class level: N.S Order level: N.S Family level: N.S Genus level: ↑ <i>Bacteroides</i> and <i>Parabacteroides</i>; ↓ <i>Dehalobacterium</i> in both male and female. In females only, ↑ <i>Prevotella</i>, <i>Coprobacillus</i>, <i>Sutterella</i>, <i>Akkermansia</i>, and unclassified genera of <i>Desulfovibrionaceae</i> and <i>Enterobacteriaceae</i> families; ↓ <i>Oscillospira</i> and unclassified members of TM7 and <i>Rikenellaceae</i> families In males only, ↑ <i>Bacteroides</i>, <i>Parabacteroides</i>, <i>Lactobacillus</i>, <i>Coprobacillus</i> and unclassified genus of the <i>Helicobacteraceae</i> family; ↓ <i>Dehalobacterium</i>, <i>Ruminococcus</i> and <i>Desulfovibrio</i> Species level: N.S</p>	[52]
	M	Cecal content	16S rRNA seq	<p>α-diversity: ↓ β-diversity: Modulated Phylum level: ↑ <i>Verrucomicrobia</i>, <i>Bacteroidetes</i>; ↓ <i>Firmicutes</i> and <i>Cyanobacteria</i> Class level: N.S Order level: N.S Family level: N.S Genus level: ↑ <i>Akkermansia</i>, <i>Bacteroides</i>, <i>Bilophila</i>, <i>Enterorhabdus</i> <i>Intestinomonas</i> and S24-7; ↓ <i>Odoribacter</i>, <i>Parabacteroides</i>, <i>Rikenella</i>, <i>Blautia</i>, <i>Coprococcus</i>, <i>Bifidobacterium</i>, <i>Desulfovibrio</i>, <i>Lachnospiraceae</i> <i>Incertae Sedis</i> and RC9 gut group Species level: N.S</p>	[53]
	M	Feces	16S rRNA seq	<p>α-diversity: N.S β-diversity: Modulated Bacterial modulation were not detailed except for: Species levels: ↓ <i>L. reuteri</i></p>	[62]

N.S = Not specified. * ARISA = automated ribosomal intergenic spacer analysis

Table A2. Microbiota modulations in environmental models of ASD.

Model	Sex	Sample	Method	Difference in Microbiota Compared to Controls	Ref.
MIA	M/F	Feces	16S rRNA seq	<p>α-diversity: No changes β-diversity: Modulated</p> <p>Bacterial modulations were only detailed at OTU level: ↑OTUs from the <i>Alphaproteobacteria</i> and <i>Bacili</i> classes, <i>Bacteroidales</i> order and <i>Prevotellaceae</i>, <i>Lachnospiraceae</i> and <i>Porphyromonadaceae</i> families ↓OTUs from the <i>Actinobacteria</i> phylum, <i>Gammaproteobacteria</i>, <i>Mollicutes</i> and <i>Erysipelotrichi</i> classes and <i>Ruminococcaceae</i>, <i>Erysipelotrichaceae</i> and <i>Aligenaceae</i> families</p>	[54]
	M	Cecal content	16S rRNA seq	<p>No assessment of diversity Phylum level: N.S Class level: N.S Order level: N.S Family level: ↑ <i>Ruminococcaceae</i>, <i>Porphyromonadaceae</i>, <i>Aoerococcaceae</i> and <i>Erysipelotrichaceae</i> Genus level: ↑ <i>Candidatus</i> Species level: N.S</p>	[94]
	N.S	Feces	16S rRNA seq	<p>α-diversity: ↓ β-diversity: Modulated Phylum level: ↑ <i>Bacteroidetes</i> and <i>Verrucomicrobia</i>; ↓ <i>Firmicutes</i> Class level: N.S Order level: N.S Family level: N.S Genus level: ↑ <i>Prevotella</i>, <i>Prevotella_other</i>, <i>Akkermansia</i> and a genus of S24-7 family; ↓ <i>Oscillospira</i>, <i>Ruminococcus</i>, <i>Bacteroides</i>, <i>Dehalobacterium</i>, <i>Desulfovibrio</i>, <i>Lactobacillus</i>, and members of the <i>Clostridiales</i> order and <i>Rikenellaceae</i>, <i>Lachnospiraceae</i> and <i>Ruminococcaceae</i> families. Species level: ↑ F16 and OTUs from the <i>Bacteroidales</i> order, <i>Clostridiaceae</i>, <i>Enterobacteriaceae</i> and S24-7 familie and <i>Akkermansia</i> and <i>Prevotella</i> genera ↓ OTUs from the <i>Clostridiales</i> order, <i>Ruminococcaceae</i> and <i>Rikenellaceae</i> families and <i>Ruminococcus</i>, <i>Bacteroides</i>, <i>Dehalobacterium</i>, <i>Desulfovibrio</i>, <i>Oscillospira</i> and <i>Odoribacter</i> genera</p>	[60]

Table A2. Cont.

Model	Sex	Sample	Method	Difference in Microbiota Compared to Controls	Ref.
VPA	M/F	Feces	Total genomic DNA pyrosequencing	<p>α-diversity: no changes β-diversity: no difference Phylum level: ↑ <i>Firmicutes</i>; ↓ <i>Bacteroidetes</i> Class level: N.S Order level: N.S Family level: N.S Genus level: ↑ Uncultured genus of <i>Erysipelotrichales</i>, uncultured genera of the <i>Bacteroidales</i> and <i>Desulfovibrionales</i> orders Species level: N.S</p>	[63]
	M/F	Feces	16SrDNA seq	<p>α-diversity: ↓ β-diversity: Modulated Phylum level: only in males ↑ <i>Bacteroidetes</i>; only in female, ↑ <i>Actinobacteria</i> Class level: Only in males ↑ <i>Bacteroida</i>, <i>Alphaproteobacteria</i>; ↓ <i>Coriobacteria</i> Order level: N.S Family level: ↑ <i>Eubacteriaceae</i>, <i>Rikenellaceae</i> and <i>Staphylococcaceae</i>; ↓ <i>Enterobacteriaceae</i> Genus level: ↑ <i>Anaerofustis</i>, <i>Proteus</i>, <i>Staphylococcus</i>, and <i>Allobaculum</i> Only in females ↑, <i>Bifidobacterium</i>, <i>Odoribacter</i> and <i>Candidatus Arthromitus</i> Species level: ↑ <i>Ruminococcus flavefaciens</i>, OTUs from the <i>Clostridiales</i> order and the <i>Ruminoccus</i> and <i>S24-7</i> genera.</p>	[61]
	M	Feces	16SrDNA seq	<p>α-diversity: ↓ β-diversity: Modulated No detail of the changes in bacterial taxa</p>	[49]
MHFD mice	N.S	Feces	16SrDNA seq	<p>α-diversity: ↓ β-diversity: Modulated Phylum level: ↑ <i>Firmicutes</i>, <i>Verucomicrobia</i>, ↓ <i>Bacteroidetes</i> Class level: N.S Order level: N.S Family level: ↑ <i>Peptostreptococcaceae</i> Genus level: ↑ <i>Streptococcus</i>, <i>Akkermansia</i> ↓ <i>Lachnospiraceae_incertae_sedis</i> Species level: N.S</p>	[55]

All studies used mice except for Liu et al. (2018) [58] who used rats. N.S = Not specified.

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RESUME SUBSTANCIEL EN FRANÇAIS :

Effet du microbiote intestinal d'enfants atteints de troubles du spectre autistique sur le comportement et les marqueurs biologiques liés à ces troubles chez la souris axénique

Abréviations : GI : Gastro-intestinal/aux ; TMF : Transplantation du microbiote fécal ; TSA : Troubles du spectre autistique.

Les troubles du spectre autistique (TSA) font partie des troubles neurodéveloppementaux les plus répandus. Ils se caractérisent par une altération du comportement social et de la communication, ainsi que par la prévalence de comportements et d'intérêts répétitifs et stéréotypés. Ils s'accompagnent souvent d'une sensibilité sensorielle accrue ou réduite et peuvent également être accompagné de déficience cognitive et de troubles anxieux. Il n'existe pas de biomarqueurs définis pour les TSA, car il s'agit de troubles multifactoriels et pouvant se présenter de façon différente selon les personnes. A l'heure actuelle, les TSA ne sont diagnostiqués que par un examen comportemental effectué par un professionnel de santé, au plus tôt entre 18 à 24 mois. Bien que le diagnostic comportemental des TSA se soit amélioré au cours de la dernière décennie, il reste difficile à caractériser. Il est estimé que les TSA touchent actuellement 1 personne sur 97 au niveau mondial. Il est également établi que les TSA présentent une héritabilité génétique d'environ 50 % [1–3]. De plus, plusieurs études ont montré que des facteurs environnementaux jouent un rôle important dans ces troubles. Pour mieux les comprendre et mieux les diagnostiquer, il est donc intéressant d'étudier comment des facteurs externes, tels que l'alimentation, les médicaments ou d'autres facteurs environnementaux susceptibles de provoquer une inflammation, un stress oxydatif ou une perturbation endocrinienne, peuvent influencer sur les symptômes des TSA [4, 5].

Étant donné que ces facteurs ont également un impact sur le microbiote, il est possible que l'un de leurs modes d'action sur les TSA soit la modification du microbiote. Il est également important de souligner que la prévalence des symptômes gastro-intestinaux (GI) est quatre fois plus élevée chez les personnes atteintes de TSA que chez les neurotypiques. Ces anomalies GI sont souvent caractérisées par une perméabilité accrue de l'intestin et une fonction immunitaire anormale dans l'intestin, qui sont en corrélation avec la gravité des symptômes des TSA. Plusieurs études suggèrent que ces symptômes gastro-intestinaux font partie intégrante de la physiopathologie des TSA et qu'ils sont en interaction avec le microbiote intestinal et le système immunitaire [6–8].

De plus, de nombreuses études provenant de différents pays publiées dans la dernière décennie ont fait l'observation d'une composition distincte du microbiote intestinal chez les personnes atteintes de TSA par rapport aux personnes neurotypiques. Il y a cependant une forte hétérogénéité dans ces études, probablement en partie due à des différences expérimentales, ainsi qu'à des facteurs environnementaux (mode de vie, régime, climat etc.). Bien que l'origine de cette altération du microbiote soit inconnue et qu'elle puisse être

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influencée par les préférences alimentaires restrictives ou les anomalies GI de certaines personnes atteintes de TSA, elle pourrait aggraver les symptômes de ces troubles [9–12].

De nombreuses études précliniques sur modèles murins et quelques études cliniques montrent que cette altération du microbiote est en interaction avec le système immunitaire et pourrait jouer un rôle dans le développement de plusieurs des symptômes et des déficiences fonctionnelles observés chez les individus atteints de TSA, comme une inflammation et une perméabilité intestinale excessive, une altération du métabolisme du tryptophane et une neuroinflammation accrue. En effet, des études interventionnelles ciblant le microbiote via des traitements probiotiques et/ou prébiotiques, peuvent améliorer les altérations comportementales, cérébrales et systémiques observées chez des modèles murins de TSA [13].

Quelques études cliniques testant les effets de traitements probiotiques chez des individus atteints de TSA ont également observé des améliorations des symptômes comportementaux et GI. Cependant, là aussi, les résultats sont assez variables selon les études [14]. Deux études pilotes ont également effectué une transplantation du microbiote fécal (TMF) provenant de donneurs neurotypiques chez des enfants atteints de TSA et troubles gastro-intestinaux et ont observé une amélioration à long terme des symptômes comportementaux et gastro-intestinaux chez ces enfants [15–17].

Cependant, des études plus contrôlées et sur de plus grands effectifs sont nécessaires pour conclure sur le potentiel thérapeutique d'une TMF dans le cadre des TSA. De plus, pour mieux comprendre les mécanismes impliqués dans ces améliorations, des études sur modèles murins sont encore nécessaires.

Un modèle murin très utilisé dans les études sur le microbiote intestinal, est le modèle axénique, qui sont des rongeurs dépourvus de microbiote, et sont donc un bon candidat pour les études de TMF. A ce jour, 2 études ont été publiées comparant l'effet d'une TMF de donneurs humains atteints de TSA, ou neurotypiques, sur des souris axéniques. Dans ces études, les souris ou la descendance des souris ayant reçu le microbiote du groupe TSA ont montré un déficit du comportement social, une augmentation des comportements répétitifs et/ou une plus grande anxiété que celles ayant reçu le microbiote du groupe neurotypique [18, 19].

Dans une approche similaire, nous avons, dans la présente étude, étudié l'effet chez des souris axéniques d'une TMF d'enfants atteints de TSA sur le comportement et les marqueurs cérébraux, immunitaires et GI liés aux TSA et pouvant être influencés par le microbiote intestinal. Nous avons choisi deux groupes distincts d'enfants atteints de TSA comme donneurs de microbiote : un groupe sans symptômes GI (groupe A) et un groupe présentant des symptômes GI (groupe AG), à savoir une constipation sévère et chronique. Cette distinction nous paraît importante, car des études ont montré que la présence de troubles GI

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avait un impact sur la composition du microbiote intestinal. De plus, pour les deux groupes de TSA, sans ou avec troubles GI, les enfants donateurs témoins étaient leurs frères et sœurs neurotypiques (groupes S-A et S-AG), contrairement aux deux études citées précédemment chez qui le groupe neurotypique n'était pas apparenté au groupe TSA. Choisir les frères et sœurs limite l'effet potentiel des facteurs génétiques et environnementaux sur la composition du microbiote entre les groupes de donateurs atteints de TSA et neurotypiques. Enfin, nous avons réalisé cette expérience sur deux lignées de souris qui présentent des différences génétiques et comportementales, à savoir les lignées BALB/c et C57BL/6J.

Quatre groupes de souris ont reçu une TMF d'un mélange des microbiotes des groupes de donateurs (4 donateurs par groupe) à l'âge de 3 semaines. Les groupes de souris ont été nommés A, S-A, AG ou S-AG en fonction du microbiote reçu : A : microbiote d'enfants ayant des TSA dans troubles GI; S-A: microbiote de leurs frères et sœurs; AG: microbiote d'enfants ayant des TSA avec troubles GI; S-AG: microbiote de leurs frères et sœurs. Le comportement des animaux a été étudié à l'âge de 9 semaines.

Dans les deux lignées, nous avons d'abord étudié la composition et l'activité métabolique du microbiote fécal et caecal, respectivement 6 et 9 semaines après la TMF. Chez les deux lignées, au niveau fécal comme caecal, une étude de la β -diversité a montré une composition distincte du microbiote entre les souris des groupes S-A et A, et S-AG et AG. Chez les souris BALB/c, la diversité α du microbiote fécal était plus importante dans le groupe AG que dans le groupe S-AG. Cependant, chez les souris C57BL/6J, elle a augmenté dans le groupe A par rapport au groupe S-A et a diminué dans le groupe AG par rapport au groupe S-AG. En termes d'abondance relative des différents phylum et familles bactériennes, de nombreuses différences entre les groupes ont été observées mais de façon assez hétérogène selon le type d'échantillon et la lignée.

Il y avait cependant quelques similitudes entre les deux lignées. Au niveau du phylum, on a observé une augmentation de l'abondance relative des Actinobacteriota dans le microbiote fécal du groupe A par rapport au groupe S-A. Au niveau famille, dans le microbiote fécal, une augmentation de l'abondance relative des *Ruminococcaceae* dans le groupe A par rapport au groupe S-A. Dans le microbiote caecal une diminution de l'abondance relative des *Tannerellaceae* dans le groupe A par rapport au groupe S-A. Enfin, dans le microbiote fécal comme caecal, une diminution des *Prevotellaceae* a été observée chez le groupe AG, par rapport au groupe S-AG. De plus, le profil d'acide gras à chaîne courte caecal était distinct entre les groupes, témoignant d'une différence dans l'activité de fermentation du microbiote. Certaines différences étaient retrouvées dans les deux lignées, à savoir une diminution des acides gras ramifiés et à longue chaîne (4 à 6 carbones) dans le groupe A par rapport à S-A, et une diminution du propionate et une augmentation du butyrate et des acides gras ramifiés et à longue chaîne chez le groupe AG par rapport au groupe S-AG. Chez les souris BALB/c uniquement, la diminution du propionate et l'augmentation du butyrate étaient également présentes chez le groupe A par rapport à S-A.

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Malgré ces fortes différences en termes de microbiote, nous n'avons observé que peu de différences comportementales, et uniquement chez les souris C57BL/6J. Dans cette lignée, la TMF du groupe A a diminué l'activité locomotrice, et la TMF du groupe AG a augmenté les comportements répétitifs (toilettages plus nombreux) et a altéré la mémoire spatiale, par rapport à la TMF des groupes frères et sœurs respectifs. Les souris C57BL/6J du groupe AG avaient donc certains comportements retrouvés chez les modèles murins de TSA, cependant, leur comportement social n'était pas altéré.

Davantage de différences ont été observées au niveau systémique, intestinal et cérébral, mais avec des distinctions en fonction de la lignée. Chez les deux lignées, la TMF du groupe A a réduit l'expression de gènes codant pour des cytokines pro-inflammatoires dans l'intestin, par rapport à la TMF du groupe S-A. Cependant, ces différences étaient localisées dans le côlon chez les souris BALB/c et dans l'iléon chez les C57BL6/J.

Chez les C57BL/6J, il y avait également une diminution de la proportion des populations de lymphocytes T pro-inflammatoires dans la rate. De plus, nous avons observé une augmentation de l'expression de ZO-1 dans l'iléon du groupe A par rapport au groupe S-A suggérant une perméabilité paracellulaire de l'iléon potentiellement diminuée dans ce groupe. En revanche, l'augmentation des niveaux d'expression de MLCK dans l'iléon du groupe AG par rapport au groupe S-AG pourrait suggérer une perméabilité paracellulaire accrue dans le groupe AG. Dans une étude complémentaire, la perméabilité intestinale des souris S-AG et AG sera mesurée en chambre de Ussing, pour mieux conclure sur l'impact de la TMF sur ce paramètre.

Chez les souris BALB/c, la TMF des groupes A et AG a réduit le nombre de neurones sérotoninergiques dans le noyau du raphé, par rapport à la TMF des frères et sœurs.

Nous avons également fait une étude de corrélation de Spearman entre les différents paramètres comportementaux et biologiques observés et la diversité et composition du microbiote. Certaines de ces corrélations étaient retrouvées dans les deux lignées, même pour des paramètres qui ne variaient pas de la même façon entre lignées. Une étude plus approfondie sur ces corrélations est prévue.

Cette étude a permis d'enrichir la littérature sur la TMF provenant d'individus atteints de TSA sur des souris axéniques. Contrairement aux deux études précédemment citées, nous n'avons pas observé d'altération du comportement social à la suite de la TMF avec du microbiote "TSA". Cependant, nous retrouvons chez le groupe AG des souris C57BL/6J, l'augmentation des comportements répétitifs rapportés par une de ces études. Chez ces souris, nous avons également observé une altération de la mémoire spatiale, qui n'avait pas été testée dans ces études. Globalement, la TMF des groupes A et AG semblent avoir réduit l'inflammation, ce qui était inattendu, mais suggère que le microbiote pourrait avoir un effet régulateur de l'augmentation de l'inflammation intestinale chez les patients atteints de TSA.

Résumé

Cette étude a été réalisée sur le modèle axénique, chez qui des altérations comportementales et cérébrales ont été observées [20–25]. Il est possible que certaines de ces altérations ne soient pas réversibles par la recolonisation par TMF, celle-ci ayant eu lieu quand les souris étaient âgées de trois semaines (âge de sevrage). Ainsi, le microbiote "sain" des groupes S-A et S-AG pourrait ne pas avoir amélioré certaines altérations. Afin de tirer des conclusions plus précises de ces différences comportementales, nous avons donc évalué les caractéristiques comportementales des souris axéniques C57BL/6J dans les mêmes tests d'anxiété, de comportement social, de comportements stéréotypés et de mémoire spatiale que ceux utilisés dans l'étude de TMF, ainsi que dans deux tests supplémentaires mesurant l'anxiété et les comportements répétitifs.

Tout d'abord, nous avons observé une forte diminution de l'activité locomotrice chez les souris axéniques C57BL/6J en comparaison aux souris conventionnelles. Cette observation a déjà été faite dans plusieurs études sur cette lignée. De plus, les souris axéniques se toilettaient plus tôt et plus longtemps que les souris conventionnelles ce qui peut témoigner d'une anxiété accrue. Les souris axéniques montraient également une tendance à une moindre mémoire spatiale que les souris conventionnelles. Des déficits de mémoire ont déjà été décrits dans quelques études chez les souris axéniques. Enfin, les souris axéniques avaient tendance à avoir de meilleures performances aux tests de comportement social que les souris conventionnelles. Cette observation diffère de ce qui est décrit dans certaines études de la littérature sur cette lignée, ce qui pourrait être dû à des différences expérimentales. En dehors de cette caractérisation comportementale, nous avons également observé une augmentation de la perméabilité paracellulaire au niveau du colon chez ces souris. Des expérimentations sont en cours pour approfondir ces observations en analysant l'expression de divers gènes au niveau intestinal et cérébral.

En prenant en compte les deux expériences de ce projet, nous pouvons émettre l'hypothèse que le microbiote du groupe S-AG aurait amélioré la mémoire spatiale déficiente chez les souris axéniques C57BL/6J contrairement au microbiote du groupe AG.

Globalement, malgré l'absence d'altération du comportement social, le groupe AG chez les souris C57BL/6J récapitule certaines caractéristiques liées aux TSA, avec une augmentation des comportements répétitifs et une altération de la mémoire spatiale. De plus, ce groupe présente des marqueurs de perméabilité iléale accrue. Il est donc intéressant de poursuivre les recherches dans ce groupe. Pour poursuivre cette expérience, notre équipe teste actuellement les effets d'un traitement probiotique et prébiotique sur les mêmes marqueurs systémiques, intestinaux, centraux et comportementaux liés aux TSA chez des souris GF C57BL/6J qui ont été colonisées avec le microbiote des groupes S-AG ou AG.

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