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Microbiota-Gut-Immune-Brain Communication Across the Lifespan

Thesis presented by

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Appendix A - Supplementary Material for Chapter 2
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Abbreviations List

3β-HSD	3β-Hydroxysteroid Dehydrogenase
4-EPS	4-Ethylphenylsulfate
ACTH	Adrenocorticotropic Hormone
AD	Alzheimer's Disease
AHR	Aryl Hydrocarbon Receptors
ANOVA	Analysis of Variance
ASD	Autism Spectrum Disorder
AUC	Area Under the Curve
AVP	Arginine Vasopressin
BAMs	Border Associated Macrophages
BBB	Blood-Brain Barrier
BDNF	Brain-Derived Neurotrophic Factor
BH	Benjamini-Hochberg Adjusted Q-Value
BORIS	Behavioural Observation Research Interactive Software
CCK	Cholecystokinin
CCR2	C-C Motif Chemokine Receptor 2
CD11b	Integrin Alpha M
CD11c	Integrin Alpha X
CD3	Cluster Of Differentiation 3
CD4	Cluster Of Differentiation 4
CD44	Cluster Of Differentiation 44
CD69	Cluster Of Differentiation 69
CD8	Cluster Of Differentiation 8
CFU	Colony-Forming Unit
CLR	Centered Log Ratio
CML	N6-Carboxymethyllysine
CNS	Central Nervous System
CNTF	Ciliary Neurotrophic Factor

CRF	Corticotropin-Releasing Factor
CSDS	Chronic Social Defeat
CSF	Cerebral Spinal Fluid
CSF1R	Colony-Stimulating Factor 1 Receptor
CT	Cardiotrophin
CXCL1	C-X-C Motif Chemokine Ligand 1
DAMPs	Damage-Associated Molecular Patterns
DAPI	4′,6-Diamidino-2-Phenylindole
DC	Dendritic Cell
E12	Embryonic Day 12
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ENS	Enteric Nervous System
EPM	Elevated Plus Maze
FBS	Faecal Bovine Serum
FDR	False Discovery Rate
fMet	N-Formylmethionine
FMT	Faecal Matter Transplantation
FOS	Fructo-Oligosaccharides
FST	Forced Swim Test
FXR	Farnesoid X Receptor
GABA	Gamma Aminobutyric Acid
GDNF	Glial Cell Line-Derived Neurotrophic Factor
GF	Germ-Free
GI	Gastrointestinal
GLP-1	Glucagon-Like Peptide-1
GOS	Galacto-Oligosaccharides
GRs	Glucocorticoid Receptors
GUI	Graphical User Interface
HPA	Hypothalamic-Pituitary-Adrenal
IBD	Inflammatory Bowel Disease
IDO	Indoleamine 2,3-Dioxygenase
IFN	Interferon

IgA	Immunoglobulin A
IgE	Immunoglobulin E
IL-1	Interleukin-1
IL-1	Interleukin-1
IL-10	Interleukin-10
IL-11	Interleukin-11
IL-17a	Interleukin-17a
IL-17R	Interleukin-17 Receptor
IL-1R1	Interleukin-1 Receptor Type 1
IL-22	Interleukin-22
IL-34	Interleukin-34
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
ILC3	Type 3 Innate Lymphoid Cells
ILCs	Innate Lymphoid Cells
LAMP1	Lysosomal Associated Membrane Protein 1
LIF	Leukemia Inhibitory Factor
LIFRβ	Leukemia Inhibitory Factor Receptor B
LPS	Lipopolysaccharide
Ly6Chi	Lymphocyte Antigen 6
MatABX	Maternal Antibiotics
MDD	Major Depressive Disorder
MHCII	Major Histocompatibility Complex Class II
MIA	Maternal Immune Activation
MLNs	Mesenteric Lymph Nodes
MRI	Magnetic Resonance Imaging
MS	Multiple Sclerosis
mTOR	Mammalian Target of Rapamycin
MUC2	Mucin 2
NAD	Nicotinamide Adenine Dinucleotide
NDS	Normal Donkey Serum
NF-κB	Nuclear Factor-Kb

NLPR6	NOD-Like Receptor Pyrin Domain-Containing Protein 6
NLRP3	NOD-Like Receptor Pyrin Domain-Containing Protein 3
NMDA	N-Methyl-D-Aspartate
NTS	Nucleus Tractus Solitarius
OF	Open-Field
OFT	Open Field
PAMP	Pathogen-Associated Patterns
PBS	Phosphate-Buffered Saline
PERMANOVA	Permutational Multivariate Analysis of Variance
PFA	Paraformaldehyde
PFC	Prefrontal Cortex
PFC	Prefrontal Cortex
PKA	Protein Kinase A
PND	Postnatal Day
PRR	Pattern Recognition Receptors
PUFAs	Polyunsaturated Fatty Acids
PVN	Paraventricular Nucleus
PYY	Peptide YY
RA	Retinoic Acid
ROR	Retinoic Acid Receptor–Related Orphan Nuclear Receptor
SCFA	Short-Chain Fatty Acids
SEM	Standard Error of The Mean
SFB	Segmented Filamented Bacteria
SimBa	Simple Behavioural Analysis
SIRT1	Sirtuin 1
SNPs	Single Nucleotide Polymorphisms
SNS	Sympathetic Nervous System
SSRI	Selective Serotonin Reuptake Inhibitors
STAT3	Signal Transducer and Transcription 3
TGFβ	Transforming Growth Factor B
TLR	Toll-Like Receptor
TLR4	Toll-Like Receptor 4
TMAO	Trimethylamine-N-Oxide

TMAO	Trimethylamine-N-Oxide
TNF	Tumor Necrosis Factor
TNFR2	Tumor Necrosis Factor Receptor 2
TRAIL	Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand
TSLP	Thymic Stromal Lymphopoietin
WHO	World Health Organization

Declaration

This thesis submitted is my own work and has not been submitted for any other degree, either at University College Cork or elsewhere.

Signed:

Author Contributions

All of the work conducted in this thesis was performed independently by the author with the following exceptions:

Chapter 2: Dr. Marcel van de Wouw and Nathaniel Ritz assisted with the cell isolation and flow cytometry protocols. Dr Serena Boscaini performed the cytokine quantification. Dr Thomaz Bastiaanssen carried out the bioinformatic analysis and associated statistical analysis.

Chapter 3: Dr. Marcel van de Wouw performed the cell isolation and flow cytometry protocols. Dr. Serena Boscaini performed the cytokine quantification.

Chapter 4: Dr. Serena Boscaini performed the cytokine quantification.

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"Para ser grande, sê inteiro: nada
Teu exagera ou exclui.
Sê todo em cada coisa. Põe quanto és
No mínimo que fazes.
Assim em cada lago a lua toda
Brilha, porque alta vive."

"To be great, be whole; don't exaggerate
Or leave out any part of you,
Be complete in each thing.
Put all you are into the least of your acts.
So too in each lake, with its lofty life,
The whole moon shines"

Ricardo Reis

Publications and Presentations

Peer-reviewed publications

Cruz-Pereira JS, Moloney GM, Bastiaanssen TFS, Boscaini S, Fitzgerald P, Clarke G, Cryan JF. Age-associated deficits in social behaviour are microbiota-dependent. Brain Behav Immun. 2023 Feb 13; 110:119-124. doi: 10.1016/j.bbi.2023.02.008

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Cruz-Pereira JS, Moloney GM, Boscaini S, van de Wouw M, Fitzgerald P, Clarke, G, Dinan, TG, Cryan JF. Targeting the gut microbiome reverses age-associated social deficits in the mouse. Targeting Therapeutics for Brain Disorders. Cork, Ireland. June 2, 2021

Cruz-Pereira JS, Moloney GM, van de Wouw M, Kiousi DE, Fitzgerald P, Clarke, G, Dinan, TG, Cryan JF. Antibiotics failed to alter age-associated social deficits under baseline and stress conditions. EBPS Biennial Meeting, Braga, Portugal. August 28-31, 2019

Abstract

While the exploration of the gut microbiome in health and disease evolves, the implications of the microorganisms that inhabit the gut for host brain health also multiply. As we grow up and grow old, the gut microbiota along with the host physiological systems, undergoes significant remodelling. The influences of the gut microbiome on host physiology are relevant across the lifespan, with continuous communication between the gut microbiome and the central nervous system (CNS) representing an important aspect of this host-microbe dialogue. A growing body of research into dissecting the involvement of the gut microbiota in the behaviour and functioning of the CNS raises the need to understand how these integrated systems communicate throughout the lifespan of the host, and how it is phenotypically reflected. Understanding the gut-brain axis across the lifespan is imperative, as these insights can be used to further support healthy brain aging, along with the development of better biomarkers towards the development of personalized therapeutic strategies.

In this thesis, we aimed to investigate the influence of the gut microbiome in immune and behavioural features throughout the lifespan of the host: in early-life and aging. To this end, we assessed the effect of microbiota depletion in aged mice and demonstrated for the first time that the gut microbiome is associated with social behaviour and restricts the accumulation of T-helper cells in the choroid plexus in aged mice. This was accompanied by modulation of caecal metabolite levels, and in particular, some metabolites previously associated with age-dependent processes, namely argininosuccinic acid and N-formylmethionine.

To further examine the involvement of the gut microbiome in aging, we explored whether supplementation with the prebiotic FOS-Inulin could alter behavioural and physiological aspects along the gut-brain axis in stressed aged mice. We demonstrated that FOS-Inulin supplementation can ameliorate the disrupted social behavioural responses that arise following a stress exposure, including the alterations in social interaction with a non-aggressive mouse and social novelty, while promoting the remodelling of caecal and prefrontal cortex metabolite levels. More specifically, dietary supplementation with FOS-Inulin promotes the amelioration of the levels of 4-Hydroxybenzaldehyde and spermine in the prefrontal cortex of stressed aged mice. Additionally, we evaluated if FOS-Inulin supplementation could alter adult social, depressive- and anxiety-like behavioural and immune markers in offspring exposed

to early life microbial disruption. In our study, we observed altered intestinal immune markers and subtle behavioural changes following this intervention.

Taken together, these results provide novel insights on time sensitive critical windows for the gut microbiome, and its impact in behaviour and immunity outcomes in the host. While further investigation into the mechanisms underlying these effects is crucial, these findings highlight the involvement of gut microbial signalling on host behaviour and immunity. This research paves the way for the future development of therapeutic options that target the gut microbiome to modify these age-dependent behavioural and metabolite alterations.

Chapter 1
General Introduction

1. Gut Microbiome

The mammalian gut plays host to a myriad of microorganisms collectively referred to as the microbiota with important implications in health and disease (Gilbert et al 2018). Over 100 trillion inhabit the gastrointestinal (GI) tract, including bacteria, viruses and archaea (Gomaa 2020). Interestingly, recent studies estimate that microbial cells outnumber human cells in a 1.3:1 ratio (Sender et al 2016). At the genetic level, over 99% of the genes in our body are microbial, surpassing 10 million (Cryan et al 2019b), reflecting the opportunities to modify the microbiome to support human and animal health.

1.1. Biogeography of the Gut Microbiota

The gastrointestinal tract presents distinct microbial habitats at different sites (Clarke et al 2019, Donaldson et al 2016). Physiological adaptations according to region-dependent nutrient and chemical gradients, along with compartmentalization of host immune cells largely contribute to the regionalized gut microbial diversity (Donaldson et al 2016). Despite the variability along the gastrointestinal tract, there is more phylogenetically variability between individuals than across the gastrointestinal sampling location (Stearns et al 2011). Furthermore, the gut mucosal and luminal microbiome are distinct, and can present distinct bacterial profiles in response to stress, and consequent depressive-like behaviour in macaques (Teng et al 2022a).

The small intestine, where the levels of oxygen and antimicrobial molecules are higher, along with a more acidic environment, is mainly inhabited by fast-growing facultative anaerobes that compete with the host for nutrients (see Figure 1) (Donaldson et al 2016).

The caecum and colon harbour the most dense and diverse microbial communities. In the mouse caecum, polysaccharides that resist digestion in the ileum are metabolized by microorganisms, which contrastingly in humans takes place mostly in the colon (Nguyen et al 2015). Due to the lower concentrations of antimicrobials, slower transit time, and lack of available simple carbon sources, the expansion of fermentative polysaccharide-degrading anaerobes is promoted (Donaldson et al 2016), which is intrinsically linked with gut microbial composition in humans (Müller et al 2019). The caecum and colon harbour the most dense and diverse microbial communities. In the mouse caecum, polysaccharides that resist digestion in the ileum

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As the colon folds on itself, it is compartmentalized between folds that are divergent from the central luminal compartment. These discrete regions present distinct microbial profiles in the mouse (Nava et al 2011, Pédron et al 2012), namely Bacteroidetes families were enriched in the central lumen, while Firmicutes families were enriched between folds (Nava et al 2011).

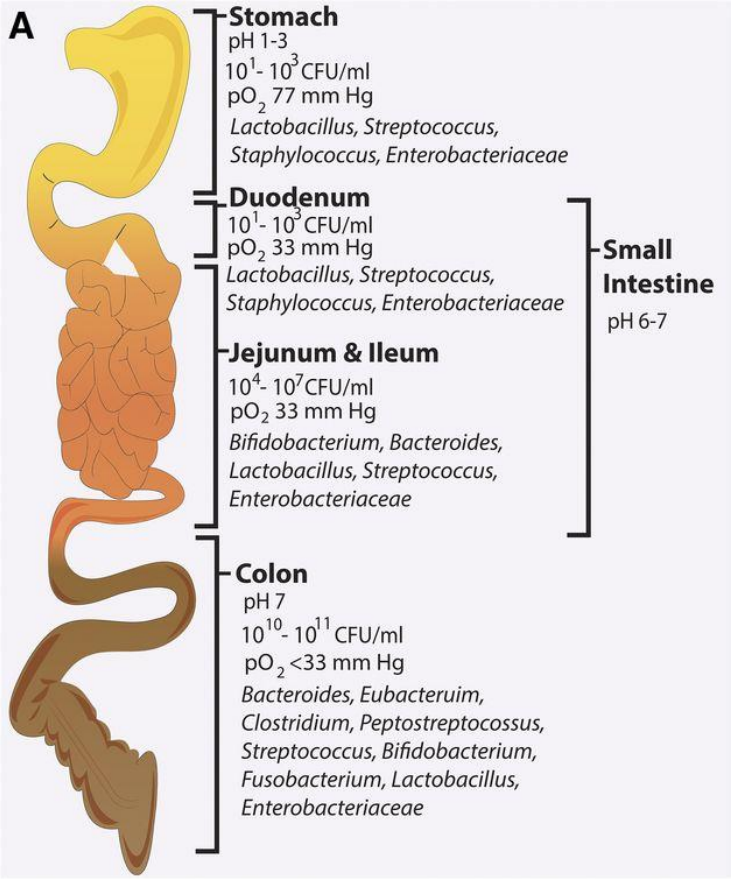

Figure 1 – Dynamic regionalization of the gut microbiota across the gastrointestinal tract. *The localization and spatial organization of gut microbial populations is adapted to the physical, chemical and immune properties of each region, resulting in diverse populational characteristics as well as distinct bacterial loads. Abbreviations: CFU – colony forming unit; mL- milliliter; mm Hg – millimetres of mercury; IgA – Immunoglobulin A. Adapted from (Clarke et al 2019).*

1.2. Microbiota and Health & Disease

The composition of the gut microbiome has been associated with health and disease markers and can be associated with health markers that reflect the dietary and lifestyle patterns of the host (Huttenhower et al 2012, Manor et al 2020) (see Figure 2 below). A cross-disease meta-analysis has shown that shifts in the human gut microbiome can be associated with disease, by reflecting either depletion of health-associated bacteria or enrichment in pathogenic or disease-associated microbes (Duvallet et al 2017). Further, a mathematical index has been developed to evaluate health status using gut taxonomic signatures as a predictor of having a disease – namely cardiovascular diseases, metabolic disorders, cancer (Gupta et al 2020) - thus opening doors to personalized medicine.

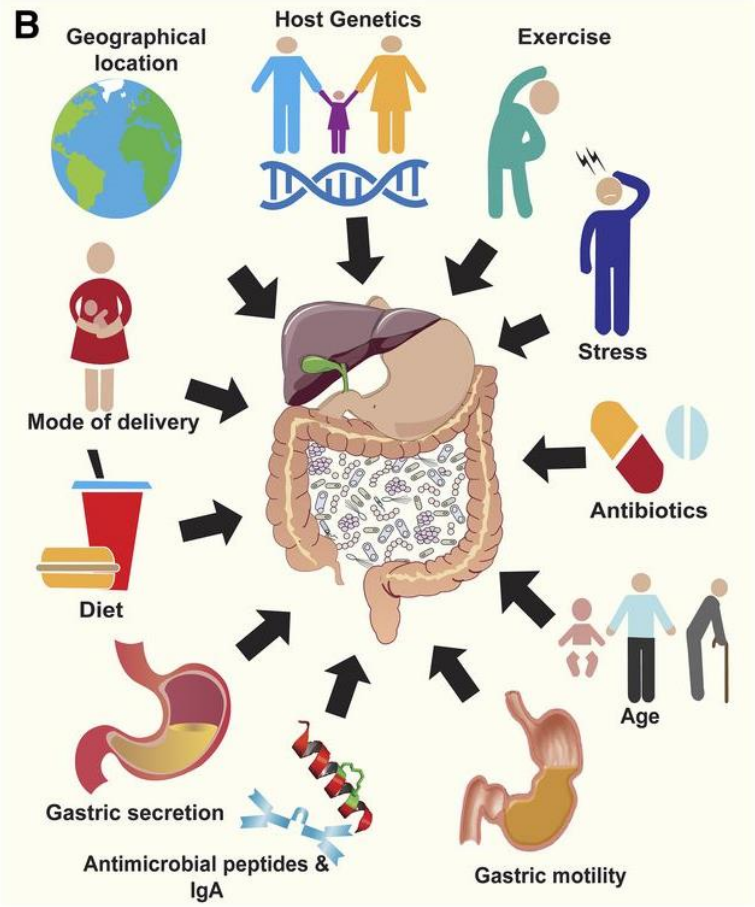

Figure 2 - Different factors that impact the function and composition of the gut microbial niches in the gastrointestinal tract. *Adapted from (Clarke et al 2019).*

1.3. Microbiome-Gut-Brain Axis

Emerging evidence supports a role for the bidirectional communication of these gastrointestinal microbiota with the endocrine and immune systems to mediate key brain processes including neuroinflammation, activation of stress axes, neurotransmission and neurogenesis (Cryan & Dinan 2015, Rhee et al 2009). Moreover, in psychology there is a growing appreciation of the role of the microbiota-gut-brain axis in psychopathology (Allen et al 2017, Cryan 2019, Sarkar et al 2018).

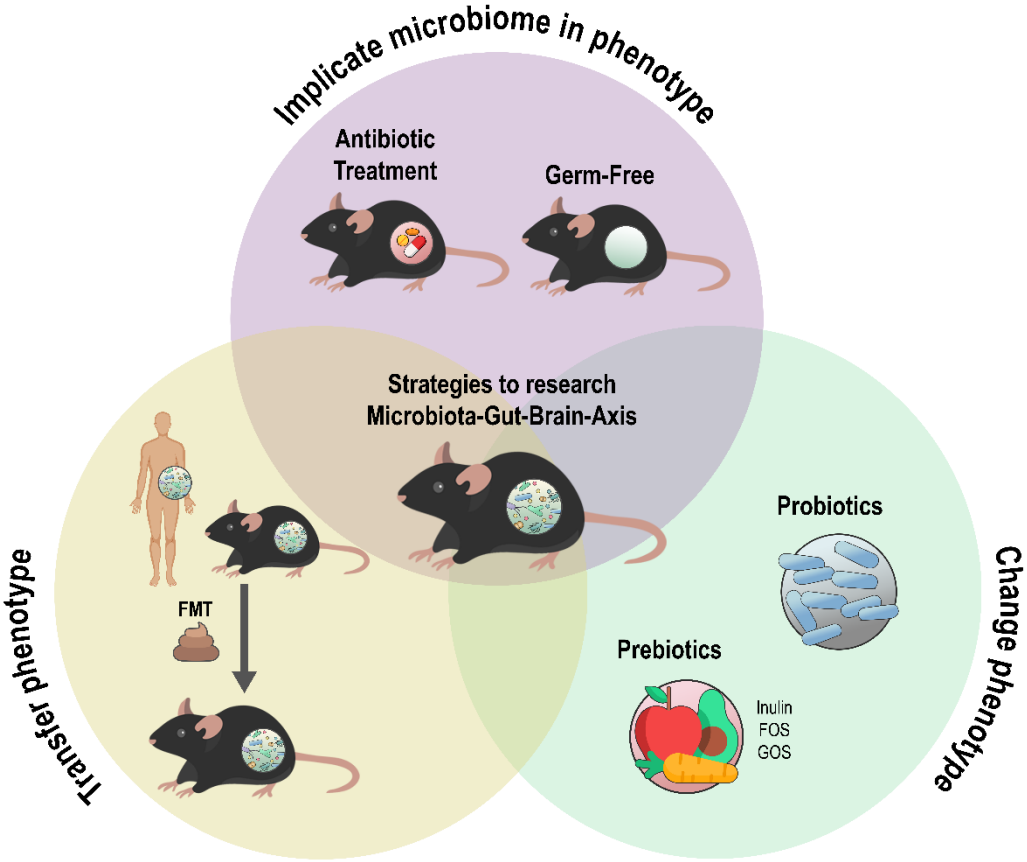

Figure 3 - Strategies for studying the influence of the gut microbiome. *Deleterious approaches such as the use of antibiotics or germ-free animals allow to infer the impact of bacterial signalling in the gut-brain axis. Faecal matter transplantation, on the other hand, allows the investigation of such bacterial signalling through the potential of transferring the phenotype and the potential causality through exposure to specific bacterial communities, or faecal samples from diseased states. Particular gut microbial groups can be enhanced through administration of either prebiotics or probiotics, and hence can be used to evaluate the potential benefit of boosting the activity of such populations. Abbreviations: FMT - Faecal Matter Transplantation.*

1.4. Strategies Used to Investigate the Role of the Microbiota–Gut–Brain Axis in Health and Disease

To understand and dissect the role of the gut microbiota in host physiology, different manipulations of gut microbial populations can be employed (Figure 3).

1.4.1. Germ-Free

Germ-free (GF) animals which lack exposure to microorganisms since birth, represent an invaluable tool into dissecting the intricate impact of microbial signals in host physiology (Kennedy et al 2018, Williams 2014) (Figure 3). Successive generations of GF animals were first generated in the mid-20th century (Gustafsson 1946). Currently, cesarian section is performed to avoid mother-pup microbiota transmission, and subsequently pups are hand-raised in an aseptic isolator, and further maintained on sterile water, food and bedding. Later generations can be bred and raised in isolators, and GF pups can be vaginally born (Cryan et al 2019b).

As consequences of lacking commensal microbes, GF animals present disrupted hormone signalling, dysregulated metabolism, immune system impairments, and altered neurotransmission and behaviour (Kawase et al 2017, Pan et al 2018a, Sudo et al 2004, Weger et al 2019, Woting & Blaut 2016).

Despite representing an important tool to evaluate host-microbe interactions, GF mice present multiple limitations, namely considering impaired immunity, altered neurodevelopment and aberrant physiology, while displaying limited translatability to human scenarios, considering they do not recapitulate the human host-gut microbiome communication, and the microbiota depletion throughout lifespan is not comparable to the human situation (Nguyen et al 2015). Nevertheless, GF models still represent an important initial assessment to screen whether the microbiota is implicated in a particular process or not (Luczynski et al 2016).

1.4.2. Antibiotics

Antibiotics, antimicrobial agents with vast medical implications (Hutchings et al 2019), represent an invaluable pharmacological tool for assessing the function of distinct bacterial populations (Figure 4). These provide the flexibility to selectively target

microbial populations and they can be administered in different stages across the lifespan (Cryan et al 2019b). This flexibility allows for adaptable experimental designs that can incorporate (Figure 4):

- a single use of any given antibiotic, to allow for the elimination of a specific group of bacteria;
- antibiotic cocktails that can eliminate different groups of bacteria, or the majority of most bacterial groups;
- non-absorbable antibiotics that infer the local impact of the gut microbial populations, therefore surpassing any potential secondary effects of the antibiotic in question in the circulation or the CNS.
- diverse administration timepoints, allowing the dissection of acute and chronic effects of gut microbiota depletion during critical time window times during development or at old age.

Antibiotics allow preclinical investigation while modelling clinical scenarios in humans, given the widespread use of these pharmaceutical compounds.

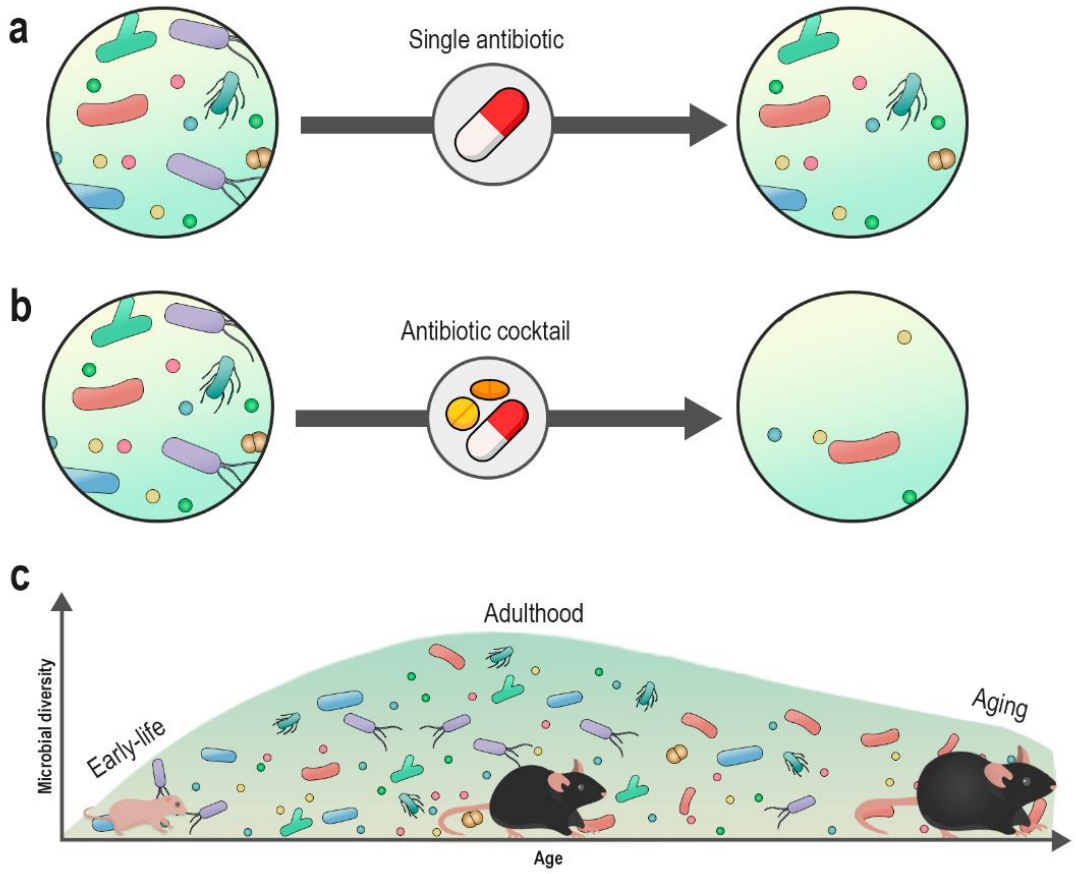

Figure 4 - Different experimental strategies for using antibiotics to study the gut microbiome. *A) Single antibiotic to deplete a specific bacterial group; B) Antibiotic cocktail to eliminate two or more groups of bacteria; C) Antibiotic use at different time periods across the lifespan to evaluate the effects of gut microbial depletion during established periods.*

1.4.3. Diet

Diet represents a decisive modulator of gut microbial assembly and is therefore becoming an appealing option for microbiota manipulation. Nutrients can directly impact the microbiota, by inhibiting or promoting their growth, by inducing a selective pressure in the gut microbial community that consequently reshapes that ecological system (Zmora et al 2019). Remarkably, the human gut microbiome rapidly changes in response to dietary interventions (David et al 2014); in fact, some dietary interventions (from high-fat/low-fibre to low-fat/high-fibre diet) can change the microbiome composition within 24 hours (Wu et al 2011). Diet represents a decisive modulator of gut microbial assembly and is therefore becoming an appealing option for microbiota manipulation. Nutrients can directly impact the microbiota, by inhibiting or promoting their growth, by inducing a selective pressure in the gut microbial

community that consequently reshapes that ecological system (Zmora et al 2019). Remarkably, the human gut microbiome rapidly changes in response to dietary interventions (David et al 2014); in fact, some dietary interventions (from high-fat/low-fibre to low-fat/high-fibre diet) can change the microbiome composition within 24 hours (Wu et al 2011).

Over the last number of decades, there has been a massive shift in lifestyle and, in particular, diet. Essentially, a great proportion of the human population adopted “Western” diets, which are high in processed foods, sugar, and fat, which are linked to the growing prevalence of obesity, diabetes and cardiovascular disease (Pavillard et al 2018). On the other hand, healthy diets such as the Mediterranean diet providing essential macronutrients such as flavonoids, omega-3 fatty acids, and omega-6 polyunsaturated fatty acids (Willett et al 1995) are associated with better life expectancy and health status (Sofi et al 2013). However, reports have also linked the consumption of western diets with cognitive and mood disorders (Jacka et al 2010, Kanoski & Davidson 2011), while the Mediterranean diet has been associated with better cognitive function (Feart et al 2010, Firth et al 2019, Lassale et al 2018). Furthermore, a recent systematic review concluded that adhering to a healthy diet, in particular, a traditional Mediterranean diet, or avoiding dietary patterns with higher inflammatory indices appears to confer some protection against depression in observational studies (Feart et al 2010, Firth et al 2019, Lassale et al 2018). Such studies provide clear evidence for a nutritional approach to prevent depression. Understanding the mechanisms underpinning such an effect will be important moving forward especially at the microbiota-immune-stress level.

In fact, recent studies showed that higher intake of unhealthy foods and lower consumption of nutrient dense foods were associated with decreased hippocampal volume (Jacka et al 2015) and inversely, that healthier diets are linked with larger hippocampal volume in humans (Akbaraly et al 2018). In preclinical models and models, different dietary interventions have been implicated in neuronal plasticity in the hippocampus hippocampal region, which further highlights the paramount importance of a healthy diet in the promotion of homeostasis and function of the brain. Furthermore, a modified Mediterranean diet, when added to normal antidepressants or psychotherapy, can have a marked positive effect in depression (Jacka et al 2017). Given that the Mediterranean diet markedly affects the composition of the microbiota by increasing levels of SCFAs and enrichment in *Prevotella* (De Filippis et al 2016) it

is tempting to speculate that the microbiota may play a role in the beneficial effects of such dietary interventions.

Dietary changes seem therefore an interesting intervention in order to promote general health. In fact, there is a growing interest in lifestyle changes, particularly adopting distinct diets such as paleolithic, ketogenic, and plant-based diets, among others, which are thought to promote health benefits, which in turn might be related to modulation of inflammation and/or gut microbiome (Dinu et al 2017, Whalen et al 2016, Williams & Cervenka 2017, Zopf et al 2018).

1.4.3. Prebiotics

A prebiotic is defined by the International Scientific Association for Probiotics and Prebiotics as “a substrate that is selectively utilized by host microorganisms conferring a health benefit” (Gibson et al 2017). Dietary fibre represents one of the main classes of prebiotics, being often described as “carbohydrates with a degree of polymerization greater than 2, which fail to be hydrolyzed or absorbed in the small intestine” (Stephen et al 2017). Fruits, vegetables, and grains constitute important sources of fibre in the diet. The drastic reduction in prebiotic intake due to increased consumption of Western-style diet has led to the coincidental rise in the incidence of obesity, inflammatory diseases, metabolic syndrome and anxiety, stress, and other "lifestyle" disorders. However, prebiotics do not always significantly change intestinal microbial activity and composition in a selective and predictable manner (Bindels et al 2015), and can also present a direct effect in the immune system (Pujari & Banerjee 2021)

1.4.3.1. Inulin

The dietary fructan inulin can be found in different vegetables, such as jerusalem artichokes, chicory roots, asparagus and bananas, and its breakdown generates frutooligosaccharide, another common prebiotic (Ramirez-Farias et al 2008, Shoaib et al 2016). Multiple studies have found that inulin supplementation favours the growth of *Faecalibacterium prausnitzii a*nd *Bifidobacterium* spp., while promoting the secretion of butyrate (de Preter et al 2008, Kolida et al 2007, Ramirez-Farias et al 2008). Additionally, maternal inulin/GOS prebiotic supplementation protects against the development of food allergies by reducing histamine levels and intestinal permeability in mice (Bouchaud et al 2016), thus suggesting that this prebiotic mixture

can modulate immune intestinal responses. Interestingly, in an animal model of mild traumatic brain injury, inulin supplementation mitigates the abundance of pathogenic bacteria, while promoting the abundance of *Bifidobacterium* spp, and *Akkermansia muciniphila*, increased the levels of short-chain fatty acids (SCFA) and improved cerebral brain flow (Yanckello et al 2022), implying beneficial effects of inulin in mitigating the long-term impact of traumatic brain injury.

1.4.3.2. FOS

Fructo-oligosaccharides (FOS), together with Galacto-oligosaccharides (GOS), are among the most explored prebiotic supplements (Tandon et al 2019). FOS can not only alter the gut bacterial composition, but also induce the production of SCFAs (Nakanishi et al 2006, Yamamoto et al 2016), and increase the secretion of immunoglobulin A (IgA) (Jangid et al 2022). FOS supplementation in humans has resulted in an increased abundance of *Bifidobacterium* and *Lactobacillus,* which are associated with better health outcomes (Tandon et al 2019). Dietary supplementation with short-chain GOS and long-chain FOS reduces anxiety-like behaviour and improves social behaviour in male BALB/c mice, while reshaping the serotonergic system in the PFC (Szklany et al 2020). Likewise, FOS combined with GOS has been reported to decrease stress-induced corticosterone secretion, along with reduced anxiety-like behaviour (Burokas et al 2017), thus implying a beneficial effect of the modulation of the gut microbiota by prebiotics on behavioural outcomes in the host.

1.4.4. Probiotics

The concept of psychobiotics was recently coined (Anderson et al 2017, Dinan et al 2013). Its definition has been expanded to refer to any targeted intervention of the microbiome that facilitates brain health (Cryan et al 2019b, Sarkar et al 2016). Although still early days, there is a growing body of data especially in healthy volunteers supporting the concept. For example, treatment with the probiotics *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175 (Messaoudi et al 2011) and galactooligosaccharide prebiotic (Schmidt et al 2015) resulted in the reduction of cortisol, thus promoting the subject's resilience to stress, and ameliorating emotional responses in healthy subjects. Additionally, ingestion of a probiotic cocktail resulted in altered brain activity and processing of emotional information in a functional magnetic resonance imaging study (Tillisch et al 2013), while attenuating negative thoughts associated with a negative mood (Steenbergen

et al 2015) supporting the importance of gut microbiota in stress and emotional responses.

Consumption of *Bifidobacterium longum* 1714 was associated with a reduction in stress and cognitive improvement in healthy subjects (Allen et al 2016). Contrastingly, some bacterial strains that have shown promising results in preclinical models did not show the same performance when administered to human populations (Kelly et al 2017) or show partial success when applied in humans (Schellekens et al 2021).

However, the exact mechanisms that make up the communication between the microbiota and the stress response remain to be elucidated. More recently, treatment with probiotics was shown to improve stress response (Nishida et al 2017, Papalini et al 2019) and depression scores in major depressive disorder (MDD) (Kazemi et al 2018) and in irritable bowel syndrome (Pinto-Sanchez et al 2017) patients and in postpartum depression (Slykerman et al 2017). In a separate study, 8 weeks of probiotic adjuvant treatment in combination with antidepressants in treatment-resistant depressive patients led to significant improvements in depression, thus improving response to treatment (Miyaoka et al 2018). Similarly, increased probiotics with *Lactobacillus plantarum* 299v improved cognitive function and decreased kynurenine concentration in MDD patients undergoing SSRI treatment (Rudzki et al 2019). However, with respect to probiotics as a preventive measure for MDD, there is no clear consensus on their overall efficacy. Over the last decade, an important discussion has started in the scientific community, particularly in the field of social psychology, about the reproducibility and replication of science (Baker 2016). As to the great potential presented by psychobiotics and their large popularity not only within the science community but also within the general public, caution is needed when drawing conclusions from what is still a limited amount of evidence. Therefore, it is clear that strain selection and much more longitudinal large-scale studies are required to demonstrate a specific role of any potentially psychobiotics (Dinan & Cryan 2019). Furthermore, improved evaluation and manufacturing advancements that will further contribute to the progress of the application of psychobiotics as well as advanced regulatory processes are key to deliver microbiome-based therapeutics in a safe and efficient fashion to the final consumer (Cunningham et al 2021).

1.5. Pathways of Communication

1.5.1. Vagus Nerve

The vagus nerve is a key component of the gut microbiome to brain signalling pathways (Fülling et al 2019) (Figure 5). The nerve endings of the vagus nerve are sensitive to neural communication from the enteric nervous system and the release of inflammatory factors from the gut and relay this sensory information through the brainstem to higher centres in the brain. Specifically, the vagus nerve innervates mainly the upper intestinal tract, and through afferent nerves enter the brainstem through the tractus solitarius and communicate with neurons in the nucleus of the tractus solitarius through glutamatergic signalling (Browning et al 2017). Even though, vagal afferent fibres do not directly access gut microbiota, gut microbial signals can still be detected by intrinsic primary afferent neurons in the enteric nervous system, or enteroendocrine cells (Han et al 2022). Recent studies using vagotomy reported a key role for gut microbiota in regulating neurogenesis possibly through mechanisms involving BDNF (O'Leary et al 2018). These findings support earlier work where the positive effect of the probiotic *Lactobacillus rhamnosus* JB1 on stress-induced depressive and anxiety-like behaviour (Bravo et al. 2011), as well as the effects of *Bifidobacterium longum* NCC3001 on anxiety-like behaviour (Bercik et al 2011b) were ablated with vagotomy. However, chronic gastrointestinal inflammation has bene shown to induce anxiety-like behaviour, in a vagus-independent manner (Bercik et al 2010). The precise mechanisms underlying these effects remain to be disentangled but given the role of vagal nerve stimulation as a treatment in some cases of depression, the role for vagal stimulation in the mechanism of action of certain probiotics seems evident.

In addition to playing a role in the positive effects on neurogenesis, communication from higher centres in the brain through efferent vagal impulses suppresses peripheral inflammatory cytokines, such as TNF-α and IL1-β in the spleen (Rosas-Ballina & Tracey 2009) to influence local events in the adrenals (Pavlov & Tracey 2017); thus, plays a role in regulating stress response.

In a recent study, faecal matter transplantation (FMT) of faeces from an aged donor to the gastrointestinal tract of a young recipient, not only leads to hippocampal alterations and defects in hippocampal-dependent tasks, but also to reduced neuronal activity in the nucleus tractus solitarius (NTS), an ascending vagus nerve pathway, and that inversely, activation of this ascending pathway results in better hippocampal function (Rei et al 2022).

Faecal matter transplantation (FMT) from chronic social defeated (CSDS) mice or *Faecalibaculum rodentium* can induce a depressive-like phenotype in Ephx2 knockout mice, a transgenic mouse line otherwise resistant to CSDS - however, this effect is reversed with vagotomy (Wang et al 2021), therefore implying a role of the vagus nerve in the gut microbial-dependent manifestation of stress. Further, colonic C/EBPβ/δ-secretase signalling is temporally activated in the gut, contributing to the formation of Tau and amyloid-β fibrils that later accumulate in the brain; however, vagotomy selectively blunts this signalling, limiting Tau and AB pathology and resulting in better cognitive outcomes, therefore unravelling a crucial role for vagal signalling in the progression of Alzheimer's pathology, in a gut-dependent process (Chen et al 2021).

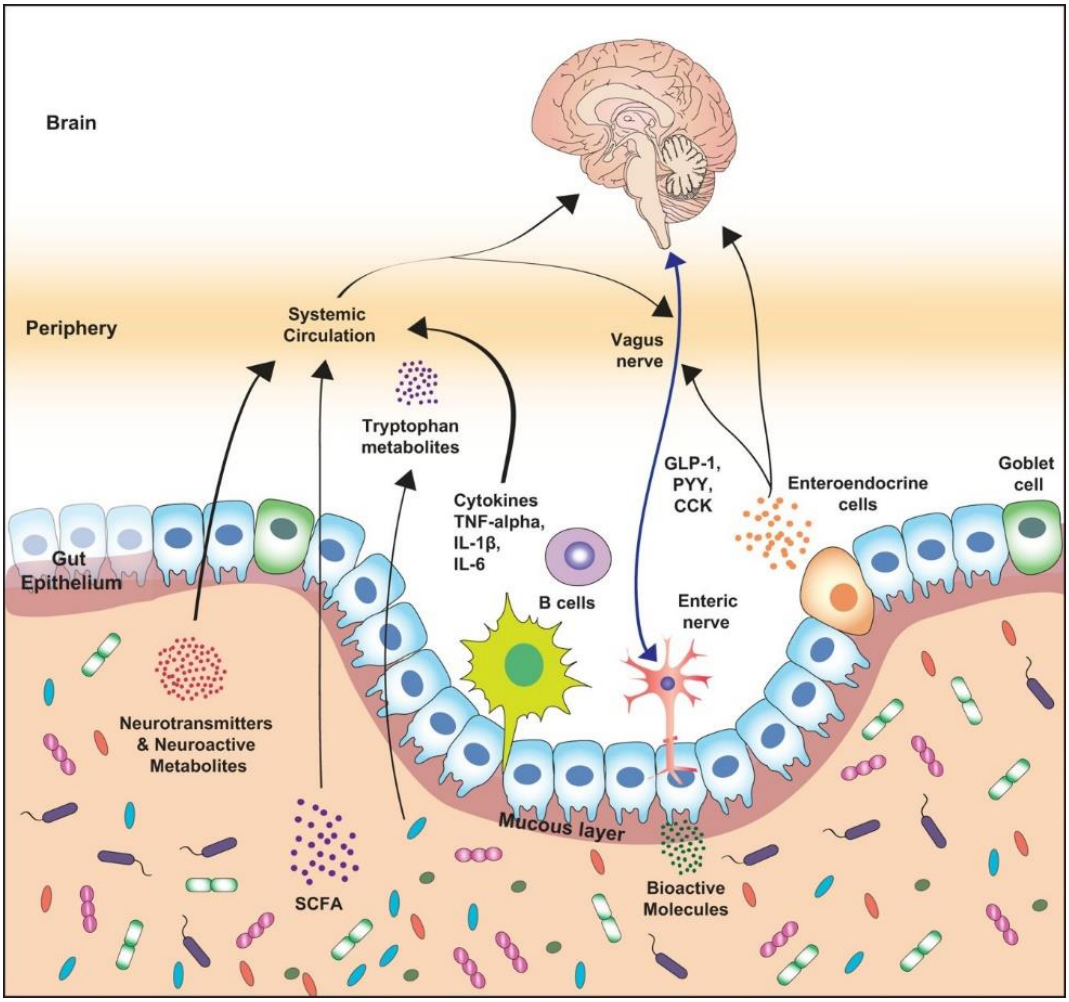

Figure 5 – Pathways of bidirectional communication between the intestine and the brain. *The gut and the brain are in constant communication through different proposed pathways such as immune signalling, neuronal innervation, liver and gallbladder metabolites, microbial metabolites and enteroendocrine signalling. Abbreviations: CCK, cholecystokinin; GLP-1, glucagon-like peptide-1; IL, interleukin; PYY, peptide YY; TNF, tumor necrosis factor; SCFA, short-chain fatty acid. Adapted from (Cryan et al 2019b).*

1.5.2. Hypothalamic-Pituitary-Adrenal Axis

The hypothalamic-pituitary-adrenal (HPA) axis represents a central neuroendocrine system and therefore a key route of communication within the gut-brain axis (Figure 5).

Microbial colonization is required for an appropriate stress response, as GF animals display increased levels of plasma corticosterone, indicating hyperresponsivity of the HPA axis (Clarke et al 2013, Sudo et al 2004). GF animals display decreased expression of NMDA and 5HT1A receptors (Neufeld et al 2011), which considering the relevance of both receptors in the modulation of hypothalamic release of corticotropin-releasing factor (CRF) could explain the altered HPA response in GF mice (Cryan et al 2019b).

1.5.3. Microbial Metabolites

The gut microbiota can greatly influence host physiology through the secretion of a wide range of metabolites that can be absorbed along the host gut and can be detected in the host circulation (Bar et al 2020, Chen et al 2022, Diener et al 2022, Krautkramer et al 2021), faeces, urine, liver and cerebrospinal fluid (Lavelle & Sokol 2020) (Figure 5).

Short chain fatty acids (SCFAs), which can promote gut barrier integrity and exhibit immunomodulatory potential, have also been increasingly recognized as key players in the microbiota-gut-brain axis. Indeed, altered levels of SCFA have been reported under different contexts where behaviour and brain physiology are altered, such as Parkinson's disease (Unger et al 2016), anorexia nervosa (Morita et al 2015), autism spectrum disorder (Liu et al 2019) and schizophrenia (Zhu et al 2020). Further, given the importance of SCFAs in regulating intestinal immunity, it is feasible to hypothesize that modulation of immune factors may impact the host. In fact, a recent study shows that intestinal-derived SCFA, and in particular propionate, reduces IL-17 secretion by intestinal γδ T cells by inhibiting histone deacetylase in mouse and human cells from patients with inflammatory bowel disease (IBD) (Dupraz et al 2021). The effects of SCFAs in gut-brain communication are suggested to take place by epigenetic remodelling, modulation of SCFA receptors, induction of the vagus nerve activation, enteroendocrine signalling and modulation of the host immune system (Cryan et al 2019b).

Tryptophan, an essential amino acid, has also been identified as a key player in microbiota-gut brain axis communication (Kennedy et al 2017, O'Mahony et al 2015). It can be metabolized in the gut through two metabolic pathways: the kynurenine pathway and the serotonin pathway (Gao et al 2018). Curiously, the gut microbiome can impact tryptophan availability, and consequently its metabolites. Kynurenine levels have been associated with depressive-like behaviour (Marx et al 2021), and probiotic administration has been shown to modulate kynurenine levels in MDD patients, suggesting that kynurenine metabolites may be pivotal for CNS pathology and can be targeted through the gut microbiome (Gheorghe et al 2019). More specifically, serum concentrations of kynurenic acid, that is known to be neuroprotective, and 3-hydroxykynurenine and quinolinic acid, that have been described as putatively neurotoxic have been shown to be altered in the context of MDD, as the kynurenic acid/ quinolinic acid ratio, a putative neuroprotective ratio tends to be lower in subjects with MDD (Savitz et al 2015). Furthermore, serotonin, which is a neurotransmitter that is reduced in the context of depression (Erritzoe et al 2022) - given that gut microbiota can actively modulate serotonin availability, it is plausible to suggest that the gut microbiome can regulate host pathways that impact brain function by regulating the availability of serotonin (Gheorghe et al 2019).
Bacterial metabolization of tryptophan can also produce indole, a metabolite that is essential for various bacterial functions but also, it can also activate aryl hydrocarbon receptors (AHR) that can modulate the host immune response (Gheorghe et al 2019, Lavelle & Sokol 2020, Roager & Licht 2018).

The gut microbiota tightly regulates the transformation of primary bile acids, predominantly produced in the liver, to secondary bile acids via key gut microbial enzymes generated in the distant gut (Hofmann 2004). Secondary bile acids have numerous effects on host health, including enhancing mucosal immunity (Sun et al 2021b). In particular, the activation of the farnesoid X bile acid farnesoid X receptor (FXR) resulted in decreased gut permeability and reduced expression of colonic proinflammatory cytokines in the context of colitis in mice (Gadaleta et al 2011). FXR and other bile acid receptors have been implicated in the regulation of immune cells, suggesting that microbiota-dependent bile acid metabolism regulates the intestinal and peripheral immune response (Cipriani et al 2011, Gadaleta et al 2011, Massafra et al 2016). Concomitantly, inflammation affects bile acid metabolism by impairing cholesterol transport out of immune cells (McGillicuddy et al 2009). Interestingly, bile acid enzymes and bile acid receptors can also be found in the brain. In the context of multiple sclerosis (MS), circulating secondary bile acids were altered along with bile

acid receptors in glial and immune cells in the brains of individuals with MS (Bhargava et al 2020). Considering that bile acids impact CNS function through glial and immune cells present in the brain, the implications for the gut microbiota in the regulation of these metabolites could be an approach to target the gut-bile acids-brain axis for neuropsychiatric disorders.

1.5.4. Immune System

Considering the intrinsic bidirectional communication between the immune cells and the gut, it is unsurprising that the immune system has been put forward as a key pathway in the gut-brain axis crosstalk (Cryan et al 2019b). The underlying mechanisms into how this communication takes place is outlined in detail in the following sections.

1.6. Microbiome-Immune Interactions

1.6.1. Immune Cell Regulation of Gut Microbiota

In homeostasis, the host immune response to the gut microbiota is tightly compartmentalized to the intestinal mucosal surface (Konrad et al 2006). Commensal microbes are separated by a thick mucus layer organized around the hyperglycosylated mucin MUC2, which promotes tolerogenic cytokines by imprinting antigen-sampled dendritic cells with anti-inflammatory signals, allowing for immunological tolerance to commensal bacteria (Shan et al 2013). Microbial metabolites, including indoles, bile acids, and SCFAs (Lavelle & Sokol 2020) can upregulate genes involved in the strengthening of the mucosal barrier by regulation of tight junctions and mucin production, while reducing pro-inflammatory factors (Bansal et al 2010).

Commensal bacteria also shape the immune responses by triggering the activation of regulatory T cells through direct recognition of microbial metabolites or products, such as SCFAs, by T cells or dendritic cells. Furthermore, commensals stimulate the induction of Th17 cells that in turn modulate the function and homeostasis of epithelial cells (Belkaid & Hand 2014).

1.6.2. Gut Microbiota Regulation of Immune Cells

The gut microbiota influences the relative populations, migration and phenotype of various subsets of immune cells (Dorrestein et al 2014), and several different studies have illustrated how gut microbial population can influence innate and adaptive immune responses at mucosal surfaces upon infection and autoimmunity (El Aidy et al 2015). Additionally, there is an increase in understanding of the importance of alterations in the crosstalk between intestinal microbes, the intestinal epithelium and the intestinal immune system, which in turn has been linked to modulation of systemic immunity (Belkaid & Hand 2014). In fact, it has been shown that the intestinal immune system is a sophisticated structure that has developed specific mechanisms to ensure that the commensal bacteria loaded is maintained and if bacteria cross the intestinal barrier, these will be targeted and eliminated by the immune system, preventing their possible invasion to the periphery (Hooper & Macpherson 2010).

The gut microbiota influences the structural development of intestinal-associated lymphoid tissues and shapes its immune response to maintain tolerance to commensal bacteria and trigger host defence through recognition of pathogen-associated patterns (PAMP) by pattern recognition receptors (PRR) and epigenetic regulators such as short chain fatty acids (Jiao et al 2020).

In addition, the gut microbiota also has a mutualistic relationship with the adaptive immune system. Intestinal IgA, secreted by B cells, can modify gut microbial communities and even contribute to the maintenance and diversification of the microbiome (Zheng et al 2020). Additionally, SCFAs generated by commensal bacteria are essential for induction of T regulatory cells, further indicating that bacterial metabolites are important for modulation of the host immune system (Arpaia et al 2013). Furthermore, commensal segmented filamented bacteria (SFB) elicit a quiescent state in Th17 cells, however, in pathogenic responses, Th17 cells are skewed towards a proinflammatory profile (Omenetti et al 2019). Furthermore, CD8 + T cells require microbial derived from the microorganism, namely butyrate, to promote their long-term survival as memory cells (Bachem et al 2019).

1.7. Microbiome-Immunology and Barrier Function

1.7.1. Gut Barrier

Innate lymphoid cells (ILCs) are involved in cytokine mediation of the integrity of the intestinal cell barrier while limiting pathological $CD4^+$ T cell responses to commensal bacteria, thus preventing intestinal inflammation (Hepworth et al 2013) (Figure 6). ILCs also represent the primary source of IL-22, an important cytokine for barrier immunity, as it can elicit host protective immunity against pathogens and mediate tissue protection (Sonnenberg et al 2011). In addition, commensal bacteria are involved in the regulation of intestinal barrier function by regulating tight junctions and epithelial cells (Yu et al 2012a). Furthermore, furthermore, bacterial penetration of the mucus layer depends on gut microbial composition (Jakobsson et al 2015) as GF mice display altered mucus thickness, significantly lowering colonic MUC2, while allowing bacterial sized beads intestinal penetration suggesting that mucus maturation depends on commensals (Johansson et al 2015). Additionally, loss of mucus barrier and altered goblet cell profiles can precede inflammatory profiles that can be involved in pathogenesis (Gustafsson & Johansson 2022).

In an animal model of leukaemia, intestinal barrier dysfunction contributes to accelerated leakage of lipopolysaccharide (LPS) into the circulation, and butyrate treatment, which is reduced in the faeces of acute myeloid leukaemia patients, slows leukaemia progression in mice by repairing intestinal barrier function (Wang et al 2022). In early life, immature intestinal epithelial cells promote high intestinal permeability, posing a risk for pathogenic bacterial and macromolecule infiltration, while also allowing for high transfer of maternal antibodies and environmental antigens (Weström et al 2020).

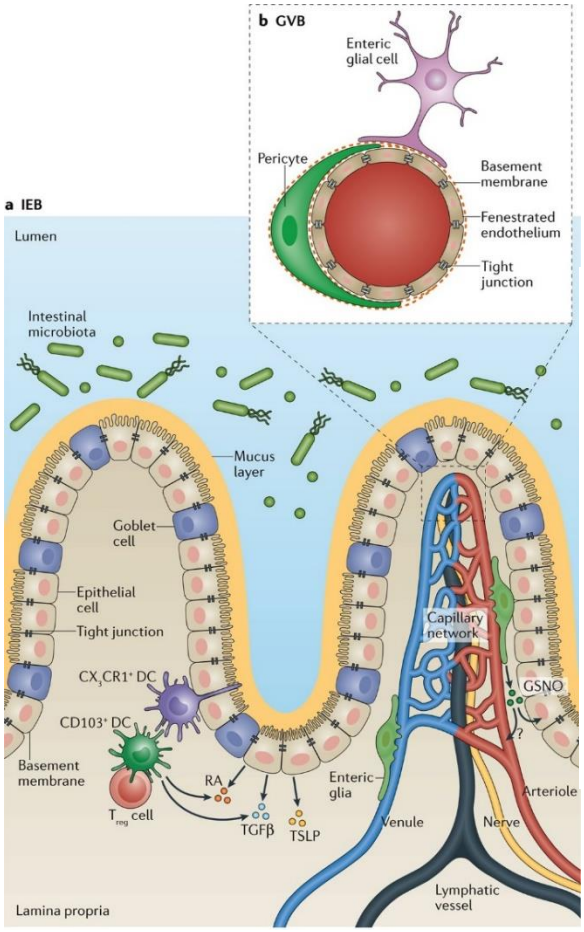

Figure 6 – The gut barrier. Goblet cells produce the mucus layer, thus supporting the mucosal barrier function. *Tight junctions seal the epithelial barrier and regulate paracellular trafficking. In the lamina propria, the mucosal immune system actively surveils food antigens, establishing oral and mucosal tolerance to such antigens. Abbreviations: DC, dendritic cell; RA, retinoic acid; TGFβ, transforming growth factor-β; Treg cell, regulatory T cell; TSLP, thymic stromal lymphopoietin. Adapted from (Spadoni et al 2017).*

Additionally, age-dependent microbial composition changes have been associated with systemic immune activation (Bosco & Noti 2021). In drosophila, age-associated chronic inflammation induces the decline of gut barrier function and compartmentalization, hence reversing the signalling pathways involved in this process reduces age-related microbial composition alterations and extends lifespan (Li et al 2016).

1.7.2. Blood-Brain Barrier

Commensal bacteria have also been shown to be important for maintaining the blood-brain barrier (BBB). The BBB is a crucial selective interface between the blood and the brain, that regulates the influx and outflux of molecules and cells into the CNS (Knox et al 2022a). Permeability of the BBB in GF mice is reduced, and this deficit is maintained through adulthood and characterized by reduced expression of the tight junction proteins occludin and claudin-5, which are important for maintaining barrier function (Braniste et al 2014). Interestingly, this effect can be reversed by colonization

with butyrate-producing bacteria or administration of butyrate in adulthood, as it reduces BBB permeability (Braniste et al 2014).

In fact, antibiotic exposure has been shown to reduce tight-junction expression and increase BBB permeability, inducing bone marrow-derived monocyte recruitment to the brain where they acquire a microglia-like state (Sun et al 2021a). Another study showed that early life antibiotic treatment impairs BBB in the hippocampus (Leclercq et al 2017), thus highlighting the relevance of the gut microbiota in BBB regulation.

A recent study has shown that immature neonatal microbiota favours colonization and translocation of group B streptococcus across the gut vascular barrier (Travier et al 2021). Furthermore, since the epithelium that lines the intestinal barrier and the choroid plexus (a component of the blood-cerebrospinal fluid barrier) is more permissive in neonates, neonatal intestinal immune immaturity contributes to susceptibility to group B *Streptoccoccus* induced meningitis (Travier et al 2021).

1.8. Microbiome, Brain Function and Behaviour

The communication between the gut microbiota and the host is increasingly evident in behavioural studies, highlighted in social behaviour, anxiety- and depressive-like behaviour (Figure 7).

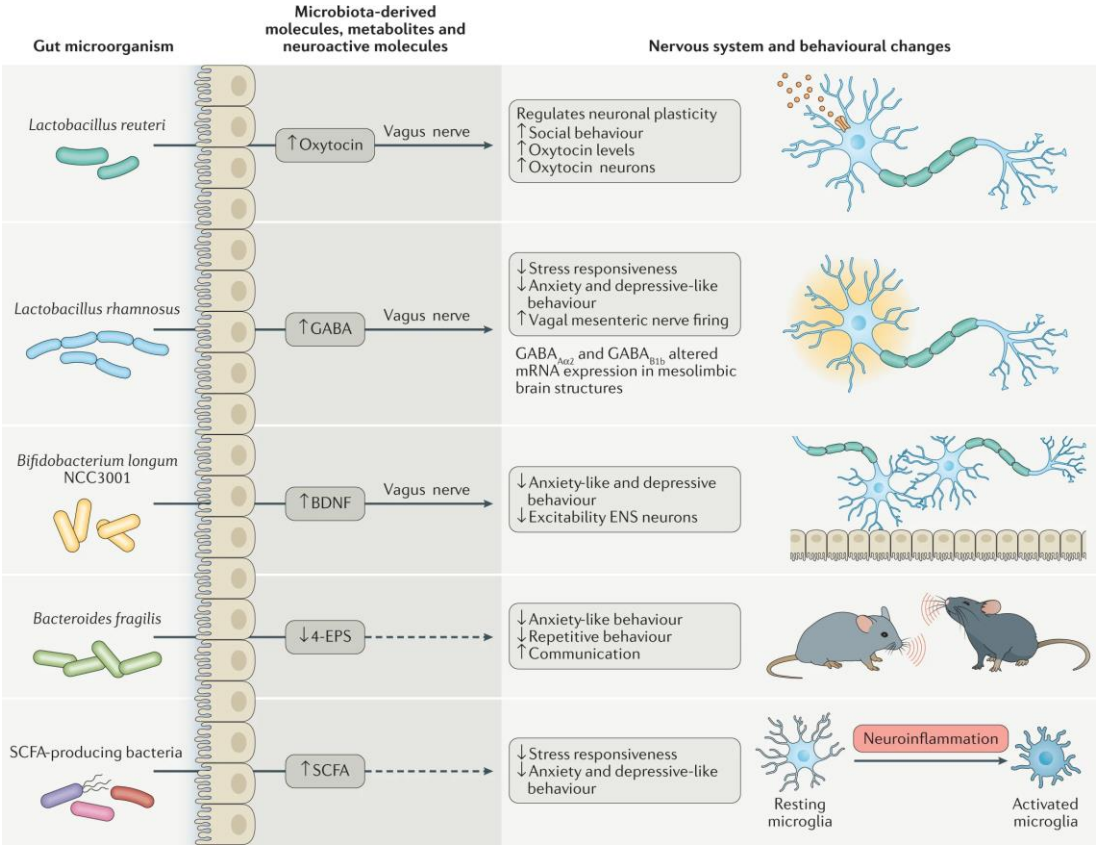

Figure 7 – Host behaviour and CNS function is shaped by microbiota and microbial-derived signalling. *Oxytocin and γ-aminobutyric acid (GABA) can be regulated by Lactobacillus species, through the vagus nerve, having beneficial behavioural outcomes. Bifidobacterium longum NCC3001 can upregulate brain derived neurotrophic factor (BDNF) and reduce depressive- and anxiety-like behaviour in mice. SCFAs can modulate stress responsiveness and depressive- and anxiety-like behaviour by alleviation of neuroinflammation. Bacteroides fragilis can improve anxiety-like behaviour in mice through reduction of 4-ethylphenylsulfate. Abbreviations: GABA - γ-aminobutyric acid; ENS – enteric nervous system; BDNF – Brain derived neurotrophic factor; 4-EPS - 4-ethylphenylsulfate; SCFA – Short-chain fatty acids. Adapted from (Morais et al 2021).*

1.8.1. Social Behaviour

Social behaviour is an essential and complex behavioural feature that can contribute to species evolution (Chen & Hong 2018) – notably, microbial signals may contribute to such an intricate behavioural network (Sherwin et al 2019). In GF animals, there are sociability and social novelty deficits, which is only recovered by post-weaning bacterial colonization regarding sociability, thus suggesting that certain social

behaviour features are influenced by gut microbial signals early in life (Desbonnet et al 2014). Likewise, mice born by Caesarean section show social novelty deficits, which can be rescued by co-housing with vaginal born mice, or supplementation with *Bifidobacterium breve* or bifidogenic prebiotics (Morais et al 2020). Furthermore, maternal high fat diet has also been shown to induce social behaviour deficits in the offspring, which can be alleviated by supplementation with *Lactobacillus reuteri* (Buffington et al 2016). Interestingly, the pervasive effects of maternal exposure to a high-fat diet on social behaviour endure across multiple generations and can be rescued post-weaning by *L. reuteri* (Di Gesù et al 2022).

Moreover, Wu and colleagues recently showed that germ-free animals, as well as antibiotic exposure, induce deficits in social behaviour that are associated with elevated levels of corticosterone (Wu et al 2021b). Curiously, *Enterococcus faecalis* improves social activity following social stress by decreasing corticosterone levels (Wu et al 2021b).

A fascinating recent study showed that mucosa-associated fungi, an essential component of the gut microbiota, promote social behaviour, possibly mediated by neuronal IL-17R-dependent signalling (Leonardi et al 2022). Taken together, a growing body of evidence demonstrates that appropriate gut microbial assembly is necessary for social behaviour, and that when disrupted, it can potentially be rescued by targeting the gut microbiome, even during adulthood.

1.8.2. Anxiety-Like Behaviour

Seminal studies showed that the gut-brain axis is involved in modulating anxiety-like behaviours in mice (Bravo et al 2011, Burokas et al 2017). More specifically, the administration of *Lactobacillus rhamnosus* JB-1 reshapes the expression of GABA receptors in the amygdala and hippocampus, and consequently regulates anxiety-like behaviour (Bravo et al 2011). In another study, prebiotic supplementation reduced anxiety-like behaviour in mice exposed to chronic stress (Burokas et al 2017). Likewise, prebiotic supplementation during early-life promotes anxiolytic effects in adulthood (Spitzer et al 2021). Interestingly, germ-free mice show reduced anxiety-like behaviour (Clarke et al 2013, Diaz Heijtz et al 2011, Luo et al 2018, Neufeld et al 2011), and this is reversed following post-weaning recolonization (Clarke et al 2013), suggesting that the gut microbiota may influence baseline anxiety-like behaviour. Furthermore, microbiota transplantation from BALB/c mice, which display a more

anxious phenotype, into germ-free NIH Swiss mice (that display a less anxious phenotype) promoted increased anxiety-like behaviours in the recipient Swiss mice (Bercik et al 2011a). Paradoxically, the inverse is observed in rats, as germ-free animals display increased anxiety-like behaviour, in a stress-sensitive strain (Crumeyrolle-Arias et al 2014b).

A microbial metabolite 4-ethylphenyl sulphate (4-EPS) that was increased in a mouse model of atypical neurodevelopment (Hsiao et al 2013) can affect oligodendrocyte function in the brain, resulting in increased anxiety-like behaviour in mice (Needham et al 2022). Furthermore, 4-EPS levels are altered in the faeces and plasma of individuals with ASD, and sequestering this metabolite by an oral small molecule decreases anxiety measures in this population (Stewart Campbell et al 2022).

1.8.3. Depressive-Like Behaviour

Another behavioural dimension that is increasingly linked to the gut microbiota is depressive-like behaviour. The absence of the gut microbiota promotes depressive-like behaviour in mice (Zheng et al 2016a), thus implying a role for the gut microbiota in the mediation of this behavioural feature, even if the increased locomotor activity in germ-free mice (Diaz Heijtz et al 2011) may interfere with the analysis of the decreased learned helplessness in the forced swim test. Interestingly, faecal transplantation from major depressive disorder donors to germ-free mice (Zheng et al 2016a) and antibiotic-treated rats (Kelly et al 2016) induces depressive-like behaviour in recipients, suggesting that exposure to these gut microbial profiles is sufficient to transfer depressive phenotypes. In fact, colonization of *Bacteroides fragilis*, *Bacteroides uniformis*, and to a lesser extent *Bacteroides caccae,* emulate the observed effects of transplanting gut microbiota from MDD patients into mice (Zhang et al 2022). Furthermore, intestinal microbial signals are required for the induction of behavioural despair in maternally separated mice, as germ-free maternally separated mice do not show depressive- and anxiety-like behaviour, despite the marked HPA axis activation and colonic cholinergic neural dysregulation (De Palma et al 2015).

A recent study found that *Mycobacterium neoaurum* isolated from testosterone-deficient patients with depression secretes a testosterone-degrading enzyme (3β-Hydroxysteroid dehydrogenase; 3β-HSD) (Jangid et al 2022). Consequently, gavage

of *M. neoaurum* or 3β-HSD lowers testosterone levels and induces depressive-like behaviour in rats (Jangid et al 2022).

1.8.4. Brain Function

Host microbiota has also been implicated in influencing brain function, as it can shape myelination, adult neurogenesis and neural plasticity.

Myelination, an essential process for the complex of neural circuits that entails the ensheathment of axons by oligodendrocytes, has been proposed to be regulated by microbial signals (Lynch et al 2021). Our group has previously demonstrated that the gut microbiota influences myelin gene expression and cortical myelination in a sex-dependent and region-specific fashion. Categorically, male, but not female, germ-free mice display hypermyelination specifically in the prefrontal cortex (PFC), a region highly involved in cognitive, decision-making and social processes (Hoban et al 2016b). Social avoidance behaviour in a nonobese diabetic mouse model was related to impaired myelination and decreased myelin-related genes in the PFC, which was associated with increased levels of a microbial-derived metabolite, *p*-cresol (Gacias et al 2016). Further, early antibiotic exposure was found to increase myelination in the PFC, which can be restored by butyrate treatment (Keogh et al 2021).

Adult neurogenesis, a CNS process is a process by which neural progenitor cells proliferate and differentiate into neuronal and glial cells, that has been largely proposed as a key pathway in the context of depression, is also suggested to be modulated by gut microbiota (Cruz-Pereira et al 2020). Specifically, germ-free mice present increased levels of adult hippocampal cell proliferation and neurogenesis, which was not prevented by microbial recolonization from three weeks of age (Ogbonnaya et al 2015). Interestingly, these effects were predominantly observed in the dorsal hippocampus rather than ventral hippocampus, which may have interesting implications for cognitive functions that predominantly engage the dorsal hippocampus such as spatial learning and memory (O'Leary & Cryan 2014). On the other hand, antibiotic treatment can result in reduced adult hippocampal neurogenesis, which was reversed by probiotic treatment and voluntary exercise but not through faecal transplantation, by modulating Ly6Chi monocyte trafficking (Möhle et al 2016).

Further, FMT from MDD patients or exposure to *Bacteroides* species impacts hippocampal neurogenesis (Zhang et al 2022). Interestingly, treatment with metformin - a widely used diabetic drug – is able to restore the high-fat diet-induced adult hippocampal neurogenesis impairments, by reshaping the gut microbial community (Ma et al 2021). Therefore, the gut microbiome seems to be involved in the modulation of adult hippocampal neurogenesis, possibly through the interaction with the immune system.

The gut microbiome can also impact brain function by influencing brain circuitry. For example, environmental enrichment, a well-established condition known to promote neural plasticity, is associated with different gut microbial composition and enhanced levels of SCFA (Lupori et al 2022). Remarkably, the faecal transplantation from environment enriched donors promotes adult ocular dominance plasticity in recipient mice, which can be replicated with SCFA administration in standard rearing mice, hence highlighting that experience-dependent modifications can shift gut microbiota composition and shape brain circuitry and plasticity (Lupori et al 2022).

2. Neuroimmunology

2.1. Influences of the Immune System in the CNS

In recent years, growing interest has been drawn to the cellular mechanisms underlying immune trafficking to the brain in the context of overall brain health. It was initially thought that the central nervous system was somewhat isolated from the peripheral immune system, since during initial observations it was observed that the engraftment of tumours or foetal tissue in the brain was successful, suggesting that the immune system was unable to act as evidenced by the lack of rejection of donor tissue.

As these observations differed from what was reported in the periphery, it was believed that the CNS failed to trigger an immune response (Engelhardt et al 2017). However, in the wake of evidence indicating that circulating cytokines can indeed influence brain and behaviour, research has shown that despite the tight regulation of immune cell migration into the CNS by the blood-brain barrier (BBB), peripheral leukocytes can infiltrate the cerebral spinal fluid (CSF), meninges, choroid plexus, perivascular spaces, and ultimately the brain parenchyma (Engelhardt et al 2017). Subsequently, specialized innate immune sentinel cells – choroid plexus

macrophages, perivascular macrophages, mast cells, meningeal macrophages, and microglia (CNS resident macrophages) – surveil the CNS under steady state conditions and are the first responders in case of potential danger, detect pathogens or tissue damage, and trigger an immunological response (Rua & McGavern 2018). Lymphocytes also accumulate in the perivascular spaces after recruitment to the CNS in response to altered levels of chemokines, to combat pathogens (Rua & McGavern 2018). Despite the constitutive presence of cytokines in the CNS under normal homeostasis and their role in synaptic plasticity, an increase in monocyte trafficking and/or an alteration in pro/anti-inflammatory cytokine balance may contribute to the etiology or progression of neuroinflammatory events (Dantzer 2018). Furthermore, recent provocative studies have shown that the CNS is in fact directly connected to secondary cervical lymph nodes by a lymphatic drainage system that can evoke peripheral immune responses (Louveau et al 2015), having far-reaching impacts in the context of multiple sclerosis (Ahmed et al 2022), stroke (Esposito et al 2019), traumatic brain injury (Bolte et al 2020), and age-associated cognitive decline and Alzheimer's disease (Da Mesquita et al 2018).

Cytokine levels in the absence of infection have been shown to regulate homeostatic behaviour. Namely, cognitive impairments have been evident in mice deficient in TNFR2 (Naude et al 2014), IL-1R1 (Avital et al 2003), and IL-6 (Hryniewicz et al 2007). Interestingly, physiological levels of IL-1β are required for hippocampal-dependent memory, and any deviation from the physiological range (excess or blockade) leads to impaired memory (Goshen et al 2007).

Early work has demonstrated that T cells are essential for behaviour as its absence results in spatial memory and cognitive impairments (Brynskikh et al 2008, Kipnis et al 2004) as well as social behavioural deficits (Filiano et al 2016) and altered emotional behaviour (Rattazzi et al 2013). Furthermore, T cell blockade to the meninges results in deficits in the Morris water maze (Derecki et al 2010) and altered social behaviour (Filiano et al 2016). Moreover, IL-4 activation and expression in $CD4^+$ T cells is essential for spatial learning (Derecki et al 2010). Likewise, IFN-γ signalling in neurons in the prefrontal cortex is essential for social behaviour (Filiano et al 2016). Furthermore, IL17-producing γδ T cells homed in the meninges have recently been shown to have an important role in short-term memory (Ribeiro et al 2019), as well as the manifestation of anxiety-like behaviour, in a process partially mediated by the gut microbiota (Alves de Lima et al 2020).

The choroid plexus is a key CNS immunological niche and is constituted by an epithelial layer comprising tight junctions covering a stromal interface that encompasses fenestrated blood vessels (Croese et al 2021). These structures sit at each of the four brain ventricles, gatekeeping the trafficking of circulating leukocytes and their molecules, while expressing receptors that integrate ‘danger’ signals such as toll-like receptors (TLR) and inflammatory cytokines (Croese et al 2021). In homeostasis, professional immune cells such as effector memory T cells, and macrophages populate the choroid plexus (Figure 8) (Baruch et al 2013, Dani et al 2021). Aside from producing CSF, the choroid plexus enables bidirectional communication between the circulation and the CNS parenchyma, as resident T cells can interact with antigen-presenting cells and can under stimulation secrete cytokines that shift the choroid plexus epithelium, and consequently impact the CNS parenchyma (Baruch et al 2013). Given its intrinsic roles in mediating neuroimmunity in the CNS and the communication with the periphery, it is unsurprising that alterations in the choroid plexus are becoming increasingly reported in association with pathological processes, such as multiple sclerosis (Ricigliano et al 2021), psychosis (Lizano et al 2019) and in particular cases of infection, such as COVID-19 (Yang et al 2021).

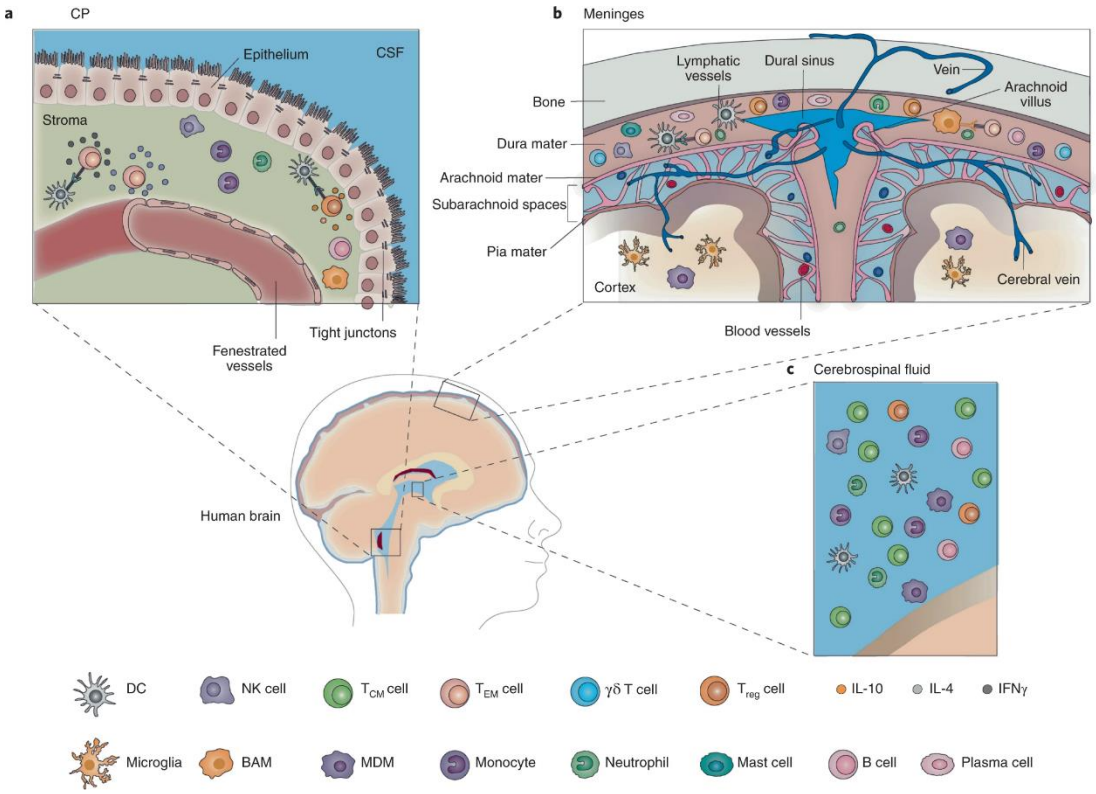

Figure 8 – Immune cells are compartmentalized in the central nervous system. *A) The choroid plexus represents an epithelial layer with tight junctions that allow the interface with blood vessels. In the stroma, dendritic cells present CNS antigens to T regulatory and effector memory cells. At the physiological level, neutrophils, natural killer cells, B cells, monocytes and border associated macrophages (BAMs) surveil the choroid plexus. B) The meninges constitute a bridge between the CSF, the CNS lymphatic vessels and the draining vessels in the deep cervical lymph nodes and are populated by different innate and immune cell populations. Abbreviations: CP, choroid plexus; CSF: cerebrospinal fluid; CNS: central nervous system. Adapted from (Croese et al 2021).*

2.2. CNS Influences in the Immune System

The communication between the central nervous system and the immune system is also evident by the expression of sickness behaviour, which can be elicited by peripheral infection in the absence of direct CNS disease (Schiller et al 2021). Cytokine signalling triggered by peripheral inflammation can impact CNS regulated processes such as hunger and sleep, in a crucial adaptation to ensure optimal use of physiological processes to guarantee survival (Schiller et al 2021). The nervous system regulates the immune response by a) synchronizing physiological processes to optimize immune responses; b) anticipating immune challenges based on past experience and sensory and visceral inputs; and c) rapidly activating and ceasing immune responses by regulation of the HPA axis and sympathetic nervous system (SNS). Recently, it was shown that abdominal inflammation triggers neuronal activation in the insular cortex and that reactivation of these neurons is sufficient to

elicit organ-specific inflammatory responses that emulate the initial inflammatory event (Koren et al 2021). Together, there is clear communication between the CNS and immune system that operate at a complex level.

3. Stress

Stress is generally defined as adverse life events; however, it is important to remember that stress is an important physiological reaction that ensures the body's adaptation to the environment (Sanacora et al 2022).

Such adverse life events are perceived by the body as a threat to its homeostasis, leading to physiological responses that promote adaptation to these challenges – allostasis. However, such adjustments may represent a great physiological cost to the organism as a result of repeated overactivity or inactivity of the systems, including the hypothalamic-pituitary-adrenal axis (HPA) leading to an increased allostatic load, which has been conceptualized to represent the biological repercussions of the wear and tear of the body after repeated stress exposure (McEwen 1998).

Overall, stress can be viewed as a necessary evolutionary response to a stimulus that results in the activation of the fight or flight mechanisms in the body which is essential for the survival of any organism (Dhabhar 2014). In mammals, this response is mediated by the HPA axis, a negative feedback system that regulates physiological responses to stress. In a matter of seconds to minutes, activation of the HPA axis enables the response of the organism to threats by prioritizing functions essential for defensive behaviours (such as cognition and energy supply) over physiological functions related to sustenance (such as digestion). Activation of the HPA axis activates neurons in the paraventricular nucleus (PVN) of the hypothalamus to secrete arginine vasopressin (AVP) and corticotropin-releasing factor (CRF), which, in turn, stimulate the production and secretion of adrenocorticotropic hormone (ACTH) in the anterior pituitary gland. As a consequence, ACTH induces the production and secretion of mineralocorticoids and glucocorticoids (corticosterone in rodents, cortisol in humans) from the adrenal cortex into the bloodstream. High levels of cortisol consequently inhibit further release of ACTH and CRF through a negative feedback mechanism. This negative feedback mechanism occurs via cortisol binding to glucocorticoid receptors (GRs) in the pituitary and PVN, as well as the hippocampus area of the brain, thus resulting in the return to the physiological state after acute activation of the system.

Glucocorticoids released by the adrenal cortex interact with glucocorticoid receptors (GRs) that are expressed not only within the HPA axis but also throughout the body, including in the gut, immune cells and in limbic brain areas such as the hippocampus where they act as transcription factors and shape the functional and structural organization of the neural circuitry that controls the behavioural response to stress (de Kloet et al 2005). In the context of depression, the HPA axis negative feedback loop is compromised, resulting in prolonged elevation of glucocorticoids (de Kloet et al 2005).

Interestingly, some symptoms of depression such as hopelessness, disrupted sleep, and alterations in appetite and body weight have been associated with HPA axis impairment, which partially explains the depressive symptoms often observed in patients with Cushing's disease (a medical condition characterized by excessive secretion of cortisol). Furthermore, chronic corticosterone treatment or chronic stress induces neuronal hippocampal atrophy (McEwen et al 2015).

Moreover, clinical neuroimaging studies have revealed volumetric reductions in the hippocampus in depression (Treadway et al 2015). Therefore, repeated and severe stress exposure, particularly during sensitive periods of neurodevelopment, promotes reprogramming of the hippocampus, inducing long-lasting alterations that could determine the response to future stressors (Marečková et al 2018), often in a sex-specific fashion (Bale & Epperson 2015), which can contribute to some stress-related depressive-like phenotypes.

Prolonged exposure to stress can result in altered gene expression as a direct result of glucocorticoids on gene transcription along with the induction of epigenetic mechanisms such as methylation and hydroxymethylation and histone modifications of DNA. These events culminate in the activation or repression of genetic factors in brain regions associated with emotion and cognition, such as the hippocampus and the prefrontal cortex (McEwen et al 2015). Particularly in the latter structures, stress and the consequent excess of glucocorticoids have been shown to lead to atrophy of dendritic processes and to the decrease of synaptic plasticity (Sapolsky 2015).

Therefore, stress-induced structural and functional abnormalities lead to impairments in cognition and affection as a result of the dysregulation of the HPA axis, which is consequently associated with an increased risk of disease, particularly depression (Belleau et al 2018). It is important to note that increased allostatic load as a result of

continued stress exposure puts a strain not only on the HPA axis but also impacts almost every other physiological system including the cardiovascular system and the immune system.

4. Microbiome-Immune-Brain Axis

Although the microbiota contributes to the priming, education, and activation of the immune response; the immune system plays a role in the maintenance and regulation of the number and diversity of gut bacteria. Current evidence suggests that in homeostatic conditions, a healthy and dynamic low-grade inflammatory tone is maintained to continuously communicate across the gut, immune and enteric nervous systems to the brain (Figure 9). As well as influencing the immune system at the level of the gut, the microbiota has also been linked to the tight regulation of microglia (Erny et al 2015, Thion et al 2018). That is, gut microbiome signals are required for the activation of conventional $CD4^+$ T cells to migrate into the brain, which in turn are essential for the appropriate microglial transition from fetal to adult state, which is crucial for the appropriate synaptic pruning and behaviour (Pasciuto et al 2020). Further, microglia maturation is modulated by gut-derived acetate, a SCFA demonstrated to also regulate microglial homeostatic metabolic state, particularly during neurodegenerative processes (Erny et al 2021).

Regarding inflammasome interactions with microbiota-gut-brain axis, NLPR6 has been shown to respond to gut microbial metabolite signalling, leading to the activation of the host immune response (Levy et al 2015). Genetic deletion of caspase-1, a key component of this signalling cascade, reduces depressive-like behaviour in mice, while resulting in gut microbiome alterations (Wong et al 2016). Antibiotic treatment of stressed mice promoted a rebalance of the gut microbiome in a similar way to that found in caspase-1 knockout mice, further implying a role for the gut microbiome in the regulation of inflammasome pathways that impact brain function (Wong et al 2016).

Microbiota transplantation from NLPR3 knockout animals suggested that microbial-derived metabolites from an inflammasome-blocking model mitigates depressive-like behaviour by attenuating astrocytic dysfunction in recipient wild-type mice exposed to chronic stress (Zhang et al 2019).

A fascinating new study has shown that a specific subset of astrocytes - LAMP1+TRAIL+ astrocytes - limit CNS inflammation by inducing T cell apoptosis, in response to IFN-γ produced by gut modulated natural killer cells, thus demonstrating a role for gut microbial modulation of CNS inflammation (Sanmarco et al 2021).

This growing evidence that microbial signals influence the immune system of the host and can therefore mediate neuroinflammation, can also be reflected in particular neuropsychiatric and neurological processes.

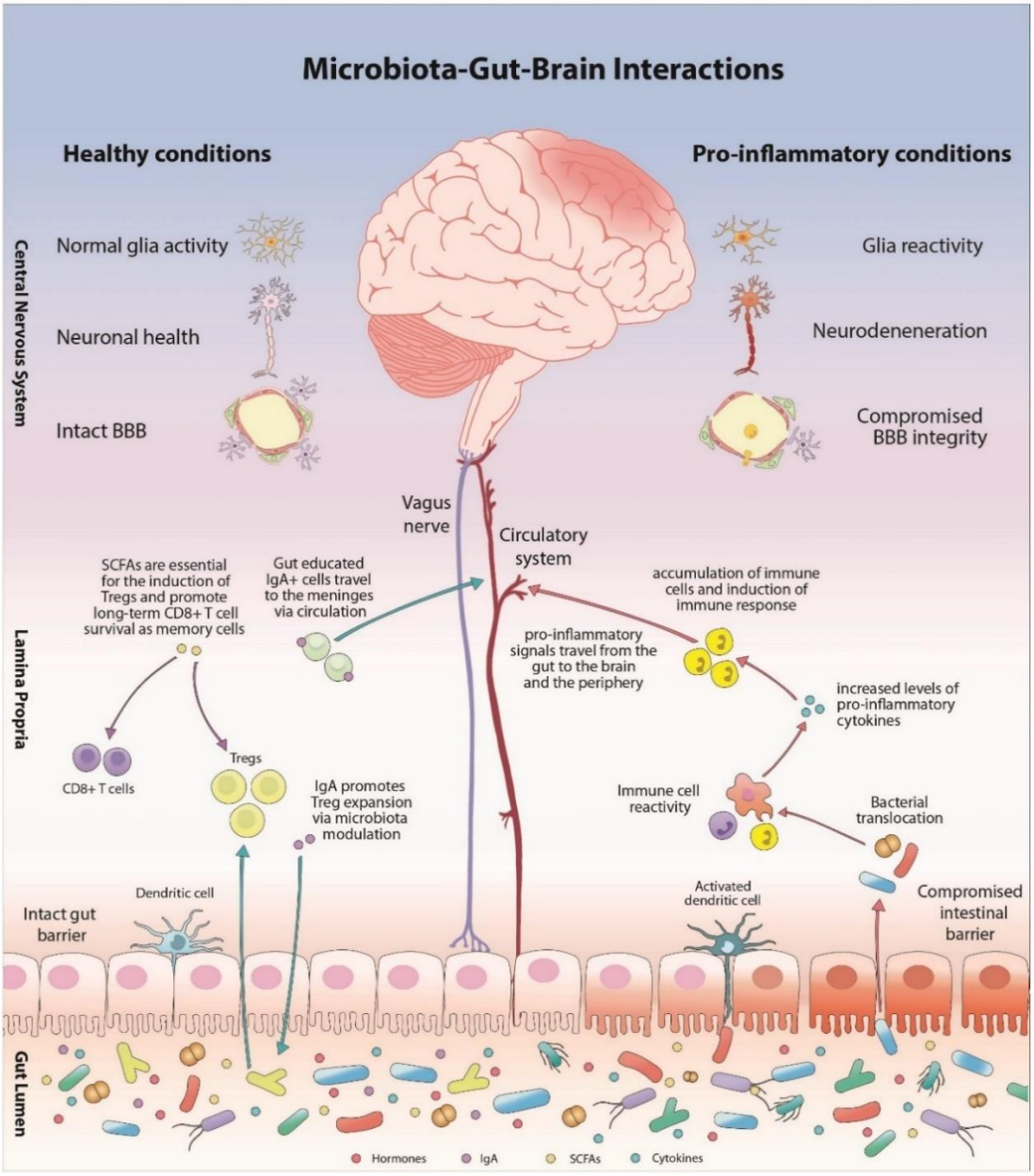

Figure 9 - Microbiota-Gut-Immune-Brain axis in healthy and proinflammatory conditions. *During homeostasis, the gut barrier is intact, circulating immune cells and signalling molecules produced from the gut microbiota reach the CNS through circulation, vagus nerve activation, and the lymphatic system. The BBB continuously controlling the flux of molecules and cells towards the CNS, whereas the glia patrol the CNS for the maintenance of normal neuronal function. Under pro-inflammatory conditions, the intestinal barrier is prone to lose integrity with microbial translocation to the lamina propria. Immune cells become reactive and produce pro-inflammatory mediators which signal to the CNS through the circulation, vagus nerve, and lymphatic system. Under these conditions, the BBB is disrupted, with compromised barrier integrity and increased immune cell infiltration to the CNS. The glia within the CNS become reactive and release molecules that together with mediators from the periphery induce neuroinflammation and neurodegeneration within the CNS. Abbreviations: BBB, blood-brain barrier; CNS, central nervous system; IgA – immunoglobulin A. Adapted from Current Opinion in Neurobiology (Ratsika, Cruz-Pereira, Lynch et al 2023).*

5. Early Life

5.1. Early Life and Neurodevelopment – Critical Windows

Significant dynamic structural and functional processes take place during the first stages of life (Figure 10). Complex neuroplastic events involve appropriate and timely division, proliferation, migration, and differentiation of glial and neural progenitor cells (Stiles & Jernigan 2010). Oligodendrocyte differentiation drastically increases axonal conduction and promotes myelination, supporting neuronal surviving and signalling (Fields 2015, Stiles & Jernigan 2010). Furthermore, given the intensified growth in these periods, programmed cell death poses a crucial process for neurodevelopment, as a way to ensure the appropriate establishment of neuronal and glial populations, thus avoiding competition for limited resources (Kristiansen & Ham 2014, Yamaguchi & Miura 2015). Elimination of apoptotic cells in the central nervous system (CNS) is mainly performed by microglia (the innate immune cells of the CNS), but can also be performed by astrocytes and oligodendrocytes, in a process that is once again critical for ensuring homeostasis and ensuring appropriate neural circuit development (Galloway et al 2019, VanRyzin 2021). Neuroplasticity is essential during neurodevelopment and disruptions, and these processes can increase susceptibility risk for developing brain diseases later in life (Codagnone et al 2019).

5.2. Microbiome & Neurodevelopment

Increasing evidence have highlighted links between neurodevelopment and gut microbiota. In fact, studies in GF mice have shown abnormal brain development in the absence of gut microbial signals, particularly in male mice (Clarke et al 2013, Neufeld et al 2011, Sudo et al 2004). Further, GF mice have also shown aberrant genetic expression related to neuronal plasticity, neurotransmission, metabolism and morphology in the hippocampus (Chen et al 2017) and the amygdala (Stilling et al 2015). Finally, a recent study has shown that maturation of early life microbiome is linked to associative learning in adulthood, while modulating microbial metabolites and glial cell line-derived neurotrophic factor (GDNF) expression in the early postnatal brain (Juricic et al 2022). Fascinatingly, in humans, increased microbial diversity at 1 year of age can predict lower cognitive performance at 2 years of age although the mechanisms are currently not well defined (Carlson et al 2018, Dinan et al 2018).

5.3. Neuroimmunology and Early Life

Pregnancy involves a remarkable complex immunological balance. The maternal-fetal interface involves an extraordinary immunological landscape, as it involves maternal immune tolerance to the fetus and, inversely, the fetus is now recognized to also present an established T-cell repertoire (Lu-Culligan & Iwasaki 2020, Miller et al 2020). Considering the intricate relationship between maternal and fetal immunity and that this is a sensitive neurodevelopmental period, it is not surprising that infections during pregnancy pose a risk for psychiatric and neurological disease in the offspring – such as cerebral palsy, epilepsy, autism spectrum disorder (ASD), schizophrenia and depression (Han et al 2021b, Knuesel et al 2014).

5.3.1. Maternal Immune Activation

Maternal immune activation (MIA) can be induced by pathogenic infection (*Toxoplasma gondii*, influenza, herpes simplex virus type 2, rubella, cytomegalovirus) that triggers immune responses that can interfere with appropriate neurodevelopment (Knuesel et al 2014, Kwon et al 2022, Lu-Culligan & Iwasaki 2020). In preclinical models, a particular subset of T helper cells - retinoic acid receptor–related orphan nuclear receptor gamma t (RORγt)–dependent effector T lymphocytes – and the effector cytokine interleukin-17a (IL-17a) have been shown to be required for MIA-behavioural deficits, while interfering with cortical development (Choi et al 2016), particularly in a subregion of the primary somatosensory cortex (Shin Yim et al 2017). Maternal injection with IL-6 can also induce behavioural abnormalities, which can be reversed with blockade by anti-IL-6 antibody (Smith et al 2007). Considering the importance of maintaining a pro-inflammatory/anti-inflammatory balance, targeting the anti-inflammatory cytokine IL-10 prevents MIA-induced behavioural deficits, by reducing circulating levels of pro-inflammatory cytokines IL-6 and TNF-α (Meyer et al 2008).

5.3.2. Cytokines and Neurodevelopment

During neurodevelopment, cytokines are involved in key processes, such as a) regulation of proliferation, apoptosis and survival mechanisms; b) induction, proliferation, maturation of neural stem cells; c) the temporal regulation of gliogenesis and neurogenesis; and d) the path integration of neuronal progenitors (Deverman & Patterson 2009). Genetic knockout models have been developed to study the

involvement of cytokines in neurodevelopment (Lu-Culligan & Iwasaki 2020). For example, transforming growth factor β (TGFβ) signalling is required for maturation of neural progenitor cells into dopaminergic neurons in the embryonic murine midbrain, as TGF-β2/TGF-β3 double-knockout mouse embryos present significantly less dopaminergic neurons in the ventral mesencephalon (Roussa et al 2006). Downstream of TGFβ, Smad proteins are involved in key neurodevelopmental processes, such as neural tube closure, oligodendrocyte differentiation, neurogenesis, astrogliogenesis, axon growth, interneuron specification and neural crest specification (Hegarty et al 2013). Different cytokine signalling pathways can induce distinct consequences – for example, TNF-α knockout mice present decreased motor and sensory neuronal death (Barker et al 2001, Sedel et al 2004).

The IL-6 cytokine family includes IL-6, IL-11, cardiotrophin (CT)-1, ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF), which present receptor families that express gp130 as a signal transducer, which in turn can activate the signal transducer and transcription 3 (STAT3). LIF receptor beta (LIFRβ) or gp130 expression deficiency results in reduced mitotic radial glial cells in the embryonic brain, forebrain hypoplasia, and deficits in astrogliogenesis (Barnabé-Heider et al 2005, Gregg & Weiss 2005, Hatta et al 2002).

Colony-stimulating factor 1 receptor (CSF1R), a receptor expressed across all macrophage development lineages, the development of microglia is not dependent on CSF-1, as these cells require IL-34 instead (Wang et al 2012)– however, $Csf1r^{-/-}$ mice show microglia depletion and impaired olfactory function (Erblich et al 2011).

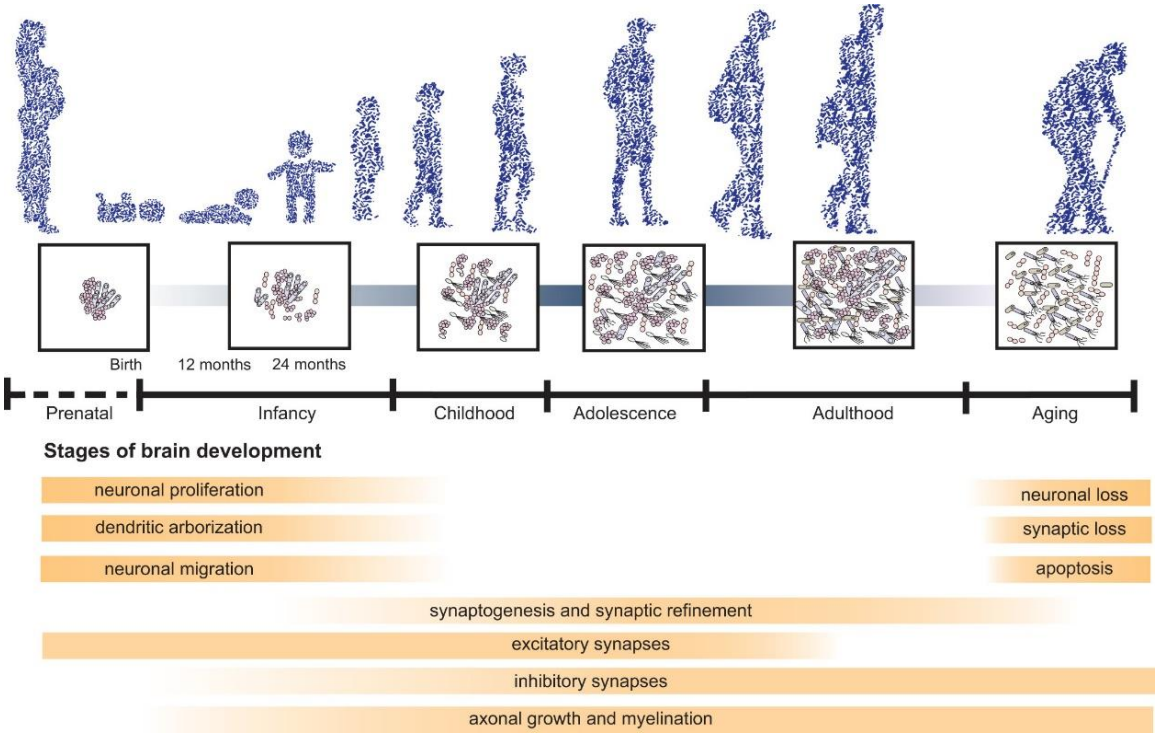

Figure 10 - Gut microbiome changes throughout lifespan matches critical windows of neurodevelopment and neurodegeneration. *From birth through infancy, adolescence, adulthood and aging, significant remodeling of the gut microbial populations takes place, concomitantly with neuronal processes that occur during defined stages of life. Adapted from (Cryan et al 2019b).*

5.2.1. Early-Life Microbiome

Early gut microbial colonization is largely influenced by mode of delivery (vaginal birth or Caesarean section) and by maternal diet, antibiotic exposure, feeding mode (breastfeeding or formula fed) and solid food introduction (Ratsika et al 2021). Early microbial colonization paves the way for long-term gut microbial colonization (Martínez et al 2018), thus reinforcing the importance of the previously stated factors in the initial microbial seeding and its lifelong impact on the host (Figure 9). Despite the fact that gut bacterial composition divergence due to mode of delivery mostly dissipates after 12 months of age, early disruption of microbial assembly impacts the offspring nonetheless (Korpela & de Vos 2018).

After birth, the gut microbiota is typically composed of a relatively low abundance of *Ruminococcaceae* and *Lachnospiraceae* and high levels of *Bifidobacteriaceae*, *Clostridiaceae* and *Enterobacteriaceae* (Bokulich et al 2016, Chu et al 2017, Yassour et al 2016). As time passes, strict anaerobes gradually become the dominant taxa,

and the diversity increases to levels closer to those observed in adulthood around the weaning and solid food introduction period (Koenig et al 2011, Palmer et al 2007, Yassour et al 2016).

During adolescence, rapid body development along with lifestyle adaptations characterize this period, therefore representing another critical period for the development (Cryan et al 2019b). Adolescents have lower relative abundances of *Sutterella* and *Prevotella*, while having a higher relative abundance of *Clostridium* and *Bifidobacterium* (Agans et al 2011) which is closer to the infantile gut microbial profile (Hopkins et al 2001) thus suggesting a more gradual transition in gut microbial composition from childhood to adulthood.

5.3. Microbiome-Immunology in Early Life

The maternal microbiome has been shown to intestinal group 3 innate lymphoid cells (ILC3) and $F4/80^{+}CD11c^{+}$ mononuclear cells in the intestine of their offspring, while shaping gene expression patterns related to the metabolism of microbial molecule metabolism and epithelial antibacterial peptides (Gomez de Agüero et al 2016). Therefore, this suggests that maternal microbiome during gestation and lactation shapes the innate immune profile of the offspring, providing pups with adequate inflammatory responses (Gomez de Agüero et al 2016). During weaning, signals from commensal microbes trigger an increase in colonic T-regulatory cells (Al Nabhani et al 2019) as well as a transient up-regulation of STAT3 in innate lymphoid cells and epithelial cells (Mao et al 2018), which can impact host metabolism and shape susceptibility to inflammatory disease.

Additionally, the intestinal microbiota shift triggered by the dietary change that accompanies weaning has been shown to trigger a ‘weaning reaction’ that is required for appropriate T regulatory imprinting, which if perturbed contributes to increased susceptibility to inflammatory disease later in life (Al Nabhani et al 2019). Furthermore, in early life, high microbial diversity is required for long-term modulation of IgE levels, which is crucial for preventing oral-induced systemic anaphylaxis (Cahenzli et al 2013). Furthermore, early colonization with intestinal commensal *Bacteroides fragilis* suppresses infiltration of natural killer T cells, which protects against the effects of colitis in adulthood (An et al 2014, Olszak et al 2012).

5.4. Stress And Early Life

During early life, the brain undergoes significant remodelling, not only prenatally, but also after birth, as crucial neurodevelopmental processes are still ongoing, posing a critical stage during which, the brain is particularly vulnerable to environmental influences, such as stress (Chen & Baram 2016). In fact, childhood adversity has been shown to have long-lasting and pervasive consequences for development (Smith & Pollak 2020). Early life chronic activation of the stress response systems – HPA axis, immune and autonomic nervous systems – has been proposed to change neural plasticity, resulting in cognitive deficits, depressive and anxiety-like behaviours and poorer health outcomes (Ganzel et al 2010, McEwen 2017). Further, early life stress has been associated with increased risk of anxiety in late adulthood (Lähdepuro et al 2019).

5.4.1. Microbiome-Stress in Early Life

Early observations from our group showed that maternal separation is associated with increased visceral pain hypersensitivity, increased anxiety-like behaviour and altered gut bacterial diversity in adulthood (O'Mahony et al 2009). The adverse impact of maternal separation on corticosterone levels and learned fear behaviours can be alleviated by probiotic administration (Cowan & Richardson 2019). Additionally, prenatal stress exposure has been shown to alter gut microbial composition in mothers and in the adult offspring, both in females (Gur et al 2017) and males (Gur et al 2019). Likewise, in humans, maternal stress impacts the vaginal (Jašarević et al 2015) and faecal offspring microbiota in a age and sex-specific manner (Jašarević et al 2017), hence establishing a direct link by which maternal stress can contribute to neurodevelopmental disorder predisposition. Additionally, maternal adverse childhood experiences are associated to altered gut microbiota during pregnancy, which are linked to altered glucocorticoid and inflammatory response to stress, which can be protected by dietary intervention with ω-3 polyunsaturated fatty acids (PUFAs) (Hantsoo et al 2019a). Taken together, this evidence implies that early life adversity can impact the microbiome, and consequently influence health outcomes in the offspring.

6. Adulthood

As previously described, early life events can impact the development and clinical manifestations of neurological and psychiatric disorders in adulthood (Han et al 2021b, Hantsoo et al 2019b).

6.1. Neuropsychiatry/ Neurological Disorders

Neuroinflammation has been increasingly recognized as a likely key contributor to the neurobiological processes involved in prevalent psychiatric disorders. A large-scale study showed that inflammation-related single nucleotide polymorphisms (SNPs) were associated with hippocampal and medial prefrontal cortex gray matter volumes, predicting the risk for development of depressive and positive psychotic symptoms in late adolescence, while this genetic signature of genes related to immunity was also present in a maternal separation model (Penninck et al 2021), highlighting the importance of neuroinflammation in the predisposition to psychiatric disorders.

6.2. Stress and Immunology

In animals exposed to social defeat stress there is an increase in neutrophils and macrophages (Hodes et al 2014, Wohleb et al 2015). Dampening of peripheral monocytes resulted in the amelioration of the depressive-like effects caused by chronic exposure to stress (Zheng et al 2016b). Moreover, deletion of pro-inflammatory cytokines such as IL-6 (Chourbaji et al 2006) and TNF-α (Simen et al 2006) resulted in the reduction of depressive-like behaviour in mice.

Glucocorticoid receptors are widely expressed on microglia throughout the brain (Sierra et al 2008), and integration of stress-induced signalling is mediated in the CNS by neuroinflammatory signalling through modulated microglia (Wohleb et al 2014). In response to stress, microglia undergo dynamic alterations in morphology and function within corticolimbic regions implicated in depressive-like symptoms. Under stressful conditions, neuroendocrine pathways fine-tune central and peripheral immune responses, resulting in monocyte trafficking and priming, subsequent changes in microglia phenotype, and ultimately culminating in neuroinflammation, which in turn can contribute to the development of several stress-related disorders, including depression (Hodes et al 2015). Exposure to cytokines released upon microglia activation has been linked to the development of aberrant behavioural phenotypes in

stress models. As a result, cumulative evidence reveals a clear link between the release of cytokines and the activity of the HPA axis. Further, exposure to social stress through a resident intruder model in rats, induces changes in the receptors of myeloid cells in the spleen, promoting persistent inflammatory alterations (Rajalingam et al 2020).

A study showed that immunization with *Mycobacterium vaccae,* a bacterium with immunoregulatory and anti-inflammatory properties, resulted in an improvement of anxiety-like behaviour caused by exposure to stress, by attenuation of microglial priming of the pro-inflammatory response (Frank et al 2018). An immunological challenge can also stimulate HPA axis activity by translocation of immunomodulators through the choroid plexus (Kunis et al 2013) and the blood-brain barrier (BBB) (Capuron & Miller 2011, Turrin & Rivest 2004). In fact, under physiological conditions, lymphocytes are present in the brain parenchyma in low numbers, accumulating mainly in the choroid plexus, meningeal spaces and the cerebrospinal fluid (Croese et al 2021).

Curiously, evidence suggests that T cells accumulate in these areas in response to signals triggered by the CNS, namely, in the meninges upon performance of cognitive tasks (Derecki et al 2010) or strikingly, in the choroid plexus after stress exposure (Lewitus et al 2008) (Figure 8).

Adaptive immunity involves the phenomenon of immunological memory whereby a particular lymphocyte (T cell or B cell) specifically recognizes unique determinants (antigens) to mount a more effective response on second and subsequent encounters with a pathogen. It is thus a highly specific defence mechanism for the body to respond to a particular pathogen. Studies in animal models have shown that glucocorticoid and stress exposure modulate T and B cell responses (Clark et al 2019, Roque et al 2011).

At a mechanistic level, increasing evidence points to a role of the adaptive immune system in gating stress responses. Studies in lymphopenic mice, that lack a functional adaptive immune system, have shown that the transfer of lymphocytes from stressed to stress-naïve animals led to the improvement of social behaviour, reduced anxiety-like behaviour and increased proliferation of hippocampal cells (Brachman et al 2015). Adoptive transfer of lymphocytes not only improved the deleterious effects of stress in animals, but also that these cells engraft the choroid plexus and the meninges of

the recipient mice exposed to stress (Scheinert et al 2016). Furthermore, a recent study reported that even though CD4+ T cells seem to not be directly involved in the response to stress, CD8+ cytotoxic T cells respond to stress not only by modulating the corticosterone and behaviour response, but also by inducing the production of pro-inflammatory cytokines possibly by the induction of monocytes and macrophages (Clark et al 2019). Additionally, it has been suggested that immunological memory to self-antigens, in a process dependent on memory T cells, might contribute to the development of stress coping mechanisms (Lewitus et al 2009).

Accordingly, with the nature and intensity of the stressful insult, an increase in immunomodulators in the circulation may result in the indirect trigger of neuroinflammation by the release of cytokines from endothelial cells of peripheral capillaries at the blood brain barrier (Spranger et al 2006), or alternatively through vagal nerve stimulation in response to peripheral inflammatory mediators (Eskandari et al 2003).

6.3. Microbiota, The Stress Response and Depression

Considering the independent synergistic effects of stress response and the immune system, and the continuous communication between the immune system and the gut microbiome, it is feasible to speculate that gut microbial signals can also impact the stress response and therefore, mental health. However, mechanistic longitudinal clinical studies investigating the role of the gut microbiome on the host mental health are lacking. There is evidence for an altered microbiota composition in depressed individuals (Huang et al 2018, Kelly et al 2016, Naseribafrouei et al 2014, Zheng et al 2016a), with the abundance of *Faecalibacterium* negatively correlated with symptom severity (Jiang et al 2015). An observational study reported that challenging the microbiota with single and recurrent antibiotic treatment augmented the risk of depression and anxiety (Lurie et al 2015). Furthermore, a large population study showed that Coprococcus and Dialister strains were not only predictors of a better quality of life, but were also consistently depleted in untreated depressed patients, while Butyricicoccus was associated with antidepressant treatment (Valles-Colomer et al 2019). Interestingly, studies demonstrated that faecal matter transplantation from MDD patients affected depressive behaviour in recipient animals (Kelly et al 2016, Zheng et al 2016a), thus suggesting further involvement of the gut microbiome in depression as well as the possibility of transdiagnostic signals (McGuinness et al 2022, Nikolova et al 2021).

6.3.1. Microbiome Manipulations in Preclinical Models of Stress & Depression

Many different stress paradigms including chronic social defeat, restraint stress, maternal separation, crowding, heat stress, and acoustic stress can impact the composition of the microbes in the gut as determined in preclinical models (Bailey et al 2011, Bharwani et al 2016, De Palma et al 2015, De Palma et al 2014, O'Mahony et al 2009). Consequently, multiple studies have focused on understanding the role of the gut microbiota in exposure to stress after positive or negative manipulation of the gut microbiota with antibiotic administration, prebiotic and probiotic intervention, faecal transplantation, and in response to stress in specific pathogen free and germ-free animals (Table 1).

Preclinical studies have demonstrated that germ-free animals – which were raised in a sterile environment from birth, and therefore lack gastrointestinal bacteria – show behavioural and phenotypical outcomes that can be reversed by colonization with bacteria and have increased sensitivity to acute stress (Clarke et al 2013, Crumeyrolle-Arias et al 2014a, Sudo 2012). Another approach to dissect the involvement of gut microbiota on stress physiology is to ablate stable core bacterial populations by exposure to antibiotics. Increasing studies show the modulation of behaviour after antibiotic treatment, suggesting that particular populations of gut bacteria could positively or negatively influence neurobehavioural outcomes (Desbonnet et al 2015a, Hao et al 2013, Hoban et al 2016a, Majidi et al 2016, O'Mahony et al 2014, Wang et al 2017).

However, further preclinical studies that examine the results of antibiotic administration on alterations in neuroendocrine levels in response to stress exposure are still lacking. Given that gut bacteria might influence stress response and emotional behaviour, multiple studies have explored the effect of prebiotic and probiotic administration in stress models.
In fact, both prebiotic and probiotic interventions have been shown to protect against the deleterious effects of stress, possibly by modulating the immune system while improving behaviour (Ait-Belgnaoui et al 2012, Bravo et al 2011, Desbonnet et al 2010, Palomar et al 2014, Savignac et al 2014) (see Table 1).

Moreover, in a chronic psychosocial stress paradigm, transplantation of faeces from non-stressed animals partially attenuated some of the deleterious effects of stress

and, likewise, faecal transplantation from stressed to naïve mice was sufficient to induce stress effects on recipient mice (Langgartner et al 2018). Further, faecal transplantation from stressed mice to naïve recipients led to anxiety-like behaviour and to the accumulation of monocytes and activated microglia in the hippocampus, while treatment with beneficial microbes from unstressed animals improves anxiety-like behaviour by amelioration of inflammation in the gut (Jang et al 2018). These studies are in line with earlier studies from Bercik and colleagues which showed that the phenotype of stress sensitive strain of mice could be transferred via the microbiome to a strain which had a normal stress and vice versa (Bercik et al 2011a). Additionally, juvenile social defeat exposure promotes later onset of irritable bowel syndrome-like symptoms, while altering noradrenaline-related metabolites in the amygdala (Matsumoto et al 2021).

Taken together, a growing body of evidence suggests that the gut microbiome is undoubtedly involved in the mechanisms underlying stress response and shaping the gut microbial communities could contribute to shed some light into the development of better and more targeted stress signalling manipulations.

Table 1: Preclinical studies of microbiota manipulations relevant to Microbiota-Immune-Stress responses

Microbiome Manipulation	Details	Species	Time/Critical Window	Stress Exposure	Physiological/ Behavioural Outcome	References
Germ-Free	Recolonization	Mice	Adulthood	Novel-Environment Stress	Hyperactivity of the HPA Axis ↑ Corticosterone ↑ ACTH ↓ Anxiety-Like Behaviour (Reversed by Colonization)	(Clarke et al 2013, Sudo 2012)
	NA	Rats	Adolescence/Adulthood	Open-Field	↑ HPA Axis Response ↑Anxiety-Like Behaviour	(Crumeyrolle-Arias et al 2014a)
	Recolonization	Mice	Adulthood	No	↓ Anxiety-Like Behaviour	(Nishino et al 2013)
Antibiotic Treatment	Ampicillin, Vancomycin, Ciprofloxacin HCL, Imipenem, Metronidazole; Pimaricin, Bacitracin, Neomycin	Rats	Adulthood/ Pre-Weaning	No	↑Visceral Sensitivity ↑ Depressive-Like Behaviour = Anxiety-Like Behaviour	(Hoban et al 2016a, O'Mahony et al 2014)
	Ampicillin, Vancomycin, Neomycin, Metronidazole	Mice	Adolescence/Weaning	Acute Restraint	↓ Anxiety-Like Behaviour Cognitive Impairments	(Desbonnet et al 2015a)
	Ampicillin, Cefoperazone	Mice	Adolescence/Weaning	No	↑ Depressive-Like Behaviour ↑ Anxiety-Like Behaviour	(Ceylani et al 2018)
	Azithromycin	Mice	Adulthood	LPS	↓ Depressive-Like Behaviour ↓ Pro-Inflammatory Cytokines	(Hao et al 2013)
	Dapsone	Mice	Aging	Surgical Stress	↓ Depressive-Like Behaviour ↓ Anxiety-Like Behaviour	(Zhang et al 2015)
	Minocycline	Mice	Adulthood/Adolescence/Weaning	LPS Early-Life Social Isolation	↓ Depressive-Like Behaviour	(Majidi et al 2016, Wang et al 2017)
	Minocycline	Mice	Adulthood	No	= Depressive-Like Behaviour = Anxiety-Like Behaviour	(Vogt et al 2016)
	Ampicillin, Gentamicin, Vancomycin, Imipenem	Mice	Adolescence/Pre-Post Weaning	No	↑Anxiety-Like Behaviour around weaning	(Lynch et al 2023)
Faecal Transplantation	Faeces from non-stressed to stressed animals; Faeces from stressed animals to non-stressed animals	Mice	Adulthood	Chronic Subordinate Colony Housing	↓ Anxiety-Like Behaviour ↓ Systemic Low-Grade Inflammation ↓ Thymus Atrophy	(Langgartner et al 2018)
	Faeces from animals exposed to stress or treated with *Escherichia coli*	Mice	Adulthood	Restraint Stress	↓ Anxiety-Like Behaviour	(Jang et al 2018)
	Faeces from young animals to aged animals	Mice	Adulthood/Aging	No	↑ Cognition in Aged Animals	(Boehme et al 2021)
	Faeces from aged animals to young animals	Mice	Adulthood/Aging	No	↓ Cognition	(Mossad et al 2021)

Probiotic Admnistration	Bifidobacterium infantis 35624	Rats	Early Life/Pre-Weaning	Maternal Separation	Immune Response Normalization ↓ Depressive-Like Behaviour	(Desbonnet et al 2010)
	Bifidobacterium animalis subsp lactis BB-12 Propionibacterium jensenii	Rats	Early Life/Pre-Weaning	Maternal Separation Restraint Stress	↑ Corticosterone ↑ ACTH ↑ IgA Improved Immune Phenotype	(Barouei et al 2012)
	Lactobacillus (L) casei CRL 431	Mice	Adulthood	Restraint Stress Food Deprivation	↑ IgA ↑ T Helper Cells ↓ IFN-γ	(Palomar et al 2014)
	Bifidobacterium longum 1714 Bifidobacterium breve 1205	Mice	Adulthood	No	↓ Depressive-Like Behaviour ↓ Anxiety-Like Behaviour	(Savignac et al 2014)
	Lactobacillus rhamnosus (JB-1)	Mice	Adulthood	No	↓ Depressive-Like Behaviour ↓ Anxiety-Like Behaviour ↓ Corticosterone	(Bravo et al 2011)
	Lactobacillus farciminis	Rats	Adulthood	Partial Restraint Stress	↓ HPA Axis Response Improved Immune Phenotype	(Ait-Belgnaoui et al 2012)
	Lactobacillus helveticus NS8	Rats	Adulthood	Chronic Restraint Stress	↓ HPA Axis Response ↓ Depressive-Like Behaviour ↓ Anxiety-Like Behaviour	(Liang et al 2015)
	Bifidobacterium pseudocatenulatum CECT 7765	Mice	Early Life/ Weaning	Maternal Separation	↓ Intestinal Inflammation ↓ Anxiety-Like Behaviour	(Moya-Perez et al 2017)
	Lactobacillus rhamnosus (R0011) Lactobacillus helveticus (R0052)	Mice	Adulthood	Water Avoidance	↑ Non-Spacial Memory ↓ Anxiety-Like Behaviour	(Smith et al 2014)
	Lactobacillus reuteri	Mice	Adulthood	Unpredictable chronic mild stress	↓ Escape Behaviour	(Marin et al 2017)
	Lactobacillus helveticus R0052, Lactobacillus plantarum R1012, Bifidobacterium longum R0175	Mice	Adulthood	Chronic Mild Stress	↓ Depressive-Like Behaviour ↓ Anxiety-Like Behaviour ↓ TNF-α and IFN-γ	(Li et al 2018b)
Prebiotic Admnistration	Fructo-oligosaccharides (FOS), Galacto-oligosaccharides (GOS)	Mice	Adulthood	Chronic Unpredictable Social Stress	↓ Corticosterone ↓ Pro-Inflammatory Cytokines ↓ Depressive-Like Behaviour ↓ Anxiety-Like Behaviour	(Burokas et al 2017)
	Galacto-oligosaccharides (GOS)	Mice	Adulthood	LPS	↓ Anxiety-Like Behaviour ↓ Cortical IL-1β	(Savignac et al 2016)
Prebiotic + Probiotic (Synbiotic) Admnistration	Polydextrose, Galacto-oligosaccharides (GOS), Lactobacillus rhamnosus GG	Rats	Early Life/ Adulthood	Maternal Separation	↓ Anxiety-Like Behaviour ↑ Spatial Learning	(McVey Neufeld et al 2017)

7. Aging

Socioeconomic advances have allowed for great improvements in healthcare services, along with improvements in quality of life have resulted in the increase of older people. WHO has reported that in 2020, the global population of people aged 60 years and above represents 13,5% of the world's population and projections show predictions that 1 out of 5 people will be 60 and above by 2050 (WHO 2020). Population aging therefore poses challenges in the context of healthy aging and adequate care to older individuals (WHO 2020).

Aging is a time-dependent physiological decline that results in progressive impaired function of biological systems and therefore increased susceptibility to death (López-Otín et al 2013). Throughout life, the body attempts to respond to diverse physiological challenges, constantly changing and adapting to reach homeostasis which results in an accumulation of biological burden, defined as allostatic load (McEwen 1998). In fact, as allostatic load is recognized as the accumulation of physiological responses to chronic stress that lead to the 'wear and tear' of the body (McEwen 2007) it has unsurprisingly been recognized as an important predictor of mortality (Robertson et al 2017). Brain aging has also been linked to higher risk of death, as at ~73 years of age, individuals with brains that appear biologically older than their chronological age presented greater risk of death as well as motor difficulties, lower cognitive function and higher allostatic load, which was independent of socioeconomic factors (Cole et al 2018).

To better understand aging, nine hallmarks that reflect the characteristic consequences of 'wear and tear' of the system have been proposed: telomere attrition, loss of proteostasis, epigenetic alterations, deregulated nutrient sensing, cellular senescence, mitochondrial dysfunction, stem cell exhaustion, genomic instability and altered intercellular communication (López-Otín et al 2013). A marked example of age-associated altered intercellular communication is 'inflammaging' – a marked low-grade proinflammatory phenotype that accompanies aging in mammals (Salminen et al 2012). Inflammaging may arise from a failure of a dysfunctional immune system to effectively eliminate pathogens and malfunctioning host cells, increased secretion of proinflammatory cytokines by senescent cells, build-up of pro-inflammatory tissue damage, increased activation of the NF-κB or an inadequate autophagy response (Salminen et al

2012). These processes lead to increased activation of the NLRP3 inflammasome among other inflammatory pathways that, in turn, increase the secretion of pro-inflammatory factors (López-Otín et al 2013).

7.1. Longevity

Longevity is invariably linked to healthy aging – it has now been shown that first degree relatives to longevous individuals live longer than direct family member of non-longevous individuals and that longevity is transmitted as a quantitative genetic trait across the top 10% of surviving individuals of their birth cohort and their families (van den Berg et al 2019). Interestingly, centenarians – individuals that reach 100 years of age – have shown to display a reduced inflammaging profile (Zhou et al 2022), better mental health status, higher cognitive functioning and lower incidence of dementia despite exposure to risk factors (Beker et al 2020, Jopp et al 2016, Shaffer 2021) - especially considering the highest risk factor for development of neurodegenerative disease is aging itself (Hou et al 2019). In addition to genetic predisposition, lifestyle factors, such as diet, have been associated with healthy longevity (Hao et al 2019, Rong et al 2019), thus reinforcing the importance of promoting healthy lifestyle interventions throughout life.

7.2. Neurodegeneration and Neuropsychiatry in Aging

Brain aging is the major risk factor for cognitive decline and the development of neurogenerative disorders (Bishop et al 2010). Age-related protein abnormalities are observed in the aging brain, such as accumulation of tau, amyloid-β or α-synuclein, which can contribute to neurodegenerative diseases (Wyss-Coray 2016). Furthermore, during the aging process, the accumulation of senescent cells which release pro-inflammatory cytokines augments chronic inflammation, inevitably resulting in tissue degeneration and contributing to the advancement of age-related diseases (Finger et al 2022). In fact, neuroinflammation has been implicated in age-associated neurodegenerative diseases such as Alzheimer's (Leng & Edison 2021) and Parkinson's (Tansey et al 2022).

7.3. Aging and Inflammation

7.3.1. Peripheral Inflammation in Aging

Aging is characterized by altered immune cell populations, such as increases in monocytes, CD4+ and CD8+ T cells, while other cell populations, such as dendritic cells, and B cells seem to be underrepresented/ decreased with age (Mogilenko et al 2021). Aging promotes low-grade systemic inflammation, represented by increases in cytokines such as IL1-β, IL-6, IFN-β, as well as chemokines and immunoregulatory factors that drive further pro-inflammatory states in immune cells (Mogilenko et al 2021, Scott et al 2017).

7.3.2. Neuroinflammation in Aging

Microglia have been shown to present marked morphological changes in aged human postmortem brain samples (Streit et al 2004). Recent studies show that with age, microglia build up lipid droplets that generate high levels of reactive oxygen species, show impaired phagocytosis and release proinflammatory cytokines (Marschallinger et al 2020), while also upregulating genes related to cytokine pathways (Pan et al 2020). In a mouse model of Alzheimer's disease, the microglial inflammatory response was aggravated by disruption of meningeal lymphatic drainage and promotion of this function could lead to improved efficacy of therapeutic targets (Da Mesquita et al 2021). In aged mice and young animal model of Alzheimer's disease a specific subset of reactive microglia is enriched in the brains of these animals (Mrdjen et al 2018). Activated microglia also increase the secretion of inflammatory cytokines which induce reactive astrocytes - which constitute a pathological CNS response in an attempt to mediate neuroinflammation - in aging and in the progression of Alzheimer's disease (Liddelow et al 2017, Pan et al 2020).

Astrocytes are non-neuronal glial cells that play a key role in modulating neurotransmission and synaptic connectivity and function that present heterogenous populations throughout brain regions across lifespan, particularly up-regulating genes of immune pathways in the aging brain (Boisvert et al 2018). In aging and Alzheimer's disease animal model, disease-specific state astrocytes were identified with a pro-inflammatory profile (Habib et al 2020).

Given that aging is characterized by altered immune cell populations, such as increases in monocytes, CD4+ and CD8+ T cells, while other cell populations such as dendritic cells and B cells seem to be underrepresented/ decreased with age (Mogilenko et al 2021), it is feasible to hypothesize that age-related brain vascular alterations makes the CNS more prone to the infiltration of peripheral immune cells (Finger et al 2022). In fact, clonally expanded CD8+ T cells have been shown to be trafficked to the cerebrospinal fluid, infiltrating the hippocampus of Alzheimer's disease patients, thus implying a role of adaptive immune cells in neurodegeneration (Gate et al 2020).

7.4. Stress in Aging and Psychiatric Risk

Late-life-depression is comorbid with other age-related medical conditions and is subject to socioeconomical and environmental factors (Prenderville et al 2015). Additionally, late-life depression is suggested to symptomatically differ from depressive manifestations in younger populations, and being generally characterized by more somatic symptoms, however evidence is still lacking to conclude that depression is indeed differently manifested in older populations (Haigh et al 2018). Further, even though psychological interventions seem to be as effective as in younger adults, antidepressant treatment is less effective in late-life depression and that late-life depression is associated with higher rate of relapse (Haigh et al 2018).

As a key feature of aging is reproductive senescence, it is no surprise that the shift in gonodal hormones with age would impact the HPA axis and stress neural responses (Bale & Epperson 2015). Further, a meta-analysis revealed that older age is associated with higher cortisol response to psychological and pharmacological challenges and that this age-dependent cortisol effect is more pronounced in women (Otte et al 2005).
In older adults, anxiety is also highly prevalent and relatively poorly understood in this particular age group, as the diagnostic criteria may not be well adjusted for this population (Bryant et al 2008, Wolitzky-Taylor et al 2010).

Acute stress in healthy older adults increases reactivity in the amygdala, suggesting a mechanism for increased vulnerability for affective disorders in aging (Everaerd et al 2017). In aged rats, chronic restraint stress did not induce dendritic

spine remodelling in the prefrontal cortex (PFC), thus suggesting age-dependent loss of dendritic spine plasticity in the aged brain (Bloss et al 2011).

In humans, meta-analyses show that social networks tend to reduce with age and that novelty seeking, particularly relating to generating new social relationships is reduced with age (Jopp et al 2016, Prenderville et al 2015). Even though social behaviour has also been shown to be decreased in aged mice (Scott et al 2017), this behavioural facet is still relatively unexplored in the context of aging (Prenderville et al 2015).

7.5. Aging Microbiome

In adulthood, gut microbiota composition is commonly stable unless perturbed, and it gradually deteriorates with age (Claesson et al 2011, Cryan et al 2019b). Lifestyle factors such as exercise and diet, which tend to reduce with age seem to have a greater impact in improved health outcomes in aged individuals (Vauzour et al 2017). While age-dependent gut microbial signatures have been identified, conveying great enthusiasm to limit age-associated disease, perhaps it will be important to focus on healthy aging approaches more than reverting to 'younger' microbial phenotypes, as the age-dependent gut microbial alterations may represent an important physiological adaptation to aging (Wilmanski et al 2022).

Most microbiome studies focused on the elder population generally focus on the a) age-dependent gut microbiome composition and b) microbiome alterations associated with age-dependent disorders (Ghosh et al 2022a). Gut microbiome signatures that are linked both with aging *per se* and unhealthy aging are defined by loss of species of the core microbiome structure and synergistic increase of specific disease-associated taxa (Ghosh et al 2022b).

Extreme aging microbiome signatures have been identified in centenarians across different studies, despite differences in geography and level of urbanization (Ghosh et al 2022a). With age, the reduction in relative abundance of dominant commensal taxa (such as *Bifidobacterium*, *Faecalibacterium*, *Prevotella*, *Eubacterium rectale*, *Coprococcus*, and *Lachnospira*) (Claesson et al 2012, Ghosh et al 2020a, Jeffery et al 2016) is accompanied by an expansion of

another group of commensals (as *Akkermansia*, *Butyricimonas*, Christensenellaceae, *Butyricicoccus* and *Odoribacter*) along with pathobionts (such as Enterobacteriaceae, *Streptococcus, Bilophila*, *Eggerthella*, and Fusobacteria) (Ghosh et al 2020a, Jeffery et al 2016).

Studies targeting disease-related gut microbiome shifts associated with unhealthy aging trajectories have reported increases in pathobionts – opportunistic pathogenic communities - (such as *Clostridium* species, *Desulfovibrio, Eggerthella* and Enterobacteriaceae family members (Ghosh et al 2022a). Contrastingly, commensal microbial profiles (such as *Akkermansia, Butyrivibrio, Butyricimonas, Oscillospira, Odoribacter,* Barnesiellaceae and Christensenellaceae) are increased and associated with healthy aging trajectories and a reduced incidence of age-related disorders (Ghosh et al 2022a, Odamaki et al 2016, Xu et al 2019).

Interestingly, a recent study showed that while showing diverging time-dependent changes with age, gut microbiome composition from healthy individuals are not only associated with increased circulation of specific microbial metabolites in the plasma, but also predict extended survival later in life reflecting healthy aging (Wilmanski et al 2021).In a rodent model of progeria (accelerated aging), the gut microbiome diverges from that observed in adult wild type mice, presenting among others, an increment of *Parabacteroides*, *Prevotella* and Enterobacteriaceae, while displaying loss of *Akkermansia* and *Dehalobacterium* (Bárcena et al 2019). Transplantation of *Akkermansia muciniphila* alone was sufficient to extend lifespan and prevent intestinal age-associated manifestations (Bárcena et al 2019), thus suggesting that reestablishment of a healthy gut microbiome can result in beneficial health outcomes.

7.5.1. Diet and Aging

Dietary interventions have been widely proposed to be a crucial mediator of inflammation in aging. In particular, the Mediterranean diet has been suggested to be associated with increased longevity and reduced age-associated disease risk by positively modulating the hallmarks of aging (Shannon et al 2021), while being associated with lower risk of cognitive impairments (Keenan et al 2020).

Furthermore, dietary interventions can be effective mediators of autophagy, a central regulator of aging (Aman et al 2021). Autophagy is a conserved catabolic pathway that degrades cellular components through lysosomes (Aman et al 2021, Hansen et al 2018). Caloric restriction is described to activate NAD- dependent protein deacetylase sirtuin 1 (SIRT1), while downregulating insulin and insulin-like signalling, the glucose signalling Ras-protein kinase A (PKA) pathway and the amino signalling target of rapamycin (TOR)-S6 kinase pathway that are key autophagy promoters while mitigating pro-inflammatory pathways (Franceschi et al 2018). Interestingly, dietary restriction in *C. elegans* delays the age-related intestinal barrier dysfunction (Gelino et al 2016), suggesting that intestinal autophagy processes may contribute to longevity (Hansen et al 2018). Spermidine, a dietary polyamine that decreases with age, when used as a dietary supplement in Drosophila melanogaster inhibited age-induced memory impairments in an autophagy-dependent manner (Eisenberg et al 2009). Autophagy is a key regulator of the NLRP3 inflammasome, a major innate immune pro-inflammatory pathway suggesting a role in the mediation of inflammation, and consequently in aging and associated neurodegenerative diseases (Aman et al 2021). A recent study has shown that short-term rapamycin treatment results in a long-lasting protective effect, inducing prolonged intestinal autophagy activation, reduction of age-related gut pathology and extension of lifespan in *Drosophila* (Juricic et al 2022).

Taken together, aging induces a pervasive intestinal and neuroinflammatory environment characterized by inflammaging. Interestingly, the potential use of dietary interventions such as polyamine supplementations and caloric and dietary restrictions may promote autophagy which has been shown to be a highly effective longevity modulator suggesting a role for the gut-brain axis in age-dependent physiological mechanisms.

7.6. Microbiome-Immune Interactions in Aging

Over the aging process, the gut undergoes remodelling, resulting compromised barrier function and in the decline of genes associated with innate and adaptive immunity including IgA, TLR4, T cell (CD3ε) and T-helper (CD4 or CD8), suggesting an overall reduction of T cell signalling pathways in the aged gut – this decline can contribute to a chronic low grade inflammation state (Sovran et al

2019). With age, there is a decline in the abundance of *Akkermansia muciniphila*, that elicits gut leakiness and consequent leak of pro-inflammatory factors that trigger a cascade involving CCR2+ monocytes that in turn interfere with B cells (Bodogai et al 2018).

Faecal microbiota transplantation from an aged donor is sufficient to induce intestinal and systemic low-grade inflammation, through increased T cell activation (Fransen et al 2017, Parker et al 2022). Conversely, transfer of microbiome from a young donor into aged mice resulted in the enhancement of M-cell maturation in Peyer's patches and elevated intestinal IgA responses (Donaldson et al 2020). Given its interface with the immune system, the gut microbiome is an important candidate in the modulation of inflammaging, as inflammatory markers have been linked to the intestinal microbiota profiles of elders in a diet-dependent manner, along with mental health markers (Claesson et al 2012).

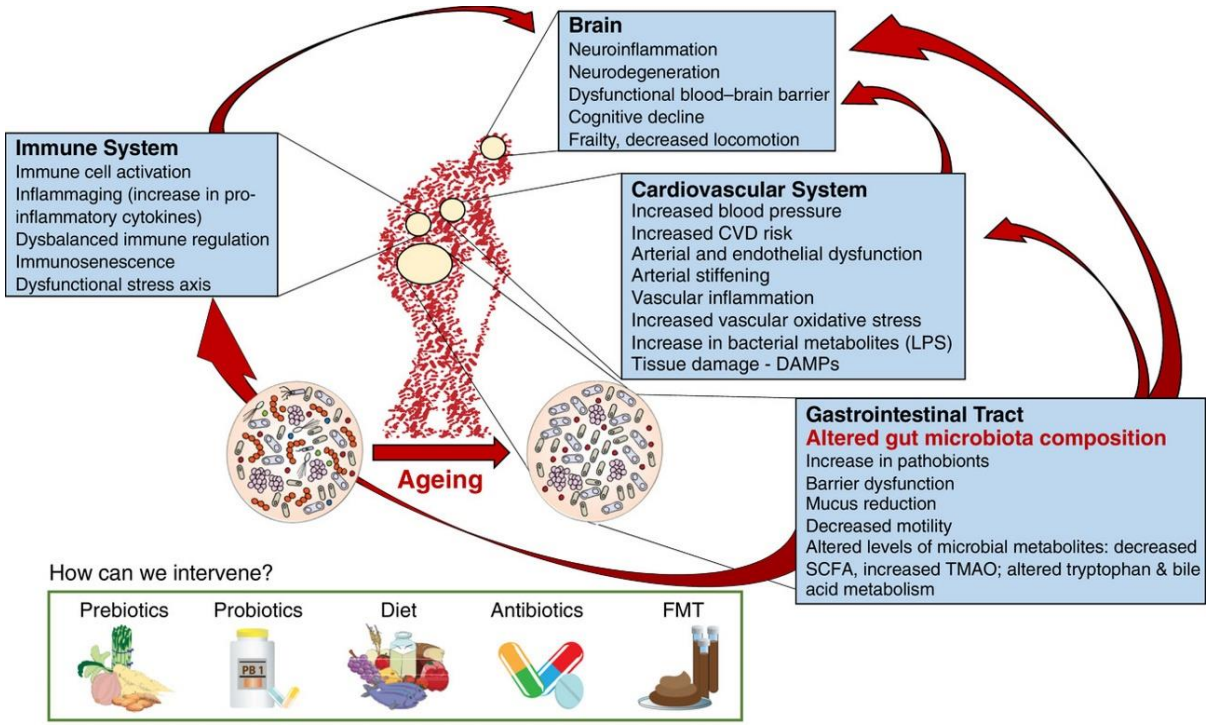

Figure 11 – Gut microbial composition in aging can influence age-related changes in gastrointestinal, immune, cardiovascular and brain health. *The microbial signaling from the aged gut microbiome can input inflammatory signals that impact systemically the health of the host. Therapeutic targeting of the microbiome can potentially provide future interventional avenues. Abbreviations: LPS, lipopolysaccharide; DAMPs, damage-associated molecular patterns; SCFA, short-chain fatty acid; TMAO, trimethylamine N-oxide; FMT, faecal microbiota transplant. Adapted from (Cryan et al 2019a).*

7.7. Aging, Microbiome and Neuroinflammation

Interestingly, the gut microbiome has emerged as a potential modulator of neuroinflammation in the context of neurodegenerative disorders such as Parkinson's and Alzheimer's, thus suggesting that targeting the gut microbiome could be an interesting therapeutic target (Bairamian et al 2022, Romano et al 2021) (Figure 11).

In aged mice and a young animal model of Alzheimer's disease a specific subset of reactive microglia is enriched in the brains of these animals (Mrdjen et al 2018). In another neurodegenerative model, it has been described that Parkinson's-derived gut microbiome is required for the manifestation of α-synuclein-induced motor deficits and microglia activation and consequent neuroinflammation, as

germ-free and antibiotic-treated mice show limited motor pathophysiology upon overexpression of α-synuclein (Sampson et al 2016). Aged microbiome also impacts microglial activation in the CNS as well as retinal inflammation, which are reversed by faecal matter transplantation from young donors by reversing intestinal permeability and systemic inflammation induced by age (Parker et al 2022).

Age-related gut microbiome composition alterations and increased intestinal permeability in mice correlate with increased circulating pro-inflammatory cytokines, which may contribute to anxiety-like behaviours and cognitive impairments (Scott et al 2017). Transplantation of faeces from aged mice induces inflammaging in young germ-free mice, as represented by increased circulating levels of pro-inflammatory cytokines such as TNF-α and increased peripheral T cell activation (Fransen et al 2017, Thevaranjan et al 2017). Faecal matter transplantation from young donor mice reshapes microbiome composition in aged mice, while positively influencing behaviour (Boehme et al 2021, Mossad et al 2021).

7.8. Aging and Microbial Metabolites

Considering the growing evidence in the dynamic involvement of the gut-brain axis in aging processes, a number of microbial-derived metabolites have been proposed to be associated with the development of neurodegenerative disorders and cognitive impairments (Connell et al 2022). Deoxycholic acid, a secondary bile acid produced by gut bacteria was found increased in the serum of AD patients, which was associated with cognitive decline (MahmoudianDehkordi et al 2019).

Trimethylamine-N-oxide (TMAO), a gut-derived microbial metabolite has been associated with brain aging and cognitive impairment (Li et al 2018a), as well as Alzheimer's disease (Vogt et al 2016); however, a mendelian randomization study found no causality for higher genetic predisposition to Alzheimer's disease and TMAO or its predecessors (Zhuang et al 2021). While the relevance of TMAO in cognitive decline is still under exploration, evidence suggests that reducing the levels of TMAO by using a pharmacological inhibitor (Gao et al 2019) or probiotic

augmentation with *L. plantarum* (Wang et al 2020) can alleviate cognitive impairment in an Alzheimer's disease mice model.
The gut microbiome largely influences age-related microglial function – more specifically, N^6-carboxymethyllysine (CML), a gut derived metabolite accumulates in the microglia of aged mice and human brains due to increased age-associated intestinal permeability, resulting in increased reactive oxygen species and disrupts mitochondrial activity in microglia (Mossad et al 2022).

Interestingly, δ-valerobetaine, a gut microbiota-derived metabolite, was altered in the serum and brain of the recipient mice in an age-dependent fashion of the FMT donor. Further, an age-associated increase of δ-valerobetaine is observed in both mice and humans, and injections of this metabolite worsen the cognitive performance of young mice while reversing the beneficial cognitive effects of faecal transplantation from young donors in aged mice, in an effect not mediated by microglia (Mossad et al 2021).

A recent study showed that intermittent fasting promotes protective effects in an Alzheimer's disease model, through enrichment of sarcosine and dimethylglycine, which are metabolites that have been previously shown to improve cognitive decline and that by modulating glial cell activity, suppress neuroinflammation (Pan et al 2022).

Isoamylamine, a gut bacterial metabolite produced by *Ruminococcaceae*, is enriched in aged mice and older humans, was shown to cause cognitive decline when administered to young mice (Teng et al 2022b). Additionally, *Ruminococcaceae* phages are reduced in aging, and their absence leads to the secretion of bacterial isoamylamine; and inversely, phage treatment is sufficient to ameliorate isoamylamine levels, which in turn contributes to boosting microglial apoptosis. Further, phage treatment also alleviates the age-dependent cognitive decline, whereas isoamylamine worsens spatial memory and learning in young mice (Teng et al 2022b).

Taken together, there is increasing evidence suggesting the presence of an age-associated gut microbial metabolite signature, that can potentially provide further insights into the impact of the gut microbiome in aging, and therefore present itself

as a potential therapeutical mitigation strategy to limit the impact of host aging (Wilmanski et al 2022).

8. Hypothesis, Aims and Objectives

The gut microbiome has arisen as a key health marker, having spanning roles in the communication with the immune system and the brain (Cryan et al 2019b). Given the continuous interchangeable signalling with the host and its intricate malleable communication that adapts throughout the lifespan of the host (Ghosh et al 2022a, Martínez et al 2018), it is imperative to understand how such communication occurs. Particularly, it is at the extremes of life – early life and aging – when the gut microbiome is predicted to exert more influence in the host health (Cowan & Cryan 2021). Considering the continuous signalling exchange between the gut microbiome and the immune system (Hooper et al 2012), and that gut microbial signals can effectively regulate CNS processes (Sanmarco et al 2021), it is necessary to explore this connection across different sensitive timepoints, and its consequent impact on behaviour. In this thesis, we aim to understand the impact of the gut microbiome in immune and behavioural outputs on the host across the lifespan.

Aim 1: What is the impact of a manipulation of the gut microbiome in social behaviour in young and aged mice? – Chapter 2 – "Age-Associated Deficits in Social Behaviour are Microbiota Dependent"

Age is increasingly linked to remodelling of the peripheral and CNS immune system along with the gut microbiome (Baruch et al 2014, Ghosh et al 2022a). Previous studies in our group have suggested that gut microbial signals in aging are linked to neuroimmunity parameters (Boehme et al 2020), which follow age-associated behavioural deficits (Boehme et al 2021). Thus, we sought to understand the impact of gut microbial signals by characterizing the effect of gut microbial depletion social behaviour, neuroimmune and caecal metabolite profiles, in young and aged mice on social behaviour, neuroimmune and caecal metabolite profiles.

Aim 2: Does prebiotic supplementation modulate stress response in aged mice? – Chapter 3 – Prebiotic Supplementation Modulates Selective Effects of Stress on Behaviour and Brain Metabolome in Aged Mice

Stress response in aging still remains relatively underexplored, despite its immense relevance for mental health in the aged population (Rimmele et al 2022). An important behavioural dimension, social behaviour, has been previously shown by our group to negatively impacted by aging (Scott et al 2017). Taken into consideration that the aged gut microbiota has also been associated with alterations in inflammatory markers (Boehme et al 2020, Scott et al 2017), and that this can be modulated by prebiotic administration (Arnold et al 2021), in this study, we evaluated the potential of a prebiotic FOS-Inulin dietary intervention in ameliorating age-related responses to stress.

Aim 3: Does prebiotic supplementation in adulthood alter behavioural and immune outputs in offspring exposed to maternal antibiotic depletion – Chapter 4 – Early-life Microbiota Depletion and Gut-Brain Axis Function: An Investigation into the Later-Life effects of FOS-Inulin Supplementation

Early life represents a sensitive period for gut microbial establishment (Martínez et al 2018) as well as for crucial neurodevelopmental processes (Stiles & Jernigan 2010), often in a sex-dependent fashion (Lynch et al 2022a). In this study, we aimed to understand if gut microbial depletion via antibiotic cocktail from mid-gestation through weaning would result in long-term deleterious behavioural and immune outcomes in male and female offspring, and if prebiotic supplementation in later life could alter such outcomes.

Chapter 2

Age-Associated Deficits in Social Behaviour are Microbiota Dependent

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Abstract

Aging is associated with remodelling of immune and central nervous system responses resulting in behavioural impairments including social deficits. Growing evidence suggests that the gut microbiome is also impacted by aging, and we propose that strategies to reshape the aged gut microbiome may ameliorate some age-related effects on host physiology. Thus, we assessed the impact of gut microbiota depletion, using an antibiotic cocktail, on aging and its impact on social behaviour and the immune system.

Indeed, microbiota depletion in aged mice eliminated the age-dependent deficits in social recognition. We further demonstrate that although age and gut microbiota depletion differently shape the peripheral immune response, aging induces an accumulation of T cells in the choroid plexus, that is partially blunted following microbiota depletion. Moreover, an untargeted metabolomic analysis revealed age-dependent alterations of caecal metabolites that are reshaped by gut microbiota depletion. Together, our results suggest that the aged gut microbiota can be specifically targeted to affect social deficits. These studies propel the need for future investigations of other non-antibiotic microbiota targeted interventions on age-related social deficits both in animal models and humans.

Introduction

Aging is an intricate process that entails complex and dynamic remodelling of different physiological systems and distinct behavioural changes. Alterations in the gut microbiome and the immune system have all been associated with aging (Cruz-Pereira et al 2022, Ghosh et al 2022a, Scott et al 2017, Wyss-Coray 2016). Moreover, aging is intrinsically linked to increased susceptibility to neurological and cognitive impairments (Wyss-Coray 2016) as well as deficits in social cognition (Moran et al 2012, Roheger et al 2022). Indeed, cognitive decline has profound impacts on social functioning (Arioli et al 2018). There has been renewed interest in linking microbiota and immune alterations with social cognition across the lifespan (Filiano et al 2016, Ratsika et al 2023, Sherwin et al 2019).

The choroid plexus - an epithelial monolayer located in each ventricle in the brain that acts as a key neuroimmunological hub (Baruch et al 2013) - represents one of the very few sites where T cells are found in the CNS (Engelhardt & Ransohoff 2005). In aged mice the choroid plexus is populated with CNS-antigen specific CD4+ T cells that promote T-helper type 2 (Th2) responses, which promote cognitive dysfunction (Baruch et al 2013). Additionally, with age the choroid plexus presents a type I interferon (IFN-I)-dependent gene profile, and in turn blockade of this signalling pathway results in amelioration of cognitive function (Baruch et al 2014).

Whether there is a relationship among gut microbiota, social behaviour and the immune system remains relatively unknown in the context of aging. Thus, we sought to understand the impact of gut microbiota depletion via antibiotic administration on social behaviour and immune readouts in aged mice, while identifying age-related metabolites that could be underlying the changes observed.

Materials and Methods

Animals

Male young C57/BL6 mice (n=20; 9-10 weeks old; Charles River, Kent, UK) and aged C57/BL6 (n=20; 18 months old; Charles River, Kent, UK) were used in this study. All experiments were conducted in accordance with European Directive 86/609/EEC, Recommendation 2007/526/65/EC, and approved by the Animal Experimentation Ethics Committee of University College Cork AE19130/P087. Animals were kept under a 12-h light/dark cycle, with a temperature of 21 ± 1 °C and humidity of 55 ± 10%. Food and water were given *ad libitum.*

Antibiotic Treatment

To deplete gut microbiota, mice were treated for 10 consecutive days with an antibiotic cocktail (ampicillin (1g/L), neomycin (0.5g/L) and vancomycin (0.35g/L)) or water (as previously described (Boscaini et al 2021)). Antibiotics were dissolved in water and changed every second day. Control animals received just water, which was also replaced every second day.

Behaviour

On day 9 post initiation of antibiotic treatment, social cognition was evaluated using the 3-chamber social interaction test, in which time spent interacting with a novel conspecific is examined against time spent with a novel object or familiar conspecific. It is based on the premise that mice are attracted to novelty, hence would prefer a novel conspecific to a familiar one (Moy et al 2004).

The test arena consisted of 3 chambers; the left and right chambers measured 13.5 x 20 x 20 cm and the centre chamber was 9 x 20 x 20 cm. A solid partition divided the chambers, with a small hole allowing access to the other chambers. During the habituation phase, the mouse was placed into the centre chamber and then allowed access to the empty left and right chambers for 10 min. For the social novelty trial, an aged-matched novel conspecific mouse was placed in a mesh cage, on the opposite chamber to a familiar conspecific mouse. Placement of the novel mouse was randomised between animals to eliminate side preferences. The 3-chamber apparatus was cleaned with 70% ethanol and allowed to

evaporate between animals. The time spent in each chamber was then measured. The animals were habituated to the room for 45 min before the test, and the test was conducted under dim light (60 lux). The time spent in each chamber was then scored using the Behavioural Observation Research Interactive Software (BORIS) (Friard & Gamba 2016).

Tissue Collection

Animals were killed by anaesthetic overdose with sodium pentobarbital and transcardially perfused in a random fashion regarding testing groups between 09.00 h and 15.00 h. The caecum and ileum were quickly removed before the transcardial perfusion, weighed, snap-frozen on dry ice and stored at −80°C. The brain was excised and kept in PFA for 24h, and then dehydrated by a sucrose gradient before being snap frozen.

Cytokine Quantification

The levels of secreted interferon gamma (IFN-γ) in the ileum were analysed with the Pro-inflammatory Panel 1 (mouse) V-PLEX Kit, and IL-17a was analysed with U-PLEX Mouse IL-17a assay, and the MESO QuickPlex SQ 120, SECTOR Imager 2400 (Meso Scale Discovery, Maryland, USA). Only data derived from duplicates with CV <15% were included in the analysis. Concentrations of cytokines were expressed in pg/mg of tissue.

Immunofluorescence

Serial coronal sections (40 um) were cut in a cryostat (Leica) and stored at -20ºC. For staining, the sections were washed three times in PBS for 5 minutes each. For antigen retrieval, slides were immersed in citrate buffer, and placed in a water bath for 15 minutes at 80ºC. Next, a blocking solution (10% normal donkey serum (NDS) in 0.3% TritonX-100 PBS) was applied once the slides achieved room temperature, for 1h30 in a humid chamber. The blocking solution was replaced with primary antibody probing solution (2% NDS, 0.3% TritonX-100 PBS and primary antibody rabbit anti-$CD4^+$ (1:500, Abcam)), and was incubated overnight at 4ºC. After primary incubation, the sections were washed four times with PBS, for 5 minutes per wash. The Alexafluor secondary antibody donkey anti-rabbit 488

(1:500, Invitrogen) was applied in a carrier solution (2% NDS, 0.1% PBS Tween) and incubated for 2h at room temperature. Following secondary incubation and 10 minutes of incubation with DAPI, the sections were washed three times with PBS, mounted with Immu-Mount (Fisher Scientific) and coverslipped. The number of $CD4^+$ cells was quantified in the wall and epithelium of the lateral ventricles and the 3rd ventricle, using Olympus BX43 optical microscope at 20x.

Caecal Metabolomics

The caecal metabolome was analysed by MS-Omics (Copenhagen) using Mass Spectrometry. Briefly, samples were acidified using hydrochloride acid, and deuterium labelled internal standards where added. All samples were analyzed in a randomized order. Analysis was performed using a high polarity column (Zebron™ ZB-FFAP, GC Cap. Column 30 m x 0.25 mm x 0.25 µm) installed in a GC (7890B, Agilent) coupled with a quadropole detector (5977B, Agilent). The system was controlled by ChemStation (Agilent). Raw data was converted to netCDF format using Chemstation (Agilent), before the data was imported and processed in Matlab R2014b (Mathworks, Inc.) using the PARADISe software described by Johnsen et. Al (Johnsen et al 2017).

Peaks were quantified using area under the curve (AUC). Biostatistics were run in R (version 4.1.2) with the Rstudio GUI (version 1.4.1717). Principal-component analysis was performed on CLR-transformed values (Aitchison et al 2000). The PERMANOVA implementation from the vegan library was used to find structural differences between treatments on a compositional level. To find metabolites that were differentially abundant based on either age or antibiotic treatment, we fitted linear models using the CLR-transformed metabolite levels with both factors as explanatory variables. To correct for multiple testing (FDR) in tests involving metabolomics features, Storey's q-value posthoc procedure was performed with a q-value of 0.2 as a cut-off (Storey 2002). Metabolomics figures were generated using ggplot2. Metabolites were filtered where the absolute β estimate of Young-Control vs Aged-Control > absolute β of Young-Control vs Aged-ABX (*i.e.*, when the difference between young and aged controls is greater than the difference between young controls and aged mice exposed to antibiotics).

Cell Isolation and Flow Cytometry

Spleens and mesenteric lymph nodes (MLNs) were dissected out of the animal, cleaned from fat tissue and stored in media (RPMI-1640 medium with L-glutamine and sodium bicarbonate (R8758, Sigma), supplemented with 10% FBS (F7524l, Sigma) and 1% Pen/strep (P4333, Sigma)) on wet ice for flow cytometry the same day.

Flow cytometry was performed as previously described ((Boehme et al 2020, Gururajan et al 2019). Spleenocytes were isolated by flushing the spleen with media using a syringe. The cell suspension was subsequently centrifuged, aspirated and incubated with 1 mL lysis buffer (Sigma, R7757) for 5 minutes. 10 mL media was added to dilute the lysis buffer and the cell suspension was poured over a 70 µm strainer, after which it was centrifuged and aspirated. $2x10^6$ cells were resuspended in 90 µL staining buffer and split into 2 aliquots for the staining procedure. MLNs were transferred onto a 70 µm strainer and disassembled using the plunger of a 1 mL syringe. The strainer was subsequently rinsed with 10 mL media, and the cell suspension was centrifuged, aspirated, $2x10^6$ cells were resuspended in 90 µL staining buffer, and split into 2 aliquots for the staining procedure.

For the staining procedure, 5 µL of FcR blocking reagent (Miltenyi, 130-092-575) was added to each sample. Samples were subsequently incubated with a mix of antibodies (CD11b - Miltenyi; CD69, Miltenyi - CD62L, Biolegend; CD8a - Miltenyi; LY6C- Miltenyi; FVS780, BD BioSciences; MHC-II - Biolegend; CD4 - Biolegend; CD44 - BD BioSciences) for 30 minutes on ice, after which they were centrifuged, aspirated and fixed using 100 µL 4% PFA for 30 minutes on ice. Samples were finally centrifuged, aspirated and resuspended in staining buffer for flow cytometric analysis the following day on the BD FACSCalibur. Data was analysed using FlowJo (Version 10).

Statistical Analysis

Statistical analyses were conducted using SPSS 27 (IBM, USA). Normality was assessed employing the Shapiro–Wilk test and for equality of variances using the Levene's test. Non-parametric data were analysed with independent-samples Kruskal–Wallis test followed by pairwise comparisons adjusted by the Bonferroni correction for multiple tests, with a 95% confidence interval. Parametric data were analysed using two-way analysis of variance (ANOVA) *post hoc* Tukey HSD. All data is represented as mean ± SEM. Social novelty was analysed using a general linear model integrating age, treatment and stimulus. Further, we utilized simple main effects to explore the individual group differences in these complex models, adjusted by the Bonferroni correction for multiple tests, as we hypothesized a priori that the Aged-control group would be significantly different from the other groups. Statistical significance was set at $p \leq 0.05$.

Results

Microbiota Depletion Restores the Age-Dependent Social Cognitive Defects

Young (9-10 weeks old) and aged (18 months old) C57BL/6 mice were assessed in the 3-chamber test after 9 days of antibiotic exposure **(Figure 1a)**. A distinct preference between either chamber was observed (Novel mouse vs Familiar mouse, ($F_{(1,35)}$=14.796, p= 0.0005), as young animals show a preference for the novel mouse chamber (Young-CTRL, p=0.039; Young-ABX, p=0.088), which is not seen in Aged-Control animals (p= 0.822) **(Figure 1b)**.

Age did not Significantly Impact Pro-Inflammatory Cytokine Levels in The Ileum

To understand how microbiota depletion influences pro-inflammatory cytokine secretion in the gut of aged animals, cytokine levels were quantified in the ileum. The proinflammatory cytokines IFN-γ and IL-17a showed no significant differences in response to aging **(Figures 1c-d)**.

T-Helper Cells Accumulate in the Aged Choroid Plexus and are Partially Restored by Microbiota Depletion

As the choroid plexus relays signals interchangeably between the CNS and the circulating immune system, and also has a prominent role in T-cell mediated neuroinflammation in aging (Baruch et al 2013), we quantified CD4+ T cells in the choroid plexus, focusing our analysis on the number of cells along the walls of the lateral and third ventricles. Considering the cells accumulated along the ventricle wall, aged animals show a significant increase in CD4+ T cells in the lateral ventricle walls, which is partially reduced towards the levels present in young animals by antibiotic treatment (Two-Way ANOVA; $F_{(1,16)}$=5.308, p=0.035; Young-CTRL vs Aged-CTRL, p<0.001; Aged-CTRL vs Aged-ABX, p=0.052) **(Figure 1f)**. Likewise, in the third ventricle aged animals show an increase in T cells, which is reversed by antibiotic treatment ($F_{(1,15)}$=6.354, p=0.024; Young-CTRL vs Aged-CTRL, p=0.001; Aged-CTRL vs Aged-ABX, p=0.023) (Figure 1g).

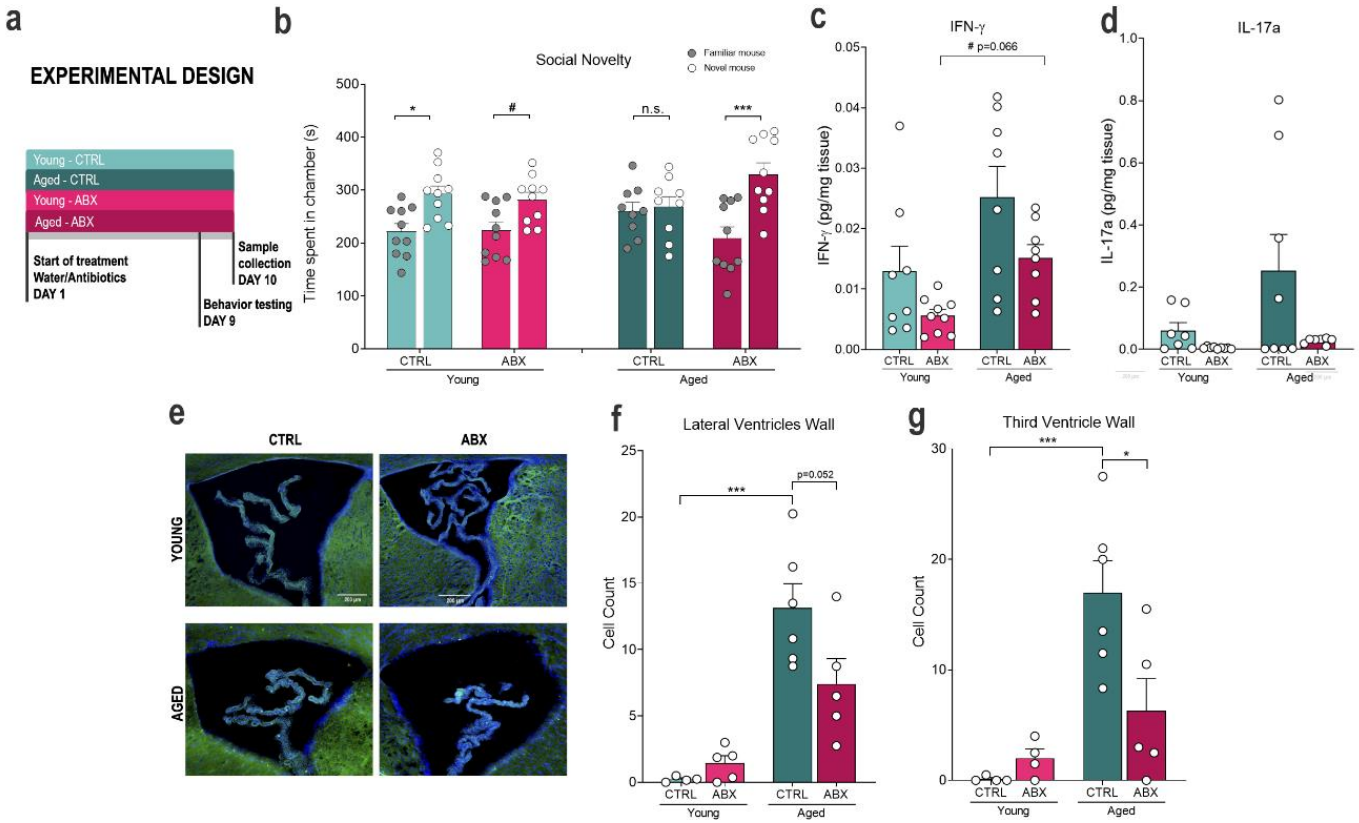

Figure 1. Microbiota Depletion Restores the Age-Dependent Social Cognitive Defects and Modulates T-Helper cell numbers in the Choroid Plexus. *a) Experimental Design. b) Social novelty assessed by 3-Chambers (n=9-10 per group). Ileal levels of c) IFN-γ and d) IL-17a. e) Representative images of the lateral ventricles (10x, scale bar – 200 µM). CD4+ T cell numbers in the wall of the f) lateral ventricles and g) third ventricle (n=4-6 per group). Results presented as mean+standard error of the mean (SEM). *p<0.05, **p<0.01, ***p<0.001.*

Age-Dependent Shifts in Caecal Metabolites can be Successfully Restored by Antibiotic Treatment

We next set out to assess the extent of which gut microbiota depletion impacts caecal metabolite concentrations in of young and aged animals with or without antibiotic exposure **(Figure 2a-b)**. Seventy metabolites were differentially altered by both age and gut microbiota depletion - after the filtering criteria (see Supplementary Methods), we focused on which metabolites if any are restored with antibiotic treatment in aged mice. After this filtering criteria, four metabolites are highlighted: 2-Methylbutyrylglicine, Argininosuccinic acid, Gentisic acid and N-formylmethionine **(Figure 2c)**. The levels of 2-Methylbutyrylglycine, an acyl glicine, a minor metabolite of fatty acids, were significantly reduced in aged animals (β=0.506, p=0.003, BH=0.028; Young-CTRL vs Aged-CTRL, p=0.001), and restored by antibiotic treatment in aged mice (Aged-CTRL vs Aged-ABX, p<0.001) as was argininosuccinic acid, an amino acid generated during the urea cycle (β=1.023, p=0.002, BH=0.019; Young-CTRL vs Aged-CTRL, p<0.001; Aged-CTRL vs Aged-ABX, p=0.03).

Gentisic acid is a metabolite that can have neuroprotective properties (Abedi et al 2020) but can also be nephrotoxic metabolite (McMahon et al 1991). In this study, gentisic acid levels were increased in the caecum of aged animals (β=-0.647, p=0.007, BH=0.042; Young-CTRL vs Aged-CTRL, p=0.003), being restored to control levels by the antibiotic (Aged-CTRL vs Aged-ABX, p=0.001). N-Formylmethionine (fMet) is a metabolite linked to mortality, potentially by promoting metabolic shift in critical illness (Cai et al 2021, Sigurdsson et al 2022). Curiously, in this study fMet levels in the caecum were significantly increased in aged mice (β=-0.674, p=0.0004, BH=0.007; Young-CTRL vs Aged-CTRL, p<0.001) and completely reduced to young animal's concentrations in aged mice receiving antibiotics (Aged-CTRL vs Aged-ABX, p<0.001). Taken together, these results suggest that microbiota depletion can reshape the metabolomic landscape, by reducing levels of metabolites previously implicated in age-dependent physiological alterations.

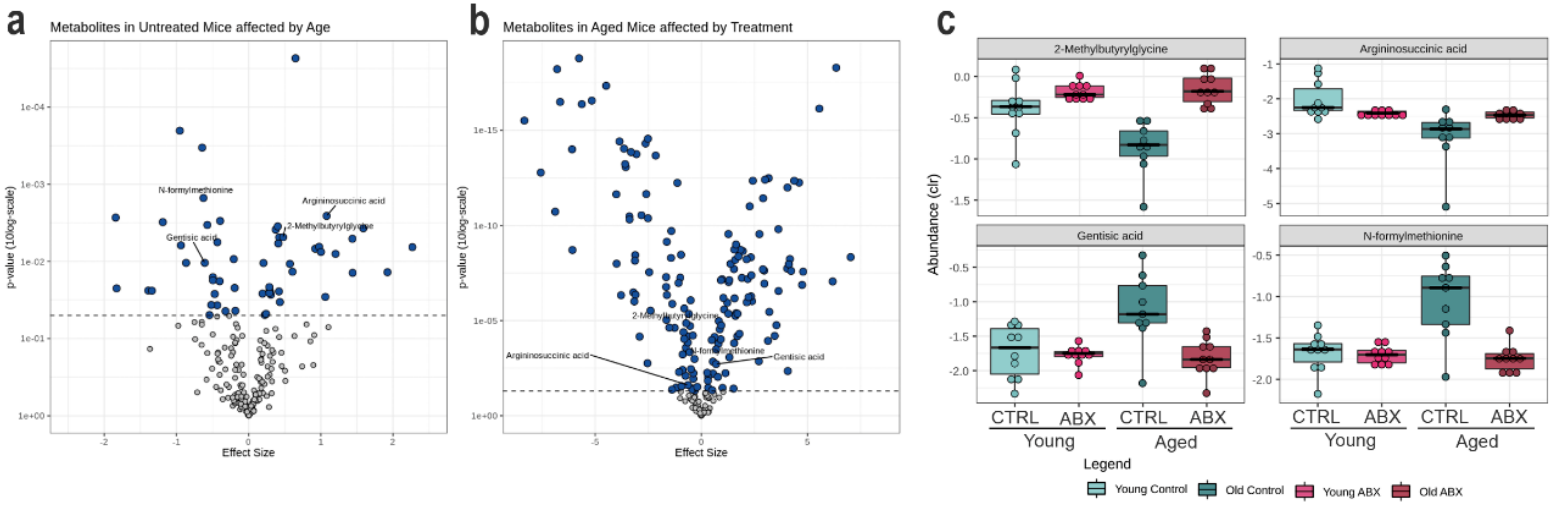

Figure 2. Age-Dependent Shifts in Caecal Metabolites can be Successfully Restored by Antibiotic Treatment. *Volcano plots showing caecal metabolites affected by age a) and by treatment, within aged animals b), respectively. The x-axis represents the estimated difference in means by groups (β), whereas the y-axis depicts p-values. Dark blue points represent metabolites that reach a p-value of < 0.05 and a Benjamini-Hochberg adjusted q-value of < 0.2. dashed horizontal lines represent the p = 0.05 mark. c) Boxplots showing the centered log-ratio transformed (clr) abundance of caecal metabolites altered by age, which are restored by antibiotic treatment. For the boxplots, boxes represent the limits of the interquartile range, the horizontal line represents the median point, and the whiskers represent the full data range.*

Discussion

As the investigation of the biological processes underlying aging continues to advance, the contributions of the immune system and the gut microbiota have gained attention. In this study, we demonstrate that gut microbiota depletion by a mild antibiotic cocktail not only modulates components of the gut metabolome and immune system features in aging, but also has a beneficial impact on social behaviour in aged mice.

In agreement with previous observations from our group (Scott et al 2017), we show that aged mice display social novelty recognition deficits. Intriguingly, now we show that antibiotic treatment in aged mice restored the preference for the novel over the familiar mouse. These data demonstrate that the gut microbiota is an important player in age-associated social impairments. Interestingly, studies have shown beneficial effects of antibiotics in improving cognitive outcomes, possibly by reducing neuroinflammation in mouse models of Alzheimer's Disease (Angelucci et al 2019).

Considering the age-dependent synergistic links between the peripheral and neural immune systems (Boehme et al 2020), we sought to explore the impact of antibiotics on the immune system by a) measuring levels of pro-inflammatory cytokines in the ileum and b) quantifying the numbers of T cells at a key neuroimmunological interface – the choroid plexus.

Aging showed a tendency for increasing IL-17a and IFN-γ in the ileum, which even though not statistically different seems to be decreased following microbiota depletion. Given that ileal pro-inflammatory cytokines are mostly impacted by microbiota depletion, it is possible that the commensal bacteria are required for driving pro-inflammatory cytokines.

Concerning the choroid plexus, with age, there was an accumulation of CD4+ T cells at the lateral and 3rd ventricles, which is in line with previous observations of a marked age-associated increase of these cells, particularly at the 3rd ventricle (Xu et al 2010). Remarkably gut microbiota depletion improved this effect, by partially reversing CD4+ T cell counts in the ventricles. This suggests that

abolishing gut microbial signals in aged animals could partially reduce the CD4+ T cell accumulation at an important neuroimmune interface in the brain.

The gut metabolome is inherently linked to the gut microbiome (Garza et al 2020, Valles-Colomer et al 2019), and indeed microbial-derived metabolites have been suggested to be involved in age-related cognitive decline (Connell et al 2022), caecal metabolomic analysis was performed to examine the metabolic impact of antibiotics in aged animals. 2-Methylbutyrylglicine and Argininosuccinic acid were reduced in the caecum of aged mice, and restored by antibiotic treatment, while inversely, gentisic acid and N-formylmethionine were found increased in the aged caecum and recovered by gut microbiota depletion. 2-Methylbutyrylglicine, a metabolite that in high concentrations has been linked to mental retardation and neuronal damage (Kanavin et al 2007, Knebel et al 2012), and which can be modulated by administration of *Akkermansia muciniphila,* was in fact reduced in the caecum of aged animals, which was eliminated following microbiota depletion, suggesting a new pattern in an aging context. Argininosuccinic acid is part of the arginine pathway, which has been previously reported to be altered in the aged brain (Rushaidhi et al 2012) and is also reduced in the aged caecum and recovered by antibiotic treatment. In contrast, gentisic acid and N-formylmethionine were increased in the aged caecum and reduced following microbiota depletion. Interestingly, gentisic acid has been suggested to inhibit angiogenesis – which in the context of tumors is beneficial as a therapeutic target (Fernández et al 2010) – however, given that angiogenesis is impaired with age (Hodges et al 2018, Reed & Edelberg 2004), perhaps the increased levels of gentisic acid in the aged caecum may reflect a maladaptive age-response, which is modulated by gut microbiota depletion. Finally, N-formylmethionine, a metabolite that has been linked to age-related disease risk and all-cause mortality (Cai et al 2021), was likewise elevated in the caecum of aged animals and restored with microbiota depletion to young-matched control levels. In light of the relevance of these metabolites in aging physiological processes, and that such levels can be improved by gut microbial depletion, it is feasible to delineate a crucial role of the gut microbiota in the mediation of age-dependent physiological alterations.

These data are in agreement with a recent study showing that antibiotics can alter the effects of social stimulus on reward behaviour (García-Cabrerizo et al 2023). Moreover, a growing literature is showing that specific strains of bacteria or microbiota-targeting prebiotics (Cruz-Pereira et al 2022) can enhance social behaviour in the mouse.

In summary, this study demonstrates that the aged gut microbiota is associated with age-dependent immune changes, which is reflected in social behavioural outputs. This suggests that during aging, a targeted gut microbiota depletion can have favourable outcomes, as particular pernicious bacterial populations may expand with age, and their metabolites can therefore have pervasive effects on the host. Future studies aiming to identify other gut microbial communities, their metabolites and their involvement in age-dependent processes would provide invaluable information that could guide new more targeted, non-antibiotic-based microbiome therapeutics.

Supplemental Material

Supplementary Table 1 – Detailed statistical reporting of the results presented in Chapter 2.

		GLM							Bonferroni adjustments			
		Within Subjects Effects				Between Subjects Effects			Young-Control	Young-ABX	Aged-Control	Aged-ABX
Figure	Outcome Variable	Stimulus	Stimulus x Age	Stimulus x Treatment	Stimulus x Age x Treatment	Age	Treatment	Age x Treatment	Novel vs Familiar	Novel vs Familiar	Novel vs Familiar	Novel vs Familiar
1b	Social Novelty	F(1,35)=14.796, p= 0.0005	F(1,35)=0.000016, p= 0.997	F(1,35)=2.243, p= 0.143	F(1,35)=3.543, p= 0.068	F(1,35)=4.618, p= 0.039	F(1,35)=0.004, p= 0.950	F(1,35)=0.651, p= 0.425	0.039	0.088	0.822	0.001

			Bonferroni Post Hoc		
Figure	Outcome Variable	Independent Samples Kruskal Wallis	Young Control vs Aged-Control	Aged-Control vs Aged ABX	Young ABX vs Aged ABX
1c	IL-17a	(H(3)= 6.599, p=0.086)	-	-	-
1d	IFN-γ	(H(3)= 13.313, p=0.004)	0.297	1	0.066
1e	TNF-α	(H(3)= 12.111, p=0.007)	1	0.036	1

		Two-Way ANOVA			Post Hoc Tukey HSD	
Figure	Outcome Variable	Age	Treatment	Age x Treatment	Young Control vs Aged-Control	Aged-Control vs Aged ABX
1g	LV Wall	F(1,16)=38.932, p<0.001	F(1,16)=2.247, p=0.153	F(1,16)=5.308, p=0.035	p<0.001	p=0.052
1h	LV Epithelium	F(1,16)=14.620, p=0.001	F(1,16)=0.161, p=0.694	F(1,16)=2.661, p=0.122	p=0.007	p=0.451
1i	3V Wall	F(1,15)=18.049, p=0.001	F(1,15)=3.123, p=0.098	F(1,15)=6.354, p=0.024	p=0.001	p=0.023
1k	3V Epithelium	F(1,15)=2.273, p=0.152	F(1,15)=1.410, p=0.254	F(1,15)=0.571, p=0.462	-	-

		Linear Model Outcome						Tukey's Post Hoc Test		Benjamini-Hochberg adjusted q-value		
Figure	Outcome Variable	Age β Estimate	Age p value	Treatment β Estimate	Treatment p value	Age x Treatment β Estimate	Age x Treatment p value	Young-Control vs Aged Control p (Tukey adj)	Aged Control vs Aged ABX (Tukey adj)	Age q value	Treatment q value	Age x Treatment q value
2c	2-Methylbutyrylglycine	-0.027916399	0.802126759	-0.713208752	3.31849E-07	0.505553364	0.003002365	0.000947502	1.93638E-06	0.939832303	6.39781E-07	0.027935045
	Argininosuccinic acid	0.057168094	0.786733805	-0.634367864	0.005712314	1.022877116	0.001686506	8.77598E-05	0.027898489	0.935339079	0.007836122	0.018995378
	Gentisic acid	0.039530619	0.803688371	0.695017134	0.000135354	-0.647390959	0.007078052	0.003432481	0.000749611	0.939832303	0.000211428	0.042075087
	N-formylmethionine	0.047696872	0.694285372	0.698044662	2.2668E-06	-0.673868446	0.000410259	7.54262E-05	1.31032E-05	0.863820172	3.94387E-06	0.006753493

Given the impact of aging in peripheral immunity, immune populations were assessed in the MLNs and spleen. Morphological changes as well as alterations to the local immune populations have been reported with aging in both the spleen and lymph nodes (Turner & Mabbott 2017), hence we aimed to understand if gut microbiota depletion in aged mice could impact such outcomes **(Figure S1)**.

In the spleen, $CD4^+$ T cells were independently influenced by age and antibiotic treatment. Splenic $CD4^+$ T cells are reduced in aged animals and further reduced by antibiotic treatment (**Figure 1a**). To further characterize the T cell populations, CD44, a marker of T cell activation (Schumann et al 2015) and CD69, an early activation lymphocyte marker (Cibrián & Sánchez-Madrid 2017) were used. Early activated splenic $CD4^+CD69^+$ T cells were altered as aged animals show a significant increase in early activated $CD4^+$ T cells, which was not reversed by antibiotic treatment (**Figure S1b**). Splenic $CD4^+CD44^+$ T cells were not altered by age or antibiotic treatment (**Figure S1c**).

Splenic $CD8^+$ T cells were altered, as aged animals treated with antibiotics showed reduced cytotoxic spleen T cells when compared to their young controls and to the aged controls (**Figure S1d**). Further, the splenic early activated $CD8^+$ $CD69^+$ T cells also presented a significant effect of age, as again this population is increased in aged control animals, which is not reversed by gut microbiota depletion (**Figure S1e**). Likewise, the percentage of splenic early activated $CD8^+$ $CD44^+$ T cells was significantly increased in aged animals, as aged control animals show increased activated cytotoxic T cells, which is not altered by antibiotic treatment (**Figure S1f**). In summary, we observed a decrease in $CD4^+$ T cells in the spleen in aged mice, while age increases the number of activated T cell populations, in a pattern that is not affected by gut microbiota depletion.

The mesenteric lymph nodes (MLNs) have been previously reported to display age-dependent changes in relation to immune cell populations (Takano et al 2020). In the MLNs, $CD4^+$ T cells were altered, despite the fact that aged animals do not show statistically significant reduction in T helper cells, that reduction is evident in aged animals exposed to antibiotics when compared to young animals administered with antibiotics (**Figure S1g**). Early activated $CD4^+CD69^+$ T cells were found altered in the MLNs, as age induces an increase in this population, which is not significantly recovered by microbiota depletion (**Figure S1h**). Further, the $CD4^+CD44^+$ population was not impacted by either age or microbiota depletion

(**Figure 1Si**). Furthermore, $CD8^+$T cells were not altered across groups (**Figure S1j**). Early activated cytotoxic T cells were impacted by age, as represented by a significant increase with age, which is not recovered by gut microbiota depletion (**Figure S1k**). Lastly, $CD8^+CD44^+$ T cells were not impacted by age or antibiotic exposure (**Figure S1l**).

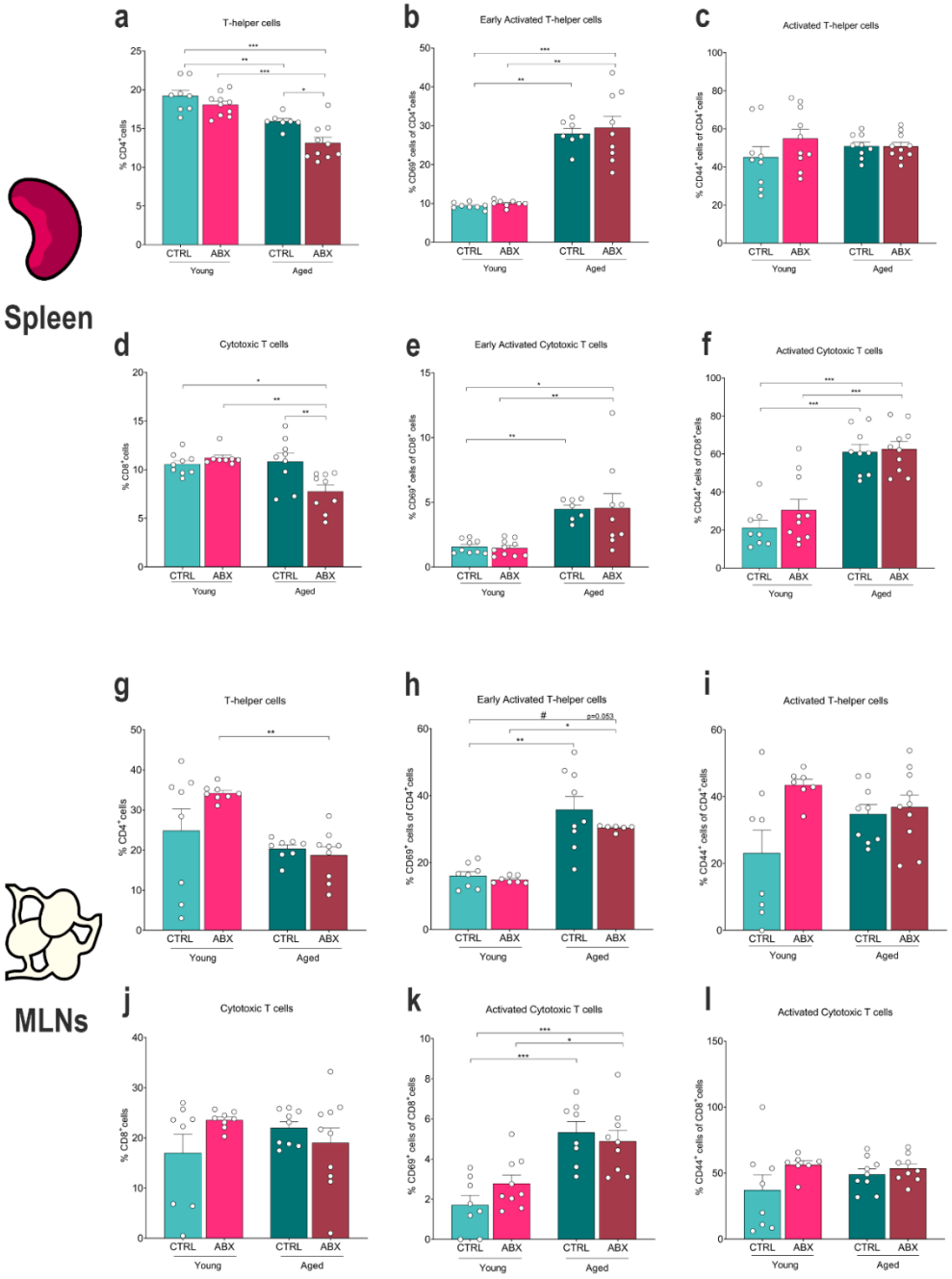

Figure S1 - Age and Microbiota Depletion Have Differential Impacts on the T Cell Profile in the Spleen and Mesenteric Lymph Nodes. *Results presented as mean+standard error of the mean (SEM). n=7-10 per group. **p<0.01, ***p<0.001*

Chapter 3

Prebiotic Supplementation Modulates Selective Effects of Stress on Behaviour and Brain Metabolome in Aged Mice

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Abstract

Aging has a significant impact on physiology with implications for central nervous system function coincident with increased vulnerability to stress exposures. A number of stress-sensitive molecular mechanisms are hypothesized to underpin age-related changes in brain function. Recent cumulative evidence also suggests that aging impacts gut microbiota composition. However, the impact of such effects on the ability of mammals to respond to stress in aging is still relatively unexplored. Therefore, in this study we assessed the ability of a microbiota-targeted intervention (the prebiotic FOS-Inulin) to alleviate age-related responses to stress. Exposure of aged C57BL/6 mice to social defeat led to an altered social interaction phenotype in the social interaction test, which is reversed by FOS-Inulin supplementation. Interestingly, this occurs independent of affecting social defeat-induced elevations in the stress hormone corticosterone. Additionally, the behavioural modifications following FOS-Inulin supplementation are also not coincident with improvement of pro-inflammatory markers. Metabolomics analysis was performed and intriguingly, age associated metabolites were shown to be reduced in the prefrontal cortex of stressed aged mice and this deficit was recovered by FOS-Inulin supplementation. Taken together these results suggest that prebiotic dietary intervention rescued the behavioural response to stress in aged mice, not through amelioration of the inflammatory response, but by restoring the levels of key metabolites in the prefrontal cortex of aged animals. Therefore, dietary interventions could be a compelling avenue to improve the molecular and behavioural manifestations of chronic stress exposures in aging via targeting the microbiota-gut brain axis.

KEYWORDS: Aging, Prebiotics, Diet, Stress, Metabolome

Introduction

Aging is a complex multiorgan process that involves considerable molecular remodelling, which is characterized by defined hallmarks, such as mitochondrial dysfunction, loss of proteostasis and altered intercellular communication which largely shapes the immune landscape due to time-dependent cellular damage accumulation (López-Otín et al 2013). Inflammaging, the accumulation of pro-inflammatory signals that follow aging in mammals have been the subject of much investigation in the context of diverse age-related pathologies (Franceschi et al 2018). Further, the allostatic load caused by the constant physiological adaptation in response to chronic stress (McEwen 2007) is an important predictor of mortality (Robertson et al 2017). Additionally, there are many neurobiological similarities between stress and aging that make it crucial to explore the effects of stress in the context of aging (Prenderville et al 2015). These changes include hyperactivation of the hypothalamic–pituitary–adrenal (HPA) axis, deficiencies in neurotrophic factors such as brain-derived neurotrophic factor (BDNF), decreases in adult hippocampal neurogenesis, increased neuroinflammation, disruption of blood brain barrier and changes in several neurotransmitters such as 5-hydroxytryptamine (5-HT or serotonin), noradrenaline, dopamine, glutamate, and γ-aminobutyric acid (GABA), all of which lead to neuronal dysfunction (Prenderville et al., 2015). Stress and HPA axis activation have been associated with a compromised profile of cognitive functions later in life (Qiu et al 2021, Rimmele et al 2022). Moreover, animal studies have highlighted a differential effect of chronic stress on dendritic spine remodelling in the prefrontal cortex (PFC) of aged compared to young rats (Bloss et al 2011).

It is worth noting that the manifestation of anxiety and major depressive disorder is substantial in elderly adults, which significantly impacts their quality of life as a result of social isolation (Cacioppo & Hawkley 2009, Prenderville et al 2015). In humans, meta-analyses show that social networks tend to reduce with age and that novelty seeking, particularly relating to generating new social relationships, is reduced with age (Jopp et al 2016, Prenderville et al 2015). Even though social behaviour has also been shown to be decreased in aged mice (Scott et al 2017), this behavioural facet is still relatively unexplored in the context of aging (Prenderville et al 2015). Taken together, the understanding of the impact of

stress on/during aging is essential to provide solutions better suited for this particular demographic.

Throughout our lifespan, the gut microbiota – the millions of microbes that inhabit the gut – has been increasingly linked to the maintenance of homeostasis (Fung et al 2017, Lynch & Pedersen 2016, Miquel et al 2018). Gut microbiome compositional changes have been reported with aging, namely a reduction of some commensal taxa (*ie Roseburia, Bifidobacterium* and *Prevotella)*, and an increase in other commensals (such as *Akkermansia* and *Christensenellaceae*) (Claesson et al 2011, Ghosh et al 2022a). Furthermore, gut microbiomes from healthy individuals, while showing diverging time-dependent changes with age, are not only followed by increased circulation of specific microbial metabolites in the plasma, but also predict extended survival later in life (Wilmanski et al 2021). These age-dependent gut microbiome alterations can reflect age-associated decline in health, but also suggest that lifestyle factors, particularly diet, offer an opportunity to shape the gut microbiome and hence contribute to better health outcomes (Ghosh et al 2022a). Diet has been shown to not only impact gut microbiome composition, but also predicts and shape inflammation and mental health markers/status and cognitive function in aged human populations (Claesson et al 2012, Ghosh et al 2020b).

The PFC is an important brain structure involved in emotional processing and social behaviour (Franklin et al 2017), also in the response to social defeat (Challis et al 2014, Covington et al 2010), and is related to emotional regulation in aged humans (van Reekum et al 2018, Winecoff et al 2011). Further in aged rats, stress has been shown to impact neural oscillations in the PFC (Takillah et al 2017). Interestingly, changes in the composition of the gut microbiome have been shown to drive microRNA expression in the PFC, along with transcriptional changes and regulation of the myelination process, thus implying a crucial role for the gut microbiome in the development and function of the PFC (Gacias et al 2016, Hoban et al 2017, Hoban et al 2016b). Additionally, the gut microbiome has been implicated in the development, programming and expression of social behaviour (Agranyoni et al 2021, Desbonnet et al 2014, Sherwin et al 2019, Wu et al 2021a).

Diet plays a prominent role in shaping the gut microbiome, making it an accessible target for microbiota modulation (Audet 2021, Sanders et al 2019). Prebiotics, substrates that are used by the gut microorganisms to confer health benefits to the host, are increasingly used as a dietary intervention to modulate the gut microbiome for health benefits (Sanders et al 2019). Inulin, a polysaccharide dietary fibre, widely used as a prebiotic, has been shown to modulate the gut microbiome and thereby shape the immune system (Han et al 2021a, Zou et al 2018). This prebiotic has been shown to modulate the gut microbiome in middle aged mice, reducing neuroinflammation and peripheral inflammation in response to stress (Boehme et al 2020). We hypothesize that a prebiotic dietary intervention can modulate age-related changes in the gut microbiome to counter the effects of stress. We assess whether any potential effects are coincident with changes in the production of microbial and host metabolites in the prefrontal cortex and caecum.

Methods

Animals

Male aged C57BL/6 mice (n=40; 18-19 months old; Charles River, Kent, UK) were used in this study. All experiments were conducted in accordance with European Directive 86/609/EEC, Recommendation 2007/526/65/EC, and approved by the Animal Experimentation Ethics Committee of University College Cork. Animals were kept under a 12-h light/dark cycle, with a temperature of 21 ± 1 °C and humidity of 55 ± 10%. Food and water were given *ad libitum*. Approximately one week before commencement of social defeat sessions, all mice were singly housed and weighed daily over the course of the experimental protocol. For the chronic social defeat stress procedure, non-experimental singly housed adult male CD1 mice (5 months old) were used as aggressors (Envigo, UK).

Study Design

Aged C57BL/6 mice (19 months old) were fed either a FOS-Inulin enriched diet or chow diet for 19 days before the beginning of the stress protocol. For 6 days, all aged animals were exposed to social defeat stress (**Figure 1a**). To understand if the prebiotic intervention acts on the gut brain axis to shape stress response in aged mice, we analysed social behaviour using social interaction (Day 25) and the three-chamber test (Day 26). Tissues and biomarkers were harvested on day 27 including for measurement of circulating levels of corticosterone, ileal pro-inflammatory cytokine levels, and prefrontal cortex and caecal metabolomics (**Figure 1b**).

Diet

Mice were fed chow (ssniff-Spezialdiäten GmbH, Soest, Germany) enriched with 10% FOS-Inulin (mixture of 92 ± 2% Inulin and 8 ± 2% Fructooligosaccharide, Orafti®Synergy1; BENEO-Orafti N.V., Tienen, Belgium) or control chow (ssniff-Spezialdiäten GmbH, Soest, Germany) for 4 weeks, as previously described (Boehme, 2019).

Stress Protocol

Mice were randomly assigned to either the stress (n = 20) or control groups (n = 20). Chronic social defeat stress was carried out daily for 6 consecutive days (see **Figure 1a** for experimental timeline) as previously described but with slight modifications (Savignac et al., 2011). Prior to the defeat sessions, all CD1 aggressor mice were tested for aggressiveness over two separate days. A CD1 mouse was exposed to another CD1 mouse until the first attack. Mice with the shortest attack latencies were selected as aggressors to be used in subsequent social defeats. For each defeat session, experimental mice were exposed to a different aggressor CD1 mouse each day over the 6-day period. The session involved a single initial exposure of the test mouse to the aggressive CD1 in the home-cage of the aggressor (33x15x13cm) and lasted until the first attack with expression of submissive posturing, or until 5 minutes had passed. The latency to attack or display a submissive posture was recorded. The mice were then separated by a perforated Plexiglas® wall that allowed non-physical contact for 2 h. Then, the separator was removed and after another defeat, mice were returned to their home-cage. Control animals were left undisturbed in their homecages during the stress protocol.

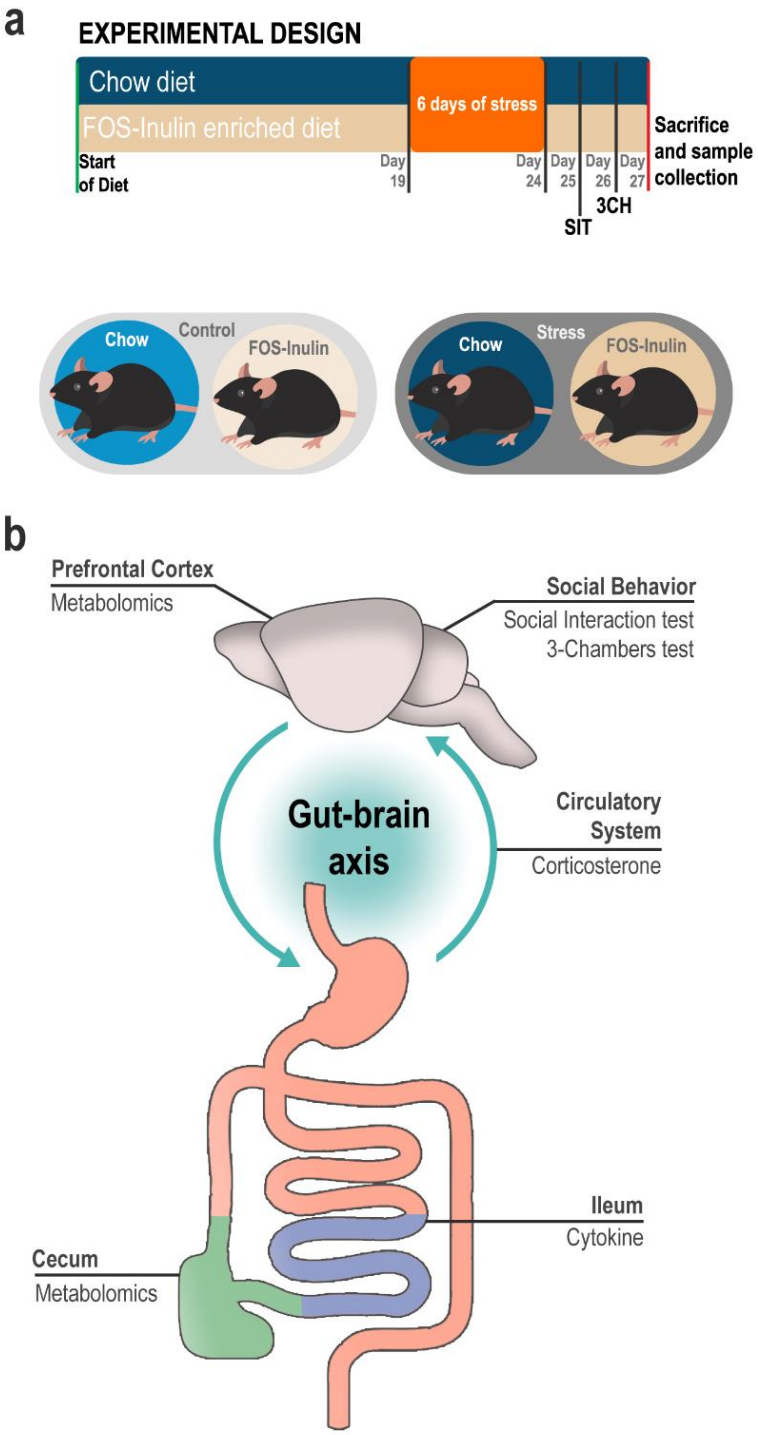

Figure 1 – Experimental design. *a) After approximately three weeks of FOS-Inulin dietary supplementation, animals were exposed to 6 days of social defeat stress. Subsequently, mice underwent social interaction test and 3-chambers sociability test, followed by sacrifice. b) Experimental outputs outlined in this experiment.*

Behaviour

Social Interaction Test

One day after the last social defeat session, the social interaction test was conducted as described before (Gururajan et al 2019). Briefly, it encompassed two trials of 150 seconds (s) each. The test was performed in an open arena (40 × 32 × 24 cm, L × W × H) containing an empty wire mesh cage (9.5 × 7.5 × 7.0 cm) placed in the middle of one of the walls of the arena. During the first trial, the chamber in the social exploration box was empty; in the second trial an unfamiliar non-aggressive CD-1 male mouse was placed inside the exploration chamber in the arena. Both mice were returned to their home-cages upon completion of the test, and the arena was wiped with 70% ethanol. All mice were habituated 45 min before testing, and testing was conducted under red light (5 lux), being recorded from the ceiling. The interaction zone is defined as a rectangular area (25cmx15cm) around the wire-mesh cage where the CD1 target mouse is placed during the interaction phase. The corner zones are defined as squared areas on both corners opposite to the wire-mesh cage (9cmx9cm). Time spent in the interaction zone, time spent in the corner zones, movement and entries in the corner zones, and time spent facing the wire-mesh were scored using a deep-learning informed software analysis coupled with the SimBa 1.1.3, an open-source toolkit for computer classification of complex social behaviours in experimental animals (Nilsson et al., 2020). Time facing the CD1 ratio was calculated as time spent facing the interaction zone during the second trial (CD1 target present) divided by the time spent facing the interaction zone during the first trial (CD1 target absent). DeepLabCut 2.2 with CUDA Toolkit 10.1 and Tensorflow 1.12.0 was used to perform behavioural analysis (Mathis et al 2018). We defined 8-body part pose configuration and labelled 200-250 frames from representative videos from each group. The system was trained using a deep neural network that was trained in 155,000 iterations as the loss relatively flattened (Mathis et al 2018, Nath et al 2019). The trained network could accurately track the position of the mice in the full sets of video segments. The labeled x-axis (i.e. left–right) and y-axis (i.e. bottom-top) positions of the pixels in each frame were stored and exported in CSV format. Further analysis was performed using SimBa 1.1.3, where the width of the arena in centimeters was compared to the width in pixels, to generate a pixel-to-centimeter ratio (Nilsson et

al 2020). Next, we defined the region of interest analysis, as described above, and extracted the metrics calculated based on the X-Y coordinates of the centre body part in a frame-by-frame basis. We followed the recommendations as described in https://github.com/sgoldenlab/simba.

3-Chamber Test

Social cognition was evaluated using the 3-chamber social interaction test, in which time spent interacting with a novel conspecific is compared to time spent with a novel object or familiar conspecific. It is based on the premise that mice will prefer to seek an animal over an inanimate object, and that they will prefer a novel conspecific to a familiar one. This test was performed 24h after the social interaction test. Mice were habituated to the room for one hour before testing.

The test arena consisted of 3 chambers; the left and right chambers measured 13.5 x 20 x 20 cm and the centre chamber was 9 x 20 x 20 cm. A solid partition divided the chambers, with a small hole allowing access to the other chambers. There were 3 trials in this test: habituation, sociability, and social novelty preference. All phases of the test lasted for 10 min, were performed sequentially, and recorded from above for later analysis. During the habituation phase, the mouse was placed into the centre chamber and then allowed access to the empty left and right chambers for 10 min. The mouse was then gently coaxed to the centre chamber and a novel mouse was placed in a mesh cage in one of the side chambers, whereas a novel object (a small rubber duck) was placed in a mesh cage in the other side chamber for the sociability trial. Placement of the novel mouse and novel objects were randomized between animals to eliminate side preferences. For the social novelty trial, an aged-matched novel mouse was placed in the mesh cage that had previously housed the novel object. The 3-chamber apparatus was cleaned with 70% ethanol between animal trials. The animals were habituated to the room for 45 min before the test, and the test was conducted under dim light (60 lux). The time spent in each chamber was then scored using DeepLabCut and SimBa, as described in the previous section.

Tissue Collection

One day after the conclusion of behavioural testing, the animals were sacrificed. Animals were killed by decapitation in a random fashion regarding testing groups between 09.00 h and 15.00 h. Trunk blood was collected in EDTA-containing tubes and centrifuged for 15 min at 10,000 g at 4°C. Plasma was collected and stored at −80°C for later analysis. Whole caecum and ileum were removed and snap-frozen on dry ice and stored at −80°C. Brain tissue was rapidly hand-dissected and snap frozen in dry ice and then stored at -80°C until further tissue processing. Spleens and mesenteric lymph nodes (MLNs) were dissected out of the animals and were processed for flow cytometry as described in the corresponding section.

Prefrontal Cortex and Caecal Metabolomics

The caecal and prefrontal cortex metabolome was analysed by MS-Omics as follows. Prefrontal cortex and caecal content were acidified using hydrochloride acid, and deuterium labelled internal standards were added. All samples were analyzed in a randomized order. Analysis was performed using a high polarity column (Zebron™ ZB-FFAP, GC Cap. Column 30 m x 0.25 mm x 0.25 µm) installed in a GC (7890B, Agilent) coupled with a quadropole detector (5977B, Agilent). The system was controlled by ChemStation (Agilent). Raw data was converted to netCDF format using Chemstation (Agilent), before the data was imported and processed in Matlab R2014b (Mathworks, Inc.) using the PARADISe software described by Johnsen et. Al (Johnsen et al 2017).

Peaks were quantified using area under the curve (AUC). Biostatistics were run in R (version 4.1.2) with the Rstudio GUI (version 1.4.1717). Principal-component analysis was performed on CLR-transformed values (Aitchison et al 2000). The PERMANOVA implementation from the vegan library was used to find structural differences between treatments on a compositional level. To find metabolites that were differentially abundant based on either stress or prebiotic supplementation, we fitted linear models using the CLR-transformed metabolite levels with both factors as explanatory variables. Linear models were also used to test for concordance or discordance of metabolite levels between the prefrontal cortex

and caecum, again including stress and prebiotic as additional explanatory variables. In order to assess differences between singular pairs of groups we used Tukey's HSD procedure. To correct for multiple testing (FDR) in tests involving metabolomics features, Storey's q-value posthoc procedure was performed with a q-value of 0.2 as a cut-off (Storey 2002). Custom scripts to analyze data can be found online at https://github.com/thomazbastiaanssen/Tjazi (Bastiaanssen et al 2022). Metabolomics figures were generated using ggplot2.

Cytokine Quantification

The levels of secreted interleukin-1β (IL-1β), interferon gamma (IFN-γ), IL-5, IL-6, IL-10 (not detected), tumor necrosis factor-α (TNFα) and CXCL1 (chemokine (C-X-C motif) ligand 1) were analysed with the Pro-inflammatory Panel 1 (mouse) V-PLEX Kit and the MESO QuickPlex SQ 120, SECTOR Imager 2400 (Meso Scale Discovery, Maryland, USA). Only data derived from duplicates with <15% CV were included in the analysis. Concentrations of cytokines were expressed in pg/mg of tissue. Limits of detection are represented in **Supplementary Table 1**.

Plasma Corticosterone Quantification

Corticosterone quantification of plasma (15 µL) collected from trunk blood at the sacrifice was performed using a corticosterone ELISA (Enzo Life Sciences) and was performed according to the manufacturer's instructions and was analysed as previously described (Bastiaanssen et al 2021, Gururajan et al 2022). A multi-mode plate reader (Synergy HT, BioTek Instruments) was used to quantify light absorbance in the assay, at 405 nm. Only data derived from duplicates with <15% CV were included in the analysis. Concentrations of plasma corticosterone was expressed in ng/mL. Limit of detection is represented in **Supplementary Table 1**.

Cell Isolation and Flow Cytometry

Spleens and mesenteric lymph nodes (MLNs) were dissected out of the animal, cleaned from fat tissue and stored in media (RPMI-1640 medium with L-glutamine and sodium bicarbonate (R8758, Sigma), supplemented with 10% FBS (F7524l, Sigma) and 1% Pen/strep (P4333, Sigma)) on wet ice for flow cytometry the same day.

Flow cytometry was performed as previously described ((Boehme et al 2020, Gururajan et al 2019). Spleenocytes were isolated by flushing the spleen with media using a syringe. The cell suspension was subsequently centrifuged, aspirated and incubated with 1 mL lysis buffer (Sigma, R7757) for 5 minutes. 10 mL media was added to dilute the lysis buffer and the cell suspension was poured over a 70 µm strainer, after which it was centrifuged and aspirated. $2x10^6$ cells were resuspended in 90 µL staining buffer and split into 2 aliquots for the staining procedure. MLNs were transferred onto a 70 µm strainer and disassembled using the plunger of a 1 mL syringe. The strainer was subsequently rinsed with 10 mL media, and the cell suspension was centrifuged, aspirated, $2x10^6$ cells were resuspended in 90 µL staining buffer, and split into 2 aliquots for the staining procedure.

For the staining procedure, 5 µL of FcR blocking reagent (Miltenyi, 130-092-575) was added to each sample. Samples were subsequently incubated with a mix of antibodies (**Supplementary Table 2**) for 30 minutes on ice, after which they were centrifuged, aspirated and fixed using 100 µL 4% PFA for 30 minutes on ice. Samples were finally centrifuged, aspirated and resuspended in staining buffer for flow cytometric analysis the following day on the BD FACSCalibur. Data was analysed using FlowJo (Version 10).

Statistical Analysis

All data are represented as mean ± SEM. Statistical analyses were conducted using SPSS 27 (IBM, USA). Normality was assessed employing the Shapiro–Wilk test and for equality of variances using the Levene's test. Non-parametric data were analysed with independent-samples Kruskal–Wallis test followed by pairwise comparisons adjusted by the Bonferroni correction for multiple tests, with a 95% confidence interval. Parametric data were analysed using two-way analysis of variance (ANOVA) and pairwise comparisons were assessed using a Tukey HSD adjustment. Time spent in interaction area, time spent in the corners, movement in the corners, entries in corners and social novelty were analysed using a general linear model integrating stress, diet and stimulus. Further, we utilized simple main effects to explore the individual group differences in these complex models, adjusted by the Bonferroni correction for multiple tests, as we

hypothesized a priori that the stress-only group would be significantly different from the other 3 groups. Statistical significance was set at $p \leq 0.05$.

Results

Prebiotic Supplementation Reverses Behavioural Impairments but not Endocrine Effects of Social Defeat Stress in Aged Animals

Following psychosocial stress, animals were tested in the social interaction test to assess the behavioural response of aged mice to stress. Stress significantly reduced the time spent in the interaction zone in Chow-fed animals ($F_{(1,34)}$=5.820, p=0.021, CTRL-Chow vs Stress-Chow p= 0.005, **Supplementary Figure 1a**), which was rescued by FOS-Inulin supplementation (Stress-Chow vs Stress-FOS-Inulin; p=0.027). An effect of diet was observed in time spent in the corner zones ($F_{(1,33)}$=6.650, p=0.015), and simple main effects showed stressed animals supplemented with FOS-Inulin spent significantly less time in the corners than stressed animals fed with chow (Stress-Chow vs Stress-FOS-Inulin; p=0.003, **Supplementary Figure 1b**). Movement inside the corner zones showed a significant effect of diet ($F_{(1,34)}$=4.594, p=0.039), as demonstrated by Stress-FOS-Inulin animals moving significantly less in the corners than Stress-Chow animals (p=0.011, **Supplementary Figure 1c).** Further, in regards to entries in the corner zone, there was a significant effect of diet ($F_{(1,33)}$=5.217, p=0.029) as FOS-Inulin supplementation in stressed animals significantly reduced entries in the corners (Stress-Chow vs Stress-FOS-Inulin, p=0.009, **Supplementary Figure 1d**). Considering a ratio of the time spent by the experimental animal facing the CD-1/interaction zone, there was an overall interaction effect of stress and diet ($F_{(1,37)}$=4.703, p=0.037) as revealed by an increase in the time facing the CD-1 by stressed animals supplemented with FOS-Inulin when compared to stressed controls (Stress-Chow vs Stress-FOS-Inulin, p=0.028, **Supplementary Figure 1e**). To further understand how our interventions shaped stress-responding in the mice we created a z-score combining the time spent in the interaction zone when the CD1 is present, time spent in the corner zones in presence and absence of CD1, the ratio of time spent facing the interaction zone, the entries and movement in the corner zones and the movement in the interaction area. Two-way ANOVA revealed an interaction effect of stress and diet ($F_{(3,37)}$=4.958, p=0.033), as revealed by a significant decrease in the Z-score in the Stress-Chow group compared to the non-stressed Control-Chow group (p=0.031) and followed by an amelioration of the stress phenotype by FOS-inulin supplementation, as shown

by an increase in the FOS-Inulin group in comparison to the Stress-Chow group (p=0.004, **Figure 2a**).

To assess the influence of FOS-Inulin supplementation on social behaviour in stressed aged animals, mice were tested in the three-chamber apparatus. While an overall significant interaction effect of stress and diet ($F_{(1,36)}$=1.364, p=0.250) was not observed, there was a significant overall preference between a novel and familiar mouse ($F_{(1,36)}$=14.914, p<0.001), which is not observed in animals fed with Chow (Control-Chow: p=0.175; Control-FOS-Inulin: p=0.006; Stress-Chow: p=0.328, Stress-FOS-Inulin: p=0.021, **Figure 2b**).

To assess the endocrine response to stress, plasma levels of corticosterone were assessed. Corticosterone in plasma collected at the time of the sacrifice showed a significant group effect (H(3)= 15.226, p=0.002). Corticosterone was elevated in Stress-Chow compared to Control-Chow (H= -17.167, p.adj = .003), which is not reversed by FOS-Inulin supplementation (H= 3.667, p.adj= 1, **Figure 2c**).

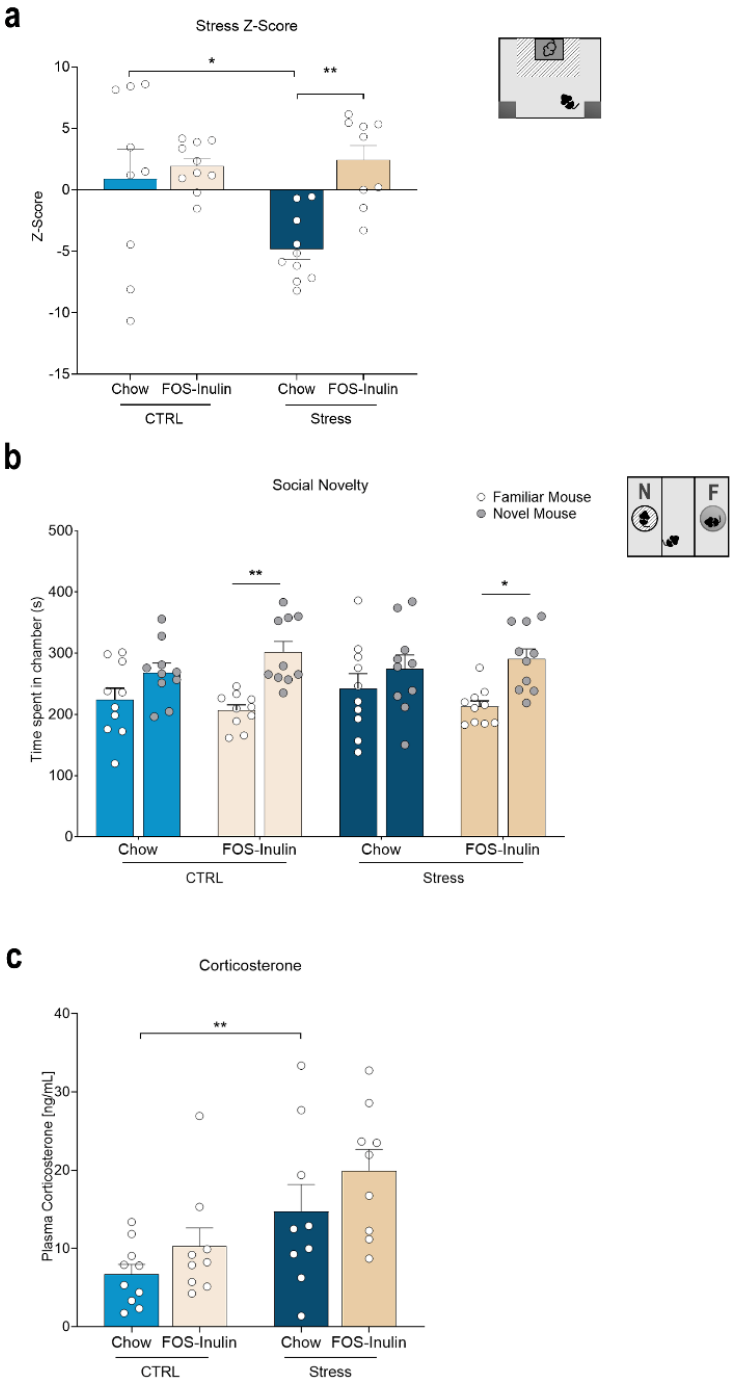

Figure 2 – FOS-Inulin supplementation ameliorates the effects of stress in aged mice. a) *Integrative Z-score of social interaction outputs; Stressed animals fed with Chow have a significantly reduced Z-score compared to control animals, which is restored by FOS-Inulin supplementation.* ***b)*** *FOS-Inulin supplemented mice, but not chow-fed animals, spend significantly more time in the novel mouse chamber.* ***c)*** *Plasma corticosterone is higher in aged animals fed with chow when exposed to stress, which is not recovered in stressed animals supplemented with FOS-Inulin. Results presented as mean+standard error of the mean (SEM). n=9-10 per group. *p<0.05, **p<0.01.*

FOS-Inulin Increases Ileal Pro-Inflammatory Cytokines

To understand how FOS-inulin supplementation impacts immune outputs in stressed aged mice, cytokine levels were assessed by MSD in the ileum, as this particular small intestine region is important in the context of immune and cytokine response to dietary factors (Toubal et al 2020). IFN-γ is a pro-inflammatory cytokine, previously linked to social behaviour (Filiano et al 2016), which is increased in aged mice (Baruch et al 2014) in the CNS, which was found to be increased by stress exposure ($F_{(1,35)}$=16.154, p=0.000332) and diet ($F_{(1,35)}$=9.653, p=0.004), though Two-Way ANOVA showed no interaction effects ($F_{(1,35)}$=2.175, p=0.150). IFN-γ was significantly increased in stressed animals supplemented with FOS-Inulin, when compared to mice treated with FOS-Inulin alone (Stress-FOS-Inulin vs Control-FOS-Inulin, p= 0.002) and when compared to control stressed animals (Stress-FOS-Inulin vs Stress-Chow, p= 0.014, **Figure 3a**), thus suggesting that stress and diet are driving IFN-γ concentrations independently. IL-6 is a pro-inflammatory cytokine that increases with age, while associated with poor cognitive function and predicts mortality in the elderly (Puzianowska-Kuźnicka et al 2016). Ileal concentrations of IL-6 were impacted by stress ($F_{(1,36)}$=14.563, p=0.001), as stressed animals fed with Chow show an increased tendency in comparison to control counterparts (Stress-Chow vs Control-Chow, p= 0.064), however this was unaltered by FOS-Inulin supplementation (Stress-FOS-Inulin vs Stress-Chow, p= 0.454), as FOS-Inulin supplementation increased IL-6 levels in stressed animals, when compared to their controls (Stress-FOS-Inulin vs Control-FOS-Inulin, p=0.04, **Figure 3b**). Notably, FOS-Inulin supplementation seems to exacerbate the levels of IFN-γ and IL-6 in the ileum of aged stressed animals. Furthermore, while IL-5 shows an effect of stress ($F_{(1,31)}$=4.644, p=0.039), there is only a non-significant trend for increased IL-5 in stressed animals supplemented with FOS-Inulin (Stress-FOS-Inulin vs Control-Chow, p=0.083, Supplementary Figure 2a). Moreover, TNF-α concentrations show a significant effect of group (H(3)= 11.110, p=0.011), as FOS-Inulin supplementation increases TNF-α ileal levels when compared to the control group (Stress-FOS-Inulin vs Control-Chow, H= -16.0175 p.adj =0.006, Supplementary Figure 2b). Finally, CXCL1 and IL-1β did not show a group effect (CXCL1: H(3)= 3.082, p=0.379; IL-1β: H(3)= 7.733, p=0.052, Supplementary Figure 2c and 2d).
Flow cytometry in mesenteric lymph nodes and spleen of general immune populations did not reveal any involvement of the peripheral immune system in

the FOS-Inulin supplementation in response to stress in aging (Supplementary Figures 3 and 4).

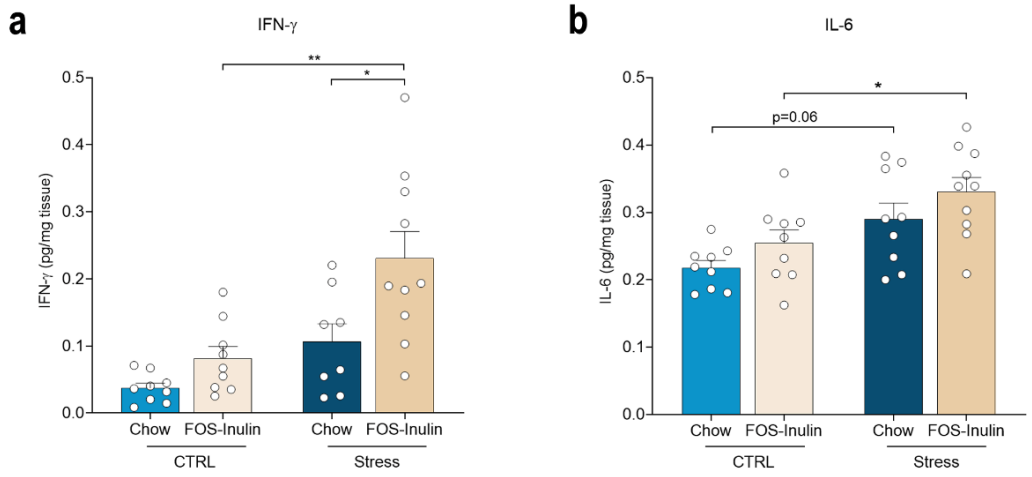

Figure 3 – FOS-Inulin supplementation increases pro-inflammatory cytokines in the ileum. a) *FOS-Inulin supplementation significantly increases the concentration of IFN-γ in the ileum of stressed aged animals, when compared with stressed animals fed with chow and control animals fed with FOS-Inulin;* ***b)*** *Stressed aged animals show increased IL-6 concentration in the ileum (non-significantly), which is further inflated by FOS-Inulin supplementation. Results presented as mean±standard error of the mean (SEM). n=8-10 per group. *p<0.05, **p<0.01, ***p<0.001*

FOS-Inulin Rescues Metabolite Levels in the Prefrontal Cortex of Aged Stressed Animals

To understand if gut microbial-derived metabolites could possibly drive the positive prebiotic response to stress in aged mice, metabolomics analysis was performed in the caecum and prefrontal cortex of stressed and non-stressed aged mice. In total, 205 metabolites were identified in the caecum and 105 were detected in the PFC. In the caecum, diet significantly impacted the caecum metabolome, whereas in the PFC, stress was the main clustering factor (**Figure 4a and 4b**). In the caecum, 139 metabolites were found altered by diet in the caecum, but no stress or interaction effects were found (**Supplementary Table 3**). In the prefrontal cortex, 8 metabolites were altered by stress or diet after post-hoc correction: 3-Methylhistidine, 4-Hydroxybenzaldehyde, apocynin, 2-hydroxy-3-methylbutyric acid, ethyl sulfate, N-acetyl ornithine, spermine and trimethylamine N-oxide. Of these, only 4-Hydroxybenzaldehyde and spermine show an interaction effect of stress and diet (**Figure 4c**). 4-Hydroxybenzaldehyde

is a naturally occurring benzaldehyde that has been shown to boost antioxidant activity and wound healing (Kang et al 2017, Wang et al 2021) – despite showing an interaction effect of stress and diet (β = -0.42, p= 0.003, q=0.0994), as this metabolite is increased in the PFC of Stress-Chow animals (Stress-Chow vs Control-Chow , p=0.019), FOS-Inulin supplementation does not significantly reverse this increase in stressed aged animals (p=0.109). Spermine, a natural polyamine reported to modulate autophagy-related age effects (Xu et al 2020), also presented an interaction effect of stress and diet (β = 0.349, p= 0.004, q=0.0994) as it was found to be decreased in the prefrontal cortex of stressed aged animals (Stress-Chow vs Control-Chow p=0.036) and presents a non-significant tendency to be recovered by FOS-Inulin supplementation (Stress-FOS-Inulin vs Stress-Chow, p= 0.072).

Further, stress impacted the prefrontal cortex concentration of apocynin (β = 0.308, p= 0.011, q=0.178), as stressed aged mice show a tendency for increase of apocynin (Stress-Chow vs Control-Chow p=0.054), which is not reduced by FOS-Inulin supplementation (Stress-FOS-Inulin vs Stress-Chow, p=0.746). Likewise, ethyl sulfate concentrations were also affected by stress (β = -0.223, p= 0.009, q=0.178), as reflected by a reduction in ethyl sulfate in the PFC of stressed aged mice (Stress-Chow vs Control-Chow p=0.045), which shows a tendency to be partially restored by FOS-Inulin supplementation (Stress-FOS-Inulin vs Stress-Chow, p=0.066).

Additionally, FOS-Inulin supplementation significantly impacted the concentrations of 3-Methylhistidine (β = 0.411, p= 0.004, q=0.178), as it was increased in control animals on a FOS-Inulin supplemented diet (Control-FOS-Inulin vs Control-Chow, p=0.02) and interestingly reduced by stress exposure (Stress-FOS-Inulin vs Control-FOS-Inulin, p=0.03). Likewise, 2-hydroxy-3-methylbutyric acid was significantly altered by diet (β = 0.197, p= 0.006, q=0.980), as it is increased in the PFC of mice on a FOS-Inulin supplemented diet in control animals (Control-FOS-Inulin vs Control-Chow, p=0.03) and in animals exposed to stress (Stress-FOS-Inulin vs Control-Chow, p=0.03). Moreover, N-acetyl ornithine was also significantly affected by diet (β = 0.189, p= 0.006, q=0.165), since FOS-Inulin supplementation increased concentrations of this metabolite in PFC of control (Control-FOS-Inulin vs Control-Chow, p=0.03) and stressed animals

(Stress-FOS-Inulin vs Stress-Chow, $p=7.72x10^{-5}$). Similarly, trimethylamine N-oxide concentrations in the PFC were largely impacted by diet ($\beta = -0.5136$, $p= 6.73x10^{-5}$, $q=0.007$), since FOS-Inulin supplementation reduces trimethylamine N-oxide in control animals (Control-FOS-Inulin vs Control-Chow, p=0.0004) and presents a trend for reduction in stressed animals (Stress-FOS-Inulin vs Stress-Chow, p=0.06).

In summary, stress mainly altered the concentration of apocynin and ethyl sulfate in the prefrontal cortex, while FOS-Inulin supplementation mainly changed the concentration of 3-Methylhistidine, 2-hydroxy-3-methylbutyric acid, and trimethylamine N-oxide, while 4-Hydroxybenzaldehyde and spermine show an interaction effect of stress and diet. This suggests that this dietary intervention modulates the levels of spermine and 4-Hydroxybenzaldehyde, in the prefrontal cortex of stressed aged mice, posing as a potential new avenue to explore age-dependent stress effects.

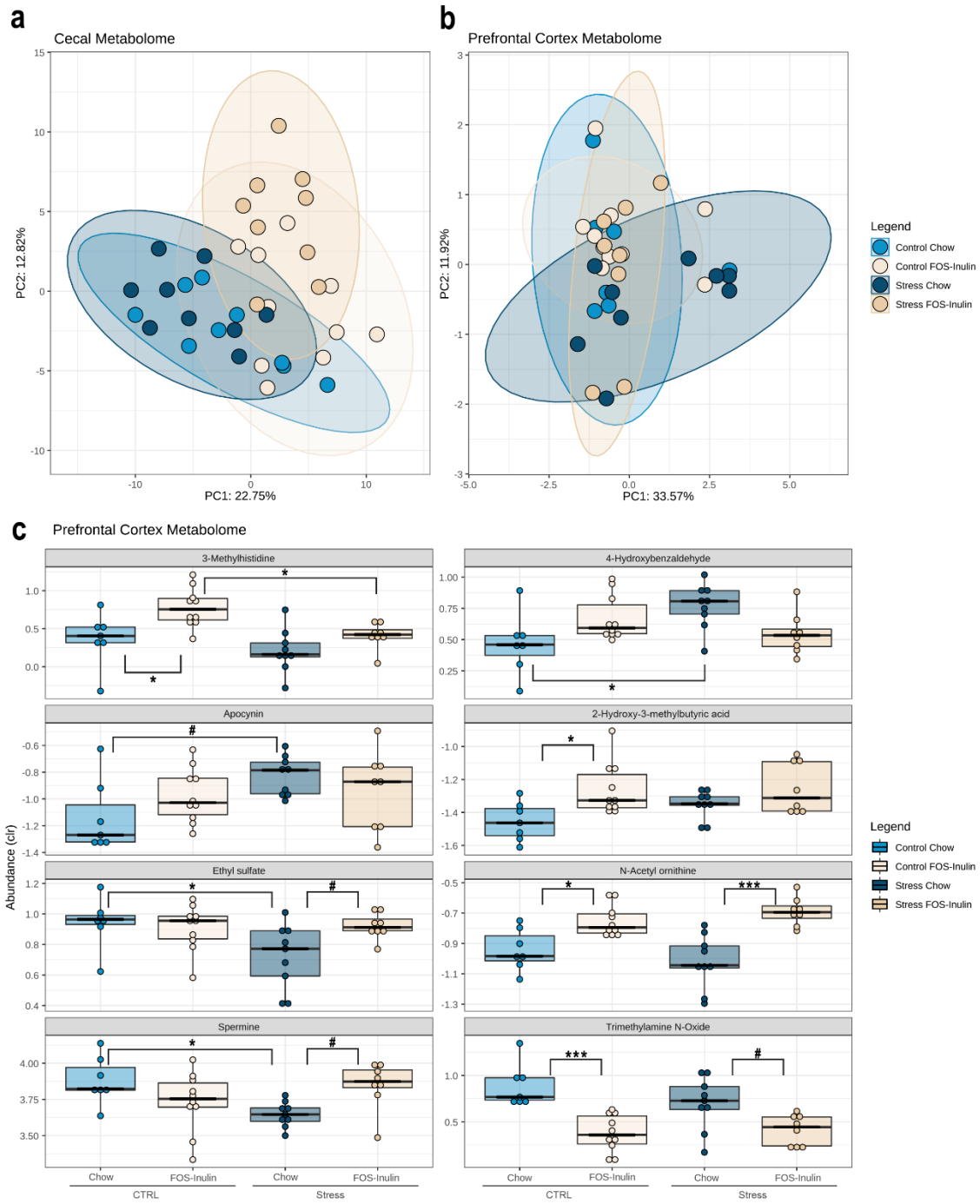

Figure 4 – Spermine and 4-Hydroxybenzaldehyde are modulated by FOS-Inulin supplementation in the prefrontal cortex of aged-stressed animals. *a-b) Principal component analysis showing the main effect of diet in the metabolome of the caecum a) and main effect of stress in the b) prefrontal cortex of stressed aged mice. c) Boxplots showing the centered log-ratio transformed (clr) abundance of prefrontal cortex metabolites that displayed a significant effect of stress, diet, or an interaction between the two. In the prefrontal cortex, 8 metabolites were altered by stress and/or diet – metabolites were altered in the PFC of stressed aged mice, which were restored in FOS-Inulin supplemented animals. For the boxplots, boxes represent the limits of the interquartile range, the horizontal line represents the median point, and the whiskers represent the full data range. n=7-10 per group. #p<0.1, *p<0.05, **p<0.01, ***p<0.001*

Discussion

An underexplored aspect of aging is the mechanisms underlying the response to stress, and its consequent behavioural outcomes. In this study, we demonstrated a clear interaction between a dietary intervention targeting the microbiome in modulating the stress response in aged mice. In particular, the present study demonstrates that FOS-Inulin supplementation improves social interaction in response to stress in aged mice and regulates spermine and 4-Hydroxybenzaldehyde concentrations in the prefrontal cortex.

In line with previous reports from our group (Scott et al 2017), aged mice exhibit social novelty deficits in contrast to their young counterparts. In this study, we further observed that stress did not exacerbate social novelty deficits, but that FOS-Inulin supplementation improves overall social recognition in aged mice, thus implying that a prebiotic dietary intervention in aging can mitigate age-dependent behavioural deficits. Social defeat exposure also elevated plasma corticosterone levels – however, prebiotic supplementation did not result in the reversal of the corticosterone levels, suggesting a dissociation between the impact of the prebiotic intervention on the behavioural and physiological response to stress.

Given the cumulative evidence that the gut microbiome is intertwined with age-related peripheral and neuroinflammation (Boehme et al 2020, Claesson et al 2012, Scott et al 2017), we explored the potential mediation of age-related effects by inflammatory factors to understand if the FOS-Inulin supplementation effects were related to inflammatory factors. In this study, analysis of pro-inflammatory cytokines was focused on the ileum, as this region of the small intestine is important for the sampling of antigens from diet, hence influencing the immune system (Brown & Esterházy 2021). Contrary to our expectations, FOS-Inulin supplementation was found to increase the concentration of pro-inflammatory cytokines in the small intestine in stressed animals. A few reports have described that FOS and inulin supplementation can increase inflammation in the colon of mice in an immune dysregulated colitis model (Singh et al 2019). Given that aging induces a low-grade inflammatory profile, and that aging interferes with the resolution of acute inflammation (Arnardottir et al 2014), it is not completely surprising that the prebiotic intervention could provoke moderate inflammation in

the small intestine of these animals. Further, the exacerbation of pro-inflammatory markers by FOS-Inulin supplementation on stressed animals could be a reflection of a 'double hit' effect as stress is a known inducing factor of inflammatory markers (Cruz-Pereira et al 2020, Marsland et al 2017). Surprisingly, in peripheral immune structures, namely the mesenteric lymph nodes and spleen, we found no changes to general immune cell populations, suggesting that the behavioural outcomes are not related to extra-intestinal modulation of inflammation by stress or FOS-Inulin (Supplementary Figures 2 and 3).

It has previously been reported that FOS-Inulin dietary intervention shapes gut microbiome in middle-aged mice (Boehme et al 2020), and that prebiotic fibres are known to modulate the gut microbiome (O'Connor et al 2020), and further that the metabolome is intrinsically connected with the gut microbiome (Garza et al 2020, Valles-Colomer et al 2019). Metabolomics were performed in the caecum and prefrontal cortex to profile the microbial metabolite levels in an intestinal structure that is reshaped by dietary fibres (Drew et al 2018) and an important brain region for emotional processing and social behaviour altered in aging (Franklin et al 2017, Yan & Rein 2022). As expected, diet was the main factor to shape the caecal metabolome, which follows consistent observations that dietary fibres change the gut microbiota in humans (Le Bastard et al 2020, Vandeputte et al 2017) and in rodents (Drew et al 2018, Fuhren et al 2021). Curiously, in an aged human cohort, despite the reshaping of the gut microbiota, inulin intake did not impact immune cell numbers in the peripheral blood (Kiewiet et al 2021), which aligns with our results (Supplementary Figures 2 and 3).

In the PFC, 4 metabolites were altered by the prebiotic supplementation alone, that may play an important role in the context of aging: 3-methyl-L-histidine, acetylornithine, trimethylamine-N-oxide and 2-hydroxy-3-methylbutyric acid. In dementia patients, 3-methyl-L-histidine has been found declined in the blood and is associated with frailty (Teruya et al 2021), while its supplementation suppresses glial inflammation and ameliorates neurovascular-unit dysfunction in an aged Alzheimer's disease model (Kaneko et al 2017), and in this study is increased with the prebiotic supplementation. Curiously, acetylornithine, also positively modulated by prebiotic supplementation in our study, has also been found elevated in the serum of dementia patients (Weng et al 2019), while being reduced

in the cerebral cortex of aged mice (Ding 2021). Trimethylamine-N-oxide, which in this study was reduced by prebiotic supplementation, is increased in both elderly humans and aged mice, and can accelerate brain cellular senescence, while enhancing cognitive impairment (Li et al 2018a). Lastly, 2-Hydroxy-3-Methylbutyric acid was identified as the metabolite responsible for the promotion of intestinal epithelial cell proliferation upon *Lactobacillus paracasei* probiotic administration (Qiao et al 2022) and is increased by FOS-Inulin supplementation in the prefrontal cortex. While it is remarkable that the levels of these metabolites in the prefrontal cortex are affected by prebiotic supplementation and have previously been reported to be involved in aging, the functional consequences of these alterations remain unclear as well as the underlying mechanism.

In our analysis, we found that stress alone, independently affected only two metabolites in the prefrontal cortex of aged animals after *post-hoc* correction: apocynin and ethyl sulfate. Apocynin suppresses reactive oxygen species, partially reversing the aging process in mesenchymal stem cells and boosts osteogenesis (Sun et al 2015), and in this study is found increased in the prefrontal cortex of aged animals exposed to stress. Ethyl sulfate is a metabolite classically linked to ethanol degradation (Helander & Beck 2005), but to the best of our knowledge this is still underexplored in the context of brain metabolome.

Here, we further show that the combination of prebiotic dietary intervention and social defeat stress in aged mice, is associated with alterations in the levels/abundance of two metabolites in the prefrontal cortex: spermine and 4-Hydroxybenzaldehyde. Spermidine, the precursor of spermine, has been found to extend the lifespan by promoting autophagy (Aman et al 2021). Spermine and spermidine delay aging-related cognitive impairments by enhancing autophagy and mitochondrial function in the brain of a mouse model of accelerated aging (Xu et al 2020). Further, spermine and spermidine levels have been found to be enhanced in the blood of centenarians (Pucciarelli et al 2012). Here, we found that levels of spermine in the aged prefrontal cortex are reduced by stress exposure, and that the prebiotic intervention recovered the spermine levels. Spermine has been shown to prevent LPS-induced memory deficits (Frühauf-Perez et al 2018) and has been found to be slightly reduced in the brains of rats after restraint stress (Hayashi et al 2004). Furthermore, spermine is also an

NMDA receptor agonist, and has been shown to be increased in the PFC of aged rats (Gupta et al 2012). Given that aging has been linked to reduced NMDA receptor function (Gramuntell et al 2021), it is feasible to associate elevated CNS spermine levels with age-dependent cognitive decline. Taken together, this could potentially suggest that stress in aged mice may increase NMDA receptor function potentially through the reduction of spermine concentration, which in turn can result in excitotoxicity, in a similar fashion to what is observed in Alzheimer's disease (Wang & Reddy 2017). Further studies are needed to dissect this hypothesis.

4-Hydroxybenzaldehyde is a relatively underexplored compound isolated from *Gastrodia elata*, a common Chinese herbal medicine that has been shown to boost acute wound healing and is altered in the plasma of middle-aged mice of a short-longevity model (Kadota et al 2021). In this study, FOS-Inulin reduced the levels of this metabolite upon stress exposure. It is challenging to draw objective conclusions from this observation, as the functional properties of this particular metabolite still remains mostly unexplored – however, it is possible that it could represent an interesting target for future experiments (or dietary modulation) as there is still a considerable number of metabolites that remain unidentified (Bar et al 2020). Taken together with the relative novelty of 4-Hydroxybenzaldehyde in this context, further studies dissecting the involvement of these metabolites in age-dependent effects will be crucial.

Conclusions

In conclusion, this study provides evidence that prebiotic supplementation with FOS-Inulin ameliorates the stress response in aged mice at a behavioural and metabolic level. This was not associated with modulation of the immune system or HPA axis but through the regulation key age-associated metabolites, spermine and 4-Hydroxybenzaldehyde, in the PFC. Further studies targeting spermine and 4-Hydroxybenzaldehyde and their metabolic pathways will be essential to uncover the specific effects and mechanisms underpinning the impact of these metabolites on the age-related stress-induced behavioural phenotypes.

Supplemental Material

To understand if FOS-Inulin supplementation could impact immune outputs, the levels of different pro-inflammatory cytokines and chemokines were assessed in the ileum. However, the levels of IL-5 (Figure 2a), TNF-α (Figure 2b), CXCL1 (Figure 2c), and IL-1β (Figure 2d) were not impacted by stress exposure or prebiotic supplementation in aged mice.

Moreover, flow cytometry was used to assess general immune populations, however stress exposure and prebiotic supplementation did not significantly impact these markers in the mesenteric lymph nodes (Figure 3) or the spleen (Figure 4).

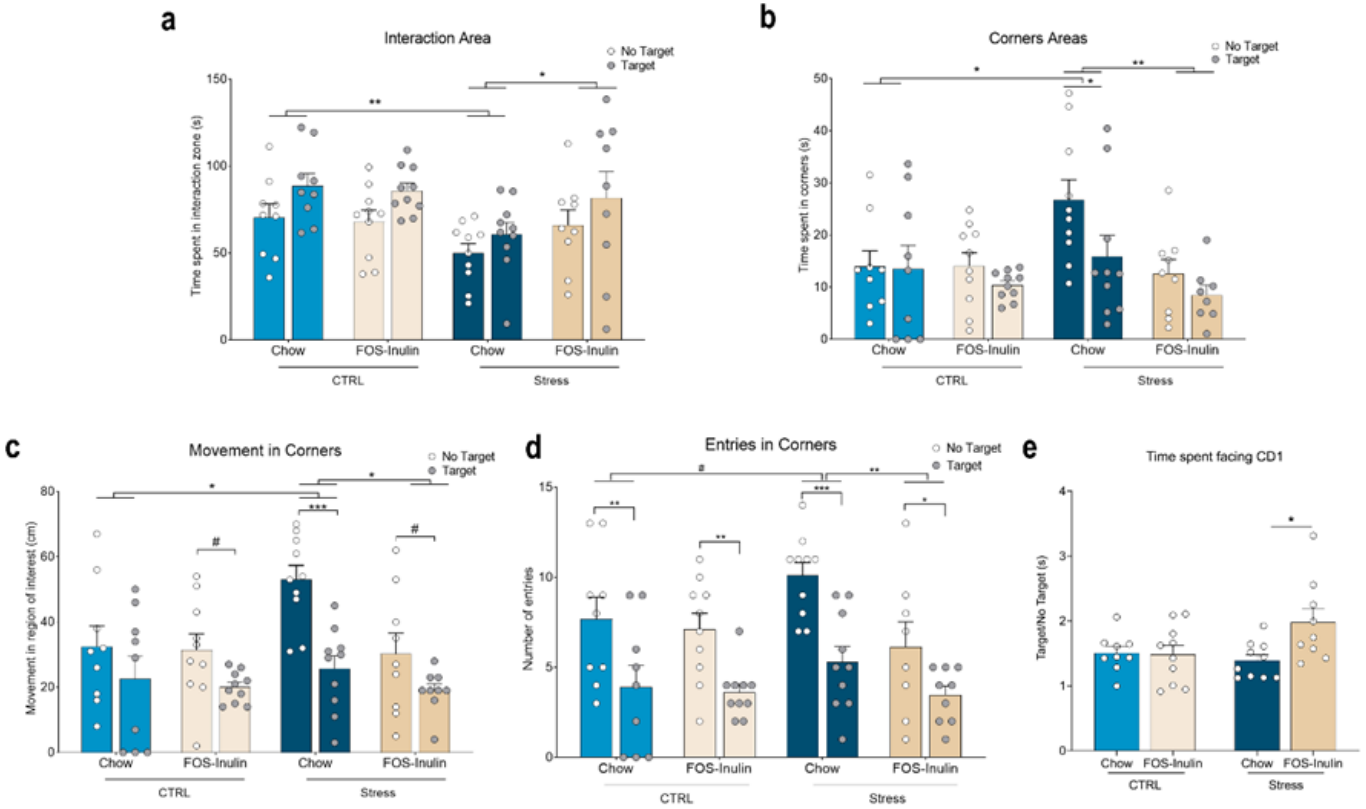

Figure 1 - FOS-Inulin supplementation effects on stress response in the social interaction test in aged mice. *a) Time spent in the interaction area. Stress-Chow animals spend significantly less time than their controls in the interaction zone. b) Time spent in the corner zones. Stressed animals fed with FOS-Inulin spend significantly less time in the corners than stressed animals fed with chow. c) Movement in corners – FOS-Inulin supplementation significantly reduces the movement in corners in aged animals exposed to stress (Stress-Chow vs Stress-Inulin). d) Entries in corners – all animals show less entries in the corner zones when the CD-1 is present in the arena. e) Time spent by the experimental animal facing the CD1 is significantly increased in stressed animals supplemented with FOS-Inulin. Results presented as mean±standard error of the mean (SEM). n=8-10 per group. **p<0.01, ***p<0.001*

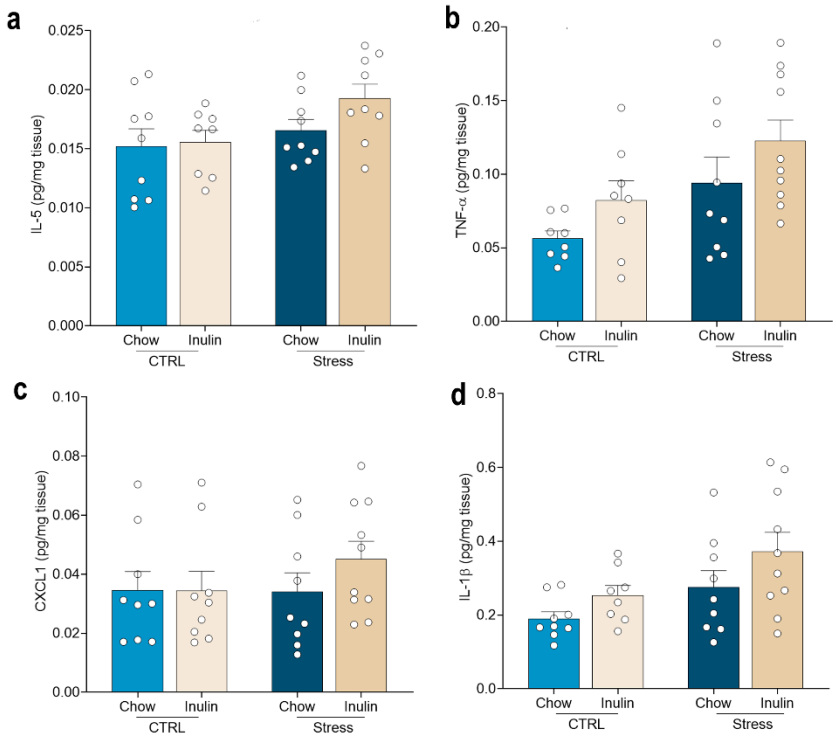

Figure 2 – Stress does not impact further ileal cytokine levels. *a) FOS-Inulin supplementation shows a trend for increased IL-5 in the ileum of stressed aged animals, when compared with control animals fed with chow. b) TNF-α is significantly increased in stressed animals with FOS-Inulin supplementation, when compared with control animals fed with chow. Stress and diet did not impact the levels of CXCL1 (c) or IL-1β (d) in the ileum of aged mice. Results presented as mean±standard error of the mean (SEM). n=7-10 per group. **p<0.01*

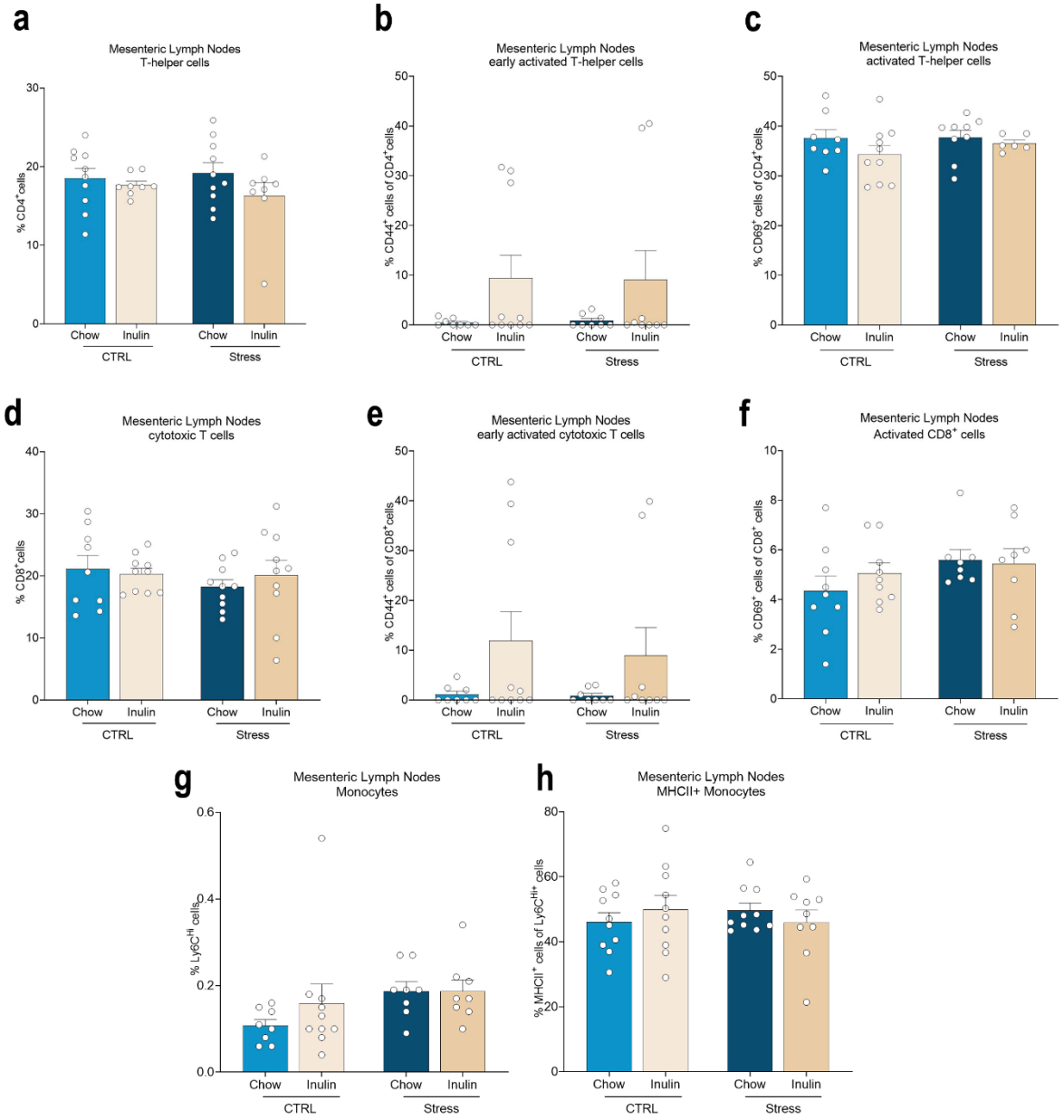

Figure 3 – Flow cytometry analysis of general populations in the mesenteric lymph nodes reveals no impact caused by stress or diet. *a) Percentage of CD4+ cells, b) percentage of CD4+CD44+ cells, c) percentage of CD4+CD69+ cells, d) percentage of CD8+ cells, e) percentage of CD8+CD44+ cells, f) percentage of CD8+CD69+ cells, g) percentage of Ly6CHi+ cells, h) percentage of Ly6CHi+ MHCII+ cells. Results presented as mean±standard error of the mean (SEM). n=8-10 per group.*

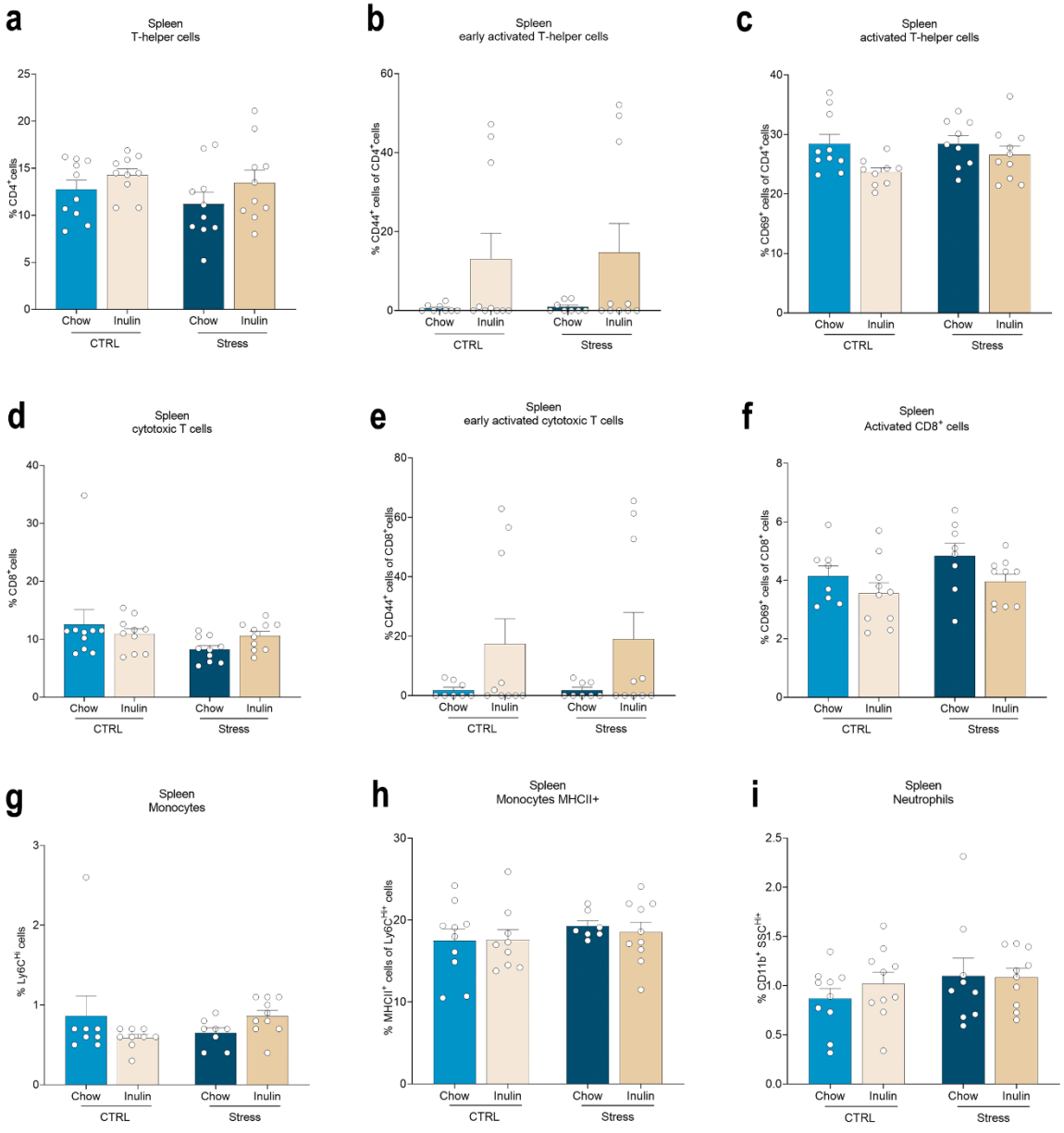

Figure 4 – Flow cytometry analysis of general populations in the spleen reveals no impact caused by stress or diet. *a) Percentage of CD4+ cells, b) percentage of CD4+CD44+ cells, c) percentage of CD4+CD69+ cells, d) percentage of CD8+ cells, e) percentage of CD8+CD44+ cells, f) percentage of CD8+CD69+ cells, g) percentage of Ly6C^{Hi}+ cells, h) percentage of Ly6C^{Hi}+ MHCII+ cells, i) percentage of CD11b+SSC^{Hi} cells. Results presented as mean±standard error of the mean (SEM). n=8-10 per group.*

Supplementary Table 1 – Limit of detection for cytokine and corticosterone quantification

	Median LLOD (pg/mL)	**LLOD Range (pg/mL)**
IL-1β	0.11	0.11-1.030
IFN-γ	0.04	0.01-0.14
IL-5	0.06	0.06-590
IL-6	0.61	0.06-1.38
TNF-α	0.13	0.13 - 403
CXCL1	0.24	0.24-1.230
CORT	27	32-20.000

Supplementary Table 2 – Antibodies used for cell sorting

Marker	***Company***
CD11b	Miltenyi
CD69	Miltenyi
CD62L	Biolegend
CD8a	Miltenyi
LY6C	Miltenyi
FVS780	BD Biosciences
MHC-II	Biolegend
CD4	Biolegend
CD44	BD Biosciences

Supplementary Table 3 – Metabolites altered in the caecum

Supplementary Table 3

Chapter 4

Early-life Microbiota Depletion and Gut-Brain Axis Function: An Investigation into the Later-Life effects of FOS-Inulin Supplementation

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Abstract

There is increasing evidence that the initial seeding of the gut microbiota is essential for the developmental trajectory of the immune and central nervous systems. Disruption of the optimal assembly of the gut microbiota has been linked to pervasive long-term effects on brain function and behaviour. Here we investigate if microbiota disruption in the mouse achieved by maternal exposure to an antibiotic cocktail (ampicillin; gentamicin, vancomycin and imipenem) in late pregnancy (E12.5-E14.5) through early postnatal life (PND21) induces long lasting effects on anxiety-like, depressive-like and social behaviour dimensions, as well as intestinal cytokine secretion in adulthood, and whether this can be rescued by prebiotic (Fructo-Oligosaccharide (FOS)-Inulin) supplementation in later life We found that maternal microbiota disruption induces sex- and region-dependent alterations in intestinal cytokine secretion, but had minimal effects on the behavioural profile in adulthood. FOS-Inulin supplementation partially countered some of these immune alterations. Given these results, it is important to further understand its impact on long-term immunity.

Introduction

From the first moments of life, microbial colonization takes place in a mutually beneficial relationship with the host (Ratsika et al 2021). Both the assembly, and the composition of the gut microbiome in early life is crucial for the priming of the immune system as well as the developmental trajectory of the central nervous system (CNS) (Gensollen et al 2016, Ratsika et al 2021). Prophylactic use of maternal antibiotics is on the rise – it is estimated that 20-25% of pregnant women are exposed to antibiotics before childbirth and approximately 80% of prescriptions to pregnant women are antibiotics (Dierikx et al 2020). Antibiotics can impact early colonization profiles via alteration of the maternal intestinal and vaginal microbiome, which initially seeds the infant microbiome highlighting the importance of evaluating its impact in early life microbiota disruption.

There has been an increasing recognition that early gut microbiota colonization patterns offer critical 'windows of opportunity' in which microbial-host interactions determine immune priming (Gensollen et al 2016). In fact, gestational colonization of germ-free (GF) pregnant mice modifies the intestinal immune populations in the offspring, conferring enhanced immune responses (Gomez de Agüero et al 2016), further accentuating the importance of appropriate gut microbial colonization in early life. Mounting evidence suggests that early life antibiotic exposure is associated with an increased risk of developing inflammatory bowel disease (Hviid et al 2011), allergy (Ahmadizar et al 2018, Baron et al 2020b), obesity (Baron et al 2020a) along with poorer childhood neurocognitive outcomes (Slykerman et al 2019). Interestingly, early life microbiota disruption during different critical windows has been linked to behavioural impairments (Desbonnet et al 2015b, Lach et al 2020, Morais et al 2020). In particular, maternal gut microbiota disruption alters social behaviour, anxiety-like behaviour and aggression in the offspring in a sex-dependent manner in mice (Champagne-Jorgensen et al 2020, Leclercq et al 2017, O'Connor et al 2021), therefore encouraging further exploration of the consequences of maternal antibiotic exposure in offspring later in life.

Diet is a key modulator of gut microbial populations (Carmody et al 2015, David et al 2014, Muegge et al 2011), therefore representing a rapid and accessible avenue for manipulation of the gut microbiota (David et al 2014). Prebiotics,

substrates that are selectively used by commensal microbes, provide health benefits to the host and represent an invaluable dietary intervention (Sanders et al 2019). Inulin-type fructan supplementation has been shown to both shape gut microbial composition in children and reduce infection rate, thus suggesting that prebiotic supplementation may modulate immune responses in young children (Lohner et al 2018). Fructo-oligosacharides (FOS) is a widely explored dietary fibre, that can effectively reshape the gut microbiota composition by promoting the growth of Bifidobacterium (Tandon et al 2019). FOS, in conjunction with GOS, have also been found to rescue selective behavioural alterations in mice delivered by Cesarian section (Morais et al 2020), thus suggesting beneficial CNS outcomes. Furthermore, dietary intervention with a complex short- and long-chain prebiotic, oligofructose-enriched inulin FOS-Inulin supplementation in middle aged mice modulates the gut microbiome, an effect which was associated with reduced neuroinflammation and altered behavioural responses (Boehme et al 2020). We recently showed that FOS-Inulin supplementation in stressed aged mice ameliorates the behavioural response to stress while modulating metabolite levels in the caecum and prefrontal cortex (Cruz-Pereira et al 2022). We hypothesize that a prebiotic dietary intervention in adulthood moderates adverse behavioural and immune outcomes in offspring from microbiota-depleted dams during pregnancy through weaning.

Materials and Methods

Animals

All experiments were conducted in accordance with European Directive 86/609/EEC, Recommendation 2007/526/65/EC, and approved by the Animal Experimentation Ethics Committee of University College Cork (approval number). 8-week-old male and female C57BL/6 were obtained (Envigo, UK) and breeding began after 2 weeks of acclimatization to the holding room. Animals were kept under a 12-h light/dark cycle, with a temperature of 21 ± 1 °C and humidity of 55 ± 10%. Mice were kept under a 12 h light/dark cycle (ON 7:30AM, OFF 7:30PM) in a temperature/humidity-controlled environment (21 °C, 55.5%) with food and water *ad libitum*. Offspring was weaned at P21 and housed in groups of 2-3/cage, from different litters to avoid litter effects.

Antibiotic Treatment

To deplete the maternal gut microbiota, pregnant female mice were exposed to antibiotics in the drinking water from mid-gestation (E12-E14) until weaning (P21). Dams were exposed *ad libitum* to an antibiotic cocktail (ampicillin (1g/L), gentamicin (1g/L), vancomycin (0.5g/L) and imipenem (0.25g/L)) or water. This was chosen as a wide-spectrum antibiotic cocktail with limited penetrance beyond the gastrointestinal tract (Maddison et al 2008, Nau et al 2018), and the doses were adjusted considering previous studies in the group delivering these antibiotics in the drinking water (Lach et al 2020, O'Connor et al 2021). Antibiotics were dissolved in water and changed every second day. Control animals received water, which was also replaced every second day.

Study Design

From approximately 12 days after the observation of a vaginal plug until offspring were weaned at PND21, pregnant and lactating mice were exposed to an antibiotic cocktail in the drinking water or vehicle. At 6 months old, when offspring were fed with chow or FOS-inulin supplemented diet for 4 weeks and underwent behavioural testing. Tests were performed in the following sequence: Three chambers test (3Ch), Open field (OF), Elevated Plus Maze (EPM) and Forced Swim Test (FST) (**Figure 1**). Animals were culled at the end of the experiment and tissue was collected for further analysis.

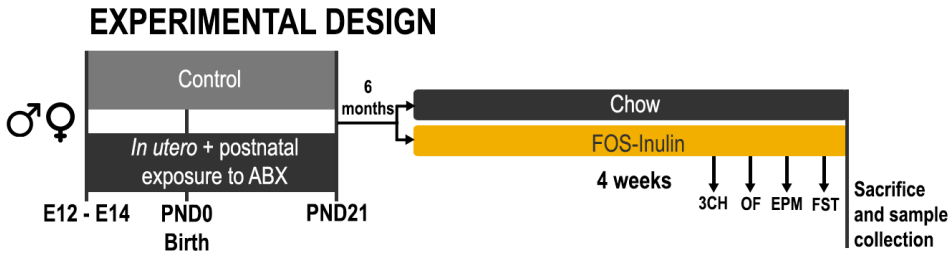

Figure 1 - Experimental Design. *Dams were exposed to an antibiotic cocktail in the drinking water from embryonic day 12-14 until weaning. Subsequently, the male and female offspring were submitted to three weeks of FOS-Inulin dietary supplementation, after which were assessed for social, anxiety-like, and depressive-like behaviour. Abbreviations: ABX – antibiotics; E – embryonic day; PND – postnatal day; 3Ch – 3-chambers test; OF – Open-Field Test; FST – Forced Swim Test*

Diet

Four weeks prior to the beginning of behavioural testing, mice were fed chow (ssniff-Spezialdiäten GmbH, Soest, Germany) enriched with 10% FOS-Inulin (mixture of 92 ± 2% Inulin and 8 ± 2% Fructooligosaccharide, Orafti®Synergy1; BENEO-Orafti N.V., Tienen, Belgium) or control chow (ssniff-Spezialdiäten GmbH, Soest, Germany) *ad libitum*, as previously described (Boehme et al 2020).

Behaviour

3-Chambers Test

Social behaviour was evaluated using the 3-chamber social interaction test, in which time spent interacting with a novel conspecific is examined against time spent with a novel object or familiar conspecific. It assumes that mice inherently prefer both novel and social stimuli, and therefore spend more time investigating these than familiar or non-social stimuli a novel conspecific to a familiar one.
The test arena consisted of 3 chambers; the left and right chambers measured 13.5 x 20 x 20 cm and the centre chamber was 9 x 20 x 20 cm. A solid partition separated the chambers, with a small opening allowing access to the other chambers. During the habituation phase, the mouse was placed into the centre chamber and then allowed to freely explore the empty left and right chambers for 10 min. Then, for the sociability phase an age- and sex-matched conspecific is placed in a mesh cage, and an object (rubber duck) is placed in the opposite mesh cage. For the social novelty trial, an aged-matched novel conspecific mouse was placed in a mesh cage, on the opposite chamber to a familiar conspecific mouse. Placement of the conspecific mice and the object were counter-balanced between animals to eliminate side preferences. The 3-chamber apparatus was cleaned with 70% ethanol and allowed to evaporate between animals. The time spent in each chamber was then measured. The animals were habituated to the room for 45 min before the test, and the test was conducted under 60 lux. The time spent in each chamber was then scored using the Behavioural Observation Research Interactive Software (BORIS) (Friard & Gamba 2016).

Open Field Test

To investigate the response to locomotor activity and a novel environment, mice were placed into open arena (40 × 32 × 23 cm) under ~60 lux lighting and allowed to explore for 10 minutes, as described before (Burokas et al 2017). Experiments were recorded using a ceiling camera for further analysis using Ethovision software (11.5 version, Noldus, UK). Distance travelled, time in centre and the latency to enter a virtual central zone (defined at 50% away from the edges) was calculated.

Elevated Plus Maze Test

To assess anxiety-like behaviour, the elevated plus maze (EPM) was used as previously described (Burokas et al 2017). The apparatus is constituted by a grey plastic cross-shaped maze, elevated 1m from the floor composed by two closed (safe) and two open (anxiogenic) arms (50 × 5 × 15 cm walls or 1 cm – 'no wall'). Experiments were performed under red light (~5 lux). Mice were individually placed in the centre of the maze facing an open arm (to avoid immediate entrance into a closed one) and were allowed to freely explore for 5 minutes. The apparatus was cleaned with 70% ethanol and allowed to evaporate between animals to avoid olfactory cues. Experiments were recorded using a ceiling camera for further analysis using BORIS (Friard & Gamba 2016). Time spent in the open arms, entries ratio (entries into open arms divided by total entries into any arm × 100), as well as stress-induced defecation were scored (entrance in an arm was defined when all four paws being positioned inside the corresponding arm).

Forced Swim Test

Mice were gently placed in a cylinder (24x21 cm diameter) containing 17-cm-depth water (23 ± 2°C). The test lasted for 6 min with activity being recorded by a ceiling camera for further analysis with BORIS (Friard & Gamba 2016). Immobility time was scored for the last 4 min of the test, with immobility being defined as lack of movement except slight motion to keep the head above the water. After removal from the cylinder, animals were gently dried and placed in a separate cage for recovery, as described before (O'Connor et al 2021).

Cytokine Quantification

Ileum and colon were homogenized with a buffer containing 10% fetal calf serum and protease inhibitors (Roche) using a tissue homogenizer (FastPrep - 24,MPBio). The levels of secreted interleukin-17a (IL17a) and interferon-gamma (IFN-γ) were analysed with the ELISA MAX Deluxe IL-17A and IFN-γ (Biolegend, USA) according to the manufacturer's instructions. Only data derived from duplicates with <15% CV were included in the analysis. A multi-mode plate reader (Synergy HT, BioTek Instruments) was used to quantify light absorbance in the assay, at 405 nm. Concentrations of cytokines were expressed in pg/mg of tissue. Limits of detection are represented in Supplementary Table 1.

Statistical Analysis

Statistical analyses were conducted using SPSS 27 (IBM, USA). Normality was assessed employing the Shapiro–Wilk test and for equality of variances using the Levene's test. Non-parametric data were analysed with independent-samples Kruskal–Wallis test followed by pairwise comparisons adjusted by the Bonferroni correction for multiple tests, with a 95% confidence interval. Parametric data were analysed using two-way analysis of variance (ANOVA) *post hoc* Tukey HSD. All data is represented as mean ± SEM. 3-Chambers tasks were analysed using a mixed factorial-ANOVA *post hoc* Tukey HSD with Bonferroni correction. Statistical significance was set at $p \leq 0.05$.

Results

Early Life Gut Microbiota Disruption does not Impact Social Behaviour or Depressive-Like Behaviour in Later Life

Mature adult mice (6 months old) from microbiota-depleted dams were fed either a FOS-Inulin enriched diet or chow diet for three weeks prior to the start of behavioural testing. Early life antibiotic exposure did not affect sociability in males ($F_{(1,34)}$=2.756, p=0.106), as all experimental groups showed preference for the animal over the object ($F_{(1,34)}$=97.776, p<0.001) (**Figure 2a**). In females, early life gut microbiota disruption influenced the time with either stimuli ($F_{(1,22)}$=4.506, p=0.045) as females from dams exposed to antibiotics in early life show a preference for a mouse over an object (Mouse vs Object; MatABX-Chow, p=0.001; MatABX-FOS-Inulin, p=0.002) which is not observed in the adult female offspring from the control groups (Mouse vs Object; H_2O -Chow, p=0.141; H_2O-FOS-Inulin, p=0.210) (**Figure 2c**).

Moreover, in the social novelty task, males present an overall chamber preference ($F_{(1,33)}$=4.924, p=0.033), even though there was no effect of early life antibiotic exposure ($F_{(1,33)}$=1.556, p=0.201) or adulthood prebiotic intervention ($F_{(1,33)}$=0.192, p=0.664). However, pairwise comparisons show that only adult control males supplemented with FOS-Inulin present a tendency towards preference for the novel mouse (Novel mouse vs Familiar Mouse; H_2O-FOS-Inulin; p=0.080), thus suggesting social deficits at this age (**Figure 2b**). Likewise, in females, social cognition deficits are evident by the lack of preference for the novel mouse ($F_{(1,22)}$=0.001, p=0.981), however there is an overall effect of early life gut microbiota disruption ($F_{(1,22)}$=5.069, p=0.035), as there is a significant difference between chow-fed female offspring exposed to early life gut microbiota disruption and control animals (H_2O-Chow vs MatABX-Chow, p=0.05), considering that female offspring seems to explore less both stimuli (**Figure 2d**).

To assess depressive-like behaviour, FST was used to determine the impact of early life microbiota disruption and adulthood FOS-Inulin supplementation on learned helplessness. Time spent immobile was not impacted by early life microbiota disruption ($F_{(1,32)}$=0.408, p=0.527) or diet ($F_{(1,32)}$=1.474, p=0.234) in male offspring (**Figure 2e**); however in female offspring, immobility time was

overall altered by FOS-Inulin supplementation in adulthood ($F_{(1,24)}$=4.433, p=0.046), even though this is not reflected in *post-hoc* comparisons (Tukey HSD; H_2O-Chow vs H_2O-FOS-Inulin, p=0.455; MatABX-Chow vs MatABX- FOS-Inulin, p=0.459) (**Figure 2g**). Stress-induced defecation in the FST was not statistically significantly altered by FOS-Inulin supplementation in adulthood ($F_{(1,33)}$=0.204, p=0.655) or early life gut microbiota disruption ($F_{(1,33)}$=0.624, p=0.435) in male (**Figure 2f**) or female (H(3)= 4.447, p=0.217) offspring (**Figure 2h**).

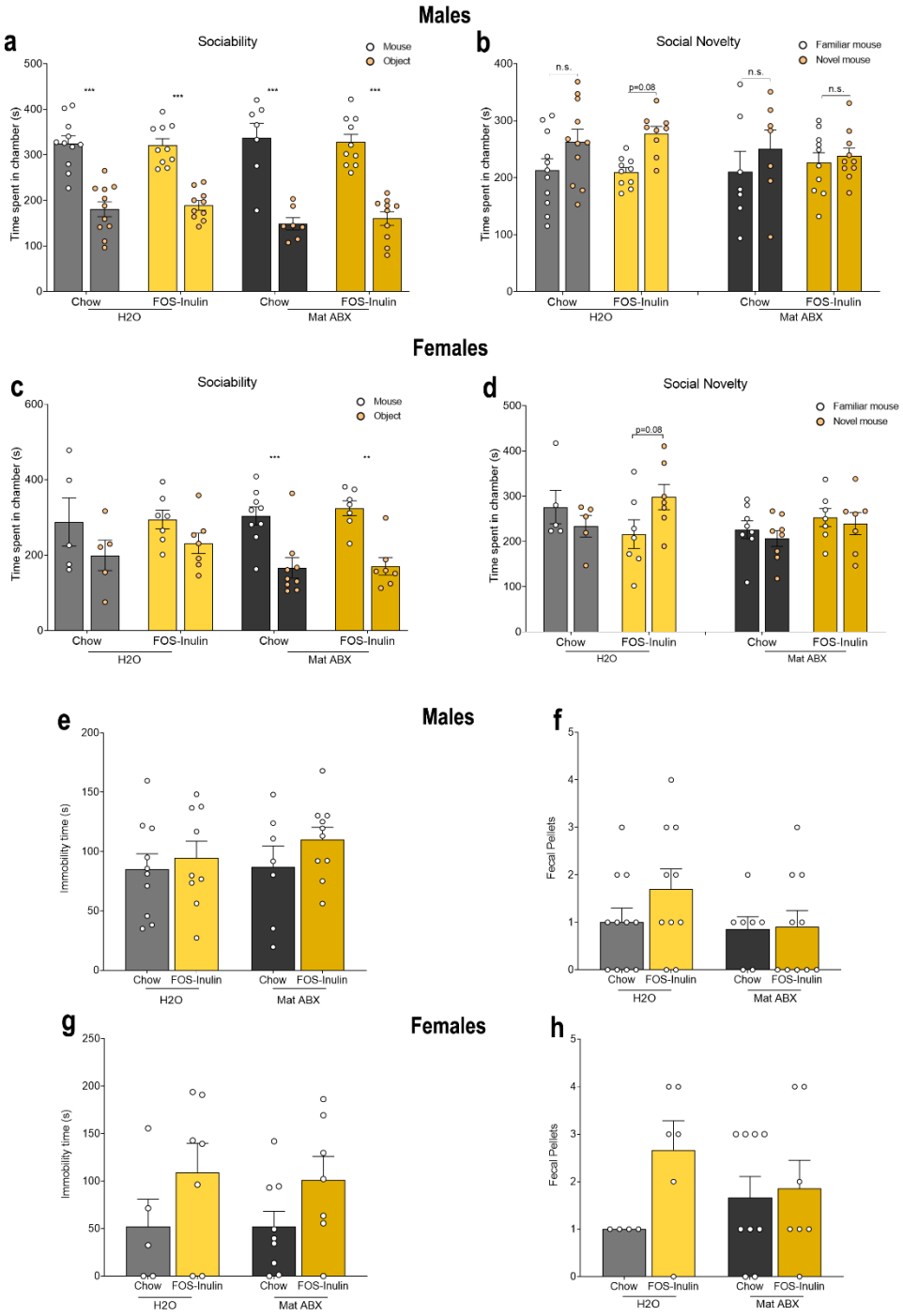

Figure 2 - Early Life Gut Microbiota Disruption does not significantly alter Social Behaviour or Depressive-Like Behaviour in Adulthood. *Sociability is not affected by early life gut microbial depletion in males a) or females c). FOS-Inulin supplementation in adulthood has a mild positive effect in social novelty in males b). Females present marked social novelty deficits, that are partially ameliorated by FOS-Inulin supplementation in the control group d). Early life gut microbiota depletion and FOS-Inulin supplementation do not affect depressive-like behaviour in males in adulthood, as it does not impact immobility time e) or stress-induced defecation f). In females, immobility time is non-significantly increased by FOS-Inulin supplementation in adulthood g), however it does not impact stress-induced defecation h). Results presented as mean±standard error of the mean (SEM). n=5-11 per group.*

Maternal Microbiota Disruption has a Mild Impact in Anxiety-Like Behaviour Later in Life with Subtle Sex Differences

Two-Way ANOVA indicates an effect of maternal microbiota disruption in male offspring with an impact on time spent in the open arms of the EPM ($F_{(1,34)}$=5.930, p=0.02), even though this was not statistically significant in the post hoc analysis (Tukey HSD; H_2O-Chow vs MatABX-Chow, p=0.238; H_2O-FOS-Inulin vs MatABX-FOS-Inulin, p=0.443) (**Figure 3a**). This can however indicate an overall impact of maternal microbiota disruption in reducing anxiety-like behaviour in males. In females, there was no effect of maternal antibiotic exposure ($F_{(1,24)}$=0.044, p=0.836) or diet ($F_{(1,24)}$= 0.002, p=0.963) (**Figure 3d**). The ratio of entries into the open arm (as described in the method section) was influenced in male offspring following maternal microbiota disruption ($F_{(1,38)}$=8.490, p=0.006) as males exposed to antibiotics in early life and supplemented with FOS-Inulin in adulthood show an increase in entries ratio compared to their controls (Tukey HSD; H_2O-FOS-Inulin vs MatABX-FOS-Inulin, p=0.033) (**Figure 3b**). In contrast, female offspring did not show an effect regarding early life antibiotic exposure ($F_{(1,24)}$=0.058, p=0.812) or FOS-Inulin supplementation in adulthood ($F_{(1,24)}$=0.031, p=0.861) (**Figure 3e**). Interestingly, stress-induced defecation in the EPM was impacted in female offspring (H(3)= 10.252, p=0.017) as FOS-Inulin supplementation normalizes stress-induced defecation (H= 11.619, p.adj = 0.022) (**Figure 3f**); however this was not observed in males (H(3)= 4.641, p=0.200) (**Figure 3c**).

In the open field test, time spent in the centre of the arena was not impacted by either early life antibiotic exposure (Two-Way ANOVA: Males - $F_{(1,35)}$=0.231, p=0.634; Females - $F_{(1,24)}$=1.192, p=0.286) or adult supplementation with FOS-Inulin (Two-Way ANOVA: Males - $F_{(1,35)}$=0.214, p=0.647; Females - $F_{(1,24)}$=2.846, p=0.105) (**Figures 3g, 3i**). Distance travelled was not significantly impacted by maternal microbiota disruption or FOS-Inulin in male (H(3)= 7.659, p=0.054) (**Figure 3h**) and female offspring (H(3)= 3.852, p=0.278) (**Figure 3j**).

In summary, we found that early life gut microbiota disruption does not significantly influence behavioural outputs, such as social and depressive-like behaviours, and has mild impacts in anxiety-like behaviours in adult mice.

Prebiotic administration in adulthood was also found to have no impact on the same measures.

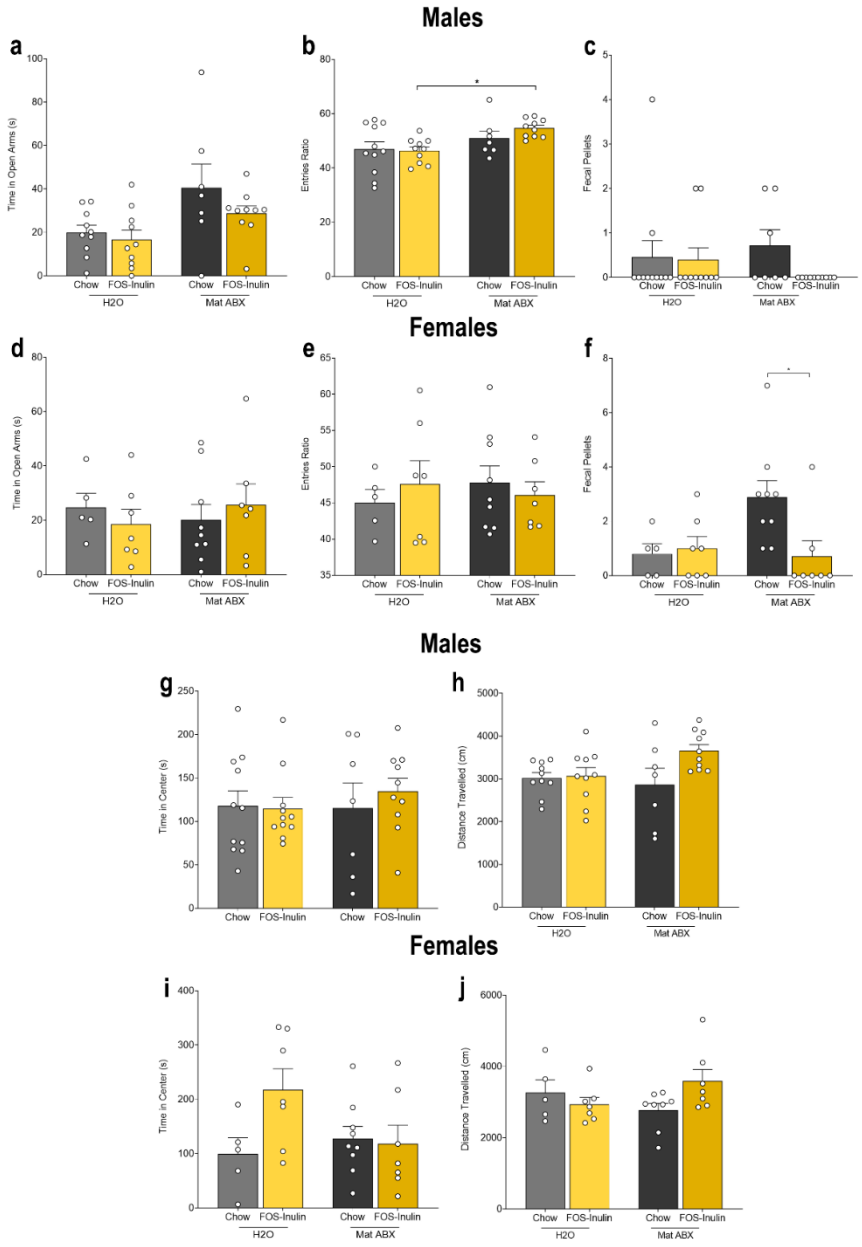

Figure 3 - Early Life Gut Microbiota Disruption has a mild impact on anxiety-like behaviour in female adult offspring. *Exploration of the open arms of the EPM was not significantly impacted by early life gut microbial depletion or FOS-Inulin supplementation in either males a) or females d). The ratio of entries into the open arm was increased in males exposed to early life gut microbial depletion and FOS-Inulin supplementation in adulthood b), which is not observed in females e). Stress-induced defecation was not altered in males c), but in females it was increased by early life gut microbial depletion and reversed by FOS-Inulin supplementation in adulthood f). Time spent in the centre of the open field was not affected in both males g) and females i). Distance travelled in the open field was also not impacted by early life gut microbial depletion or dietary intervention in adulthood in males h) or females j). Results presented as mean±standard error of the mean (SEM). n=5-11 per group.*

Prebiotic Exposure in Adulthood and Early Life Gut Microbiota Disruption Modulate Cytokine Levels in the Ileum and Colon Later in Life

In the colon, male offspring shows a non-significant trend to be impacted by early life microbiota disruption or FOS-Inulin supplementation in adulthood in regards to IL-17a concentration (H(3)= 6.554, p=0.088) (**Figure 4a**); however, female offspring revealed significantly altered colonic IL-17a concentrations following maternal microbiota disruption ($F_{(1,25)}$=24.325, p<0.001) as early life microbiota disruption is associated with reduced IL-17a levels in the colon (Tukey HSD; H_2O-Chow vs MatABX-Chow, p<0.001) while interestingly FOS-Inulin supplementation also reduces IL-17a in female control animals (Tukey HSD; H_2O-Chow vs H_2O-FOS-Inulin, p=0.043), but not females exposed to maternal antibiotics (Tukey HSD; MatABX-Chow vs MatABX- FOS-Inulin, p=0.297) (**Figure 4c**). In regards to IFN-γ in the colon, male offspring show group differences (H(3)= 9.240, p=0.026), however this is not reflected as statistically different with FOS-Inulin supplementation (H_2O-Chow vs H_2O-FOS-Inulin, H(3)= -7.700, p=0.621), and is not impacted by early-life microbiota disruption (H_2O-FOS-Inulin vs MatABX-FOS-Inulin, H(3)= 6.200, p=1.000) (**Figure 4b**). Female offspring also presented altered colonic IFN-γ (H(3)= 13.834, p=0.003) as maternal antibiotic exposure reduces the IFN-γ concentration in the colon (H_2O-Chow vs MatABX- Chow; H(3)= 16.114, p=0.002), and FOS-Inulin supplementation presents a slight tendency to improve this effect (MatABX-Chow vs MatABX- FOS-Inulin, H(3)= -9.857, p=0.095) (**Figure 4d**).

In the ileum, considering IL-17a concentrations neither male (H(3)= 6.767, p=0.08) (**Figure 4e**) or female offspring show a significant effect of either early-life microbiota disruption (Females - $F_{(1,26)}$=1.013, p=0.325) or diet (Females - $F_{(1,26)}$=2.892, p=0.103) (**Figure 4g**). Contrastingly, ileal concentrations of IFN-γ in males was altered by early life microbiota disruption and FOS-Inulin supplementation in adulthood (Males - $F_{(1,31)}$=17.169, p<0.001) as FOS-Inulin supplementation reduces IFN-γ in control animals (Tukey HSD; H_2O-Chow vs H_2O-FOS-Inulin , p<0.001) but not in animals exposed to maternal antibiotics (Tukey HSD; MatABX-Chow vs MatABX- FOS-Inulin, p= 0.576), and is further increased in offspring from dams exposed to early life antibiotics (Tukey HSD; H_2O-FOS-Inulin vs MatABX-FOS-Inulin, p<0.001) (**Figure 4f**). In females

however, ileal IFN-γ is only altered by early life disruption of the microbiota ($F_{(1,26)}$=18.482, p<0.001), as females from dams exposed to antibiotics show a significant increase in IFN-γ in the ileum (Tukey HSD; H_2O-Chow vs MatABX-Chow, p=0.001), which tends to be reduced non-significantly by FOS-Inulin supplementation in adulthood (Tukey HSD; H_2O-FOS-Inulin vs MatABX-FOS-Inulin, p=0.06) (**Figure 4h**).

In conclusion, our results demonstrate that the immune profile in the gut is altered by gut microbiota manipulation both by early life exposure to antibiotics and in adulthood, by prebiotic administration. Colonic cytokine levels are uniquely altered in adult females, indicating a possible sex-dependent mechanism. Contrastingly, ileal cytokine levels appear altered by prebiotic administration.

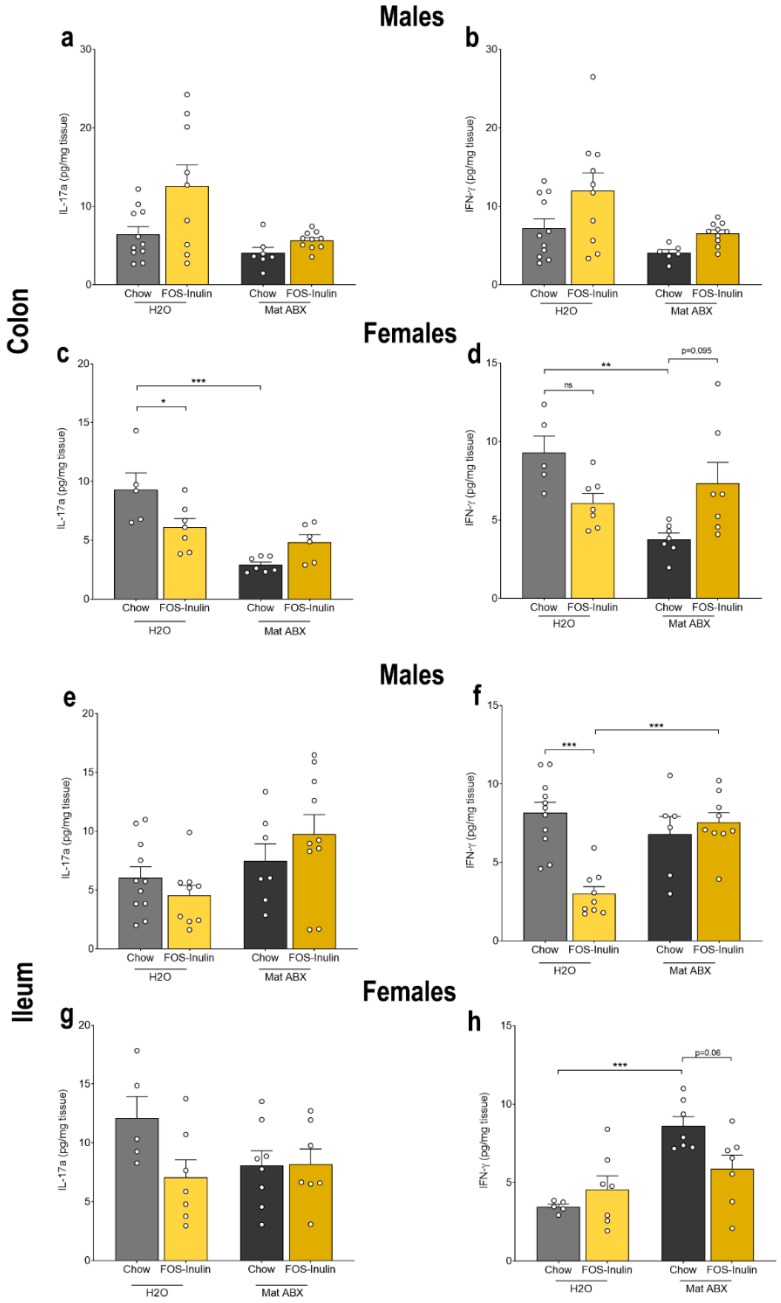

Figure 4 – Early life gut microbiota disruption and prebiotic exposure in adulthood alter cytokine levels in the ileum and colon. *Colonic levels of IL-17a a) and IFN-γ b) are not significantly altered by early life gut microbial depletion or adulthood FOS-Inulin supplementation in males. In females, FOS-Inulin supplementation reduces colonic levels of IL-17a, just in the control group, and it is further reduced by early life exposure to gut microbial depletion c). Colonic levels of IFN-γ are similarly reduced by FOS-Inulin supplementation in the control group; however, in female offspring exposed to gut microbial depletion in early life, it increases the IFN-γ levels d). In the ileum, IL-17a concentrations are not altered by early life gut microbial depletion or FOS-Inulin supplementation in males e); however, IFN-γ levels in the ileum of adult males are significantly reduced by FOS-Inulin supplementation, just in the control group f). In females, ileal levels of IL-17a are not altered by dietary intervention in adulthood or early life gut microbial depletion exposure g); inversely, IFN-γ levels are significantly increased in the ileum of females exposed to early life gut microbial depletion, which is partially reversed by FOS-Inulin supplementation in adulthood. Results presented as mean±standard error of the mean (SEM). n=5-11 per group.*

Discussion

In this study, we sought to understand the long-term consequences of early life gut microbiota disruption on behaviour and gut immune signalling, and to determine if prebiotic supplementation in adulthood can reshape such effects. Antibiotics offer a more flexible approach to negatively modulate the microbiota than germ-free animals, as they can be administered in distinct time windows or to target specific microbial populations (Champagne-Jorgensen et al 2020, O'Connor et al 2021). Interestingly and in contrast to the literature (Champagne-Jorgensen et al 2020, Leclercq et al 2017, O'Connor et al 2020) early-life antibiotic exposure failed to induce robust behavioural changes although there were some intriguing changes in intestinal immune parameters.

In the present study we found that early-life microbiota disruption impacted social preference and memory and that there were distinct profiles in male and female animals. Regarding sociability measures, in males, all groups display an expected preference for a mouse over an object in the sociability task, whereas females exposed to maternal antibiotics display a preference for the mouse over the object, which is not observed in the control group. Regarding the social novelty task, both males and females display deficits in this task, evidenced by the lack of preference of the novel mouse over the familiar. Nonetheless, adult FOS-Inulin supplementation in the offspring not exposed to the gut microbial depletion in early life, induces a trend for the reversal of this deficit. This contrasts with other studies investigating social sequaele of antibiotic exposure (Leclercq et al 2017, O'Connor et al 2021).

This may be due to the use of different antibiotics, different ages strains of mice or experimental designs used. Most previous research has examined the effect of early-life penicillin on social behaviour. For example, it has been reported that sociability and social novelty deficits emerge following exposure to low dose penicillin (31 mg/kg/day) in late pregnancy and early postnatal life (E12-14 - PND21), the same time window used here, in adult BALB/c mice (6 weeks old) (Leclercq et al 2017). Interestingly, another report using prenatal penicillin exposure (31 mg/kg/day) from E15 to birth suggested antibiotic-induced deficits in adolescent male BALB/c mice in sociability, but no deficits in social memory (Champagne-Jorgensen et al 2020). Penicillin exposure (33 mg/kg) immediately

prior to weaning (PND14-PND21) resulted in sociability deficits in males, while all female groups display deficits in sociability (Kayyal et al 2020); curiously, in our study, female control animals lack preference for a mouse over an object, except for the female offspring from dams exposed to antibiotics. Furthermore, we have previously shown maternal exposure (PND1-PND8) to penicillin (31 mg/kg/day) but not an combined antibiotic cocktail (consisting of ampicillin 1 mg/mL; vancomycin 0.5 mg/mL; metronidazole 1 mg/mL; ciproflox acin 0.2 mg/mL and imipenem 0.25 mg/mL) resulted in social recognition deficits (O'Connor et al 2021). A study from our group evaluating the same antibiotic cocktail as in this study to study the gut microbial depletion impact in early life found subtle sex-dependent social behaviour alterations during the pre-weaning period (Lynch et al 2023). This suggests that different time windows of prenatal antibiotic administration (Champagne-Jorgensen et al 2020, Kayyal et al 2020, Leclercq et al 2017) or the selective effects of antibiotic exposure (O'Connor et al 2021) can result in different social behaviour outcomes. This raises the possibility that adolescence is a sensitive period for social behaviour (Yamamuro et al 2020, Yamamuro et al 2018) and therefore the discrete effects of early life microbiota disruption in adolescence do not extend through adulthood.

Maternal antibiotic exposure and FOS-Inulin supplementation in adulthood did not impact anxiety-like behaviour in either males or females in this study. Even though different studies report reduced anxiety-like behaviour following microbiota disruption via early life exposure to antibiotics, this evidence is not consistent across different anxiety-like behaviour tasks (light-dark box, EPM and OF) (Champagne-Jorgensen et al 2020, Desbonnet et al 2015b, Leclercq et al 2017), and can be sex-dependent (Champagne-Jorgensen et al 2020). More specifically, different studies using the same prenatal low dose penicillin exposure experimental setting lead to distinct effects in the elevated plus maze, as penicillin exposure from late pregnancy through weaning increased exploration of the open arms in males (Leclercq et al 2017), while penicillin exposure from late pregnancy until birth (Champagne-Jorgensen et al 2020) or during the week prior to weaning (Kayyal et al 2020) does not impact exploration of the open arms of the EPM. Interestingly, maternal exposure to either penicillin or antibiotic cocktail during the first week of life reduced the entries into the open arms of the EPM, suggesting increased anxiety-like behaviour (O'Connor et al 2021). However, penicillin

exposure at different time periods does not affect exploratory behaviour in the open field (Champagne-Jorgensen et al 2020, Kayyal et al 2020, Leclercq et al 2017). Furthermore, Kayyal and colleagues, in line with our results, also observed no differences in the open field and elevated plus maze (Kayyal et al 2020) following microbiota disruption by exposure to low dose of penicillin V one week prior to weaning.

Microbiota depletion has been previously reported to increase immobility time in the forced swim test, thus reflecting increased stress-coping behaviours (Guida et al 2018, Hoban et al 2016a). In this study, maternal antibiotic exposure during late pregnancy through weaning did not impact depressive-like behaviour in adulthood, which is in line with previous reports from our group, where exposure to penicillin or an antibiotic cocktail for a week after birth did not alter stress-coping behaviour (O'Connor et al 2021). Interestingly, in this study, adulthood FOS-inulin supplementation increased immobility time (not statistically significant) in females, but not males, which could imply that the effects of gut microbiota manipulation could be sex-dependent – however this should be further explored in future studies.

Given the importance of the early life gut microbiome in the development of the immune system (Gensollen et al 2016) and that low dose of penicillin exposure during different time windows in this period has been linked to altered immunity (Champagne-Jorgensen et al 2020, Kayyal et al 2020, Leclercq et al 2017), we quantified two important pro-inflammatory cytokines – IL17a and IFN-γ – in the ileum and colon of adult offspring from dams exposed to antibiotics and supplemented with FOS-Inulin.

In the ileum, IL17a concentrations were not altered by maternal antibiotic exposure or FOS-Inulin supplementation in both males and females; however, maternal microbiota disruption decreased colonic IL17a specifically in females, which is not recovered by prebiotic supplementation in adulthood.

FOS-Inulin supplementation significantly reduced IFN-γ secretion specifically in the ileum of adult males from control dams, but not those exposed to maternal microbiota disruption. In females, there is a region-dependent effect, as IFN-γ is

increased in the ileum of adult females exposed in early life to antibiotics, which is partially recovered by FOS-Inulin supplementation in adulthood; in the colon, this effect is the inverse as maternal antibiotic exposure reduces the levels of IFN-γ, which is also partially recovered by FOS-Inulin supplementation in adulthood. Taken together, these results suggest a long-lasting impact of maternal antibiotic exposure in the secretion of IFN-γ at different sites in the gut, particularly in female mice. Previous reports on early life microbiota disruption during various time windows within the scope of this study have suggested that such disturbance impacts CNS cytokine profiles, but not in circulation (Leclercq et al 2017) and that maternal antibiotic exposure impacts the peripheral T regulatory profile (Champagne-Jorgensen et al 2020, Kayyal et al 2020), and are sex-dependent (Champagne-Jorgensen et al 2020). In fact, early life antibiotic exposure was shown to alter T regulatory populations in the colon in adulthood in female mice (Zhang et al 2021), which could explain the dichotomy we observed in the present study. Interestingly, another study employing maternal antibiotic exposure found that at 6 months old, the offspring shows reduced circulatory levels of IFN-γ, as well as reduced levels in the hippocampus (Madany et al 2022).

In this study, we sought to understand if a dietary intervention in adulthood could modulate behavioural and immune aspects in male and female mice from dams exposed to an early life microbiota disruption via antibiotic exposure. The long-term consequences of early life gut microbiota disruption using this approach manifests as alterations in intestinal immune markers and a subtle pattern of behavioural alterations. Future studies should also investigate the enduring consequences of antibiotic exposure on a variety of brain parameters including blood brain barrier function (Leclercq et al 2017).

Discussion

5.1. Overview and Summary

In this thesis, we explored the hypothesis that the gut microbiome can influence behavioural, immune and metabolite outputs in the host throughout the lifespan. To evaluate this, positive (FOS-Inulin) or proof-of-principle (microbiota depletion using antibiotic cocktail) strategies of studying the influence of the gut microbiota were used in different periods across the lifespan, to understand its impact on host-microbe interactions.

In **Chapter 2**, our data showed that the aged gut microbiome impacts host behaviour and physiology and therefore presents itself as a promising therapeutic target to reduce age-associated alterations. Unlike the adverse effects on behaviour of antibiotic-induced microbiota depletion in early-life and adulthood (Desbonnet et al 2015, Lach et al 2020, Tengeler et al 2021), in this study we show that gut microbiota depletion in aged mice is associated with some beneficial outcomes, such as improved social behaviour, suggesting that social behaviour is particularly sensitive to gut microbial signals in this specific time window. Furthermore, depletion of the gut microbiome impacted neuroimmunity in aged animals, reflected as an accumulation of T cells in the choroid plexus, a brain site largely impacted by age-dependent inflammatory signals (Baruch et al 2014, Baruch et al 2013). These results were also accompanied by modulation of caecal metabolites following microbiota-depletion, including metabolites previously linked to important age-dependent processes. This suggests that the age-dependent compositional remodelling of the gut microbiota is associated with gut metabolite alterations that can plausibly be linked to behavioural and neuroimmune outcomes. Future studies characterizing and understanding these specific metabolites in the behavioural and immune parameters of the aged host (both in preclinical models and large populational human studies), as well as the specific microorganisms involved in such effect will be pivotal to develop more targeted therapies.

In **Chapter 3**, using a positive manipulation of the gut microbiota in aging, we observed that prebiotic supplementation with FOS-Inulin improves the behavioural response to stress in aged mice. However, such effects were independent from modulation of the immune system or HPA axis but were associated with the regulation of key age-associated metabolites: spermine and

4-Hydroxybenzaldehyde, in the PFC. Future studies targeting spermine and 4-Hydroxybenzaldehyde specifically and their corresponding metabolic pathways will be paramount to uncover the specific outcomes and mechanisms underlying the significance of these metabolites on the age-related stress-induced behavioural phenotypes.

Lastly, in **Chapter 4**, we aimed to understand if a dietary intervention in adulthood could modulate behavioural and immune outcomes in male and female mice from dams exposed to early life microbiota disruption via antibiotic exposure. The long-term impact of early life gut microbiota disruption using this particular approach was reflected in alterations in intestinal immune markers and subtle behavioural alterations.

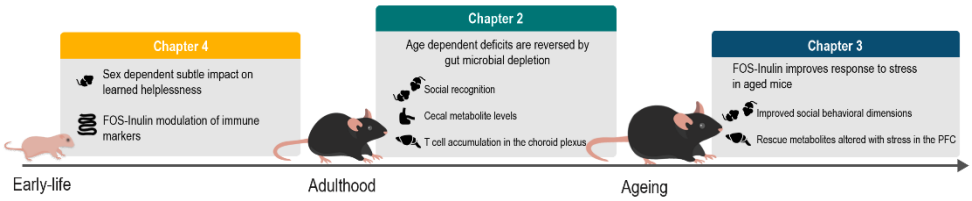

Figure 1 - Summary of the main findings in this thesis. *Gut microbial signalling differently shape behavioural and immune responses in the host throughout the lifespan, while being associated with metabolite remodelling, Targeting the gut microbiota, particularly in aging, promotes considerable adaptations, that can impact the health of the host.*

5.2. The Importance of Studying Healthy Aging: A Microbial Perspective

As access to better nutrition and healthcare has increased worldwide, so has the aged population. The increase of population aging and increases in life expectancy inspire research into ensuring healthy aging, that is the “ongoing process of developing and maintaining the functional ability that enables wellbeing in older age” (Michel et al 2021, WHO 2020). Increases in life expectancy have resulted in the extension of pension age in multiple countries; however, these policies are just considering life expectancy (Lynch et al 2022b). A recent study in the UK population, it is projected that from age 50, life expectancy is expected

to reflect a gain per calendar year averaging 6.4 weeks (0.12 years) and 0.7 weeks (0.21 years) in women and men, respectively. However, the gain in healthy working life expectancy is only 2.8 weeks (0.05 years) per year for women and 1 week per year for men (0.02 years) (Lynch et al 2022b). Therefore, socioeconomic policies should be adjusted to focus on healthy aging outcomes and, therefore, understanding the factors that promote healthy aging is imperative with the gut microbiome is increasingly coming into focus in this regard. Nonetheless, it is important to consider the gut microbiome as a feature of healthy aging, as in that context, the gut microbiome trajectory in late life could reflect an important physiological adaptation. In that context, attempts to 'turn back the microbial clock' by reshaping it into young adulthood microbiome profile might be misguided (Wilmanski et al 2022).

Aging is intrinsically linked to increased susceptibility to neurological and cognitive impairments (Wyss-Coray 2016) as well as deficits in social cognition (Moran et al 2012, Roheger et al 2022). Indeed, cognitive decline has profound impacts on social functioning, as social interaction involves decoding complex social information (Arioli et al 2018). However, healthy older adults can also face challenges in a component of theory of mind – a complex construct that involves inferring others' mental states and believes (Bottiroli et al 2016) or processing emotional recognition from facial expressions (Arioli et al 2018). Furthermore, impaired social functioning can arise as a result of age-dependent changes in brain structure and function (Hughes et al 2019). Preclinical evidence from our group also indicates that social behaviour deficits are also observed in aged mice (Scott et al 2017).

With age, another physiological function that progressively declines is the hormonal secretion. Altered hormonal secretory patterns and regulation of feedback sensitivity is characteristic of the ageing organism. A well know manifestation of such age-dependent alterations is the impact in gonadal hormones, namely progesterone, estradiol and testosterone (van den Beld et al 2018). Curiously, recent studies have linked testosterone (Li et al 2022) and estradiol (Li et al 2023) degradation to gut microbial activity, and its consequent depressive-like behavioural impact, in males and females, respectively.

Here we sought to dissect the involvement of the gut brain axis in social behaviour in aged mice, resorting to two distinct gut microbiome interventions: gut microbial depletion via an antibiotic cocktail and prebiotic supplementation.

5.3. The Gut Microbiome and Social Behaviour

In **Chapter 3**, we have shown an important role for the gut microbiome in the social novelty deficits in aged mice. This implies that in this particular context, depleting the gut microbiota can have a beneficial effect – however, further studies must explore which particular bacterial groups are involved in this response. Interestingly, some evidence suggests that certain antibiotics can improve cognitive dysfunction in Alzheimer's disease – an age-related disorder (Angelucci et al 2019). Namely, rifampicin can promote amyloid-β clearance by increasing the efflux of the blood-brain barrier (Qosa et al 2012), and also inhibits pro-inflammatory mediators (Bi et al 2011). These effects could be due to direct mechanisms of action – rapamycin can prevent cognitive dysfunction and tau pathology by targeting mTOR targeting (Wang et al 2014) and D-cycloserine, a partial NMDAr agonist, rescues age-dependent cognitive impairment when injected in the ventral hippocampus of aged rats (Portero-Tresserra et al 2018). Nonetheless, the specific effects of these antibiotics on the gut microbiota, and their consequent effects via the gut-brain axis in cognitive domains in models of age-related disorders still remain underexplored. Particularly, considering that targeting the gut microbiota in age-related disorder models can reduce neuroinflammation in said models (Sun et al 2018), it is imperative to further explore the involvement of specific gut microbial signals in the modulation of inflammation and consequent CNS function in aging.

In **Chapter 2**, we show that FOS-inulin supplementation improves social novelty in aged mice, suggesting, once again, that targeting the gut microbiome can ameliorate social behaviour in aged animals. Interestingly, exposure to social defeat did not exacerbate the social novelty deficits observed in aged mice. Mild social defeat in aged mice suggests that stress in aging can alter different behavioural phenotypes, namely increased sensitivity to anhedonic behaviour (Oizumi et al 2019). This indicates that stress response in aging can elicit different behavioural phenotypes, particularly hedonic behaviour, in comparison to young

adults; thus, stress did not further aggravate pre-existing social behaviour deficits in aged mice.

Impairments in social functioning in later life have been associated with functional disability (Henry et al 2016), mental (Jones et al 2015) and physical problems (Holt-Lunstad et al 2010), and reduced quality of life (Phillips et al 2010). Remarkably, individuals with strong social relationships show a 50% increased likelihood of survival (Holt-Lunstad et al 2010), reinforcing the direct importance of social relationships in physiology. Social cognition impairments are now recognized as a representation of dysfunctional core cognitive domains (Cotter et al 2018), that can precede further cognitive impairment manifestations (Boyer et al 2019). Additionally, it has been found that social isolation among older adults with diabetes is considerably high (Ida & Murata 2022), and is related to poorer health outcomes, namely glycemic control (Ida & Murata 2022, Kobos et al 2020). Further, given that feeding behaviour is largely influenced by social context (Higgs & Thomas 2016), and that social isolation in individuals over 50 years old is associated poorer dietary habits, reflected by reduced variety of fruit and vegetable intake (Conklin et al 2014), it is feasible to hypothesize that increased sociability too can indirectly promote a healthy gut microbiome. In turn, exploring the gut microbiota as a target for promoting social behaviour in aging is necessary, and warrants further research.

5.4. Aging and Microbiota-Derived Metabolites

Recent technological advancements have allowed the exploration of metabolomics, that comprise small endogenous or exogenous molecules that result from different physiological processes (Panyard et al). Despite the exploration of the relevance of circulating metabolites being in its early days, it is well-established that these metabolites undeniably reflect the diet and lifestyle of the host (Bar et al 2020, Chen et al 2022, Diener et al 2022). Future studies should focus on providing similar frameworks into the metabolite patterns in the CNS and how those relate to health and disease state, thus offering more insights into how the gut microbiome effectively impacts the CNS. Further, a considerable challenge for the field is to dissect whether the studied metabolites are in fact microbial, or host derived – as the exploration of the metabolome expands, new

bioinformatic tools are developing to allow the discrimination of the origins of the studied metabolites (Yu et al 2022).

Extensive changes in the metabolome due to chronological age have been demonstrated in the human blood and urine (Bunning et al 2020, Darst et al 2019, Lawton et al 2008, Menni et al 2013, Psihogios et al 2008, Rist et al 2017, Yu et al 2012b). Therefore, chronological age-associated metabolomic signatures have been identified in plasma (Darst et al 2019, Johnson et al 2019) and cerebrospinal fluid, namely metabolites associated with cellular energy (Peters et al 2021), and metabolic adaptations (Petr et al 2021).

Considering the undeniable contribution of the microbiota-gut-brain axis in age-associated physiological and disease patterns, it is unsurprising that gut-derived metabolites are proposed to represent key players in the aging process (Connell et al 2022). Recently, gut-derived δ-valerobetaine was identified as a metabolite intrinsically linked to aging physiological processes, as its increased levels in the plasma and brain of aged mice and older adults was associated with age-dependent impairments in cognitive processes (Mossad et al 2021). Moreover, the same researchers found an age-dependent accumulation of N^6-carboxymethyllysine in the serum and brain of aged mice and older adults, which interferes with microglia physiology in mice, through a microbiota-dependent increase in intestinal permeability (Mossad et al 2022). These observations reflect promising insights into the involvement of gut microbial derived metabolites in age-related physiological processes in the brain. Future studies should characterize the potential link between these microbial-derived metabolites in healthy aging and also cognitive decline. More specifically, large-scale populational studies, assessing the impact of different factors influencing the gut microbiome throughout life (mode of delivery, antibiotic use, psychobiotics) should inform on its influence in metabolites and their potential impact in the host aging physiology.

In **Chapter 2**, we observed age-dependent remodelling of the caecal metabolome, namely *a)* increased levels of gentisic acid and N-formylmethionine and, *b)* reduced levels of 2-Methylbutyrylglicine and Argininosuccinic acid, which can be restored by gut microbial depletion. Some of these metabolites and the

corresponding pathways have been previously linked with aging and age-dependent physiological alterations (Cai et al 2021, Fleszar et al 2019).

In **Chapter 3**, we evaluated the effect of prebiotic supplementation on the impact of stress exposure in the caecal and prefrontal cortex metabolome of aged mice. From the metabolites altered in the prefrontal cortex, only 2 metabolites were shaped by stress and moderated by the dietary intervention: *a)* 4-Hydroxybenzaldehyde, which was increased by stress and reduced by FOS-Inulin and *b)* spermine, that was decreased by stress exposure and ameliorated by FOS-Inulin. 4-Hydroxybenzaldehyde, while relatively underexplored, is a metabolite previously shown to block GABA activity (Tao et al 2006, Zhang et al 2017) – GABA is the main inhibitory neurotransmitter in the CNS, and its activity is suggested to be decreased with age (Maes et al 2018, Porges et al 2021). Even though in this study we observed increased levels of 4-Hydroxybenzaldehyde upon stress exposure in aged animals, it is feasible to hypothesize that perhaps prebiotic supplementation can modulate GABAergic transmission, to ameliorate a potential maladaptive response to stress (Partridge et al 2016), however this is outside the scope of this research. On the other hand, spermine, an autophagy promoter (Aman et al 2021), was reduced in the PFC of stress aged animals and recovered by FOS-Inulin. Given the growing literature on the positive outcomes associated with spermine in aging and age-dependent disease (Frühauf-Perez et al 2018, Pucciarelli et al 2012), the reversal of this metabolite in the PFC of aged animals upon exposure to stress suggesting spermine as a promising healthy aging target and warrants further research.

Both in **Chapters 2** and **3**, metabolites related to arginine biosynthesis pathway were identified as key factors that follow gut microbiome reshaping in aged animals: argininosuccinic acid and spermine. Polyamines such as spermine have been found decreased with aging and are synthesized from ornithine; on the other hand, arginine which serves as a precursor to ornithine, has also been reported to be decreased with aging (Pan et al 2018b). Considering that argininosuccinic acid is a precursor to arginine (Nagamani et al 2012), and that L-arginine is increasingly linked to healthy aging, and improvements in age-dependent cognitive decline (Gad 2010, Mone et al 2022), further exploration into how gut microbiome targeted therapies can shape arginine availability in aging could be

vital. These metabolites should be explored in future studies, evaluating its targeting in neuro behavioural outcomes in aged models, for example, by administering said isolated metabolites in young and aged mice and assessing different behavioural dimensions, while establishing the underlying mechanisms.

In conclusion, seeing as healthy *vs* unhealthy aging is increasingly associated with divergent gut microbial ecological compositions, aging is more and more associated with gut microbial derived metabolite signatures, that can mitigate the negative impacts of host aging (Wilmanski et al 2022). The aforementioned studies in this thesis further highlight the importance of the adjustment of metabolite levels in the aged host using strategies to reshape the aged gut microbiome, additionally building on to a rapidly growing body of evidence (Figure 2). Large-scale longitudinal studies are imperative to dissect the involvement of these metabolites into the healthy vs unhealthy aging patterns, and the pathways associated to such effects, in order to explore therapeutic avenues targeting such mechanisms, and alleviate such effects in the context of unhealthy aging.

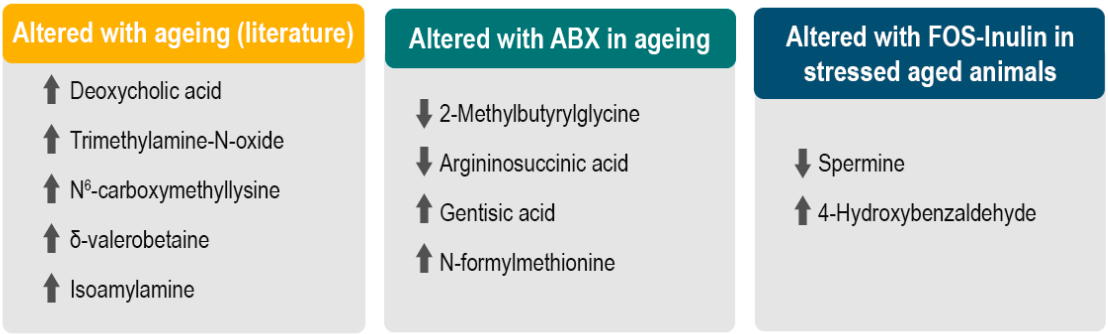

Figure 2 – Summary of reported age-dependent metabolite alterations in relation to the gut-brain axis, as previously described in the literature, and in this thesis *(Chapters 2 and 3).*

5.4. Gut Brain Axis and Prebiotics Throughout Life: When Should We Intervene?

Diet is appreciated as an easy and simple route of manipulation of the gut microbiome (Wu et al 2011), that can effectively and quickly impact it (David et al 2014, Wu et al 2011). Dietary interventions can also regulate the host immune system by reshaping host physiology adaptations that can optimize immune responses, providing an accessible tool to modulate immunological functions,

particularly during aging (Collins & Belkaid 2022). In fact, dietary interventions have been proposed to represent lifestyle factors that can mitigate the impact of neuroinflammation during aging (Muscat & Barrientos 2020).

Prebiotics are substrates that promote the growth of certain groups of bacteria, conferring benefits to the host (Tawfick et al 2022). These can include FOS and inulin, fibres that are present in dietary sources, and can effectively present immunomodulatory properties (Costa et al 2021, Tawfick et al 2022). Given the relevance of these fibres in the evidence gathered numerous dietary interventions, we assessed the use of this combined supplementation in two different timepoints – adulthood and late life.

In **Chapter 3**, dietary modulation by FOS-Inulin supplementation promoted improved behavioural stress responses in aging, while modulating key metabolite levels in the caecum and the prefrontal cortex. Future studies should address the potential of prebiotic supplementation for a longer period in aged animals, and most crucially, during middle age as a possible preventative measure for age-dependent stress responses.

In **Chapter 4**, the same dietary intervention was employed during adulthood, in an effort to understand if such supplementation could mitigate the impact of antibiotic exposure from a prenatal stage through weaning. In this study, we observed that FOS-Inulin supplementation in adulthood did not induce significant behavioural alterations, and in fact, it induced a negative impact in females in a learned helplessness task. Concurrently, FOS-Inulin supplementation altered pro-inflammatory cytokines in the gut in a region- and sex-dependent manner.

This sex-dependent dichotomy of pro-inflammatory signalling highlights the importance of studying the physiology of both sexes, especially considering these differences could underpin different behavioural responses and have consequences for neuropsychiatric disorders (Lasselin et al 2018, Shobeiri et al 2022). Interestingly, in mice, particular brain regions- such as the corpus callosum, the midbrain and the thalamus- are particularly sensitive to immune dysfunction and therefore can reflect behavioural patterns (Fernandes et al 2022).

Early life adversity can induce long-lasting impact that can be reflected in risk for neuropsychiatric and neurodegenerative disorder development in adulthood and late in life (D'Amico et al 2022, Wells et al 2014). Additionally, prenatal and early postnatal events have been proposed to impact cognitive reserve later in life, hence suggesting that developmental disturbances in early life can impact age-dependent neurological processes (de Rooij 2022). Therefore, it is imperative to study the impact of early life disturbances across the lifespan, and further understand if, how and when the gut microbiome can be targeted to ameliorate such impacts.

5.5. Gut Brain Axis and Gut Microbial Depletion Throughout Lifespan

Antibiotics represent an accessible experimental strategy to study the physiological impact of bacterial groups, that can be used at different timepoints throughout the lifespan (Cryan et al 2019b), and this flexibility allows the exploration of the dynamic inputs of gut microbial signals across life.

In **Chapter 2**, gut microbial depletion in aged mice promoted revealed a role for the microbiome in social cognition phenotypes, amelioration of age-dependent caecal metabolite levels, and mitigation of T cell accumulation in the choroid plexus.

In **Chapter 4**, early life microbiota depletion did not significantly impact behavioural profiles later in life. While antibiotic use should always be approached with caution, in particular, in early life, these results suggest that early life gut microbial depletion from mid-gestation through weaning does not induce sustained detrimental impacts on behaviour later in life. However, future studies should study the impact of such early life disturbance, upon exposure to a stressor in adulthood.

Considering that gut microbial depletion in late life had such a marked impact, it is possible to attribute the lack of an striking impact in adult offspring exposed to early life to the possibility that a) this study focused in physiological conditions, and as such, an immune challenge in adulthood, or stress exposure can potential elicit different outcomes; and b) and that the gut microbial ecosystem had been

re-established at such timepoints, and that the age-dependent gut microbial alterations emphasize late life as a more sensitive time period.

Taken together, given the growing attention given to the use of antibiotic cocktails as an experimental strategy, it is imperative to understand not only how individual groups of bacteria affect the gut-brain axis, but what are the effects of the depletion of specific gut microbial taxa at different selective life stages.

5.6. The Immune System as a Conduit for Gut-Brain Signalling: Profilling Gut Immune Signals

Through life, the immune system continuously recognizes and responds to danger, while also repairing and calibrating the system to prepare for future challenges, ensuring the return to homeostasis. The continuous exposure to insults can lead to disrupted immune homeostasis, which can shape maladaptive immune changes that can pose long-lasting consequences (Halper-Stromberg & Jabri 2022).

Aging is accompanied by significant multiorgan remodelling, as illustrated by disturbed physiological integrity of different biological systems, including the immune system (López-Otín et al 2013, Mogilenko et al 2021). In fact, low-grade systemic inflammation characterized by accumulation of pro-inflammatory factors, defined as inflammageing, has been recognized as a key factor in aging biology, as the crosstalk between dysfunctional immune cells and the pro-inflammatory factors reciprocally interchange signals, thus amplifying a pro-inflammatory and dysfunctional state (Mogilenko et al 2021). Age-dependent modifications of the gut microbiome have also been increasingly associated with age-associated inflammageing and immunosenescence, and hence been proposed to represent a promising interventional target to mitigate the effects of aging on the immune system (Conway & A Duggal 2021, Shintouo et al 2020).

In **Chapter 2**, the levels of pro-inflammatory cytokines IL-17a and IFN-γ in the aged ileum showed a non-significant increase that presents a tendency to be decreased by antibiotic treatment.

In **Chapter 3**, the FOS-Inulin dietary intervention increases the levels of pro-inflammatory cytokines IFN-γ and IL-6, which even though not surprising considering that dietary fibres can inflate pro-inflammatory markers in the gut (Singh et al 2019), shows that the beneficial effects of the prebiotic supplementation are independent of the modulation of the peripheral immune system.

In **Chapter 4**, early-life microbiota disruption is associated with sex- and region dependent remodelling of IL-17a and IFN-γ. Namely, female adult offspring exposed to gut microbial depletion in early life show opposite modulation of IFN-γ in the ileum and colon, which can be ameliorated by FOS-Inulin supplementation in adulthood.

Collectively, the studies depicted in this thesis suggest that while the gut immune status is essential for the host, in the metrics hereby assessed, it does not necessarily explain behavioural phenotypes, particularly in aging. Nevertheless, this does not role out an important involvement of the immune system in host behaviour.

5.7. The Immune System as a Conduit for Gut-Brain Signalling: Focus on the Choroid Plexus

In recent years, more and more evidence has shown that unlike previously thought, the CNS not only presents specialized immune cells that surveil their surroundings, but also that it communicates with the peripheral immune system, exchanging signals (Koren et al 2021, Rua & McGavern 2018). Furthermore, aging is associated with disruption of different physiological systems, including neuroimmunity, which can increase susceptibility to cognitive decline (Fonken & Gaudet 2022).

Given the intricate communication between the gut microbiome and the immune system, it is not surprising that immune signalling can impact brain function, in response to gut microbial shifts. In fact, recent studies show that gut-derived immune cells can in fact migrate to the CNS and impact its environment and consequent physiology (Fitzpatrick et al 2020, Sanmarco et al 2021).

The choroid plexus is an epithelial bilayer, consisting of a complex array of immune, mesenchymal and neural cells that compose the blood-cerebrospinal fluid (CSF) barrier (Cui et al 2021). Aside from secreting CSF, the choroid plexus is also a key hub gating or allowing passage of peripheral immune cells into the CNS. In fact, the choroid plexus is an essential region for immunosurveillance in the CNS, not only skewing the phenotype of immune cells, but also its cells express indoleamine 2,3-dioxygenase (IDO), an enzyme expressed in intestine cells, responsible for tryptophan catabolism, and retinoic acid metabolic enzymes, which concomitantly inhibit T cell activity and promote regulatory T cell responses (Spadoni et al 2017).

Even though the choroid plexus is still relatively underexplored as a potential reference point in the gut-brain axis, recent studies have shown that there may in fact be an intricate connection. An interesting study from our group has recently shown that germ-free animals present disrupted tight junction network in the choroid plexus, suggesting a role for the gut microbiota in the maintenance of the blood-CSF barrier (Knox et al 2022b). Gastric infection with *Helicobacter suis* promotes cognitive decline and microglia activation through inflammation of the choroid plexus, disruption of the blood-CSF barrier and increased permeability of the intestinal barrier (Gorlé et al 2018). Further, intestinal inflammation promotes the closure of the blood brain barrier and is associated with microglial phenotype modifications as well as cognitive deficits (Carloni et al 2021). This suggests that gut-derived inflammatory signals can regulate phenomena in the choroid plexus, and therefore represent key contributors in the gut-brain axis.

In **Chapter 2**, we showed that in line with the beneficial social behaviour outcomes with gut microbial depletion in aged mice, there was an associated mitigation of CD4+ T cells accumulated in the choroid plexus. While this result warrants further investigation into the causal mechanisms underpinning such effect, it represents further evidence that the gut microbiome can influence neuroimmunity in the choroid plexus, which in turn can be linked to behavioural outcomes. Future studies using viral gene delivery systems in the context of different immune challenges (stress/ immune activation) will be crucial to further understand the involvement of the choroid plexus as a conduit for neuroinflammation in the gut-brain axis.

As neuroimaging technology progresses, future studies using high-resolution MRI could help bridging the gap in understanding the gut microbiome mediation of the neuroinflammatory processes in the choroid plexus, by exploring preclinical and clinical models (Fleischer et al 2021). Such studies would allow for further characterization of the involvement of the choroid plexus in neuroinflammatory processes, and potentially constitute new biomarkers of age-related disease, such as Alzheimer's disease (Čarna et al 2023).

5.8. Future Questions to Address

What was previously known?	How did this research help advance this?	What are the open questions?
Gut microbial metabolites are potentially involved in physiological and disease states during the aging process (Figure 2)	We further provide evidence of gut microbial modulation of metabolites in aging (Chapter 2) and in the response to stress exposure during aging (Chapter 3)	Are there specific gut-derived metabolites driving the behavioural response in aging? Is there potential for targeting specific microbial populations from a personalized medicine perspective, that could be used as augmentation therapy to improve social functioning in later life and consequently, quality of life?
Aging inflammatory signals induce significant remodelling in the choroid plexus (Baruch et al 2014, Baruch et al 2013)	Gut microbial depletion in aged mice reduces the accumulation of $CD4^+$ T cells in the ventricles (Chapter 2)	What gut microbial signals are driving the age-dependent T cell accumulation? Could the same effect be obtained by targeting the specific metabolites that were highlighted in our analysis? How does this influence other behavioural features in aging?
Stress exposure impacts inflammatory markers, and can be ameliorated by prebiotic supplementation in mid-life (Boehme et al 2020)	Prebiotic supplementation improves behavioural outcomes in response to stress in aged mice, by remodelling of metabolites in the PFC, independently of the immune system or the HPA axis (Chapter 3)	Do spermine and 4-Hydroxybenzaldehyde elicit the same protective effects in aged stress mice? Could targeting of these metabolites in older adults improve social and emotional outcomes?
Early life gut microbial depletion elicits impaired behavioural outcomes in adolescence and adulthood	Gut microbial depletion in early life did not induce significant behavioural remodelling, while moderately altering intestinal pro-inflammatory markers that are modified by prebiotic administration (Chapter 4)	Would behavioural assessment earlier or later in life highlight impairments elicited by early life gut microbial exposure? Could a second hit (ie., stress exposure, inflammatory insult) trigger an aberrant behavioural and immune response in offspring exposed to early life gut microbial depletion? Would prebiotic supplementation mitigate such potential effects?

5.9. Overall Conclusions

Aging reflects well-defined physiological adaptations (López-Otín et al 2013), and as the field progresses, so thus the exploration of better biological biomarkers to monitor healthy aging. López-Otín and colleagues defined 9 hallmarks of aging, which greatly contributed for the study of the biology of aging; now, in light of the advancement in understanding the influence of the gut microbiota in health throughout the lifespan of the host, adaptation of the gut microbiota has been proposed as the "10th Hallmark" (Carter 2021, Guerville et al 2020).

In conclusion, the gut microbiota signals influence immune, neuroimmune and behavioural outcomes throughout the lifespan, thereby shaping the health of the host. Targeting the aged gut microbiota through opposing strategies (antibiotic exposure or prebiotic supplementation) can ameliorate the production of metabolites that present age-dependent alterations, while improving behavioural patterns (Figure 1).

This research opens doors to deeper investigations into the mechanisms underpinning age-dependent metabolite alterations that can be mediated through targeting the gut microbiome. Mechanistic analysis into the molecular mechanisms underlying these effects is imperative, as well as extended characterization of gut microbial metabolites specifically in the CNS, in humans.

Moreover, our research also highlights time-sensitive periods for the gut microbiome; in particular, we observed, in these particular datasets, that remodelling the gut microbiome in aging results in far-reaching outcomes, in comparison with targeting the gut microbiome in early life. This could be a consequence of an adaptation of the gut microbial architecture in the first stages of life. Nonetheless, it would be interesting to see how offspring exposed to early life gut microbial depletion would perform later in life: could it aggravate the physiological aging pattern?

As the exploration of the gut microbiome continues, identifying the mechanisms underpinning the impact of gut microbial signalling on host immunity and behaviour remains an important research objective. Large-scale populational

studies surveying those questions, combined with preclinical models that allow for the investigation of the underlying physiological processes, will help to identify, and stratify healthy and diseased populations. However, more than the identification of particular groups of bacteria, it is imperative to identify metabolites impacted by gut-microbial functions and use them for diagnosis and surveying general health of the host, thus providing the possibility to specifically reduce or amplify the levels of these metabolites in a targeted way, according to specific needs, from a personalized and precision medicine perspective. Identifying and personalizing the metabolite targets will allow for improvements in the health trajectory of the host, as well as providing augmentation therapy in disorders associated with the gut-brain axis.

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