The Effects of Flaxseed and Flaxseed Oil on the Gut-Brain Axis in Lipopolysaccharide-Challenged Male C57Bl/6 mice

Dawson Livingston

Supervisor: Dr. Krista A. Power

Thesis submitted to the University of Ottawa
in partial fulfillment of the requirements for the
Master of Science degree in Microbiology and Immunology

Department of Biochemistry, Microbiology, and Immunology
Faculty of Medicine
University of Ottawa

Abstract

Individuals living with depression and anxiety show systemic increases of the bacterial endotoxin lipopolysaccharide (LPS), which induces an inflammatory cascade, resulting in negative effects across the gut-brain axis (GBA). LPS administration in mice has previously been used as a rodent model of depression/anxiety. Flaxseed (FS) contains key bioactives, including an omega-3 fatty-acid, dietary fibre, and a poly-phenolic compound which all may attenuate the effects of LPS through modulation of the GBA. The objectives of this thesis were to examine the effects of LPS on the GBA in C57Bl/6 mice and to determine if dietary supplementation with FS and/or FS oil (FO) provided protection against the LPS challenge. The LPS-induced negative effects across the GBA were partially attenuated by dietary supplementation with FS, but not FO, through changes in microbiota composition/function and systemic-/neuro-inflammation. Therefore, the potential benefits of FS are independent of the oil or are synergistic of all bioactives.

Acknowledgements

I would like first and foremost thank Dr. Krista. A. Power for her profound and endless support throughout this degree, the time and effort you have spent with me on this project has greatly improved my ability as a scientist, and I am greatly looking forward to learning much more from you.

I am incredibly grateful for the funding from the Nutrition and Mental Health Master's Scholarship for allowing me to truly focus on this research, and to NSERC for funding the costs associated with this research. The flaxseed used in this study were generously supplied by Natunola, and the flaxseed oil was kindly extracted by Dr. Farah Hosseinian's lab at Carleton University.

I'd like to thank the members of the Power lab for their consistent help throughout this study. To Dr. Lalit Kishore and Alexane Rodrigue, I am very appreciative of all you have taught me so far, I would not have been able to complete this study and its analysis without you guys. I recognise the important role of Allison Sweet, Julia Loftus and Farida Ghali for their contributions to my research and am incredibly grateful.

To my thesis advisory committee consisting of Dr. Marie-Claude Audet and Dr. Erin Mulvihill, I want to thank you for your consistent guidance and support throughout this project, I truly appreciate the contributions you have made to my academic journey thus far.

Lastly, to my partner, Brianna Raven, thank you for your unwavering support throughout this degree and in life, you consistently make me a better person.

Table of Contents

List of Abbreviations

ALA- Alpha (α)-linolenic acid
BBB- Blood brain barrier
BW- Body weight
CNS- Central nervous system
DHA- Docosahexaenoic acid
EPA- Eicosapentaenoic acid
EPM- Elevated plus maze
FO- Flaxseed oil
FS- Flaxseed
GBA- Gut brain axis
HIP- Hippocampus
i.p- Intraperitoneal
IFN- Interferon
IL- Interleukin
LPS- Lipopolysaccharide
mPFC- Medial prefrontal cortex
n3-PUFA- n3-poly-unsaturated fatty acid
NF-κB- Nuclear factor kappa B
OFT- Open field test
PFC- Prefrontal cortex
SAL- Saline
SCFA- Short chain fatty acid
SDG- Secoisolariciresinol diglucoside
TJ- Tight junction
TLR- Toll-like receptor
TNF- Tumour necrosis factor
TST- Tail suspension test
ZO- Zonula-occludens

List of Figures

List of Tables

Chapter 1. Introduction

The burdens of mental illnesses (e.g. depression, anxiety) are estimated to cost roughly $51 billion per year to the Canadian economy[1]. While approximately half of the economic burden to Canada is due to healthcare costs associated with mental illness, the remaining half is comprised of factors including decreased quality of life, and decreased work productivity[1,2]. In 2017 it was estimated that 1 in 10 Canadians aged 15-24 were experiencing some form of depression[3], while 2.5% of Canadians over the age of 15 reported symptoms common in generalized anxiety disorder[4]. Importantly, over the COVID-19 pandemic, mental health has been further deteriorated[5], implying an increased need for both systematic and scientific intervention.

Depression and anxiety, linked through common co-morbidity[6], are associated with an increase in inflammation in the brain (henceforth, neuroinflammation)[7–9]. Neuroinflammation can arise from various sources (e.g. stress, infection), and results in an increased activation of microglia, thus producing pro-inflammatory cytokines (e.g. IL-1β, TNF-α, IL-6)[10]. This has been further linked to changes across the gut-brain axis (GBA), which refers to the bi-directional link between the gastrointestinal tract and the central nervous system (CNS)[11,12]. This gut-brain connection is highlighted by the co-morbidity of mental (i.e. depression, anxiety) and intestinal illnesses (i.e. ulcerative colitis, abdominal pain, diarrhea)[13,14]. In individuals living with depression or anxiety, there is a proposed connection between dysbiosis of the gut microbiota and neuroinflammation in the brain[15], emphasizing the *microbiota*-gut-brain axis (mGBA)[16]. Despite many hypothesized interactions between the GBA[17], this thesis will be focusing on communication through the bacterial endotoxin lipopolysaccharide (LPS).

LPS is a component of the cell wall of Gram-negative bacteria, and is increased in the plasma of individuals living with depression or anxiety[18]. Consequently, in humans, endotoxin

(LPS) administration has been established to induce depression[19], with one instance of endotoxin-induced anxiety[20]. The implication of LPS is far greater than depression and anxiety alone, as it is also increased in neurodegenerative diseases such as Alzheimer's[21], and Parkinson's[22,23]. Thus, attenuating the impact of LPS on the GBA may be important in attenuating mental illnesses and neurodegenerative diseases. Diet is an important factor when discussing mental illnesses since poor nutrition has been identified as a potential confounding factor in these disorders[24,25]. In this thesis, I will be investigating the potential benefits of dietary supplementation with flaxseed (FS) and flaxseed oil (FO) on the GBA in LPS-challenged mice.

Chapter 2. Literature review

2.1 LPS and its role in inflammation

Bacterial LPS is commonly extracted from members of the *Enterobacteriaceae* family, primarily *E. coli* and *Salmonella* as their outer membranes are comprised of roughly 75% LPS[26]. Different serotypes of LPS may induce alternative effects on inflammation[27], however the general mechanism remains the same. LPS is the ligand for toll-like-receptor-4 (TLR-4), the binding of which induces an innate immune response through the NF-κB pathway[28] (Figure 1). Briefly, following the LPS/TLR-4 binding, there are two potential pathways: MyD88-dependent and MyD88-independent. The MyD88-dependent pathway involves production of pro-inflammatory cytokines (IL-1β, TNF-α, IL-6) both through NF-κB activation and other pathways. The MyD88-independent pathway involves the production of type 1 interferons (e.g. IFN-β). Importantly, the anti-inflammatory cytokine IL-10 can be produced through either of the above pathways either through TNF-α [29] or through IL-27[30].

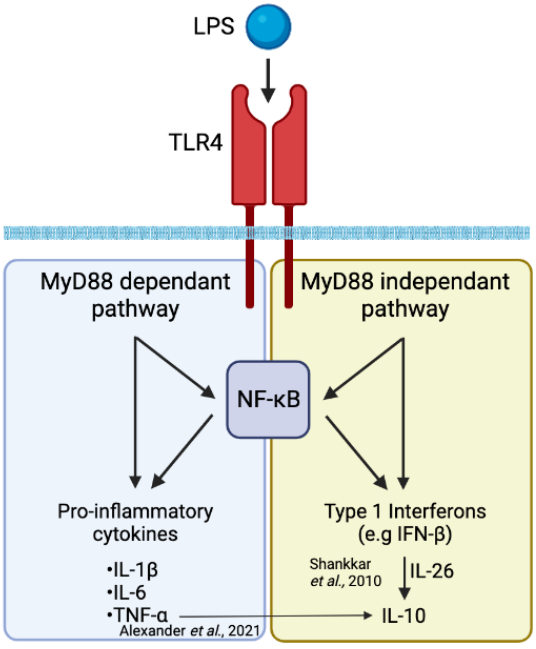

Figure 2-1. Downstream pathways following binding of LPS to its receptor TLR4
Figure adapted from[28].

Similar to the effects of LPS administration in humans[19], LPS administration in murine models is established to induce negative effects in the brain [31–35] and systemically[33,36]. The effects of LPS administration on the gut have not been examined in a human context, however in murine models, there are known negative effects on the gut[37–39], however the effects on the gut microbiota are still underexplored[40,41]. The majority of studies investigating the effects of LPS on aspects the GBA have been conducted with *in vivo* and *in vitro* rodent models, and thus will be the focus of this literature review.

2.2 LPS and the microbiota, intestinal inflammation and barrier integrity

2.2.1 The role of the gut as the source and target of LPS

A dysbiotic intestinal microbiota has been observed in individuals living with mental illnesses suggesting a possible target site for therapeutic interventions[42]. Importantly, a fecal transplantation study (transferring feces from individuals living with depression to microbiota-deficient rats) showed an increase in depressive- and anxiety-like behaviours, linking mental illness to gut dysbiosis[43]. Microbial dysbiosis is defined as an imbalance of the gut microbiota, and generally refers to a decrease in commensal or beneficial microbes and an increase in harmful or inflammatory microbes[44], which is highly relevant in relation to the co-morbidity of gut and brain diseases[16]. A dysbiotic microbiota often displays reduced diversity, specifically, α- and β-diversities. α-diversity is described as the species richness (number of different microbial species) and/or evenness (distribution of microbe abundance)[45]. ß-diversity is defined as the change in diversities between samples/environments[45]. Inflammation in the intestine results in microbial dysbiosis driven by increased abundance of *Enterobacteriaceae* for example[46,47], resulting in

increased presence of LPS within the gut. This increased LPS can cause damage to the epithelium, resulting in an increase in intestinal permeability and systemic inflammation[48].

There is also evidence that LPS can influence the microbiota composition. For example, in one study, an intraperitoneal (i.p) LPS administration in male C57Bl/6 mice induced a decrease in α-diversity, along with significantly different β-diversity[41]. This LPS-induced microbial dysbiosis was also associated with specific microbial changes, such as decreased abundance of *Actinobacteria* and *Firmicutes* and increased abundance of *Alloprevotella* and *Butyricimonas*[41]. Another study in hamsters suggested that the impact of i.p LPS on the immune system are independent of the gut microbiota[40], however, this may be related to the different sources of LPS; *E. coli*[41] vs *Salmonella enterica*[40]. A third study involving i.p LPS (from *E. coli*) in piglets also showed a decrease in α-diversity and a significant difference in β-diversity, highlighted by increases in *Enterobacteriaceae* and decreases in *Clostridium* and *Lachnospira*[49]. Taken together, there is some evidence of an effect of i.p LPS on the gut microbiota in mice and piglets, however there is limited overlap in specific taxonomic changes, likely related to species differences.

2.2.2 Implication of LPS in intestinal health

In a variety of animal models, i.p LPS has been shown to negatively impact intestinal health through factors such as intestinal barrier integrity, morphological changes, and inflammation. Intestinal barrier integrity is a critical measure of intestinal health, since when permeability is increased, there may be an increased paracellular translocation of immune-stimulating and potentially pathological bacterial metabolites and endotoxins (i.e. LPS)[50]. Tight junctions (TJ) are regulators of paracellular transport, and generally force nutrients and ions to cross through transcellular mechanisms[50]. Studies in *in vivo* models (mice, rats, piglets) show that i.p LPS decreases both the protein[37,38,49,51–53] and gene[39] expression of TJs (occludin, claudin and zonula-

occludens-1 (ZO-1)) in the colon and small intestine. TJs are affected in a TLR-4 dependant manner (expressed on the basolateral surface of enterocytes[54]) through activation of the MyD88-dependant pathway[55]. The MyD88-dependant pathway results in an increase of pro-inflammatory cytokines as described above (section 2.1) (i.e. IL-1β, TNF-α, IFN-γ, IL-6), which have all been documented to be increased following i.p LPS in a variety of models[38,49,51,56–58].

The importance of the gut, specifically the microbiota and intestinal health have been outlined here as important in the response to LPS. These changes discussed, including the altered microbiota, and increased intestinal permeability, may suggest an increase in systemic inflammation through increased translocation of LPS across the gut barrier. Therefore, interventions which may attenuate the negative effects in the gut may result in benefits across the GBA to attenuate neuroinflammation and mental health disorders.

2.3 Acute and chronic systemic immune responses to LPS

Systemic inflammation is an important aspect of mental illness[59,60], especially as a meta-analysis described an association between systemic inflammation and depressive symptoms across 15 studies[61]. Consequently, i.p LPS is well established to induce systemic inflammation[36,62–64]. For example, 6-8 week old male CD-1 mice were subjected to a single i.p injection of LPS, and there was evidence of increased pro-inflammatory cytokines (IL-1β, TNF-α) in the serum 28-hours post-injection[64]. To examine the more acute impact of i.p LPS on systemic inflammation, 6-8 week old CD-1 male and female mice were given a i.p injection of 1 mg/kg LPS, then euthanized 2 hours post-injection[63]. They found increased splenic concentrations of IL-1β, TNF-α and IL-6, indicating an increase in systemic inflammation, which was further increased in the male mice compared to female mice. Similarly, in 8 week old C57Bl/6 male mice, they found an increased in serum TNF-α 1-hour following 3 mg/kg LPS i.p injection[65]. This increase in systemic

inflammation is a result of monocyte activation[66], through mechanisms described previously (Section 2.1). Therefore, i.p LPS induces systemic inflammation as measured by serum and splenic cytokines. This increase in systemic inflammation may be linked to an increase in neuroinflammation[36,67,68], highlighting the importance of interventions that may attenuate these LPS-induced negative effects systemically and in the brain.

2.4 *Neuroinflammation and the effects of LPS on the brain*

Neuroinflammation is highly relevant when discussing mental illnesses, specifically depression and anxiety. Many brain regions are potentially implicated in mental illnesses, but of most relevance in this thesis are the hippocampus (HIP) and the prefrontal cortex (PFC), both of which are implicated in depression[69,70] and anxiety[71,72]. The established effects of i.p LPS injection on the brain include the induction of neuroinflammation, generally characterized by an increase in pro-inflammatory cytokines (i.e IL-1β, TNF-α) in the HIP and PFC[31,33,73,74]. To elaborate, in one study, 8 week old C57Bl/6 male mice were given an i.p injection of 1 mg/kg LPS and euthanized ~26 hours post-injection, and there was evidence of increased IL-1β and TNFα in the frontal cortex, and increased IL-1β and IL-6 in the HIP[73]. In aged (2-years old) C57Bl/6 male mice, an i.p injection of 0.15 mg/kg LPS increased IL-1β, TNF-α, IL-6 and IFN-γ 24-48 hours post-injection in the HIP and PFC[31]. Thirdly, a study with male Sprague-Dawley rats involved chronic i.p LPS (0.5 mg/kg every 2 days for a total of 7 injections), and 48-hours after the last injection, there was evidence of increased IL-1β, TNF-α and IL-6 in both the HIP and PFC[74]. These pro-inflammatory cytokines are produced by glial cells within the brain (i.e. astrocytes and microglia)[75] following binding of LPS to TLR-4 (enriched in microglial cells)[76,77]. Importantly, LPS has been shown to increase blood brain barrier (BBB) permeability[34,35,78], which could lead to increased LPS

translocation into the brain, increased presence of systemic-derived cytokines in the brain, and increased microglial activation. The specific role of microglia in mental illnesses are still controversial but there could be relations to microglia-neuron interactions[79] and the kynurenine pathways[80], but these are out of the scope of this thesis. Therefore, LPS effectively induces neuroinflammation, a symptom characteristic of depression/anxiety[7–9], suggesting a requirement for therapeutics to alleviate the LPS-driven effects across the GBA.

2.5 Diet as potential modulator of LPS effects on the GBA: Flaxseed as a target food

2.5.1 Flaxseed and its components

Flaxseed (*Linum usitatissimum*) (FS) is an oilseed comprised of 40% oil (~40g/100g), over half of which is the n3-polyunsaturated fatty acid (n3-PUFA) α-linolenic acid (ALA) (50-62%), 25-30% dietary fibre, and 1-2% polyphenolic lignan , secoisolariciresinol diglucoside (SDG) [81,82]. Each of these individual bioactives have anti-inflammatory and/or antioxidant effects as described in this section, and can modulate aspects of the GBA, such as the gut microbiota.

2.5.2 Role of n3-PUFAs on the gut microbiota composition and function

Omega-3 fatty acids, or n3-PUFAs as described henceforth, are long-chain fatty acids that have are associated with various health benefits[83]. There is evidence that n3-PUFAs can modulate the gut microbiota composition. Increased microbial α-diversity has been observed in some studies[84,85], with microbial β-diversity commonly changed[84–89]. Although there are differences based on model, sex, dose etc., there are consistent increases in *Bacteroidetes*[84,86,87], *Lactobacillus*[84–86], and *Lachnospiraceae*[84,85], and decreases in *Firmicutes*[86,88]. These studies involved supplementation with fish oils which include the n3-PUFAs eicosapentaenoic acid (EPA)

and docosahexaenoic acid (DHA). FS derived n3-PUFAs, specifically ALA, can be converted into DHA and EPA within the body (Figure 2). Despite low conversion rates of ALA to EPA and DHA (0.2-7%) dependant on factors such as species, dose, age and sex [90–93], there remains evidence that FS oil (FO) can also modulate the diversity and composition of the intestinal microbiota.

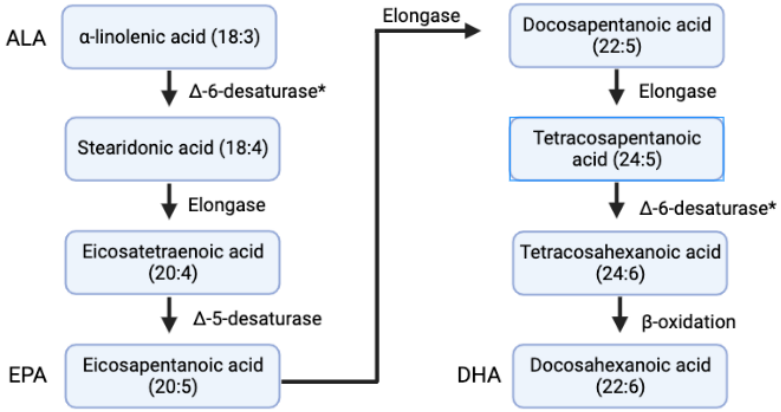

Figure 2-2. Metabolism of ALA into EPA and DHA.
An asterix suggests a rate-limiting step.

To elaborate, female C57Bl/6 mice fed with 3.67% FO for 3 weeks showed no change in α- nor β- diversity, however there were a few specific taxonomic changes such as increased *Parabacteroides* and *Deferribacterales*[94]. In another study, C57Bl/6 male mice fed high fat diets supplemented with 18.4% FO (45% energy from fat, 35% energy from FO) (~552mg/day for 8 weeks), demonstrated both significantly increased α-diversity, a clear difference in β-diversity, decreased levels of *Firmicutes* and *Clostridiales*, and increased levels of *Allobaculum* and *Coreobacteriaceae*[95]. FO supplementation has also been shown to decrease *Firmicutes* and increase *Bacteroidetes* in a mouse model of diabetes mellitus[96].

Only one study to date has examined the effects of n3-PUFA supplementation on tight junctions in the gut. In this study, following induction of intestinal dysbiosis by ceftriaxone sodium, mice were given a daily gavage of either 30, 60 or 90 mg of n3-PUFAs for 3 weeks, and

the 90 mg administered mice had increased occludin (tight junction protein) in the ileum, suggesting a beneficial impact on the integrity of the barrier[85]. Therefore, mechanistically FO may have beneficial impacts on the gut integrity and the microbiota community composition, which may attenuate the negative effects induced by LPS.

2.5.3 Implication of n3-PUFAs on systemic inflammation

As highlighted in section 2.1, when LPS translocates across the gut barrier, there is an increase in systemic inflammation. With the potential increase in gut barrier integrity with n3-PUFAs (section 2.5.2), this may relate to a decrease in systemic inflammation due to a decrease in LPS crossing the gut barrier. To elaborate on this link, in the study investigating different doses of n3-PUFAs in the previous section, they also found that the 90 mg dose was sufficient to decrease serum IL-1β, TNF-α and increase IL-10[85]. Another previously described paper with 1% EPA or DHA supplementation, found a decrease in serum LPS which highlights the potential benefits of n3-PUFA supplementation[84]. With FO supplementation, studies have shown potentially anti-inflammatory effects[97,98]. For example, one study used a post-partum model of depression in Sprague-Dawley rats, and found that FO supplementation (43.2 mg/day for 15 days) decreased serum IL-1β, TNF-α and IL-6, suggesting an anti-inflammatory effect of FO[98]. Another study using Sprague-Dawley rats with drug (streptozotocin-nicotinamide) induced diabetes, showed decreased plasma concentrations of these same cytokines with 10% FO supplementation[96]. Therefore, n3-PUFAs may have beneficial anti-inflammatory effects systemically, which may attenuate the LPS-induced systemic inflammation.

2.5.4 Role of n3-PUFAs in modulating neuroinflammation

In relation to the potential benefits on the gut, n3-PUFAs are also characterized by having beneficial effects in the brain. The fish oils, EPA and DHA, are well characterized to combat the negative effects of i.p LPS in the brain[31,73,74,99]. The study in aged C57Bl/6 mice (section 2.4.1) also involved supplementation of 50μl of fish oil daily for 26 weeks, and resulted in decreased IL-1β, and TNF-α in both the HIP and PFC[31]. A study in 8-week old C57Bl/6 male mice gave mice a daily gavage of 50 μl of fish oil for 2 weeks followed by i.p injection of LPS (Section 2.4.1), and again there was significant attenuation of IL-1β, TNF-α and IL-6 in the frontal cortex and HIP[73]. A study from the same group following the same experimental design (measuring different outcomes aside from inflammation) demonstrated the same findings (decreased IL-1β, TNF-α, IL-6)[99]. Lastly, a study in Sprague-Dawley rats (Section 2.4.1) had supplemented their mice with 1.5 g/kg of fish oil per day for 3 weeks (2 latter weeks overlapping with LPS challenge), and they found decreased IL-1β and IL-6 in both the PFC and HIP[74]. Importantly, none of these studies discuss attenuation of inflammation in the serum. Therefore, current research has shown that fish oil derived n3-PUFAs have anti-inflammatory effects in the brain, however no study to date has examined the anti-inflammatory potential of FO. Nonetheless, these positive effects on the HIP and PFC may translate to potential therapeutic effects of n3-PUFAs with respect to mental illness.

2.5.5 *FS fibre and gut microbiota composition and function*

FS fibre is rich in fermentable non-digestible carbohydrates and is comprised of roughly 2/3 insoluble and 1/3 soluble fibre[100]. This fibre is primarily digested in the large intestine where it is fermented into short chain fatty acids (SCFAs) such as acetate, butyrate and propionate[101] by intestinal commensal bacteria[102]. Many taxonomic groups of bacteria are involved in fibre

fermentation, notable groups include *Bifidobacteria*, *Prevotella*, *Alistipes*, *Roseburia* and *Lactobacillus* amongst many others[103]. SCFAs are associated with beneficial health effects, in particular, anti-inflammatory effects via suppression of pro-inflammatory cytokines (e.g. IL-6, TNF-α). Locally (in the gut), SCFAs have been described to help maintain intestinal barrier integrity, and decrease inflammation[104] through binding to G protein-coupled receptors (GPRs), specifically GPR 41 and 43 which results in decreased inflammatory effects[105]. There has not been much research on the isolated effects of FS fibre, however one study has shown that FS fibre is capable of modulating the gut microbiota compared to a high fat diet shown by changes in β-diversity, and increasing certain taxonomic groups such as *Actinobacteria, Verrucomicrobia, Bifidobacterium, Lactobacillus*[106]. They also noted increased butyrate with FS fibre supplementation, but no effect on the other SCFAs[106]. Other studies using *in vitro* models of fibre fermentation show that FS fibre effectively increases SCFA levels[107,108]. The primary function of interest of the gut microbiota with respect to fibre supplementation is SCFA production, which is shown to be increased with FS fibre supplementation. Therefore, dietary fibre is an important aspect of FS, as it can modulate the gut microbiota, and increase SCFA concentrations which could potentially attenuate systemic- and neuro-inflammation.

2.5.6 FS fibre and systemic-/neuro-inflammation

The relationship between dietary fibre and the brain may be related to the production of SCFAs, which specifically attenuates microglia activation/function[109–111]. SCFAs that reach systemic circulation can cross the BBB through monocarboxylate transporters (MCTs), after which they have widespread beneficial effects such as reduced inflammatory signaling by glial cells and increasing the BBB integrity[104]. In a study where butyrate was given as a pre-treatment

to LPS-exposed rat primary microglial and hippocampal slice cultures, butyrate successfully prevented the LPS-induced inflammation, thus suggesting that *in vitro*, butyrate could attenuate neuroinflammation[112]. This was verified by an *in vivo* study which showed a significant attenuation of LPS-induced inflammation in aged Balb/c mice given butyrate supplementation[113]. Although no study has explicitly examined the effects of FS fibre on either systemic or neuroinflammation, we can extrapolate from the known benefits of SCFA.

2.5.7 FS-derived SDG and the gut microbiota composition and function

FS is the richest source of the plant lignan secoisolariciresinol diglucoside (SDG) consisting of ~1g/100g FS depending on the cultivar[114]. The effects of SDG on the fecal and cecal microbiota have been examined in female C57Bl/6 mice[94]. The mice received 0.15% SDG dietary supplementation (physiologically relevant dose) for 3 weeks, and at the surface level there were no differences in α- nor β- diversity, although there was increased abundance of *Mucispirillum* and *Ruminococcus.* Interestingly, short-term (10 days) oral administration of a synthetic version of SDG (LGM2605) further altered the cecal microbiota shown by changes in β-diversity[115]. LGM2605 induced changes at the phylum level by increasing *Bacteroidetes* and *Proteobacteria*, and decreasing *Firmicutes*[115].

The changes in microbiota composition also may relate to changes in microbiota function, most abundantly through metabolism of SDG into its downstream metabolites enterolactone (ENL) and enterodiol (END)[94,116]. SDG and its metabolites have been shown to have beneficial effects on the cardiovascular system, mental stress, and have anti-diabetic and anti-cancer effects[116]. The potential benefits of SDG may be more impactful in females since ENL and END are phytoestrogens; compounds that can bind with the estrogen receptor[117]. Therefore, SDG is

capable of altering the gut microbiota, and produces downstream metabolites which may induce beneficial effects.

2.5.8 Role of FS-derived SDG on systemic-/neuro- inflammation

The effects of daily SDG (oral gavage, 1.2 – 4.8 mg/day) for 21 days were investigated in chronically-stressed ovariectomized ICR mice, and determined that SDG was capable of reducing proinflammatory IL-1β and IL-6 in the frontal cortex, suggesting an anti-inflammatory effect[118]. Interestingly, a more recent study examined the effects of a single dose of SDG (4mg/mouse) on LPS-induced blood brain barrier (BBB) permeability and TNF-α induced neuroinflammation in mice, and showed that SDG is capable of attenuating LPS-induced BBB permeability and TNF-α-induced immune cell infiltration across the BBB[35]. This increase in BBB integrity by SDG also decreased the translocation of inflammatory signals to the brain, thus attenuating the neuroinflammatory state[35]. These studies thus suggest an anti-inflammatory role of SDG in mice, which could lead to attenuation of LPS-induced neuroinflammation. However, these studies utilized a dose of SDG (4mg/mouse) that is more than the amount achievable from FS consumption. Typically, in animal model FS is supplemented into diets at a level of 10% which is equivalent to ~ 2 tablespoons per day for humans. This supplementation level of FS would deliver approximately 0.3mg SDG/day/mouse. Therefore, although these studies are promising, it is still unknown if SDG at concentrations achievable by diet could lead to the reduced neuroinflammation and improved BBB integrity.

2.5.9 The effects of whole FS on the gut microbiota composition and function

No studies have examined the effects of whole FS supplementation on the murine GBA, however, one study demonstrated changes to the gut microbiota given 10% FS (~ 120 mg oil, 75 mg fibre, 4.5 mg SDG/day for 3 weeks) dietary supplementation in male C57Bl/6 mice[119]. Whole FS-supplementation had no significant effect on the α-diversity, however the β-diversity was significantly changed[119]. They found an increase in butyrate-producing bacteria (*Roseburia*), and a near elimination of *Akkermansia muciniphila* (present in the mucus layer, and generally a beneficial bacteria[120]). A similar study with 10% FS dietary supplementation in female C57Bl/6 mice, showed an increase in α-diversity and significantly different β-diversity[94]. Specific taxonomic changes also include increased *Roseburia* and *Lactobacillus* and *Coriobacteriaceae*, the latter two which may be indicative of sex-dependant effects.

In addition to the effects of FS supplementation on the gut microbiota, there was also evidence of increased SCFA concentrations in male mice[119], however there is no evidence in female mice. Therefore, whole FS can modulate the mouse microbiota (both male and female), increase SCFA production (male), which may suggest a potential benefit of FS dietary supplementation across the GBA.

2.5.10 Role of whole FS on systemic-/neuro-inflammation

Although no studies have shown the effects of whole FS on the rodent GBA, one study has shown anti-depressive/anxiolytic effects given FS supplementation. In a chronic stress study supplementing Sprague-Dawley rats with either 10% FO (~ 750 mg oil/day for 4 weeks) or 20% whole ground FS (~ 600 mg oil, 375 mg fibre, 22.5 mg SDG/day for 4 weeks), there was an attenuation of depressive-like behaviours[97]. These anti-depressive-like behaviours may be

indicative of anti-inflammatory effects, however in this study they only examined the effects of FS and FO on brain derived neurotrophic factor (BDNF) and discovered no significant effects. To my knowledge, there are no studies investigating the effects of whole ground FS supplementation directly on systemic or brain inflammation. The increase in ENL and END previously described may be a key feature however, as *in vivo* analysis of human blood lymphocytes showed that ENL and END decreased activation of the NF-κB pathway[121]. Therefore, the effects of whole FS on the murine GBA have yet to be thoroughly investigated with respect to the GBA, however there remains potential for critical gut-brain communications.

Chapter 3. Rationale, Hypotheses, and Objectives

3.1 Rationale:

Mental illnesses, specifically depression and anxiety are an increasingly growing problem in Canada, highlighted in the past two years with the COVID-19 pandemic. These problems are likely exacerbated by the lack of resources available, including the availability of family doctors. Thus, the requirement for scientific interventions of mental illnesses is very high, which suggests the need for an easily accessible option.

In a rodent model of depression and anxiety, specifically through i.p administration of LPS, the effects on anxiety/depressive-like behaviours is variable between studies and is affected by many factors including LPS source, dose, mouse strain, age, etc[31,41,73,122,123]. Therefore, it is important to validate the LPS response in C57Bl/6 male mice. Additionally, there has not been previous research investigating the involvement of the GBA in LPS-induced neuroinflammation, specifically highlighting the potential link between LPS-induced gut dysbiosis and neuroinflammation. Understanding these interactions will allow us to identify key therapeutic targets for mental illnesses.

Diet has been indicated as a factor in mental illnesses in children and adolescents[124]. This is highly relevant especially in young adults, who may just be starting to have some food independency and choice. Health Canada's food guide recommends eating more plant-based foods, such as nuts and seeds and FS is a readily available crop in Canada, and has been shown to beneficially alter the gut and the microbiota in C57Bl/6 mice[94,119]. FO has been shown to beneficially alter systemic inflammation and depressive-like behaviours[98]. Therefore, FS may be an accessible source of food which may attenuate some of the effects of mental illness across the GBA.

To date, no studies have examined the ability of FS and FO to attenuate depression/anxiety via modulation of the GBA. Based on the evidence that fish oil can effectively attenuate LPS-induced neuroinflammation (2.5.4), while purified FS fractions (FO (2.5.4) and SDG (2.5.8)) have been shown to induce beneficial anti-inflammatory/anti-depressant/anxiolytic effects, this MSc research thesis will determine the impact of whole ground FS and its purified FO on the mouse GBA.

3.2 Overall Thesis Hypotheses

Based on the knowledge gaps identified in the literature review and rationale above, it is hypothesized that:

1. Mice injected with a single dose of LPS (i.p) will develop symptoms of sickness and anxious/depressive-like behaviours driven in part through modulation of the GBA (microbial dysbiosis, and systemic and neuroinflammation).
2. Mice consuming FS- and FO-supplemented diets will display an attenuated response to LPS, in part through modulation of the GBA (microbiota, systemic and neuroinflammation)
3. Mice consuming diets supplemented with FS will have a greater attenuation of the LPS-induced negative effects compared to mice consuming FO alone, due to the combined effects of dietary fibre, lignans, and ALA-rich FO.

3.3 Overall Thesis Objectives

To address my first hypothesis, in Study 1 I will

1. Examine the effects of an acute i.p administration of LPS in C57Bl/6 male mice on the GBA, including sickness and depressive-/anxiety-like behaviours, gut microbiota composition, systemic inflammation, and HIP and PFC neuroinflammation.
2. Determine if there is a relationship between LPS-induced neuroinflammation and gut microbiota dysbiosis by correlating changes in diversity and taxa abundances with behaviours and inflammatory cytokine expression.

To address my second and third hypotheses, in Study 2 I will

1. Measure the LPS response (systemic inflammation, neuroinflammation, and sickness behaviours) in mice fed control, FS or FO diets and determine the role of the gut microbiota (composition and function) in the diet-induced effects.
2. Compare the outcomes of FS and FO on the LPS-induced negative effects to determine if FS induces additive effects compared to FO.

Chapter 4: The effects of i.p LPS on the GBA and depressive-/anxiety-like behaviours in male C57Bl/6 mice

4.1 Introduction

Mental illnesses are highly prevalent in Canada, with 50% of individuals by the age of 40 living with (or had lived with) a mental illness[2], with the greatest representation in individuals between the ages of 15-24[125]. The COVID-19 pandemic has further worsened mental health, specifically depression and anxiety[5] indicating an increased need for systematic and scientific intervention. Mental illnesses such as depression and anxiety, linked by common co-morbidity[6], are known to induce negative effects across the bi-directional gut-brain axis (GBA), highlighted by dysbiosis of the gut[15] and increased inflammation in the brain (neuroinflammation)[7–9]. Similarly, mouse models of depression and anxiety such as the immune activation model, through i.p injection of the bacterial endotoxin LPS, have been shown to induce negative effects across the GBA[41,65,73].

In the gut, i.p LPS in male piglets has been shown to negatively impact the gut microbiota through decreased diversity and increased relative abundance of harmful bacteria such as *E.coli*[49]. Systemically, i.p LPS in male CD-1 mice resulted in increased pro-inflammatory cytokines (IL-1β, TNF-α) in the serum[64]. In the brain, many studies in male C57Bl/6 mice highlight increased pro-inflammatory cytokines in key brain regions implicated in depression and anxiety such as the HIP and PFC[31,73,74]. Despite the LPS-induced negative changes in the across the GBA, no study has investigated the link between the effects of i.p LPS on the gut and the brain. Therefore, the goal of this study was to determine if there is an association between LPS-induced neuroinflammation and microbial dysbiosis to establish potential therapeutic targets for mental well-being.

In this study, we hypothesized that mice administered i.p LPS will exhibit characteristics similar to depression and anxiety, highlighted by microbial dysbiosis, systemic, and neuroinflammation and anxiety- and depressive-like behaviours. Consequently, the objectives of this study were to 1) characterize the effects (microbiota, systemic and neuro-inflammation), and phenotypic and behaviour responses of an acute LPS (i.p.) administration in C57Bl/6 male mice; and 2) Determine if there were relationships between the LPS-induced effects in the gut and brain.

4.2 Methods:

4.2.1 LPS preparation

LPS from *Escherichia coli* O26:B6 (Sigma, L3755-100MG) was dissolved in 0.9% sodium chloride saline (Fresenius Kabi Canada, 02395150), to create a of 5 mg/mL stock solution. The stock solution was aliquoted into DNAse, RNAse-free microcentrifuge tubes, and stored at -80°C until needed. On the day of injection, one aliquot was removed from the freezer and diluted to 0.2 mg/mL with the 0.9% sodium chloride saline mentioned previously.

4.2.2 Animals and study design

Male C57Bl/6 mice (5 weeks old) were purchased from Charles River, (Kingston, NY, USA), and were acclimatized for 2 weeks on a modified, purified AIN-93G basal diet (BD) with 7% corn oil (see Table 3 for composition). The mice were individually caged with corn cob bedding and cotton nesting material and had access to food and water *ad libidum.* The mice were maintained on a 12 hr:12 hr light:dark cycle, with room temperature at 23°C, and humidity at 40%. All experimental conditions were reviewed and approved by the uOttawa Animal Care Committee (Animal use protocol: #HSe-2857-R3).

Figure 4-1. Experimental design Study 1.

After acclimatization, 24 mice were randomized by BW into 2 experimental groups 1) LPS, or 2) saline (SAL) and given an intraperitoneal (i.p.) injection of 1 mg/kg BW LPS or an equal volume of SAL. The old nests were removed, and 2 new squares of nesting material were added to the front of the cages. The mice were monitored at defined timepoints throughout the day to measure i) sickness behaviours, ii) BW loss, iii) diet intake, and iv) nest building behaviours, as described below. Twenty-four hours following injection, mice underwent two behaviour tests each (one depressive-like and one anxiety-like), either the open field test (OFT) + splash test (ST), or the elevated plus maze (EPM) + tail suspension test (TST), as described below. Forty-eight hours following injection, the mice were euthanized by decapitation and blood and tissues were collected and stored for later molecular analyses, as described below.

4.2.3 Analysis of Sickness behaviours

Sickness is the first response to systemic inflammation[126], thus, sickness behaviours were assessed as a potential primary outcome that could be attenuated by future dietary interventions. It is also important to monitor the sickness of mice following LPS injection to ensure the sickness does not coincide with the behaviour tests. In this study, three measures of sickness were assessed i) Diet intake and BW loss, ii) sickness behaviour scores, and iii) nest building behaviours.

4.2.3.1 Diet and BW measures:

Approximately 30 minutes prior to injection, BW and diet were weighed using an electronic balance. The BW and diet remaining was weighed at 8-, 24-, and 48-hours post-injection. Diet intake was calculated by Diet weight (pre-injection) – Diet weight (8-, 24- or 48-hours post-injection). The percent BW change was calculated by (BW2-BW1)/BW1 * 100.

4.2.3.2 Sickness behaviour scores

Following the methods outlined by Kolmogorova et al., 2017, the mice were closely monitored, and select sickness behaviours were recorded 30 minutes, 2, 4, 6, 8, 12, and 24 hours post-injection. The behaviours measured include pilo-erection (hair standing up), lethargy (slow-moving), ptosis (squinted eyes), and huddling (hunched back). The behaviours were scored based on the presence (1) or absence (0) by two blinded observers, and the score for each mouse was considered an average of both observers.

4.2.3.3 Nest building scores

Nest building is a natural behaviour for mice, and is said to estimate the integrity of hippocampal functions[128], and is an indicator of sickness following LPS injection[129]. Immediately before injection of LPS or SAL, the used cotton nesting material was removed and discarded. Two new pieces of cotton nesting material were added to the front of the cage, and videos of the nests were recorded 8- and 24- hours post-injection. The videos were scored by two blinded observers with focus on 4 key attributes: i) nesting material shredding, ii) nest shape, iii) nest location, iv) nest walls. The maximum score is 6.5. The scoring system is outlined in the appendix (Table S1).

4.2.4 Anxiety-like behaviour tests:

4.2.4.1 Open field test:

The open field test (OFT) is often used to assess the fear of open spaces which is reminiscent of anxiety-like behaviours[130]. Mice were habituated in for a minimum of 30 minutes at 100 lux in their home cage in a quiet room adjacent to the testing room. The light in the testing room was set to 300 lux. A mouse was placed in the centre an empty white box with no lid (45cm x 45 cm x 45 cm). The mice were automatically tracked using Ethovision XT 15 (Noldus) for 10 minutes, after which the mice were returned to their respective cages in their housing room. The resulting data was analysed to determine the following: time spent in the centre of the box, time spent in the corners of the box, total distance travelled, and latency to enter centre/corners.

4.2.4.2 Elevated Plus Maze:

The elevated plus maze (EPM) is characteristically used to assess exploratory behaviours and the fear of open space, which are reminiscent of anxiety-like behaviours in rodents[131]. The EPM apparatus has 2 perpendicular and intersecting platforms (~6cm wide and ~75cm in length) with an open space where they meet, and is set up ~74cm off the ground. Two of the arms (1 cross-section) are open platforms, the other two arms were enclosed with ~20cm tall walls. Prior to testing, the mice were habituated in the room adjacent to the testing room for a minimum of 30 minutes at 100 lux in their home cage. A mouse was placed into the centre of the EPM apparatus and were automatically tracked using Ethovision XT 15 (Noldus) for 10 minutes, which determines time spent in the open vs closed arms, latency to enter each arm, and total distance moved. Mice were then immediately returned to their respective home cage in their housing room.

4.2.5 Depressive-like behaviour tests

4.2.5.1 Splash Test:

The splash test (ST) is used to measure grooming (cleaning itself) which is reminiscent of depressive-like behviours [132]. Mice were habituated in their cages for minimum 30 minutes in the testing room. A spray bottle was prepared with mouse drinking water instead of a 10% sucrose solution to minimize the potential for sucrose-induced changes in the microbiota. A mouse was quickly, yet forcefully sprayed twice with water on its lower back (one spray on either side of the tail), then was transferred to a clean cage in front of a video camera. The mice were recorded for 10 minutes, then moved back to their respective cages. The videos were scored by two trained and blinded observers using the BORIS software (University of Torino), which resulted in the following outputs: total time spent grooming, latency to groom. A secondary metric was also assessed in these videos, specifically rearing (mouse is on two legs), which is a measure of exploratory behaviour and may be reminiscent of anxiety-like behaviours. The rearing was scored similar to the grooming, and resulted in the following outputs: total rearing time, latency to rear, and mean duration of grooming. total rearing time, latency to rear mean duration of rearing,

4.2.5.2 Tail Suspension Test:

The tail suspension test (TST) is a measure of behavioural despair, which is reminiscent of depressive-like behaviours [133]. Mice were habituated for minimum 30 minutes in the testing room. The tail of the mice were put through a small plastic tube (~2cm long) to prevent the mice from escaping, the mice were then attached to the tail suspension interface cabinet (Med Associates, DIG-735) with a piece of tape on the upper third of the tail. The mice were kept on the apparatus

for 6 minutes, after which they were returned to their respective cages. The interface cabinet and the load cell amplifier (Med Associates, ENV-505TS) provided the data which could determine the mobility and immobility time, where increased immobility time was indicative of an increase in depressive-like behaviours[31].

4.2.6 HIP and mPFC isolation:

After euthanasia by decapitation, the brain was promptly isolated. With the brain on a chilled surface, the olfactory bulbs were removed and discarded. A 1 mm section of the frontal cortex (prefrontal cortex) was excised and stored in a microcentrifuge tube on dry ice. To extract the hippocampi, the cerebellum was removed and stored, and the brain was then cut to separate the two hemispheres. With the medial surface facing up, the midbrain, striatum and thalamus were peeled back to reveal the HIP which was then collected and stored in a microcentrifuge tube on dry ice. This procedure was completed for both hemispheres of the brain, then tissues were stored at -80°C until further analysis.

4.2.7 HIP and mPFC mRNA expression:

4.2.7.1 *RNA extractions*

RNA was extracted and purified using Total RNA Purification Plus Kit (Norgen Biotek, #48400). Five 5 ceramic beads (VWR, #10158-554) were placed into reinforced bead mill tubes (VWR, #10158-556), and 300 µl of Buffer RL was added, containing 0.01% 2-mercaptoethanol. One HIP or the mPFC was added to each of the bead mill tubes, then samples were run in a Bead Mill 24 (Fisherbrand, #15340163) for 25 seconds at 2500 RPM. The homogenate was then transferred to an RNAse free microcentrifuge tube and centrifuged for 2 minutes at 2000 RPM.

The supernatant was transferred to the genomic DNA (gDNA) removal columns and was centrifuged for 2 minutes at 8000 RPM. For every 100 µL of flow-through, 60 µl of 100% ethanol was added, and the RNA was isolated using the RNA purification columns, and the columns were centrifuged for 1 minutes at 6000 RPM. Following RNA isolation, 400 µl of wash solution A was added to the column and centrifuged for 1 minute at 6000 RPM, this process was repeated a total of three times. Purified RNA was then eluted using 22 µl of elution solution A, and RNA concentration and quality was measured by Nanodrop (Thermo scientific, #ND-ONE-W). RNA was stored at -80°C until further use.

4.2.7.2 Complementary DNA preparation:

The purified RNA was used to prepare complementary DNA (cDNA) using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, #4368814). A master mix was created with 10X RT buffer, 25X dNTP mix (100 mM), 10X RT random primers, Multiscribe reverse transcriptase, and nuclease free water. The master mix was added to 0.2 mL PCR tubes (Eppendorf, #951010006), followed by 1 µg of RNA. The cDNA was synthesized in a T100 Thermal cycler (Bio-rad, #1861096) with the following program: 10 minutes at 25°C, 120 minutes at 37°C, and 5 minutes at 85°C. The resultant cDNA was diluted (1:10) with nuclease free water and stored at -20°C until further use.

4.2.7.3 Quantitative PCR

Quantitative PCR (qPCR) was performed using the CFX96 Touch (Bio-rad CFX96) real time system. A master mix was created using SYBR green (Applied Biosystems, 4#367659), 10 mM primers (Table 4-1), and nuclease free water, and 6 µl was added to each well of a Low-

Profile, Semi-Skirted Hard-Shell, PCR Plate (Bio-rad, #HSL9601). The RNA was added to the respective wells (4 µl), then the plate was sealed using the Microseal 'B' adhesive seal (Bio-rad, #MSB1001). Relative gene expression was then calculated using the ΔΔCq method, using the housekeeping gene RPLP0 as a baseline.

Table 4-1. Primers used for qPCR for measurement of relative gene expression.

Gene	Forward Primer 5' to 3'	Reverse Primer 5' to 3'
RPLP0	ACTGGTCTAGGACCCGAGAAG	TCTGACCTCTGTTCCACCCT
IL-1β	AGTTGACGGACCCCAAAAG	GGACTACTCTCGTAGGTCGA
TNF-α	CATCTTCTCAAAATTCGAGTGACAA	CCCAACATGGAACAGATGAGGGT

4.2.8 Cecal DNA extractions

Cecum content was collected at euthanasia and immediately placed in a pre-chilled microcentrifuge tube on dry ice then was stored at -80°C. Cecal DNA was extracted using QIAamp Fast DNA Stool Mini Kit (Qiagen #51604, Germany) with the recommended protocol. Briefly, small portions of cecal content (~50 mg) were placed into reinforced bead mill tubes (VWR, #10158-556) containing five 5 ceramic beads (VWR, #10158-554) and 1 mL of InhibitEX buffer and were placed into a Bead Mill 24 (Fisherbrand, #15340163). Samples were homogenized for 1 minute at 4,000 x g (more if required), after which the homogenates were transferred to G-tube microcentrifuge tubes (VWR, #21150-486) and incubated on a hot plate (Fisher Scientific, Isotemp #11-715-125D) at 95°C for 6 minutes. The samples were vortexed for 15 seconds at high speed, then centrifuged at RT for 2 minutes at 14,000 RPM, then 600 µl of the supernatant was placed into a new G-tube containing 25 µl of proteinase K. The new G-tubes were vortexed for 15 seconds, then 600 µl of Buffer AL was added, followed by another 15 second vortex. The samples were then incubated at 70°C for 10 minutes, then 600 µl of 100% ethanol was added, and then vortexed for 15 seconds. Next, 600 µl of lysate was added to the QIAamp spin columns and were centrifuged at RT for 1 minute at 14,000 RPM, the filtrate was discarded. This was repeated until all the lysate

had passed through the filter. Buffer AW1 (500 µl) was added to each of the spin columns, followed by centrifugation for 1 minute at 14,000 RPM. The spin columns were carefully transferred to new collection tubes, then 500 µl buffer AW2 was added, and the spin columns were centrifuged for 3 minutes at 14,000 RPM. The empty spin columns were transferred to a new collection tube and were centrifuged for 3 minutes at 14,000 RPM. Spin columns were transferred to fully labeled microcentrifuge tube and 10-20 µl of buffer ATE (depending on size of sample) was added directly onto the spin column membrane (with care taken not to touch the membrane). Spin columns were incubated for 1 minute at RT, followed by centrifugation for 1 minute at 14,000 RPM. DNA concentration and quality was measured by nanodrop. DNA concentration was confirmed using the Quant-iT PicoGreen dsDNA Assay kit (Thermo Fisher Scientific, #P11496)

4.2.9 PicoGreen measurement of DNA concentration

To ensure accurate DNA concentrations for 16S library preparation, the Quant-iT PicoGreen dsDNA Assay kit was used as per the manufacturer's instructions. Briefly, 20X TE buffer was diluted (1:20) to make 1X TE buffer. DNA standards were made by a 1:50 diluted in 1X TE buffer (Table 4-2). In a 96-well black plate with a clear bottom (Millipore Sigma, # CLS3603PP), 99 µl of 1X TE was added to each well (excluding standards), followed by adding 1 µl of sample DNA and 100 µl of standards to their respective wells. PicoGreen was diluted 1:200, and was added to each well (100 µl). The plate was covered in tinfoil and incubated for 5 minutes at RT, with gentle shaking by hand in the last 30 seconds. The fluorescence was read in a Spark Multimode Microplate Reader (Tecan, Switzerland).

Table 4-2. Standard curve for Quant-iT PicoGreen dsDNA Assay kit.

Standard	Volume of 1 X TE	Volume of Stock DNA (2 µg/ml)	Concentration
Standard 1	0	500	2 µg/ml
Standard 2	100	400	1.6 µg/ml
Standard 3	200	300	1.2 µg/ml
Standard 4	350	150	0.6 µg/ml
Standard 5	450	50	0.2 µg/ml
Standard 6	475	25	0.1 µg/ml
Standard 7	495	5	0.02 µg/ml
Standard 8	499.5	0.5	0.002 µg/ml
blank	500	0	0 µg/ml

4.2.10 16S library preparation:

The 16S rRNA library (V3-V4 regions) was prepared following Illumina guidelines. DNA (extracted in section (4.2.8), was normalized to 5ng/ml with 10 mM TRIS (pH 8.5) based on the concentrations determined by PicoGreen (4.2.9). Of the normalized DNA, 2.5 µl (12.5 ng) was combined with 5 µl of Amplicon PCR Forward Primer (1 µM), 5 µL of Amplicon PCR Reverse Primer (1 µM) (Table 4-3), and 12.5 µL 2x KAPA HiFi HotStart ReadyMix (Cedarlane # 07958935001) in a hard shell 96-well 0.2 ml low-profile PCR plate (Bio-Rad, #HSL9601). The plate was sealed with Microseal 'B' PCR plate sealing film (Bio-Rad, #MSB1001), and put in a thermal cycler with the following program: 95°C for 3 minutes, 25 cycles of (95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds), then 72°C for 5 minutes. The PCR product was verified on a 1.5% agarose gel.

Table 4-3. 16S Amplicon PCR Primer Sequences

Primer	Sequence (5' to 3')
16S Amplicon PCR Forward Primer	TCGTCGGCAGCGTCAGATGTGTATAAGA GACAGCCTACGGGNGGCWGCAG

16S Amplicon PCR Reverse Primer	GTCTCGTGGGCTCGGAGATGTGTATAAG AGACAGGACTACHVGGGTATCTAATCC

The amplified 16S V3-V4 region was then purified using AMPure XP beads (Beckman Coulter Genomics, Cat # A63881). Specifically, using a multichannel pipette, all of the PCR product from the previous step was transferred to a hard shell 96-well 0.2 ml high-profile PCR plate (Bio-Rad, #HSS9601), 20 µl of the AMPure XP beads (RT) were added to each well and mixed well by pipetting. The plate was covered with a plate seal (Bio basic, #TB418), briefly (and carefully) mixed by vortex, centrifuged for 1 minute at 1,000 x g, and incubated at RT for 5 minutes. The plate was placed on a magnetic stand (Life Technologies, #AM10027) for 2 minutes until supernatant was clear. With the plate on the magnetic stand, the supernatant was discarded by multichannel pipette. The beads were washed by adding 200 µl of 80% ethanol to each well, then incubated on the magnetic plate for 30 seconds, the supernatant was then discarded. This wash was repeated once more. The beads were left to air dry on the magnetic stand for 10 minutes, after which the plate was taken off the magnet, and 52.5 µl of 10 mM Tris pH 8.5 was added to each well, and was mixed by pipetting until the beads were fully resuspended. The plate was incubated at RT for 2 minutes, then returned to the magnetic stand and once the supernatant was clear (~2 minutes), 50 µl was transferred to a new low-profile PCR plate, and was stored at -20°C until the next day

Dual indices and Illumina sequencing adaptors were then added to the purified 16S V3-V4 regions, by transferring 5 µl of sample from the previous step into a new low-profile PCR plate, then adding 5 µl of Nextera XT index primer 1, and 5 µl of Nextera XT index primer 2 to their respective wells (Table 4-4). Next, 25 µl of 2x KAPA HiFi HotStart ReadyMix was added to each well, followed by 10 µl of PCR grade water (Thermo Fisher Scientific, #10977015), and the

contents of the well were mixed well by pipetting. The plate was sealed with Microseal 'A' Sealing Film (Bio-Rad, #MSA5001, and centrifuged for 1 minute at 1,000 x g at RT. The plate was then put into a thermal cycler with the following program: 95°C for 3 minutes, 8 cycles of (95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds), then 72°C for 5 minutes. The PCR product was washed using AMPure XP beads and 2 ethanol washes as described above. After air-drying the beads for 10 minutes, the plate was removed from the magnetic stand, and the beads were resuspended in 27.5 of 10 mM Tris. The plate was returned to the magnetic stand for 2 minutes until the supernatant had cleared, and 25 µl of the supernatant was transferred to a new low-profile PCR plate.

Table 4-4. Index kits used in Illumina 16S sequencing

Index Kit	Catologue number
Nextera XT Index Kit	#FC-131-1001
Nextera XT Index Kit v2 Set C	#FC-131-2003
Nextera XT Index Kit v2 Set D	#FC-131-2004

DNA concentration of all samples were measured by PicoGreen assay as described previously (4.2.9) and were normalized based on the standard curve to 4 nM with 10 mM Tris. Following normalization, 5 µl from each well was pooled in a microcentrifuge tube and the DNA concentration was confirmed using Qubit as described by the manufacturer (Life Technologies, MAN0002325|MP32850 Revision: A.0). Briefly, in a 500 µl Qubit tube, 198 µl of Qubit solution and 2 µl of pooled DNA were mixed, then read on the Qubit machine, the pooled library was further diluted with 10 mM Tris if required. The 4 nM pooled DNA was then denatured by adding 5 µl of the pooled library in a new microcentrifuge tube with 5 µl of 0.2 N NaOH. The solution was briefly vortexed and centrifuged for 1 minute at 280 x g at RT, then was left to incubate at RT for 5 minutes. Pre-chilled hybridization buffer (HT1) (900 µl) was added to the denatured DNA library (20 pM denatured library in 1 mM NaOH) and placed on ice until next dilution.

The denatured DNA library was further diluted to 4 pM by mixing 120 µl of 20pM denatured DNA library and 480 µl of pre-chilled HT1 in a new microcentrifuge tube, then placed on ice. The 10 nM PhiX library (Illumina, #FC-110-3001) was diluted to 4 nM by combining 2 µl of the 10 nM PhiX library with 3 µl of 10 mM Tris. This library was denatured by mixing 5 µl of 4 nM PhiX and 5 µl of 0.2N NaOH, briefly vortexed and incubated at RT for 5 minutes. The PhiX library was diluted to 20 pM by adding 990 µl of pre-chilled HT1 to the denatured 4 nM PhiX library. This library was further diluted to 4 pM by combining 120 µl of 20 pM denatured PhiX library and 480 µl of pre-chilled HT1. In a microcentrifuge tube, 90 µl of the denatured 4 pM PhiX library and 510 µl of the denatured 4 pM amplicon library prepared previously were combined and incubated at 96°C for 2 minutes. The tube was inverted several times following incubation and kept in an ice water bath for 5 minutes. The samples were then loaded into the Miseq reagent kit V3 600 cycle cartridge (Illumina, MS-102-3003), and placed in the Illumina Miseq (Illumina, Miseq) for sequencing.

4.2.11 Post-Sequencing Metagenomics

The resultant dual-index paired-end reads were analyzed using Qiime2 (version 2022.2) [134], specifically the sequences were subjected to quality control specifically denoising and chimaera filtering using the Divisive Amplicon Denoising Algorithm 2 (DADA2) Qiime2 plugin[136]. Resultant features were aligned with MAFFT[141] and the phylogenetic tree was generated using FastTree analysis[138]. The resulting taxonomy was classified using GreenGenes classifier 13.8 and the sklearn naïve Bayes classifier[143,145,147]. Features were filtered to include only features with a minimum of 500 frequency and 5 samples. Alpha diversity metrics (Shannon's Diversity[149], Faith's Phylogenetic Diversity[150], and Observed Features), and a Beta diversity metric (Bray-

Curtis[152]) were analysed using the Qiime2 core-metric method at a sampling depth of 34000. The Bray-Curtis differences were assessed by PERMANOVA[153] in Qiime2. Taxa-bar plots of relative abundances were created for visualization in GraphPad Prism 9 (v9.4.0). The differential enrichment of taxonomic groups were assessed by Linear discriminant analysis effect size analysis (LEfSe) [139] through the Galaxy Server (https://huttenhower.sph.harvard.edu/galaxy/).

4.2.12 Serum Isolation and Multiplex

Immediately following blood collection at euthanasia, blood was kept at room temperature to allow clotting, then put on ice before centrifugation. The blood was centrifuged for 10 minutes at 10,000 x g at 4°C, after which the supernatant (serum) was collected and separated into 50 µl aliquots and stored at -80°C until use.

The serum concentrations of IL-1β, TNF-α, and IL-10 were measured by a custom Milliplex Mouse High Sensitivity T Cell Magnetic Bead Panel (Millipore, #MHSTCMAG-70K-03), as per the manufacturer's instructions. Briefly, the lyophilized serum matrix (Millipore, #MXMSM-11) was re-constituted (10 minutes) with 2.0 mL assay buffer (Millipore, #L-AB) and mixed well. To prepare the antibody-immobilized beads, each individual vial of beads (Anti-Mouse IL-1β bead (Millipore, #MIL1B-MAG), Anti-Mouse TNF-α (Millipore, #MCYTNFA-MAG), Anti-Mouse IL-10 (Millipore, #MIL10-MAG)) was sonicated for 30 seconds, vortexed for 1 minute, then 60 µl was added to the provided mixing bottle, followed by 2.82 mL of assay buffer. The beads were mixed well by vortex. The standards were made by first reconstituting the High Sensitivity T Cell Standard (Millipore, #MHSTC-8070) with 250 µl of serum matrix, inverted several times to mix, then vortexed for 10 seconds. After 10 minutes, the standard (Standard 1) was transferred to a microcentrifuge tube, and a serial dilution was prepared by adding 50 µl of

Standard 1 and 150 µl serum matrix to a new microcentrifuge tube (Standard 2), this process was repeated until Standard 7 was made. The wash buffer was prepared by mixing the 60 µl of 10X wash buffer (Millipore, #L-WB) with 540 µl of MilliQ water. In the provided 96-well plate, at RT (all reagents should be at RT), 200 µl of wash buffer was added to each well by multichannel pipette, the plate was sealed and mixed on a plate shaker at 600 RPM for 10 minutes. The wash buffer was decanted by inverting the plate and lightly tapping it onto paper towels. To the appropriate wells, 50 µl of each standard or control was added, followed by 25 µl of assay buffer to all the sample wells, then 25 µl of neat serum. The mixing bottle containing the beads was vortexed, and 25 µl was added to all wells, after which the plate was sealed, covered in aluminium foil, and incubated overnight (18 hours) with shaking at 4°C.

The next day, the plate was placed on a magnetic plate, and the well contents were gently removed by inverting then drying on paper towels (while attached to the magnetic plate). The plate was then washed three times by removing the plate from the magnetic stand, adding 200 µl of wash buffer to each well, shaking for 30 seconds (600 RPM) at RT, reattaching the plate to the magnet and allowing the beads to settle for 1 minute. The well contents were then decanted as described above, and the wash was repeated twice more. Following the washes, 25 µl of the detection antibodies (Millipore, #MHCTC-1070) were added to each well, and the plate was sealed, covered in aluminium foil, and incubated for 1 hour with shaking at RT. Next, 25 µl of Streptavidin-Phycoerythrin (Millipore, #L-SAPE3) was added to each well, and the plate was sealed, covered in aluminium foil, and incubated for 30 minutes with shaking at RT. With the plate on the magnetic stand, the well contents were decanted, and the plate was washed 3 times as discussed above. To each well, 150 µl of Drive Fluid PLUS (Thermo Fisher, #4050030) was added, and the beads were resuspended by shaking for 5 minutes at RT. The plate was then read by the

Bio-Plex MAGPIX Multiplex Reader (Bio-Rad), and data was acquired with Bioplex manager software (Bio-Rad, #171015001) and was presented as concentration (pg/mL).

4.2.13 Statistical analysis

Unless specified, all the statistical tests were performed in GraphPad Prism 9 (v9.4.0). BW changes were analyzed with unpaired t-tests at each time point. Sickness scores were analyzed by repeated measure (RM) two-way ANOVA with Šídák's post-hoc test. The nest building and the behaviour tests were also analyzed by unpaired t-test. The diversity metrics were analyzed through Qiime2, specifically, the α-diversity was analyzed by Kruskal-Wallis test by ranks, and the β-diversity was analyzed by PERMANOVA. The taxonomy was analyzed using LEfSe analysis through the Galaxy server (https://huttenhower.sph.harvard.edu/galaxy/) with an LDA threshold of 2, and p-value cut-off of 0.05. Measures of systemic inflammation (relative spleen weight, serum cytokines) were analyzed by unpaired t-test. Similarly, the gene expression in the brain was analyzed by unpaired t-test after transforming the data by square root to normalize based of the Kolmogorov-Smirnov distance. Correlations were performed using the Spearman method.

4.3 Results:

4.3.1 LPS negatively impacted BW and DI in the 24-hours post-injection

Mice body weight (BW) was measured prior to injection, and the %BW change at 8-, 24-, and 48- hours post-injection, compared to time 0, was calculated. As shown in Figure 4-2A, there was a significant effect of time and treatment on BW loss such that mice that received LPS, had significant BW loss at the 8- ($P<0.05$) and 24-hour ($P<0.0001$) timepoints, compared to the SAL mice. At 48-hours post-injection, the LPS mice had recovered from their BW loss ($P=0.0734$). The diet intake (DI) of the mice was also measured at the time points mentioned above. As shown in Figure 4-2B, there was a significant effect of time and treatment on DI such that mice that received

LPS consumed significant less diet at 24-hours post-injection compared to the SAL mice (P<0.0001). At 48-hours post-injection, the LPS mice had the same DI as the SAL mice suggesting they had recovered at this point. The DI prior to injection was 3.061 ± 0.057g (data not shown), similar to the 24-hour SAL mice (3.033 ± 0.102g), suggesting the SAL injection had no effects on DI.

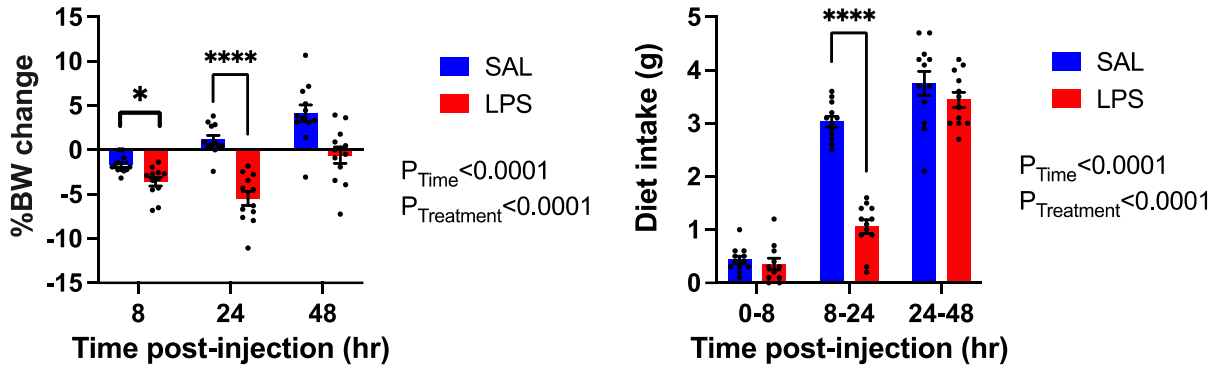

Figure 4-2. The effects of i.p LPS on body weight (BW) and diet intake (DI) in the 48-hours post-injection.
A. Percent BW change. **B.** Diet intake (g). BW data was analyzed by unpaired t-tests at each time point (n=12/group). DI Data was analyzed by mixed-effects two-way ANOVA with Šídák's post-hoc test (n=11-12/group). * (P<0.05), **** (P<0.0001)

4.3.2 LPS injection resulted in an increase in sickness behaviours

Sickness behaviours were measured at specific time points (0.5, 2, 4, 6, 8, 24 hours post-injection), and included four specific behaviours; 1. Lethargy (slow moving), 2. Huddling (arched back), 3. Piloerection (hair sticking up), 4. Ptosis (Squinted eyes). There was a significant effect of time on sickness behaviour, with the greatest sickness score (mice were most sick) at 4-6 hours post-injection (Figure 4-3A). There was an overall increase in sickness score with LPS challenge (P<0.05), although there was only significance between LPS and SAL at 12-hours post-injection (P<0.05) (Figure 4-3A). When assessed indivdiually, lethargy, huddling, and ptosis was

significantly increased by time and LPS ($P<0.05$), however, piloerection (Figure 4-3D) was not significantly increased by LPS ($P=0.12$).

A additional measure of sickness behaviour (nest quality scores) was measured at 2 time points (8 and 24 hours post-injection) (Figure 4-4). Unpaired t-tests showed a significant difference between SAL and LPS mice at 8-hours post-injeciton, but not at 24-hours post-injection ($P=0.754$).

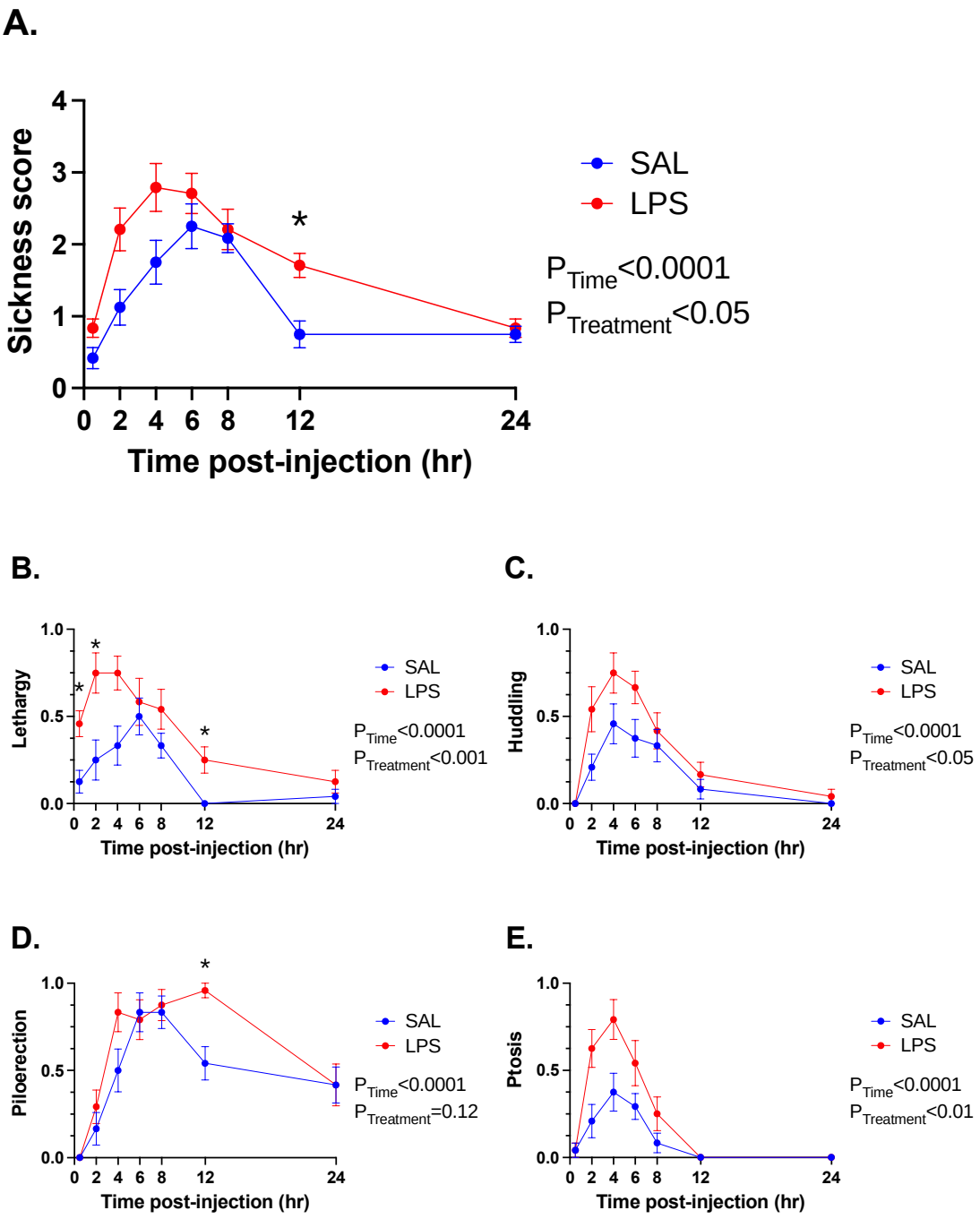

Figure 4-3. Specific sickness behaviours following i.p LPS challenge.
A. Total sickness scores for the 24-hours post-injection. **B.** Lethargy. **C.** Huddling. **D.** Piloerection. **E.** Ptosis. Data was analyzed by repeated measures (RM) two-way ANOVA, and the total sickness score was further analyzed by Šídák's post-hoc test (n=12/group). * (P<0.05).

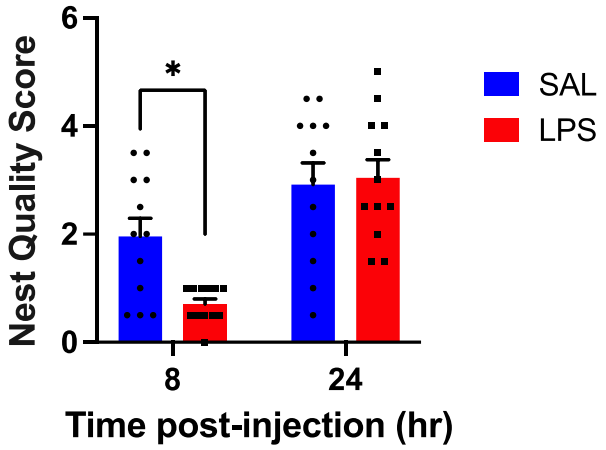

Figure 4-4. Nesting scores for SAL and LPS mice in the 24-hours post-injection.
Nesting score was analyzed by unpaired t-tests at each time point (n=12/group). * (P<0.05).

4.3.3 LPS did not consistently induce depressive- or anxiety-like behaviours

Twenty-four hours post-injection of SAL or LPS, depressive and anxiety-like behaviours were measured. Anxiety-like behaviours (measured by open field test (OFT) and elevated plus maze (EPM)) were not affected by LPS challenge (Figure 4-5A-D). Specifically, in the OFT, there were no differences in time spent in the corners (P=0.6922), or total distance moved (P=0.6371) (Figure 4-5A,B). In the EPM, there were no differences in time spent in the closed arm (P=0.7919), or total distance moved (P=0.1257) (Figure 4-5C,D). Depressive-like behaviours (measured by the splash test and tail suspension test (TST)) were generally unaffected by LPS challenge (Figure 4-5E-H). In the splash test, there was no difference in total grooming time (P=0.4930), however there was a significant decrease in total rearing time (P<0.05), which is more representative of anxiety-like behaviours (Figure 4-5E,F). In the TST, there were no differences in either immobility (P=0.9502), or mobility (P=0.5743) time (Figure 4-5G,H).

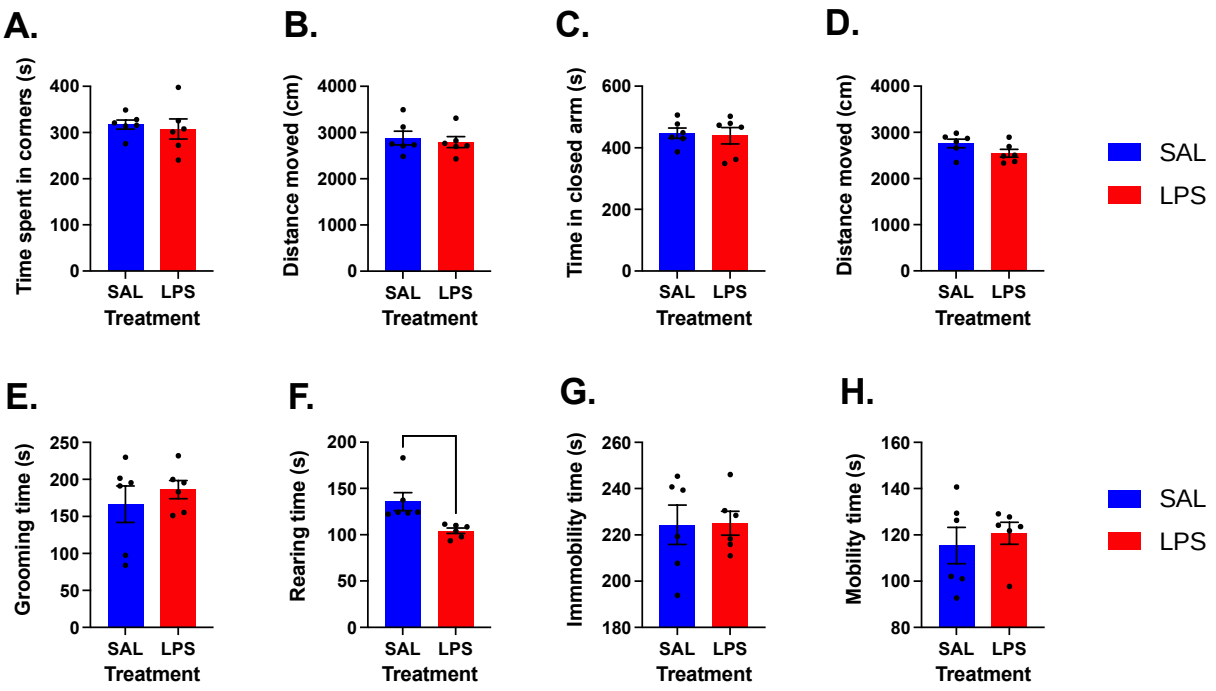

Figure 4-5. The effects of LPS on depressive- and anxiety-like behaviours.
A-B. Open field test, (A. Time spent in corners, B. Distance moved). **C-D.** Elevated plus maze (C. Time spent in closed arm, D. Distance moved). **E-F.** Splash test (E. Total grooming time, F. Total rearing time). **G-H.** Tail suspension test (G. Immobility time, H. Mobility time). Data was analyzed by unpaired t-test (n=6/group). * (P<0.05).

4.3.4 Administration of i.p LPS resulted in changes to the cecal microbiota diversity and composition

The cecal microbiota α-diversity was assessed using the Shannon diversity metric, Faith's phylogenetic diversity (PD) metric, and pielou's evenness metric. Neither Shannon diversity (P=0.094) nor Faith's PD (P=0.862) were affected by LPS challenge, however Pielou's evenness was significantly greater in the LPS-challenged mice (P<0.05) (Figure 4-6A-C). The cecal bray-curtis β-diversity, was significantly different in the LPS mice compared to the SAL mice (PERMANOVA q<0.05).

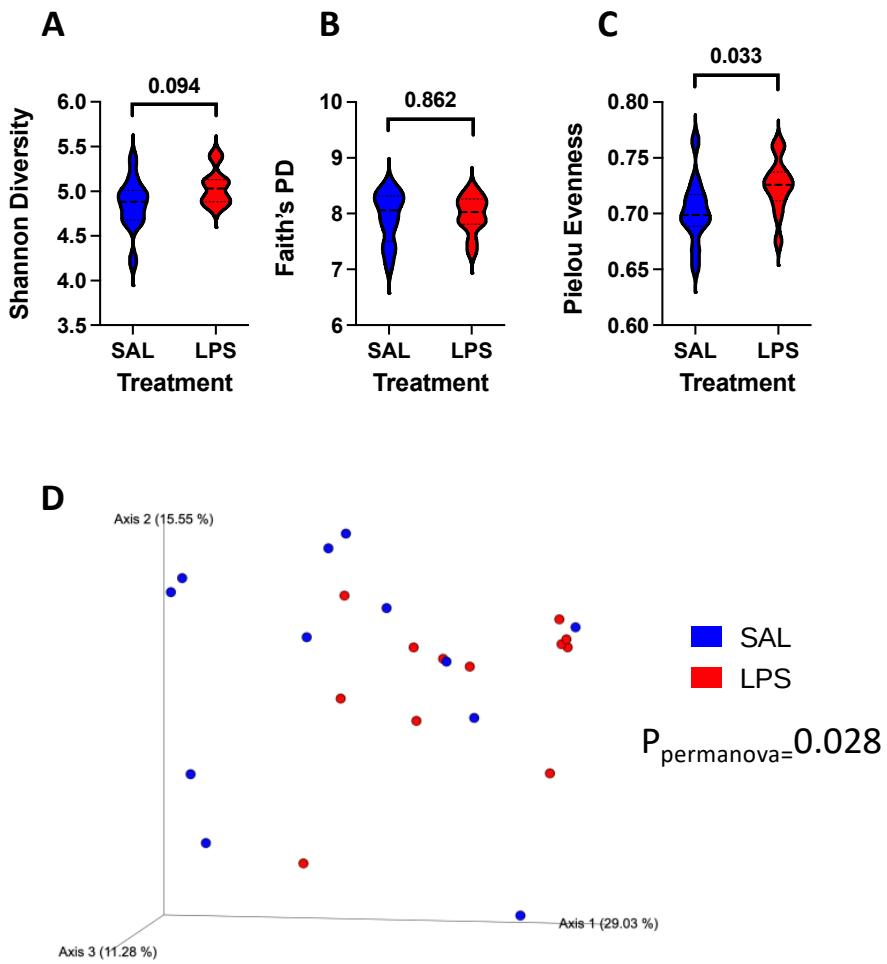

Figure 4-6. Effect of LPS on cecal microbiota diversity.
A-C. Alpha diversity metrics (A. Shannon diversity, B. Faith's phylogenetic diversity, C. Pielou's Evenness). **D.** Bray-Curtis beta diversity visualized by principal component analysis (PCoA). The α-diversity data was analyzed by Kruskal-Wallis test by ranks. The β-diversity data was analyzed by PERMANOVA.

In addition to the measures of α-diversity and β-diversity, the cecal microbiota was also examined for changes in specific taxonomic ranks (Figure 4-7A-B; Table 4-5). There was a decrease in *Bacteroidetes* at the phylum level following LPS challenge (Figure 4-7A), and there were a few changes at the genus level (Figure 4-7B). Specifically, LPS-challenged mice exhibited increased *Roseburia* and *Ruminococcaceae*, and decreased *rc4-4*, and *Herbaspirillum* amongst a few other changes (Table 4-5).

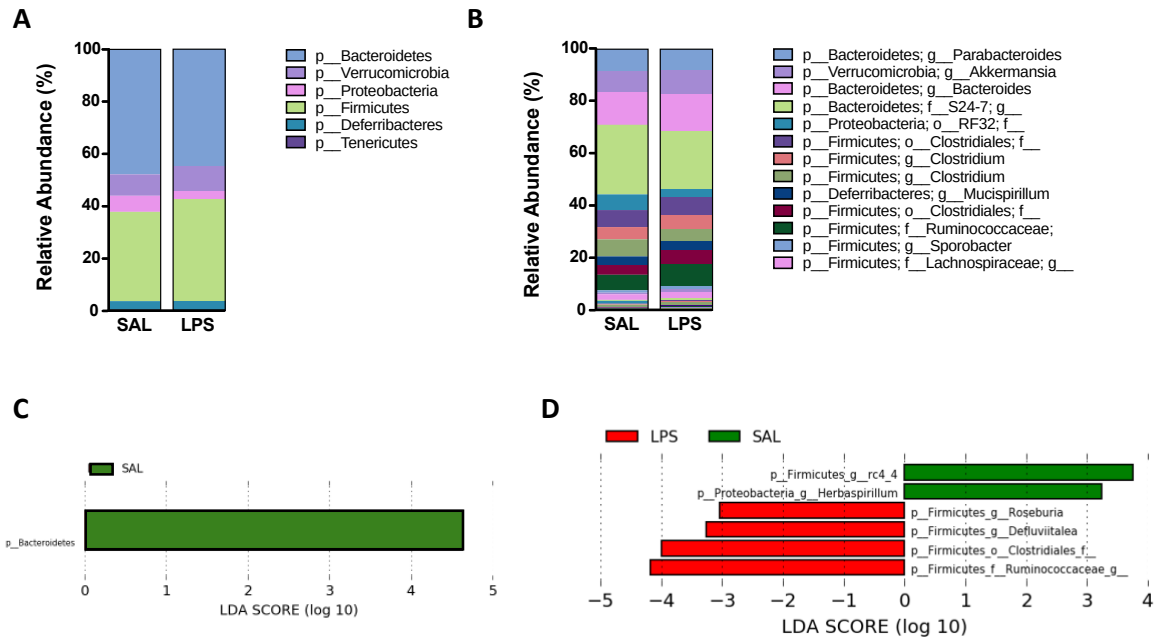

Figure 4-7. Taxa-specific effects of LPS on the cecal microbiota.
A. Relative abundance at the phylum level. **B.** Relative abundance at the genus level. **C-D.** Lefse plots showing the effect of LPS on the cecal microbiota (C: Phylum level changes, D: Genus level changes). The legend in the taxa bar plots includes taxonomic groups with relative abundance greater than 1%. (n=12/group).

Table 4-5. Relative abundance of the cecal microbiota in SAL and LPS mice.

Taxonomy	Relative abundance (%) ± SEM	
	SAL	**LPS**
Bacteroidetes	47.88 ± 1.528	44.97 ± 1.222
g__Parabacteroides	8.429 ± 1.306	8.232 ± 0.811
g__Bacteroides	12.38 ± 1.378	13.87 ± 0.711
f__S24-7; g__	26.63 ± 1.823	22.39 ± 0.848
Firmicutes	34.18 ± 2.019	39.02 ± 1.716
f__Ruminococcaceae; g__	2.257 ± 0.299	3.39 ± 0.214
o__Clostridiales; f__	3.791 ± 0.390	5.56 ± 0.532
f__Ruminococcaceae; g__	3.410 ± 0.240	4.88 ± 0.405
g__Sporobacter	0.870 ± 0.159	1.35 ± 0.225
g__Defluviitalea	0.609 ± 0.048	0.882 ± 0.076
f__Lachnospiraceae; g__	2.203 ± 0.197	2.56 ± 0.100
g__Dorea	0.825 ± 0.104	0.883 ± 0.103
g__Oscillospira	0.334 ± 0.052	0.414 ± 0.141
g__rc4-4	1.021 ± 0.239	0.167 ± 0.050
f__Streptococcaceae; g__	0.236 ± 0.040	0.321 ± 0.086
g__Clostridium	0.262 ± 0.040	0.453 ± 0.166
f__Peptosteptococcaceae; g__	0.107 ± 0.048	0.295 ± 0.141
g__Roseburia	0.089 ± 0.031	0.233 ± 0.053
g__Lactobacillus	0.238 ± 0.118	0.390 ± 0.144
Verrucomicrobia	8.23 ± 0.549	9.31 ± 0.679
g__Akkermansia	8.156 ± 0.546	9.213 ± 0.677
Proteobacteria	6.13 ± 2.246	3.10 ± 1.780
o__RF32; f__	6.025 ± 2.226	3.007 ± 1.766
Deferribacteres	3.27 ± 0.312	3.37 ± 0.353
g__Mucispirillum	3.24 ± 0.308	3.32 ± 0.352
Tenericutes	0.217 ± 0.211	0.159 ± 0.153
g__Anaeroplasma	0.215 ± 0.209	0.159 ± 0.153

Mean relative abundance (% ± SEM) of taxonomic groups in the cecal microbiota at the Phylum level (grey), or the order, family, or genus level (white) with mean abundance greater than 0.2% in either group (n=12/group).

4.3.5 LPS had differential effects on systemic inflammation 48-hours post-injection

Forty-eight hours post-injection, mice were euthanized and blood and spleen were collected, and serum was isolated. Systemic inflammation was measured by multiplex, and serum levels of IL-1β, TNF-α and IL-10 were assessed (Table 4-6). Serum IL-1β in both the SAL and LPS mice was below the minimum detectable limit. Serum TNF-α and IL-10 were increased with LPS but not significantly (P>0.05). As a gross measure of systemic inflammation, spleen weight relative to BW was significantly increased with LPS challenge (splenomegaly).

Table 4-6. Measures of systemic inflammation.

Measure	SAL	LPS	p-value
Serum IL-1β (pg/ml)	<4.4	<4.4	N/A
Serum TNF-α (pg/ml)	8.466 ± 1.346	10.62 ± 1.268	0.2583
Serum IL-10 (pg/ml)	37.57 ± 6.506	48.38 ± 7.780	0.3107
Spleen weight (%BW)	0.2493 ± 0.0066	0.4102 ±0.0089	<0.0001

Data was analyzed by unpaired t-test (n=10-12/group). Minimum detection limits: IL-1β= 4.4 pg/ml, TNF-α=0.488 pg/ml, IL-10=1.34 pg/ml.

4.3.6 Neuroinflammation was increased by LPS 48-hours following injection

Neuroinflammation was measured by quantitative PCR (qPCR), specifically through measurement of pro-inflammatory cytokine mRNA (IL-1β and TNF-α) in both the hippocampus (HIP) and prefrontal cortex (PFC). LPS challenged mice showed increased IL-1β (P<0.0001) and TNF-α (P<0.01) mRNA in the HIP (Figure 4-8A-B). Alternately LPS increased IL-1β (P<0.05) but not TNF-α (P=0.3851) in the PFC (Figure 4-8C-D).

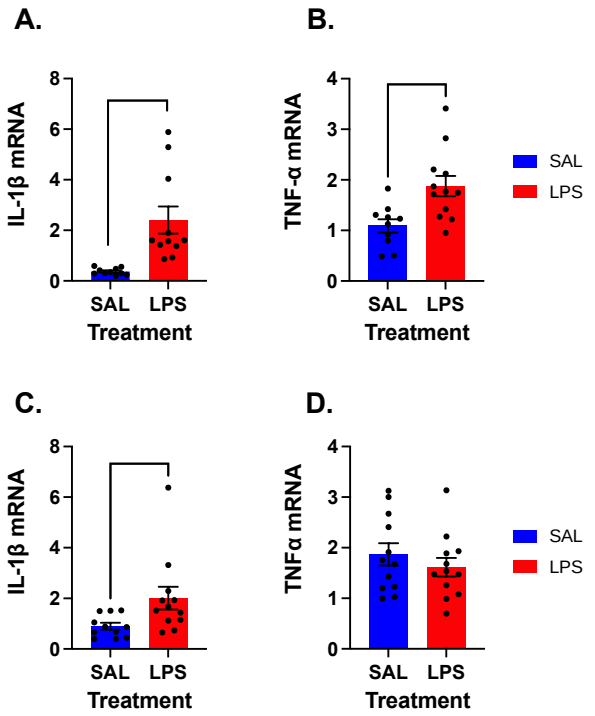

Figure 4-8. Gene expression of pro-inflammatory markers in the hippocampus and prefrontal cortex.
A-B. Hippocampus gene expression (A. IL-1β, B. TNF-α). **C-D.** Prefrontal cortex gene expression (A. IL-1β, B. TNF-α). Data was analyzed by unpaired t-test (n=10-12/group). IL-1β data was transformed by square root to normalize based on the Kolmogorov-Smirnov distance. *(P<0.05), **(P<0.01), ****(P<0.0001).

4.3.7 Correlations linking the effects of LPS across the GBA

Correlations were assessed to determine if there were any links between the gut, the brain and systemic inflammation measures, and there were multiple examples of this throughout the data, but only the most relevant were included here (Figure 4-9, Table S2). As expected, the hallmark phenotypic and behavioural responses associated with LPS exposure (e.g. sickness, reduced DI, BW loss) were correlated with biomarkers of systemic (spleen weight) and neuroinflammation (HIP IL-1β and TNF-α). Interestingly, these outcomes were also associated with the microbiota suggesting a potential role of the gut in mediating LPS responses. For example, alpha diversity (Pielou's evenness) was significantly associated with BW loss (r= -0.42; P<0.05),

nesting behaviours (r= -0.41; P<0.05), and sickness AUC (r= 0.45; P<0.05). In connection with this, biomarkers of systemic inflammation (spleen weight) and neuroinflammation were also significantly associated with specific microbiota (Figure 4-9). For example, IL-1β expression in both the HIP and PFC were negatively associated with the relative abundance of Firmicutes genera, *rc4-4* (HIP: r= -0.64; P<0.01) (PFC: r= -0.63; P<0.01) and Proteobacteria genera *Herbaspirillum* (HIP: r= -0.5; P<0.05) (PFC: r= -0.57; P<0.01). On the other hand, Firmicutes genera, *Roseburia* was positively associated with spleen weight (r= 0.59; P<0.01) and hippocampal IL-1β (HIP: r= 0.53; P<0.05) and TNF-a expression (HIP: r= 0.53; P<0.05). Collectively, although preliminary, these data highlight a potential role of the gut microbiota in mediating LPS responses, and thus may be a target to attenuate the negative health consequences of LPS on mental health.

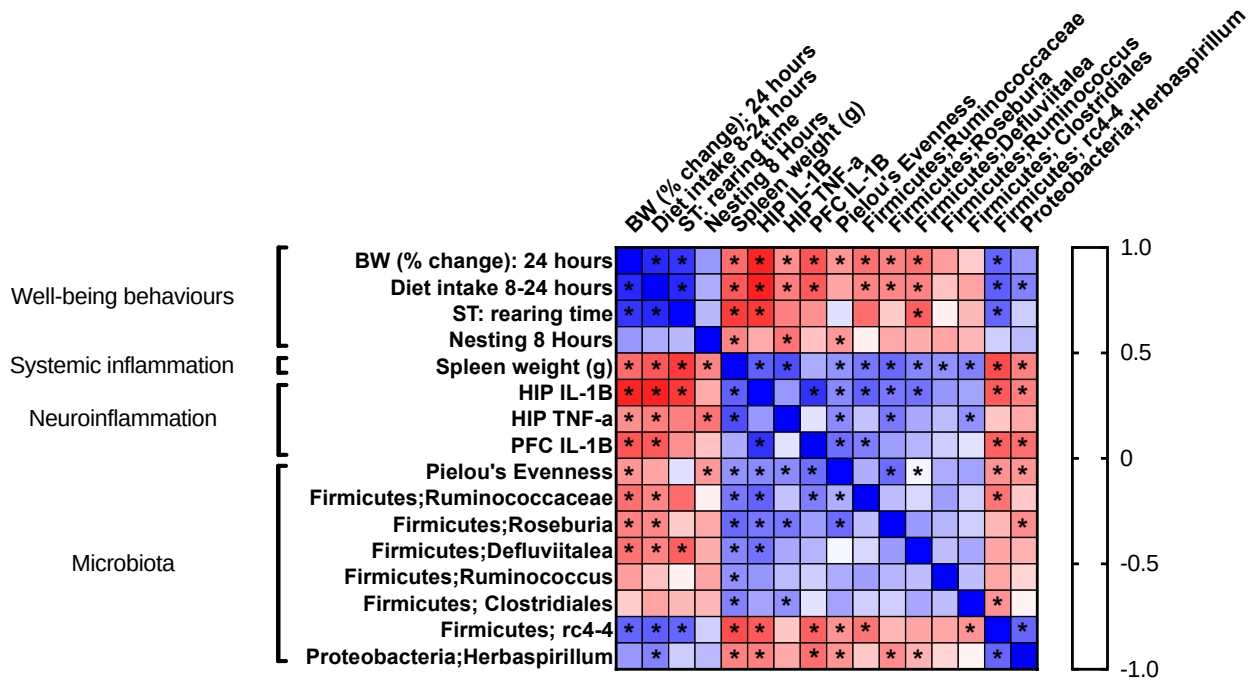

Figure 4-9. Spearman correlation matrix highlighting specific interactions throughout the GBA.
The colour of the boxes represents the level and direction of the correlation, with blue (score of 1) indicating a perfect positive correlation, and red (score of -1) indicating a perfect negative correlation. An asterix identifies a significant correlation P<0.05.

4.4 Discussion

The objectives of this study were to 1) characterize the effects (microbiota, systemic and neuro-inflammation), and phenotypic and behavioural responses of an acute i.p LPS administration in C57Bl/6 male mice; and 2) determine if there were relationships between the LPS-induced effects in the gut and brain. Overall, this study demonstrated that an acute exposure to i.p LPS (1 mg/kg) altered the gut microbiota composition in male 7-week-old C57Bl/6 mice, impacted systemic inflammation; altered specific behavioural changes (sickness behaviours, nest building behaviors, and rearing); and induced neuroinflammation in key brain regions (HIP and PFC). On the other hand, expected alterations in anxiety- and depressive-like behaviours as measured in the OFT, EPM, and TST were not evident 24-hours post LPS administration. This data suggests that although this model did not consistently modulate all anxiety and depressive-like behaviours measured, there remained important GBA implications, which could potentially be targeted with dietary supplementation with FS and FO in Study 2.

LPS is known to induce sickness as demonstrated by decreased BW, DI[135], and increased specific sickness behaviours[137]. Consistent with the literature, results from this study show LPS induced sickness highlighted by the loss of BW, decreased DI, increased sickness scores, and worse nest building. The LPS-challenged mice were not fully recovered 24-hours post-injection highlighted by the loss of BW. On the other hand, by 48-hours post-injection, the LPS mice had almost fully recovered based on this measure. Consequently, with other measures of well-being, such as the sickness behaviours and nest building, there was evidence of a recovery compared to the SAL mice 24-hours post-injection. The measures of sickness behaviour in this study were rather subjective as we were still learning, however the protocol was refined prior to Study 2. The nest building results were consistent with a previous study showing no differences between LPS

(1 mg/kg) and SAL in female C57Bl/6 mice at 24-hours post-injection[129], suggesting a lack of sex differences in this measure, although this could be verified in future studies. Taken together, i.p LPS at this dose induced negative effects on well-being, with partial but not complete recovery 24-hours post-injection.

Depressive- and anxiety-like behaviours have previously been established 24-hours post i.p LPS in C57Bl/6 male mice with doses ranging from 0.15 – 1 mg/kg[41,73,99,122,135,140]. Despite the previous evidence, in this study there were no consistent effects of LPS on depressive- or anxiety-like behaviours. The inconsistent anxiety- or depressive- like behaviours induced by LPS in this study, may be potentially related to factors such the age of mice[142], source of LPS[27], and animal facility conditions[144]. Additionally, a recent systematic review/meta-analysis on the reliability of anxiety-like behaviour tests suggested a lack of sensitivity these tests[146]. To my knowledge, there has not been a thorough investigation into the sensitivity of depressive-like behavioural tests, however one study highlights the difficulty in reproducing behaviour tests, based on factors such as housing conditions, vendors, circadian rhythm, and/or seasonal effects to name a few[148]. Therefore, there were no consistent anxiety- or depressive- like behaviours induced by LPS in this study.

Only one study to date has investigated the effects of i.p LPS on the mouse gut microbiota, where the fecal microbiota was examined 25 hours post-injection following 0.5 mg/kg LPS[41]. This current study is the first to examine the cecal microbiota 48-hours post LPS injection, and there was an increase in α-diversity (Pielou's evenness metric), and a significant difference in β-diversity (bray-curtis) (Figure 4-6C,D). In the previous studies investigating i.p LPS on the microbiota, there had been decreased α-diversity induced by LPS in C57Bl/6 mice[41], and in piglets who also underwent an i.p LPS-challenge[49]. Thus, the increase in evenness by LPS was unexpected, but

may be related to the decrease in the highly abundant *Bacteroidetes*, and the increases in the less abundant *Clostridiales* and *Ruminococcaceae*. The change in β-diversity with LPS administration suggests that LPS alters the microbial community compared to SAL, which was consistent with these previous studies[41,49]. In this study, the relative abundance of *Roseburia*, an established SCFA producer (specifically butyrate)[151] was increased in LPS mice, which is the opposite effect seen in the feces of in piglets (12 hours post LPS injection)[49]. The difference in timeline here could suggest that the increased *Roseburia* may be related to the recovery from the LPS-challenge. Importantly, this increase in relative abundance of *Roseburia* is not entirely novel in models of neuroinflammation, as studies involving other disease states (e.g. Parkinson's Disease, Diabetes) also show increased *Roseburia*[154]. None of the other significant microbial changes in this study were altered in the previous studies involving i.p LPS. The genera *rc4-4*, decreased by LPS, has been labelled as a potentially pathogenic bacteria[155], but has also been associated with obesity[156,157], which could be related to the LPS-induced BW loss. Furthermore, *rc4-4* was negatively correlated to spleen weight and HIP IL-1β, potentially suggesting a link between inflammation and *rc4-4* abundance. Therefore, i.p LPS, 48 hours induces changes in the cecal microbiota, specifically through altering the α- and β-diversity and increasing relative abundance of *Roseburia* and decreasing relative abundance of *rc4-4*.

When LPS is injected, it induces a cytokine storm derived from monocytes (amongst other sources), with IL-1β and TNF-α being among the first cytokines released[66]. IL-1β plays an important role in sickness behaviours specifically through induction of fever and HPA axis activation[158], and when IL-1β is deficient, TNF-α may also induce sickness behaviours[159,160]. Importantly, production of IL-1β and TNF-α peaks 1-4 hours following LPS injection[33,36,64], which likely explains the peak in sickness behaviours at 4 hours post-injection (Figure 4-8A). In this

study, systemic inflammation was assessed 48-hours post-injection. Despite no effect on serum cytokines, the LPS-induced splenomegaly suggests a significant immune response[161], and critically that the inflammatory effects of LPS are longer lasting in the spleen rather than the serum. Furthermore, there were significant correlations between sickness behaviours and spleen weight ($r=0.64$; $P<0.001$) suggesting a potential relationship between systemic inflammation and the resulting sickness behaviours (Figure 4-9). Taken together, we can use sickness behaviours as a predictor of systemic inflammation until euthanasia after which, splenomegaly can be used as an estimate of the immune response.

Although there was no increase in systemic inflammation measured by serum cytokines 48-hours post-injection, there is evidence that IL-1β and TNF-α expression remain elevated for longer in the brain than in the serum[33,64]. Notably, there was increased IL-1β in both brain regions, however only increased TNF-α in the HIP, as LPS injections are known to differentially induce inflammation in different brain regions[73,162], which could be related to the presence of certain cell populations (e.g. microglia, astrocytes) in these brain regions[162–164]. The lack of serum response, and differential inflammatory effects in the HIP vs PFC suggests that an earlier time-point (24-hours) may be more appropriate to assess the extent of the inflammatory response, which will be done in Study 2. Despite the increase in neuroinflammation, there was only 1 significant behavioural outcome (total rearing time in the splash test), which correlated to HIP IL-1β expression ($r= -0.78$; $P<0.01$), indicating that it may be more than inflammatory cytokines that would typically drive the rest of the depressive- and anxiety- like behaviours. Therefore, the effect of i.p LPS is longer lasting in the brain than the serum where the effect seems more transient, which has been previously identified following LPS challenge in C57Bl/6 male mice[33].

In conclusion, in this study the i.p LPS model was validated with respect to the gut (altered gut microbiota), systemically (increased spleen weight), and the brain (increased neuroinflammation). However, there were no consistent behavioural changes induced by LPS, with only 1 test showing signs of depressive/anxiety- like behaviours (splash test). In the 24-hours post-injection, LPS induced significant BW loss, decreased DI, increased sickness (specific sickness behaviours and poor nest building). After euthanasia, 48 hours post-injection, there was some evidence of systemic inflammation through an increase in spleen weight, which translated to increased neuroinflammation in the HIP and PFC. Taken together, there is ample evidence to state that LPS has effects across the GBA, however with the lack of consistent behavioral effects, it may be better to euthanize mice 24-hours post injection to capture the more transient impacts of LPS across the GBA.

Chapter 5. The effects of dietary supplementation with flaxseed (FS) and flaxseed oil (FO) on LPS-induced gut dysbiosis and systemic- and neuro-inflammation

5.1 Introduction

The GBA is emerging as a key factor in mental illnesses, such as depression and anxiety. The gut microbiota, and the resulting metabolites can have beneficial/negative impacts both systemically and the brain[165]. One potential source of this interaction is the bacterial endotoxin LPS, which is a component of the bacterial cell wall of Gram-negative bacteria, and outside the gut, it can cause both systemic- and neuro- inflammation[65]. This theory has been extrapolated into an animal model of depression/anxiety, through i.p injection of LPS.

LPS administration results in changes across the GBA, including altered microbiota composition and function[41,49], poor intestinal health[35,36], and increased inflammation systemically[65], and in the brain, specifically the PFC and HIP[73,74]. Although the PFC has been established as a key brain region implicated in mental illnesses, there is a growing body of litertature identifying the medial PFC (mPFC) as a hub for distinct pathways related to depression and anxiety[166–168]. FS is a rich source of dietary fibre, an n3-PUFA (ALA) and a key lignan SDG, all which have been shown previously to have beneficial effects in some aspect of the GBA[35,98,119]. Specifically, FS dietary supplementation for 3 weeks has previously demonstrated beneficial effects on intestinal health and the gut microbiota[94,119]. Furthermore, a daily gavage with FO for 2 weeks has previously resulted in decreased inflammatory mediators systemically[98]. A single administration of SDG has been shown to attenuate the LPS-induced increase in BBB permeability and neuroinflammation[35]. Based on the presence of each of the bioactive compounds, FS may be able to attenuate the negative effects of LPS across the GBA.

I hypothesized that like Study 1, mice receiving an i.p LPS injection will exhibit characteristics similar to depression and anxiety, highlighted by microbial dysbiosis, systemic, and

neuroinflammation. Secondly, mice fed diets supplemented with FS or FO will improve the response to i.p LPS through modulation of the gut microbiota, and the systemic/brain immune response. Finally, compared to mice consuming FO alone, mice consuming diets supplemented with FS will further attenuate the microbial dysbiosis, systemic- and neuro-inflammation due to the combined beneficial effects of dietary fibre, lignan, and ALA-rich FO. Thus, the objectives of this study were to measure the LPS response (systemic inflammation, neuroinflammation, and sickness behaviours) in mice pre-fed control, FS or FO diets and determine the role of the gut microbiota (composition and function) in the diet-induced effects. Additionally, the outcomes of FS and FO on the LPS-induced negative effects will be compared to determine if FS induces additive effects compared to FO.

5.2 Methods

5.2.1 FS nutrient composition, FO extraction, and fatty acid composition

The brown FS used in this study was generously donated by Natunola (Winchester, ON, CAN). FS was ground and sieved and sent to Bureau Veritas (Mississauga, ON, CAN) for proximate analysis (fat, lipid, carbohydrate), and dietary fibre (soluble and insoluble) content, the results of which are shown in Table 5-1.

Table 5-1: Macronutrient composition of whole ground FS

Nutritional parameter	Whole ground FS (g/100g)	Method
Protein	17.08	AOAC 992.15
Available carbohydrates	3.6	Calculation
Fat	39.7	AOAC 996.06
Saturated fatty acids	3.89	AOAC 996.06
Cis-monounsaturated fatty acids	6.87	AOAC 996.06
Cis-polyunsaturated fatty acids	27.1	AOAC 996.06
Trans-fatty acids	0.13	AOAC 996.06
Omega-3 polyunsaturated fatty acids	20.7	AOAC 996.06
Omega-6 polyunsaturated fatty acids	6.40	AOAC 996.06
Total dietary fibre	30.6	AOAC 991.43, 985.29
Soluble dietary fibre	9.7	AOAC 991.43
Insoluble dietary fibre	20.9	Calculation
Moisture	5.53	AOAC\ADPI methods
Ash	3.5	AOAC 923.03
Energy (kcal)	501	Calculation

FO was generously extracted from FS by Dr. Hosseinian's laboratory at Carleton University using an automatic oil cold press machine (Huanyu, CZR 309 110V). The resulting oil was filtered and was left at -4 °C to winterize overnight to remove impurities. The supernatant was collected and was centrifuged at 1500 RPM for 15 minutes, and the supernatant was collected and stored at 4°C until use. The fatty acid profile of the extracted FO was analysed by the Lipid Analytical Laboratory (Guelph, ON, CAN) (Table 5-2, S3 and Figure 5-1).

Table 5-2. Key fatty acid profile of FO, for full list see Appendix A. (Table S3)

Common Name	Systematic Name	Lipid numbers	Fatty acid content	
			%	mg/g
Palmitic acid	Hexadecanoic acid	**C16:0**	5.46	52.63
Stearic acid	Octadecanoic acid	**C18:0**	3.98	38.30
Oleic acid	Octadecenoic acid	**C18:1 (C9)**	16.64	160.30
Linoleic acid	Octadecadienoic	**C18:2N6**	15.97	153.82
α-linolenic acid (ALA)	Octadecatrienoic acid	**C18:3N3**	55.03	530.17
EPA	Eicosapentaenoic acid	**C20:5N3**	0.03	0.32
DHA	Docosahexaenoic acid	**C22:6N3**	0.09	0.84

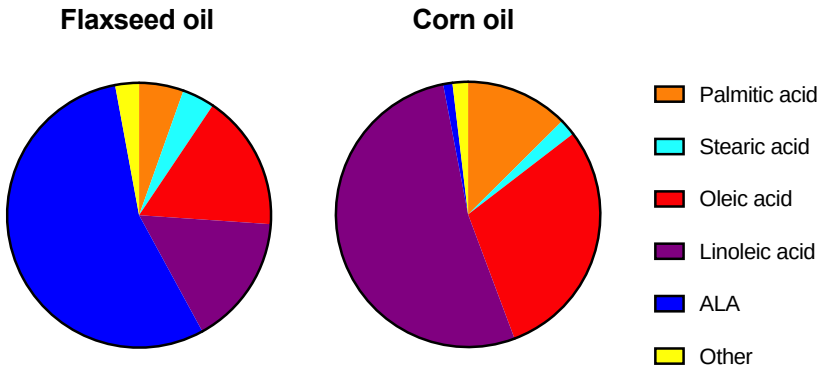

Figure 5-1. Primary fatty acid profile of FO used in this study compared to the fatty acid profile of corn oil (adapted from Carrillo et al., 2017).

5.2.2 Diet preparation

Three isocaloric experimental diets were prepared by Envigo (Wisconsin, USA) using nutrient content data (Table 5-3): 1) Modified, purified basal diet (BD) (AIN-93G with 7% corn oil), 2) BD supplemented with 10% whole ground FS, and 3) BD supplemented with 3.97% FO. The amount of FO in both the FS and FO diets is matched. For complete nutritional composition

of the diets, see Table 5-3**.** The experimental diets were stored at -20°C to prevent fatty acid oxidation.

Table 5-3. Experimental diet composition.

Ingredients	BD (g/Kg)	BD + 10% FS (g/Kg)	BD + 3.97% FO (g/Kg)
Casein	200	173	200
L-Cystine	3	3	3
Corn Starch	397.49	387.22	397.48
Maltodextrin	132	132	132
Sucrose	100	100	100
Corn Oil	70	30.5	30.3
Cellulose	50	19.4	50
Mineral Mix (AIN-93G-MX)	35	35	35
Vitamin Mix (AIN-93-VX)	10	10	10
Choline Bitartrate	2.5	2.5	2.5
TBHQ[a]	0.014	0.014	0.014
Ground Flaxseed	0	100	0
Flaxseed Oil	0	0	39.7

Diets adapted from [119]. All diets contain 20% protein, 7% fat, and 5% fibre.
[a] Tertiarybutylhydroquinon

5.2.3 LPS preparation

The LPS was prepared as outlined in Study 1 (section 4.2.1)

5.2.4 Animal and experimental design

Male C57Bl/6 mice (5 weeks old) were purchased from Charles River, (Kingston, NY, USA), and were individually caged with corn cob bedding and cotton nesting material and had access to BD and water *ad libidum* for a 1-week acclimatization period. The mice were maintained on a 12 hr:12 hr light:dark cycle, with room temperature at 23°C, and humidity at 40%. All experimental conditions were reviewed and approved by the uOttawa Animal Care Committee (Animal use protocol: #HSe-2857-R3).

Following acclimatization, 72 mice were randomized by BW into three experimental diet groups; 1) BD, 2) BD supplemented with 10% whole ground FS, and 3) BD supplemented with 3.97% FO. The mice were fed their respective diets for 3 weeks, during which the BW and diet intake was measured 3 times a week using a digital scale. Importantly, the diet was replaced 3 times a week to prevent excessive oxidation of the fatty acids in the diets. After 3 weeks, feces were collected, then the mice were given an i.p injection of LPS or SAL as define above (Section 4.2.2). Directly after injection, the old nests were removed from the cage and 2 new squares of nesting material were added to the front of the cage. The mice were monitored throughout the day to measure sickness behaviours, BW loss and diet intake, and nesting behaviors as described above (Section 4.2.3). Twenty-four hours post-injection, feces were collected, and the mice were euthanized by decapitation, and their tissues were collected for molecular analysis as defined in Study 1 (Section 4.2.2). The PFC was further dissected to excise the medial PFC by collecting the medial 2 mm (the inside of the white matter).

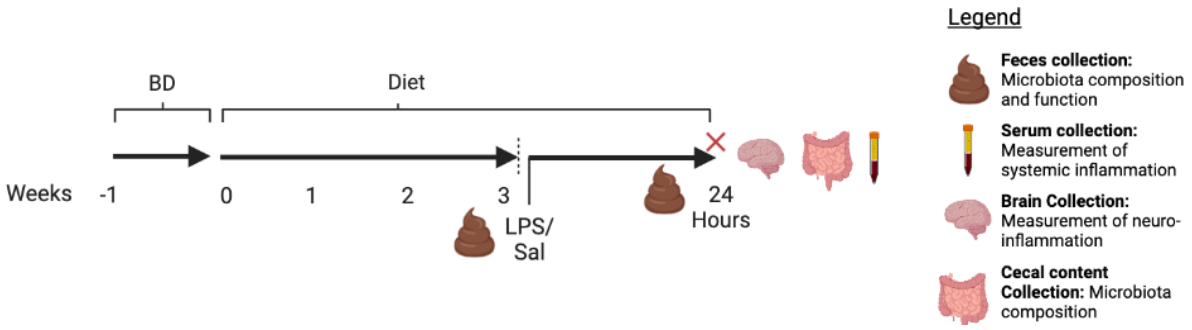

Figure 5-2. Experimental design for Study 2.

5.2.5 Analysis of Sickness behaviours

The sickness behaviours were monitored at distinct time intervals in the 24 hours following injections as described in study 1, without monitoring the 12-hour time-point (Section 4.2.3.2)

5.2.5 Nest scoring:

New nesting material was added to the cage prior to injection, and videos were taken at 8- and 24- hours post-injection then scored at a later point as described in study 1 (Section 4.2.3.3)

5.2.6 Diet and BW measures:

Diet and BW following injection was measured 8- and 24- hours post-injection, as previously described (Section 4.2.3.1)

5.2.7 HIP and mPFC inflammatory gene expression:

RNA extraction, cDNA preparation and qPCR were performed as outline in Study 1 (Section 4.2.7) with one addition. IL-10 was also measured in these brain regions using the following primers: F(5'-3') CCTGGGTGAGAAGCTGAAGAC and
R(5'-3') CCTTGTAGACACCTTGGTCTTGG.

5.2.8 DNA extractions

DNA was extracted as outlined in Study 1 (Section 4.2.8), with the exception that both cecal and fecal DNA were extracted.

5.2.9 16S rRNA sequencing and inferred microbiota function

Illumina 16S rRNA sequencing was performed as outlined in Study 1 (Section 4.2.10). Inferred microbiota function was assessed using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt)[170] on the Galaxy server (https://huttenhower.sph.harvard.edu/galaxy/). Specifically, the full pipeline of PICRUSt included

normalizing by copy number, predicting the metagenome, and categorizing by function. The resulting Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs[183] were then visualized using Statistical Analysis of Taxonomic and Functional Profiles (STAMP)[171].

5.2.10 SCFA extractions and GC

The feces were weighed and placed into the FreeZone 12 Liter Console Freeze Dry System (Labconco, #7759030) for 18-20 hours. After freeze-drying, the tubes were re-weighed to calculate the moisture content of the fecal material. The dried feces were mixed with MilliQ water (10 x dry weight) and vortexed thoroughly for 15 seconds. The samples were incubated at RT for 3 hours, while vortexing every 30 minutes for 15 seconds. The samples were then centrifuged for 30 minutes at 14,000 x g at 4°C, the supernatant was collected and centrifuged again for 30 minutes at 14,000 x g at 4°C. The pH of the supernatant was recorded (Thermo Scientific Orion Star A111 pH Benchtop Meter, Thermo Scientific ROSS MICRO PH ELECTRODE), then 10 µl of internal standard (IS) (comprised of 5.5 mM 2-ethylbutyric acid (Sigma-Aldrich, #109959-100ML) in 100% Formic acid (Sigma-Aldrich, #33015-1L) was added to 100 µl of each sample (10% IS), after which, pH was recorded again. The samples were filtered using 13 mm syringe filters (0.20 µm pore size) (VWR #CA28145-491), then placed into glass inserts (Thermo Fisher Scientific, #C4010-630) and into 11mm amber glass Snap-it Target LoVials (Thermo Fisher Scientific, #C4011-6W) with 11mm autosampler vial Snap-it Snap Caps (Thermo Fisher Scientific, #C4011-50).

To quantify the SCFA concentrations, a standard curve was created for the gas chromatograph (GC) (Shimadzu, Nexis GC-2030) using a serially diluted Volatile Free Fatty Acid Mix (Millipore Sigma, 46975-U) to make the following concentrations: 0.078 mM, 0.156 mM,

0.313 mM, 0.625 mM, 1.25 mM, 2.5 mM, 5 mM and 10 mM. The GC was equipped with the following: a 0.25 μm column (Restek, #RSK-10226), a 10 μl syringe with 0.63mm OD (Shimadzu, SZ-221-34618-00), and a wool deactivated split glass insert (Shimadzu, SZ-220-90784-5). The samples were injected at a volume of 1 μl, the column flow rate was set at 1.5 mL/minute at a 20:1 split ratio, helium was the carrier gas. The initial oven temperature was 100 °C, then at a rate of 10°C/minute was increased to 200°C. Injector temperature was set to 200°C, and the detector temperature was set to 300°C. Samples were run in triplicate (~20 minutes per run), and SCFA concentrations were automatically calculated by comparison to the standard curve.

5.2.11 Serum Multiplex

Analysis of serum cytokines was performed as outlined in Study 1 (Section 4.2.12).

5.2.12 Statistical analyses:

Unless specified, all the statistical tests were performed in GraphPad Prism 9 (v9.4.0). Final BW was analyzed by Kruskal-Wallis test with Dunn's multiple comparisons test. Total DI was analyzed by one-way ANOVA with Tukey's post-hoc test. BW and DI in the 24- hours post-injection were analyzed by two-way ANOVA with Tukey's multiple comparison's test. Sickness scores were analyzed by RM two-way ANOVA with Dunnett's multiple comparison test (compared to BD-SAL), and sickness AUC and nesting score were analyzed by two-way ANOVA with Tukey's Post-hoc test. Microbiota diversity was assessed as in study 1. Microbiota composition was analyzed by LEfSe similar to Study 1. In this study, LEfSe analysis in the cecum was performed within diet (SAL vs. LPS) and within SAL mice (between diets). The fecal microbiota prior to injection was compared between BD and FS/FO fed mice respectively. To

compare the fecal microbiota before and after LPS challenge, comparisons were made only within diet (pre-post LPS). The inferred function was analyzed in STAMP using Welch's two-sided t-test with Benjamini-Hochberg multiple test correction (q<0.1). SCFA concentrations were log-transformed to normalize based on the Kolmogorov-Smirnov distance, then were analyzed by t-tests within diet. Measures of systemic inflammation (spleen weight, serum cytokines) were measured by one-way ANOVA, with Dunnett's multiple comparison test compared to BD-SAL. Brain gene expression was transformed using the following formula: $y = -1 \times \log(y)$, to normalize based on the Kolmogorov-Smirnov distance then was analyzed by one-way ANOVA, with Dunnett's multiple comparison test compared to BD-SAL.

5.3 Results

5.3.1 Dietary supplementation with FS and FO for 3 weeks did not impact BW or DI

BW and DI were measured 3 times per week during the 3 week dietary supplementation period, and there were no differences in final BW between diets (P=0.1667) (Figure 5-3A). The total DI was also not affected by diet (P=0.0839) (Figure 5-3B).

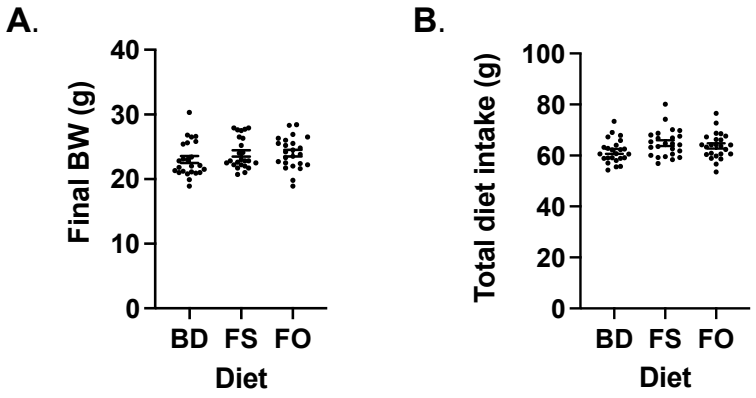

Figure 5-3. Body weight and diet intake following the three week dietary supplementation

A. Body weight following dietary supplementation. **D.** Total diet intake. Final BW was analyzed by Kruskal-Wallis test with Dunn's multiple comparisons test. Total DI was analyzed by one-way ANOVA with Tukey's post-hoc test. n=24/group.

5.3.2 LPS-induced BW and DI loss were differentially impacted by diet

BW and DI were measured at 8- and 24-hours post-injection. At 8- hours post-injection, LPS-challenged mice lost more weight than the SAL mice, irrespective of diet (Figure 5-4A). At 24-hours post-injection, all LPS-challenged mice continued to lose weight, more so in the FS-fed mice ($P<0.05$ vs BD-LPS), compared to the SAL group (Figure 5-4B). There was an overall treatment and diet effect on DI 8-hours post-injection, with LPS reducing DI, and FS-LPS showing decreased DI compared to FS-SAL ($P<0.001$) (Figure 5-4C). While at 24-hours post injection, there was only a treatment effect on DI, where all LPS treated mice had reduced diet intake compared to the SAL group regardless of diet (Figure 5-4D).

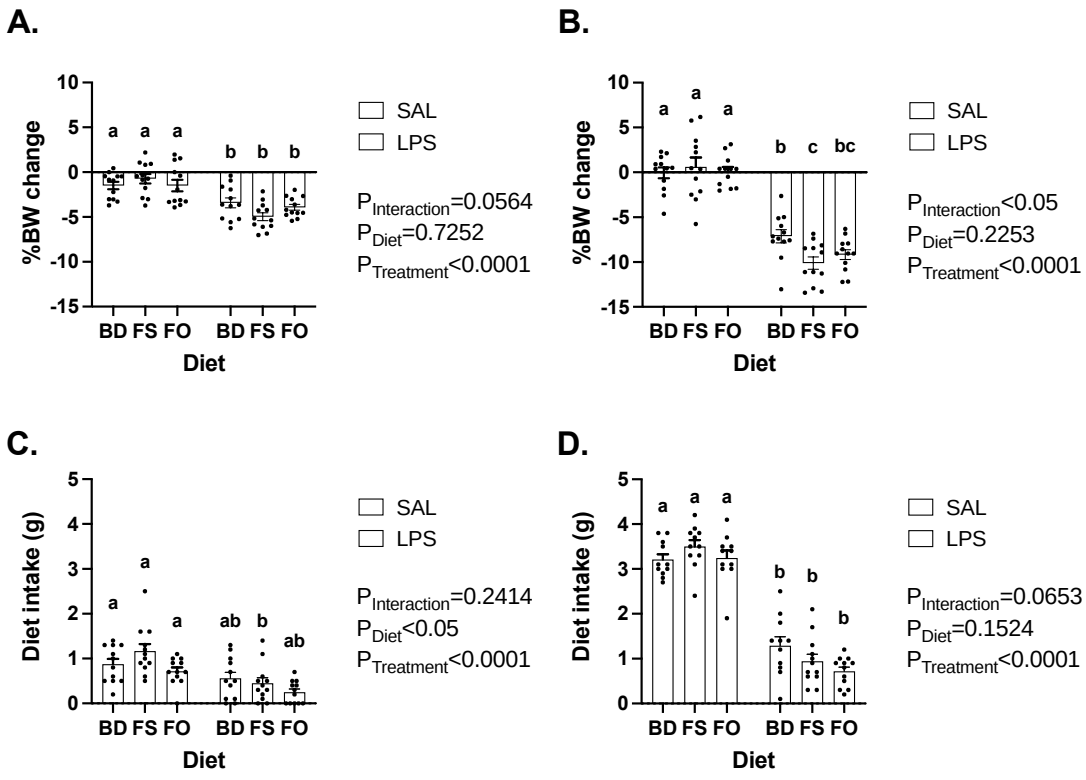

Figure 5-4. The effects of diet on the LPS-induced decrease in BW and DI.
A-B. Percent body weight change 8- (A) and 24- (B) hours post-injection. **C-D.** Diet intake 8- (C) and 24- (D) hours post-injection. Data was analyzed within treatment and/or within diet, by two-way ANOVA with Tukey's multiple comparison's test. Data points not sharing a letter are significantly different.

5.3.3 Sickness behaviours were not significantly impacted by dietary supplementation with FS or FO.

Sickness behaviours were measured during the 24-hours post-injection. There was an overall effect of time and group on the sickness behaviours, driven by a significant increase in sickness within all LPS groups compared to BD-SAL (Figure 5-5A). This increase was seen across all time-points except at 24-hours post-injection, where no differences were noted. Area under the curve (AUC) analysis equally showed significant increases in sickness behaviours with LPS-challenge, irrespective of diet (Figure 5-5B).

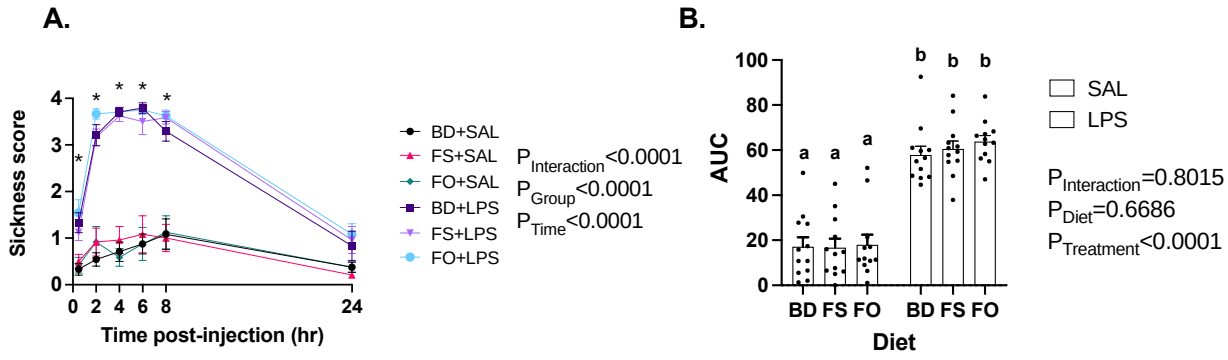

Figure 5-5. The effects of FS and FO on LPS-induced sickness behaviours
A. Sickness scores for the 24-hours post-injection. **B.** Area under the curve (AUC) analysis of sickness scores in A. Sickness scores were analyzed by RM two-way ANOVA with Dunnett's multiple comparison test (compared to BD-SAL) (n=12/group). An asterix denotes significance between the LPS groups and BD-SAL. The AUC data was analyzed by two-way ANOVA with Tukey's Post-hoc test. Data points not sharing a letter are significantly different.

As the second measure of sickness, nest building behaviours were assessed 8- and 24- hours post-injection, and there was an overall effect of LPS at both time points, but no effect of diet. At 8-hours post-injection, the LPS-challenged mice had quite low nesting scores (indicative of poor-

quality nests), which was not altered by diet (Figure 5-6A). The SAL mice at this time point had moderate nests (max score = 6.5), with quite a large variation within each group (0-6.25) (Figure 5-6A). This spread was still evident 24-hours post-injection with nest scores from 0 to 6.5 in this case (Figure 5-6B). Importantly, the LPS-challenged mice, 24-hours post-injection showed improved nesting scores from the 8-hour time point, however they did not recover to the level of the SAL mice (Figure 5-6B).

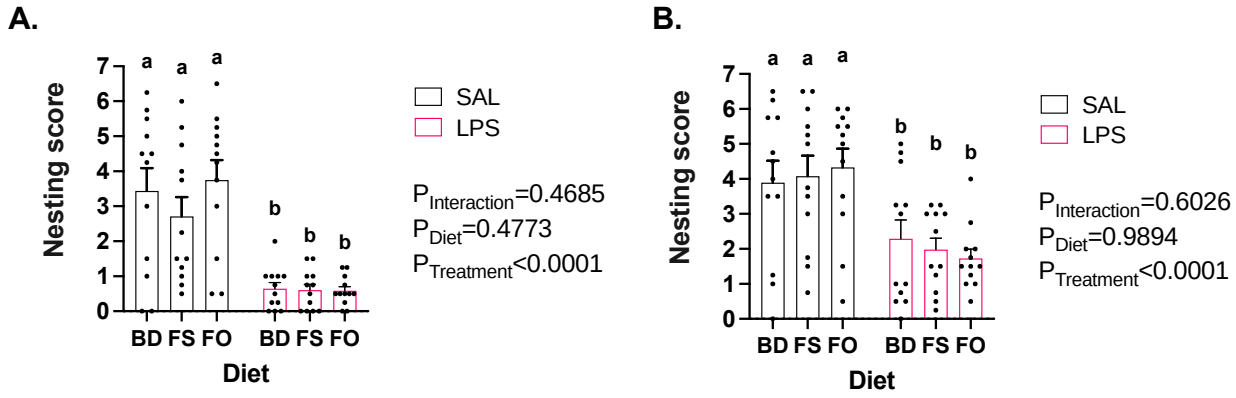

Figure 5-6. The effect of diet on nest building
A. Nest score 8-hours post-injection. **B.** Nest score 24-hours post-injection. Data was analyzed by two-way ANOVA with Tukey's post-hoc test (n=12/group). Data points not sharing a letter are significantly different.

5.3.4 The cecal microbiota composition was differentially impacted by FS and FO in SAL and LPS injected mice

The cecal microbiota diversity and composition were examined 24-hours post-injection (SAL and LPS). A shown in Figure 5-7, there was no effect of LPS or diet on cecal α-diversity including Shannon's diversity (Figure 5-7A), Faith's phylogenetic diversity (Figure 5-7B), Pielou's evenness (Figure 5-7C), and observed features (Figure 5-7D) as assessed by Kruskal-Wallis test. Despite the general lack in effects on α-diversity, there were changes in β-diversity as measured by Bray-Curtis dissimilarity index. Firstly, within treatment groups (SAL or LPS), FS significantly altered β-diversity compared to both BD and FO, with no difference between FO and

BD (Figure 5-7E, Table 5-4). Secondly, there were significant effects of LPS on β-diversity, such that there were significant differences between SAL and LPS within each diet (BD: P<0.05, FS: P<0.01, FO: P<0.01) (Table 5-4).

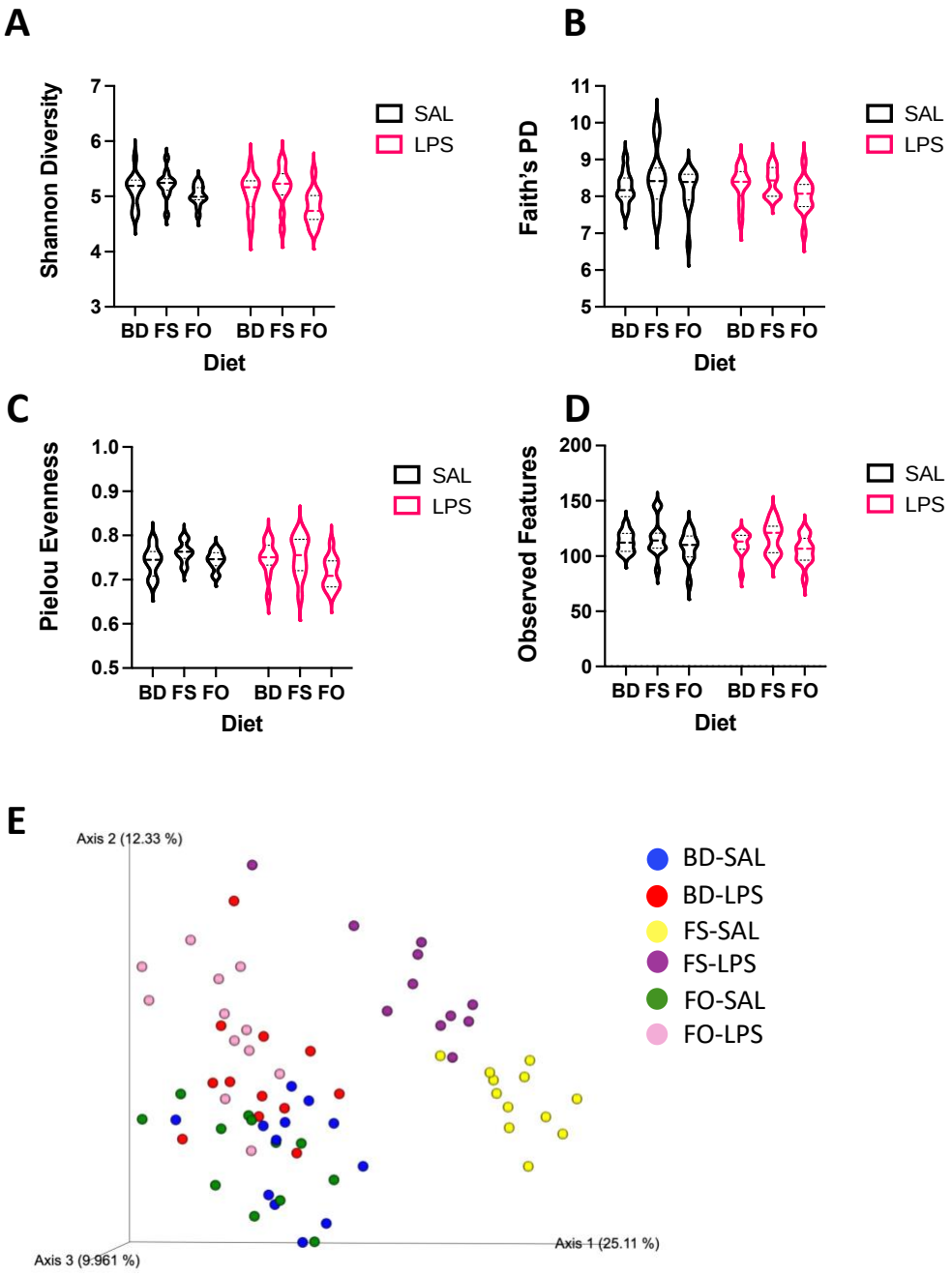

Figure 5-7. Effects of LPS, and dietary supplementation with FS/FO on the cecal microbiota diversity.

A-D. Alpha-diversity A. Shannon diversity, B. Faith's phylogenetic diversity, C. Pielou's evenness, D. Observed features. **E.** Bray-Curtis β-diversity of all mice visualized by PCoA. Alpha diversity metrics were analyzed by Kruskal-Wallis test by ranks (n=11/12/group). Beta-diversity was assessed by PERMANOVA (n=12/group).

Table 5-4. Cecal Bray-Curtis PERMANOVA results

Group A	Group B	PERMANOVA q value
BD-SAL	FS-SAL	<0.01
	FO-SAL	0.251
	BD-LPS	<0.05
FS-SAL	FO-SAL	<0.01
	FS-LPS	<0.01
FO-SAL	FO-LPS	<0.01

PERMANOVA statistics matching to Figure 5-7E, n=12/group

The changes to the cecal microbiota community outlined by β-diversity are further corroborated by changes in specific taxonomic groups, at both the phylum (Figure 5-8A) and genus level (Figure 5-8B, Table S4). Within the SAL groups, there were significant effects of diet on the cecal microbiota, largely driven by FS supplementation (Table S4). At the phylum level, FS-SAL mice had greater relative abundance of *Firmicutes* and lower *Tenericutes* and *Deferribacteres* compared to BD-SAL. There were no differences between BD-SAL and FO-SAL at the phylum level, but relative abundance of *Roseburia* was higher in FO-SAL mice at the genus level. FS significantly altered the cecal microbiota at the genus level compared to BD mice, shown by increased relative abundance of *Lachnospiraceae*, *Bacillaceae*, *Clostridium* (family *Lachnospiraceae*) and *Ruminococcus*, and lower *Anaerotruncus*, *Defluviitalea*, *Oscillispira*, *Dorea*, *Clostridium* (family *Ruminococcaceae*), *Anaeroplasma*, *Mucispirillum* and *Parabacteroides* (Figure 5-8H). Thus, FS had a far greater impact on cecal microbiota composition than FO.

In addition to the changes seen in the SAL mice, indicating the effects of diet, there were also collective changes induced by LPS (Figure 5-8C,D, Table S4). The Venn diagrams outline the number of genera that overlap between diets that are decreased (Figure 5-8C) and increased (Figure 5-8D) by LPS. Of particular interest, 4 genera (or lowest known rank) were similarly altered following LPS challenge; *Anaerotruncus* and *Bacteroides* (increased), *S24-7 and Streptococcaceae* (decreased). The FO-LPS mice had the most genera (or lowest known rank) uniquely decreased by LPS (*Clostridium* (family *Lachnospiraceae*), *rc4-4* (like Study 1), *Peptostreptococcaeae* and *Clostridiales)*, whereas the FS-LPS mice had the most genera (or lowest known rank) uniquely increased by LPS (*Oscillospira, Parabacteroides, Defluviitalea* and *Ruminococcaceae* (like Study 1). Overall this data shows that LPS alters the composition of the cecal microbiota 24-hours post-injection (as shown 48-hours post-injection in Study 1). In healthy mice, supplementation with FS, more-so than FO, has a greater effect of the cecal microbiota, however there were differential effects of diet on the LPS response despite some similarities.

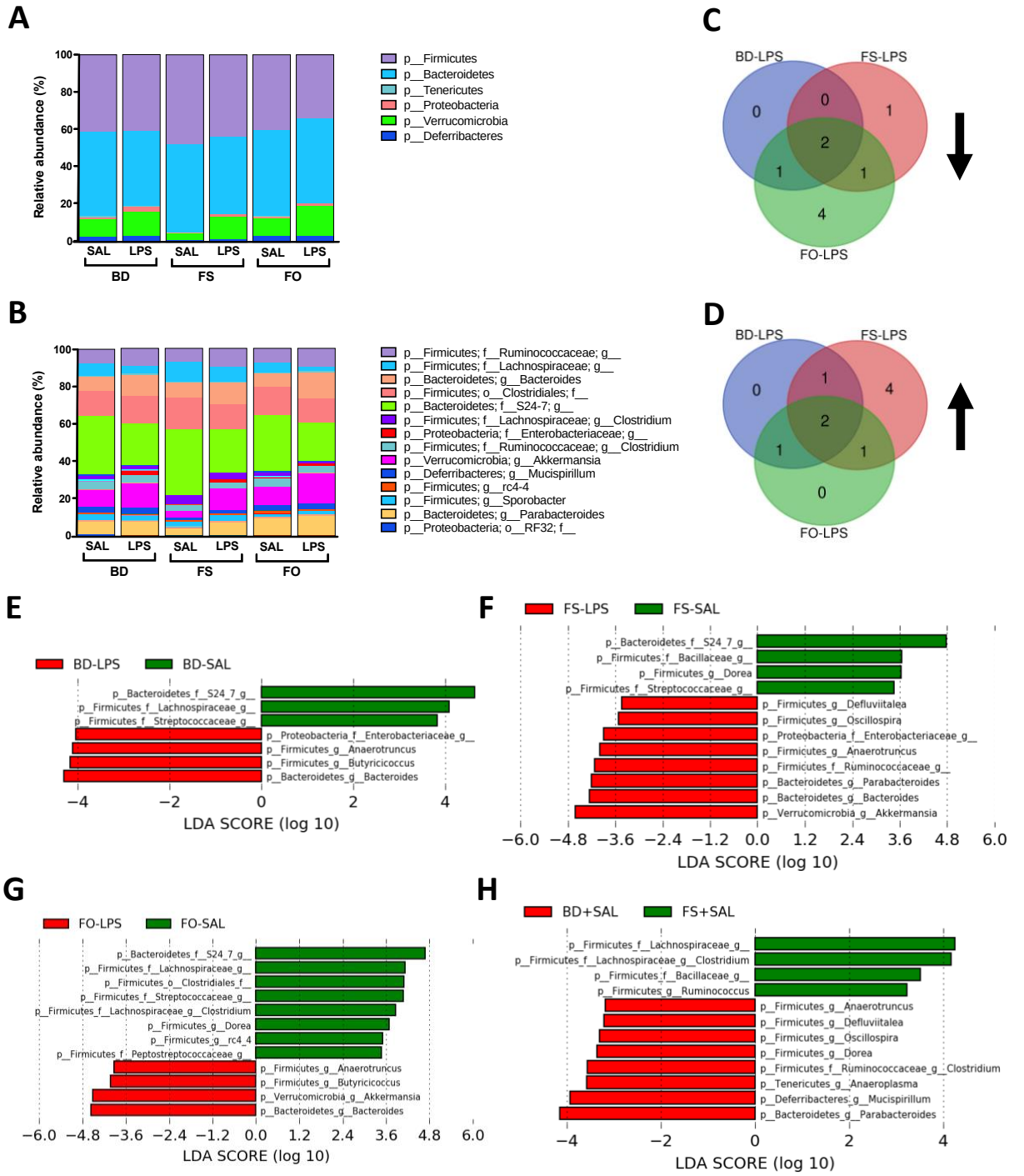

Figure 5-8. Taxonomic changes in the cecal microbiota at the phylum and genus level. **A.** Phylum level changes. **B.** Genus level changes. **C-D**. Significant changes in genera showing overlapping (decreased (C) or increased (D) effects of LPS between diets. **E-G.** Lefse plots showing the effect of LPS on the cecal microbiota within diet. **H.** Lefse plot showing the effect of FS on the cecal microbiota composition.

5.3.5 Fecal microbiota composition and function are differently affected by FS and FO supplementation

After 3 weeks of dietary supplementation, pre-LPS, FS increased α-diversity compared to both BD in each of the four metrics shown (Shannon Diversity, Faith's PD, Pielou's Evenness, and Observed Features) (Figure 5-9A-D), while there was no effect of FO. The β-diversity (Bray-Curtis Dissimilarity index) in FS-fed mice was significantly different from both the BD and FO mice, however there were no differences between BD and FO in this metric (Figure 5-9E).

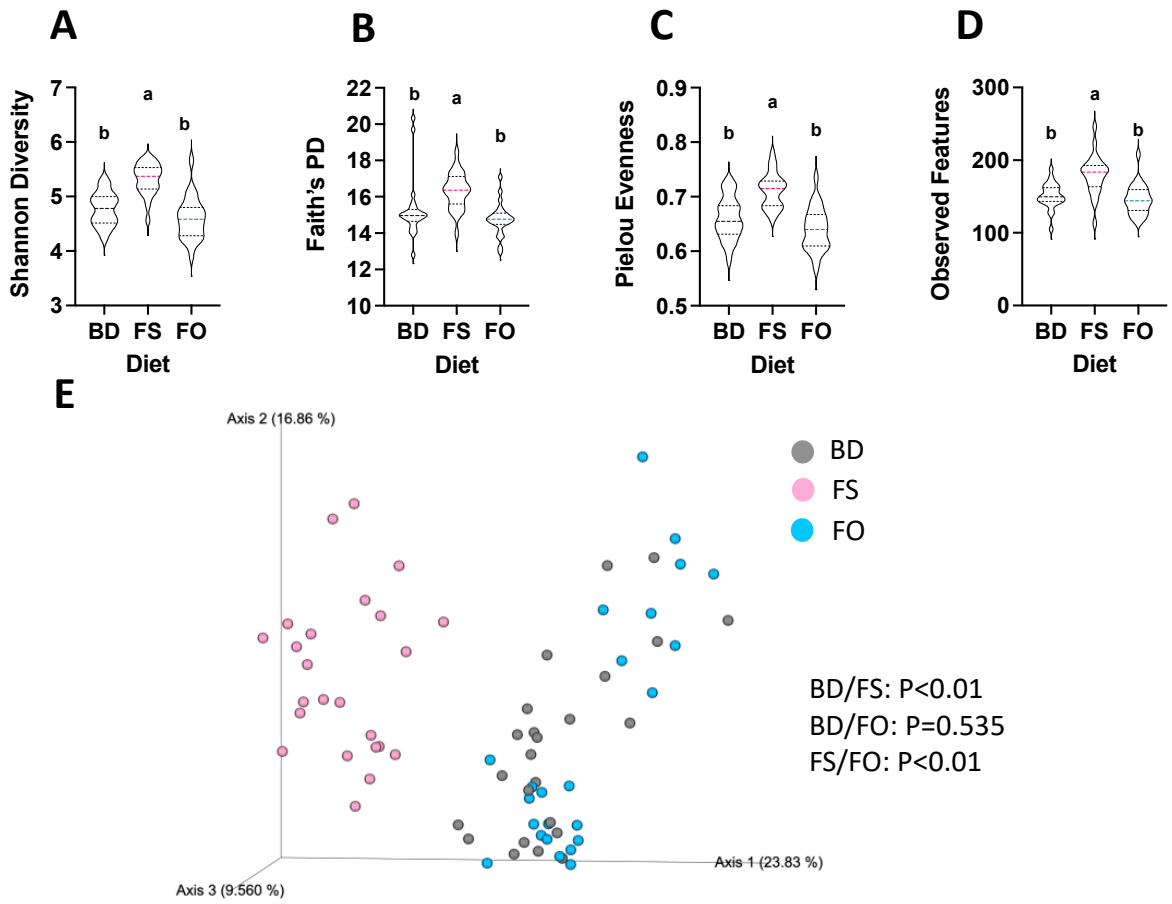

Figure 5-9. The effects of FS and FO on the fecal microbiota diversity
A-D. Alpha-diversity (A. Shannon diversity, B. Faith's phylogenetic diversity, C. Pielou's evenness, D. Observed features). **E.** Bray-Curtis β-diversity of all mice visualized by PCoA. Alpha diversity metrics were analyzed by Kruskal-Wallis test by ranks (n=24/group). Beta-diversity was assessed by PERMANOVA (n=24/group).

Furthermore, FS impacted the fecal microbiota composition with taxonomic changes at both the phylum (Figure 5-10A,C, Table 5-6) and genus level (Figure 5-10B,D, Table 5-6). FO induced no changes at either the phylum nor genus level. FS had greater relative abundance of 3 phyla compared to BD (*Firmicutes*, *Actinobacteria, Cyanobacteria*), and 3 lower abundant phyla (*Tenericutes, Defferibacteres, Verrucomicrobia*) (Figure 5-10C).

At the genus level, FS diet induced a stronger effect on the fecal microbiota composition (Figure 5-10D) compared to the effects of FO, highlighted by 25 genus level differences (BD vs FS) such as greater abundance of *Lachnospira, Allobaculum*, *Bifidobacterium and Enterobacteriaceae,* and lower abundance of lower abundance of *Akkermansia* and *rc4-4* (Figure 5-10, Table 5-6). Therefore, similar to the cecal microbiota, FS dietary supplementation alters the fecal microbiota, however FO does not.

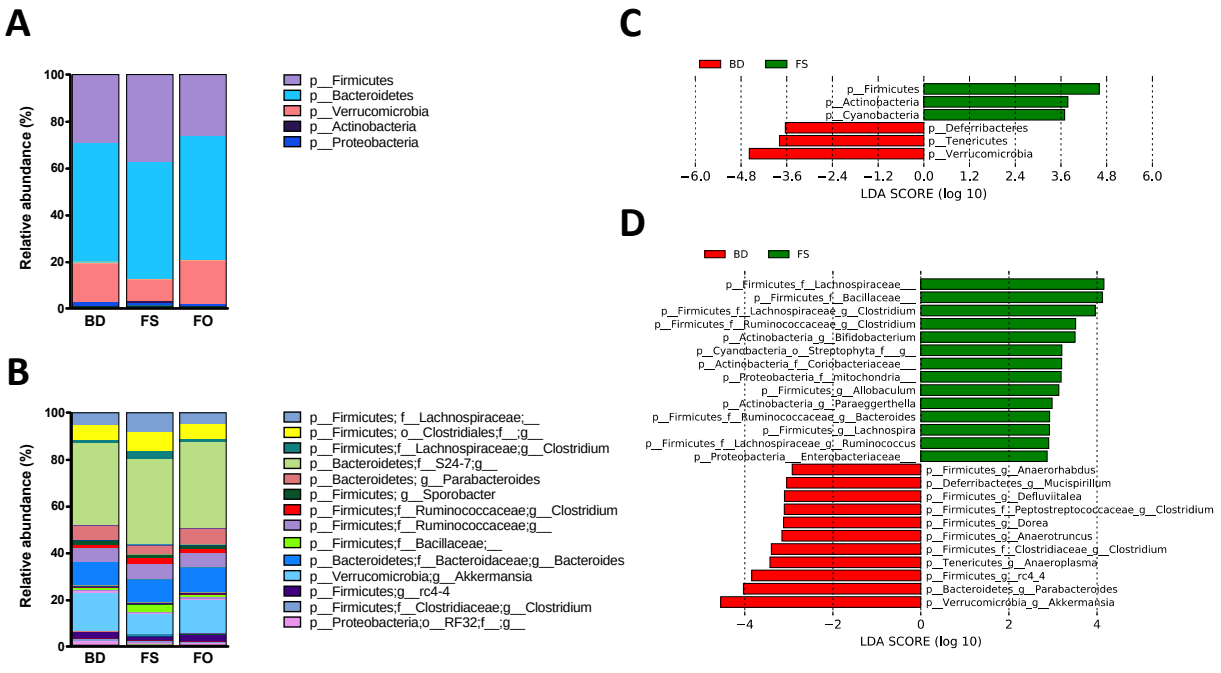

Figure 5-10. The effects of FS and FO on the fecal microbiota composition
A. Phylum level changes. **B.** Genus level changes. **C-D**. Significant changes in phylum (C) or genus (D) in FS-fed mice. Significance was determined by Lefse analysis between groups (n=24/group).

Table 5-5. The relative abundance of the fecal microbiota pre-injection

Taxonomy	Relative abundance (% ±SEM)		
	BD	FS	FO
p__Firmicutes	29.591 ± 1.414	37.706 ± 1.244	26.644 ± 1.365
f__Ruminococcaceae; g__	5.67 ± 0.419	6.614 ± 0.51	6.109 ± 0.436
f__Lachnospiraceae; g__	5.264 ± 0.614	8.25 ± 0.62	4.859 ± 0.641
o__Clostridiales; f__	6.517 ± 0.485	8.370 ± 0.684	6.705 ± 0.427
g__rc4-4	2.791 ± 0.349	1.362 ± 0.207	2.973 ± 0.333
g__Clostridium (f__Ruminococcaceae)	1.747 ± 0.206	2.372 ± 0.163	1.986 ± 0.239
g__Sporobacter	1.436 ± 0.29	1.291 ± 0.249	1.153 ± 0.186
g__Clostridium (f__Clostridiaceae)	1.013 ± 0.15	0.553 ± 0.111	0.826 ± 0.158
g__Oscillospira	0.542 ± 0.1	0.372 ± 0.069	0.564 ± 0.105
g__Dorea	0.401 ± 0.044	0.185 ± 0.018	0.46 ± 0.05
g__Defluviittalea	0.380 ± 0.046	0.184 ± 0.023	0.421 ± 0.043
g__Lactobacillus	0.499 ± 0.177	0.405 ± 0.216	0.354 ± 0.1
f__Bacillaceae;__	0.768 ± 0.201	3.335 ± 0.547	0.968 ± 0.225
g__Clostridium (f__Peptostreptococcaceae)	0.266 ± 0.065	0.03 ± 0.013	0.199 ± 0.061
o__Lactobacillales; f__	0.196 ± 0.017	0.156 ± 0.02	0.206 ± 0.02
g__Clostridium (f__Lachnospiraceae)	1.266 ± 0.144	3.141 ± 0.198	1.279 ± 0.161
g__Ruminococcus (f__Lachnospiraceae)	0.114 ± 0.023	0.223 ± 0.034	0.127 ± 0.022
g__Allobaculum	0.193 ± 0.073	0.447 ± 0.081	0.213 ± 0.07
p__Bacteroidetes	51.019 ± 1.441	50.279 ± 1.428	53.058 ± 1.002
f__S24-7;g__	35.188 ± 1.127	36.852 ± 0.873	36.662 ± 1.336
g__Bacteroides	9.919 ± 0.708	9.667 ± 0.851	10.376 ± 0.721
g__Parabacteroides	5.912 ± 0.417	3.76 ± 0.443	6.467 ± 0.398
p__Deferribacteres	0.478 ± 0.101	0.299 ± 0.092	0.307 ± 0.066
g__Mucispirillum	0.478 ± 0.101	0.299 ± 0.092	0.428 ± 0.099
p__Tenericutes	0.608 ± 0.138	0.147 ± 0.065	0.44 ± 0.115
g__Anaeroplasma	0.608 ± 0.138	0.147 ± 0.065	0.6 ± 0.146
p__Verrucomicrobia	16.38 ± 1.576	9.019 ± 1.219	18.339 ± 1.239
g__Akkermansia	16.38 ± 1.576	9.019 ± 1.219	14.397 ± 1.576
p__Actinobacteria	0.074 ± 0.022	0.978 ± 0.313	0.264 ± 0.209
g__Bifidobacterium	0.018 ± 0.018	0.643 ± 0.305	0.019 ± 0.018
f__Coriobacteriaceae	0.003 ± 0.002	0.202 ± 0.030	0.003 ± 0.002
p__Proteobacteria	1.831 ± 0.722	1.023 ± 0.391	0.885 ± 0.454
f__Enterobacteriaceae; g__	0.088 ± 0.02	0.204 ± 0.05	0.124 ± 0.045
o__RF32 ;f__	1.743 ± 0.715	0.755 ± 0.391	0.574 ± 0.357
p__Cyanobacteria	0.019 ± 0.016	0.548 ± 0.275	0.064 ± 0.043
o__Streptophyta; f__	0.019 ± 0.016	0.335 ± 0.251	0.048 ± 0.034

Mean relative abundance (% ± SEM) of taxonomic groups in the fecal microbiota pre-injection at the Phylum level (grey), or the order, family, or genus level (white) with mean abundance greater than 0.2% in any group (n=24/group).

Dietary supplementation with FS induced many significant effects on the inferred function of the microbiota, when compared to BD (Figure 5-11A), while there were no differences in inferred function between BD and FO. Notably, FS increased mean proportion of transporters, decreased biosynthesis of LPS, and decreased the mean proportion of LPS biosynthesis proteins (Figure 5-11A). The measured function of the gut microbiota was assessed by measuring SCFAs through GC. FS increased total SCFAs compared to BD (Figure 5-11B), specifically acetate (Figure 5-11C) and butyrate (Figure 5-11E), but not propionate (Figure D). There were no differences in fecal SCFA concentrations between BD and FO, indicating that the changes in fecal microbiota composition by FS did not translate to altered microbiota function (inferred and measured).

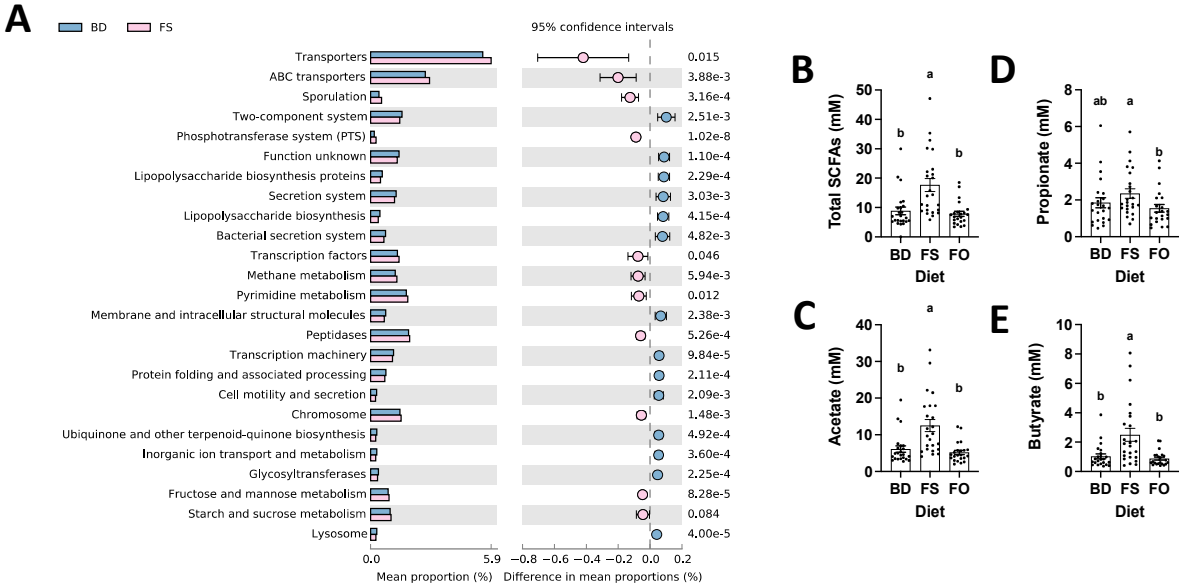

Figure 5-11. Pre injection microbiota inferred and measured function.
A. Inferred function of the fecal microbiota in FS-fed mice. **B-E.** Fecal SCFA concentration (B. Total fecal SCFA, C. Acetate, D. Propionate, E. Butyrate). The inferred function was analyzed in STAMP using Welch's two-sided t-test with Benjamini-Hochberg multiple test correction ($q<0.1$). SCFA concentrations were log-transformed to normalize, then were analyzed by one-way

ANOVA with Tukey's post-hoc test. Data points not sharing a letter are significantly different (n=24/diet).

5.3.6 LPS alters the fecal microbiota composition and function differently depending on background diet composition

To determine the impact of i.p. LPS on the fecal microbiota, diversity was measured, and comparisons were made between pre-LPS and post-LPS samples within each dietary group (e.g. pre-LPS BD vs post-LPS BD). Overall, there were minimal effects of LPS on measures of α-diversity, with the exception of Pielou's evenness which was decreased by LPS in FS-mice ($P<0.05$) (Figure 5-12C). On the other hand, while LPS-administration did not alter the β-diversity in the BD mice ($P=0.169$) (Figure S2A), there were significant effects of LPS on fecal β-diversity in FS-fed mice (Figure 5-12E) and FO-fed mice ($P<0.05$) (Figure S2B).

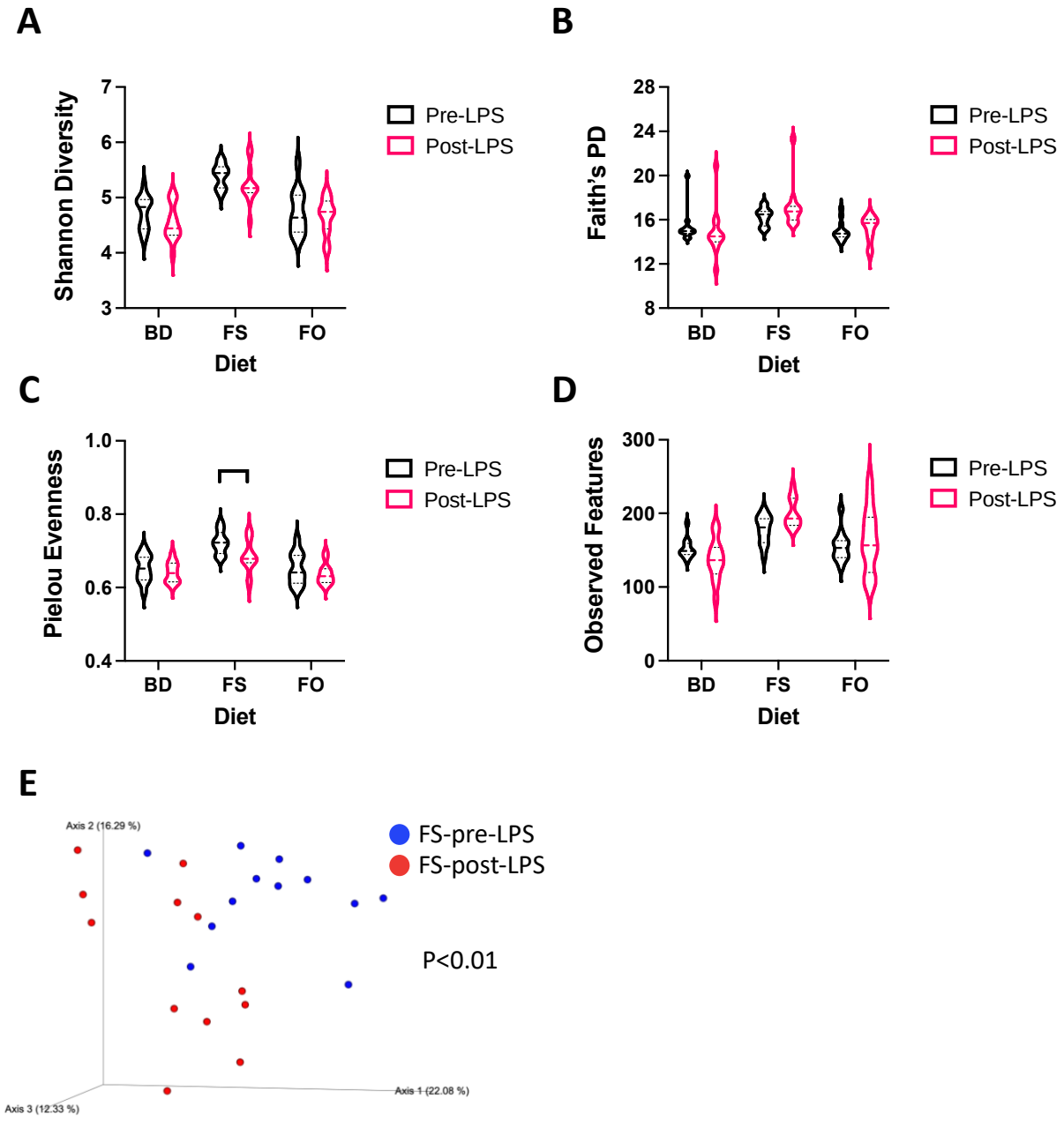

Figure 5-12. The effect of LPS on the fecal microbiota of FS- and FO- fed mice.
A-D. Alpha-diversity (A. Shannon diversity, B. Faith's phylogenetic diversity, C. Pielou's evenness, D. Observed features). **E.** Bray-Curtis β-diversity visualized by PCoA showing the FS mice pre and post LPS β-diversity**).** Alpha-diversity was analyzed using the Kruskal-Wallis test by ranks (n=12/group), significance within diet between pre-post LPS is denoted by an asterix (*). Beta-diversity was analyzed by PERMANOVA (n=12/group).

The fecal microbiota composition was then examined at both the phylum at genus level, comparing the relative abundances before and after LPS injection within diet groups. At the phylum level, there were no common effects of LPS that were shared in all dietary groups. The effects of LPS differed depending on the background diet composition including a decrease of *Firmicutes* in the BD and FS mice, and an increase in *Proteobacteria* in the FS and FO mice (Table S5) (Figure 5-13A). Furthermore, in the FS-fed mice only, LPS resulted in an increase in Deferribacteres and Verrucomicrobia and a decrease in Bacteroidetes. At the genus level, irrespective of the changes highlighted by the pre-injection feces (section 5.3.5), LPS induced some overlapping effects between diets (Figure 5-13C-D). *Enterobacteriaceae* was increased in with LPS in all diets which was also increased in FS mice following SAL injection, however much less abundant in the SAL mice (0.603 ± 0.195) than the LPS mice (9.368 ± 3.09) (Table S5 and S6). On the contrary, *Lachnospiraceae* was unanimously decreased following LPS injection regardless of diet. LPS challenge in both BD and FS mice resulted in lower abundance of *RF32, Clostridium* (family *Clostridiaceae*), and the family *Bacillaceae*. FS and FO mice showed increased relative abundance of *Clostridium* (family *Erysipelotrichaceae*) and decreased *S24-7* indicating a potential role of FO in the microbiota-mediated LPS response. As previously discussed, the FS-induced decrease in *Akkermansia* (*Akkermansia muciniphila*) was unexpectedly increased following injection of LPS. FS mice post-LPS also uniquely showed increased *Mucispirillum* and decreased *Paraeggerthella*. FO mice post-LPS uniquely decreased *rc4-4*, but there were no genera uniquely increased by LPS in this dietary group. BD-LPS mice post-injection differentially showed increased *Bacteroides* and decreases in *Clostridium* (family *Peptostreptococcaceae*) and *Ruminococcus* (family *Ruminococcaceae).* Therefore, similar to the cecal microbiota, LPS induced some changes in the fecal microbiota 24-hours post-LPS injection

irrespective of diet (*Enterobacteriaceae, Lachnospiraceae*). FS and FO also differentially altered the fecal microbiota following injection highlighted by unique taxonomic changes when comparing the fecal microbiota pre and post LPS injection.

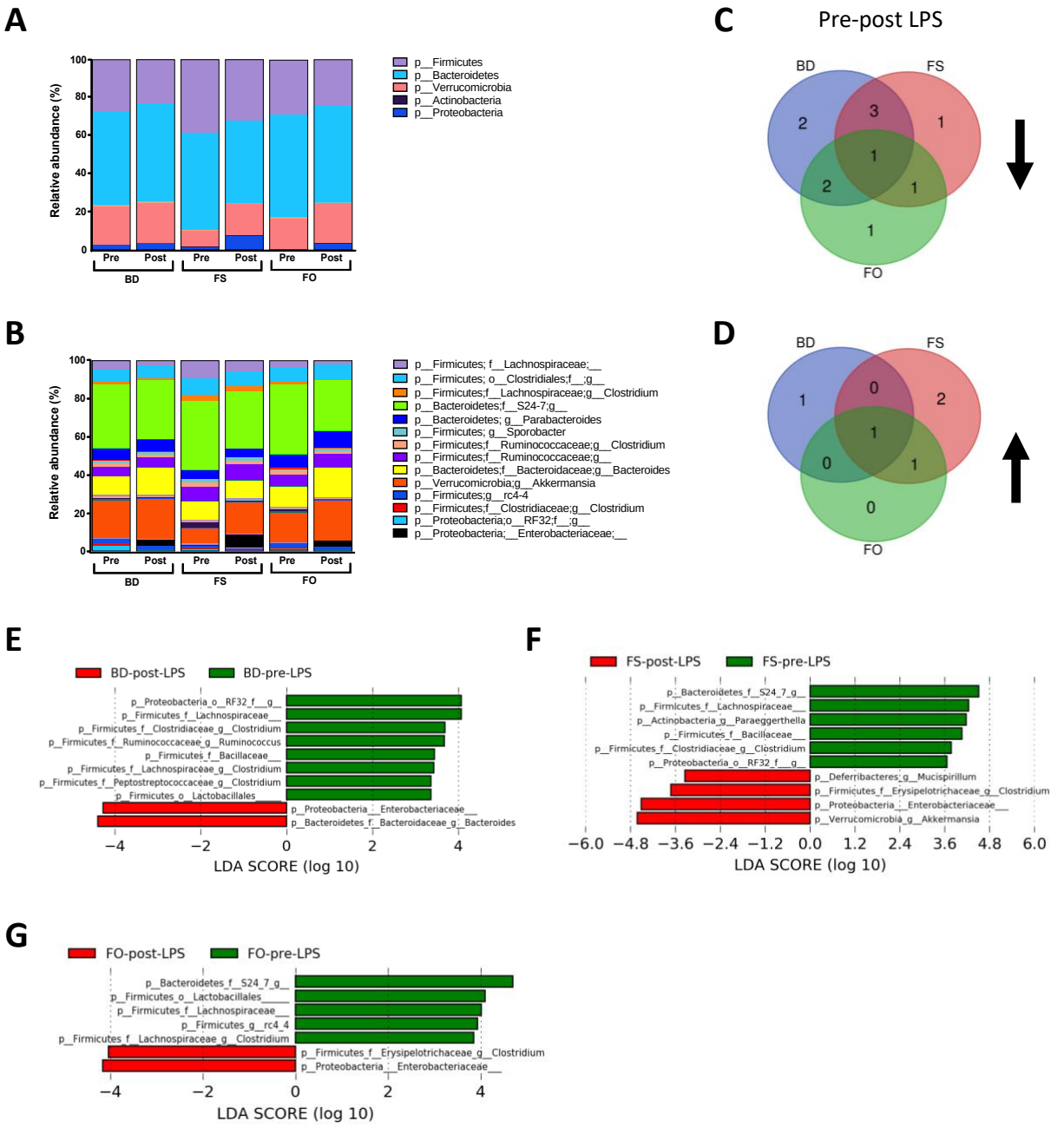

Figure 5-13. Taxonomic specific effects in the fecal microbiota before and after LPS administration.

A. Bar plots showing the Phylum level relative abundances in each dietary group pre- and 24 hours post-LPS injection. **B.** Bar plots showing Genus level relative abundances in each dietary group

pre- and 24 hours post-LPS injection. **C-D.** Venn diagrams depicting the number of unique and shared genera that were significantly decreased (Figure 5-13C) or increased (Figure 5-13D) by LPS in each dietary group. **E-G.** Lefse plots depicting the changes in the fecal microbiota before and after LPS challenge in each diet (E: BD, F: FS, G: FO). (n=12/group).

The inferred function of the microbiota following LPS injection was visualized in STAMP. There were no differences in inferred function pre vs. post LPS in BD mice indicating the taxonomic changes highlighted in the previous section do not translate to changes in inferred microbiota function. There were however many significant changes in both FS (Figure 5-14A) and FO groups (Figure S3). In FS group, LPS increased mean proportion of LPS biosynthesis and LPS biosynthesis proteins and decreased various measures of metabolism, such as pyrimidine metabolism, methane metabolism, starch and sucrose metabolism, and galactose metabolism. There were some similarities between FS and FO with respect to the LPS response, such that 20 inferred functions were shared between them were decreased, and 2 were increased, but FS had far greater unique effects on the inferred function of the microbiota (Figure 5-14B,C). This suggest that FO was driving some of the effects of FS on the microbiota function, however the other components of FS or the combination of all components results in a greater effect on microbiota function

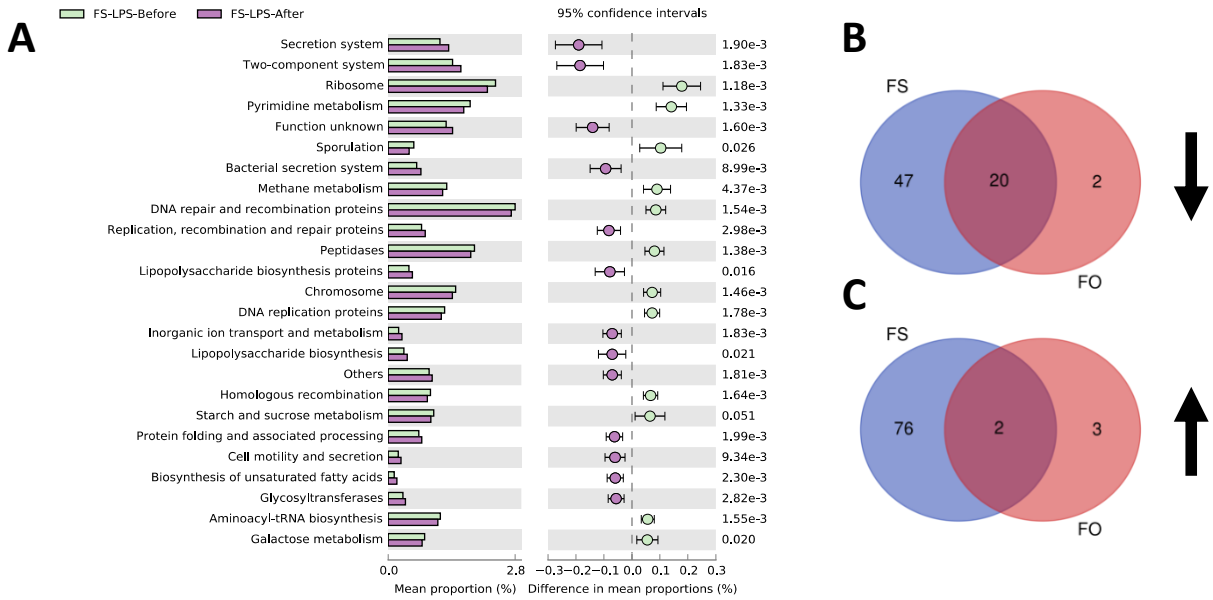

Figure 5-14. Inferred function before and after LPS administration in FS and FO mice
A. Inferred function of the fecal microbiota before and after LPS in FS-fed mice. **B-C.** Venn-diagram analysis of decreased (B), and increased (C) inferred pathways following LPS injection in FS and FO mice. The inferred function was analyzed in STAMP using Welch's two-sided t-test with Benjamini-Hochberg multiple test correction ($q<0.1$).

The measured fecal microbiota function was assessed by measuring concentrations of SCFAs. There were no significant effects of LPS administration on total SCFA concentration BD ($q=0.1709$ for all diets) (Figure 5-15A). Similarly, there was no impact on acetate ($q=0.2294$ for all diets) (Figure 5-15B), propionate ($q=0.8129$ for BD and FO mice, $q=0.2808$ for FS mice) (Figure 5-15C), and butyrate ($q=0.124$ for BD and FO mice, $q=0.058$ for FS mice) (Figure 5-15D). Thus, there was no effect of LPS on production of SCFAs.

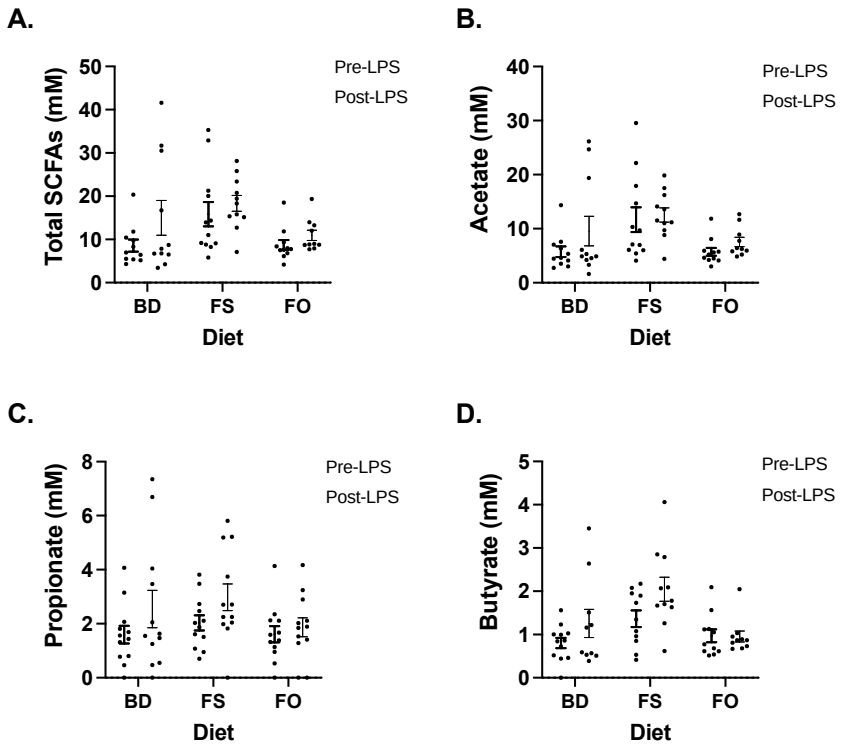

Figure 5-15. Effects of LPS on SCFA production induced by FS/FO. Effects of FS/FO/LPS on SCFA production.
A. Total SCFAs. **B.** Acetate. **C.** Propionate. **D.** Butyrate. Data was log(y) transformed to normalize based on the Kolmogorov-Smirnov distance. Normalized data was analyzed by t-tests within each diet (n=10-12/group).

5.3.7 Systemic inflammation induced by LPS was positively impacted by FS supplementation

The mice were euthanized 24-hours post-injection, and tissues and blood were collected, and serum was extracted. The serum concentrations of IL-1β, TNF-α and IL-10 were measured by multiplex (Table 5-4). Unfortunately, the serum concentrations of IL-1β were consistently below the minimum detectable limit for this multiplex. The serum TNF-α was increased in the BD-LPS and FO-LPS mice, but not in the FS-LPS mice, compared to the BD-SAL group. Similarly, serum IL-10 was increased in the BD-LPS and FO-LPS mice, but not in the FS-LPS mice, compared to the BD-SAL group. The relative spleen weight was increased in all LPS mice regardless of diet. Therefore, FS partially attenuated the systemic inflammation induced by LPS by attenuating the increase in serum TNF-α.

Table 5-6. The effect of FS and FO on LPS-induced systemic inflammation.

Measure	SAL			LPS		
	BD	FS	FO	BD	FS	FO
Serum Il-1β (pg/ml)	<4.4	<4.4	<4.4	<4.4	<4.4	<4.4
Serum TNF-α (pg/ml)	10.27 ± 1.257	7.692 ± 1.377	9.491 ± 0.838	20.74 ± 2.464*	14.22 ± 1.597	22.04 ± 2.938*
Serum IL-10 (pg/ml)	37.69 ± 6.158	29.53 ± 10.87	44.01 ± 9.820	101. 89 ± 13.72*	79.30 ± 6.826	105.45 ± 10.68*
Spleen weight (%BW)	0.280 ± 0.014	0.250 ± 0.010	0.242 ± 0.006	0.366 ± 0.015*	0.390 ± 0.025*	0.384 ± 0.017*

Serum data was analyzed by one-way ANOVA, with Dunnett's multiple comparison test (SAL: n=5-7/group), (LPS: n=10-12/group). Minimum detection limits: IL-1β= 4.4 pg/ml, TNF-α=0.488 pg/ml, IL-10=1.34 pg/ml. Spleen weight was also measured by one-way ANOVA, with Dunnett's multiple comparison test (n=12/group). An asterix (*) represents significance compared to the BD-SAL.

5.3.8 LPS-induced neuroinflammation was partially attenuated by FS supplementation

Neuroinflammation was assessed in two brain regions, the HIP and mPFC, and LPS effectively induced neuroinflammation, as shown by increased IL-1β, TNF-α and IL-10 (Figure 5-16). There were no effects of diet on the LPS-induced increase in IL-1β and TNF-α compared to BD-SAL. IL-10 was not significantly increased (P=0.0953) in the FS-LPS group compared to BD-SAL potentially suggesting an attenuation of LPS-induced IL-10 production (Figure 5-16F). Importantly, although not significant, the FS-LPS mice consistently display lower gene expression of IL-1β and TNF-α in both brain regions, suggesting a partial attenuation of the LPS-induced inflammation.

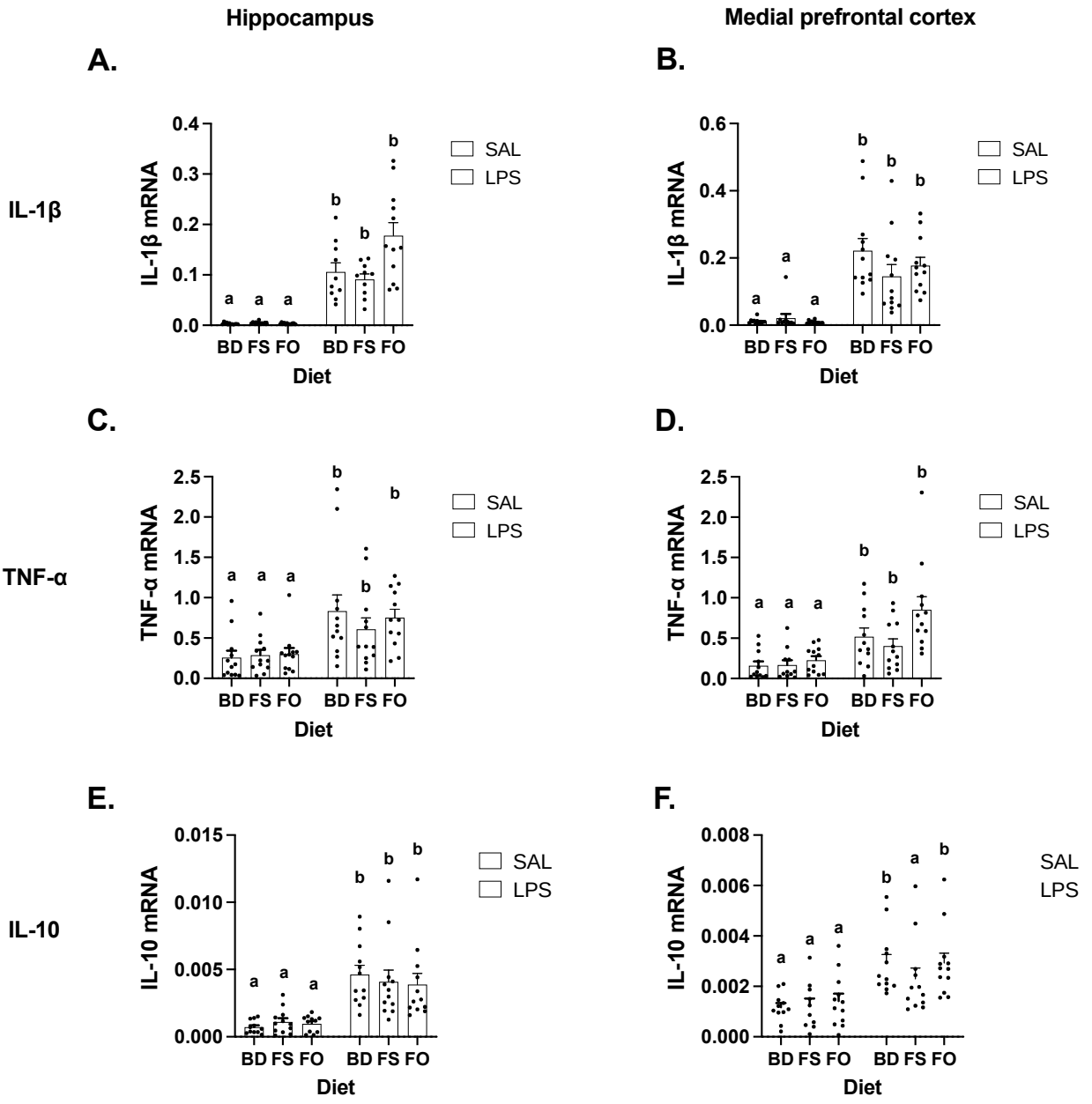

Figure 5-16. The effects of FS and FO on LPS-induced neuroinflammation in the hippocampus and medial prefrontal cortex.

A-B. Relative gene expression of IL-1β in the HIP (A) and mPFC (B). **C-D.** Relative gene expression of TNF-α in the HIP (C) and mPFC (D). **E-F.** Relative gene expression of IL-10 in the HIP (E) and mPFC (F). Data was transformed using the following formula: $y = -1 \times \log(y)$, to normalize based on the Kolmogorov-Smirnov distance. Normalized data was analyzed one-way ANOVA with Dunnett's multiple comparison test (n=12/group) compared to BD-SAL. Data points not sharing a letter are significantly different.

5.4 Discussion

The objectives of Study 2 were to further examine the effects of LPS on the GBA, and to determine the effects of dietary supplementation with FS and FO on these LPS-induced changes. Overall this study demonstrated that LPS, 24-hours post-injection, induced negative effects across the GBA, specifically through changes in the gut microbiota composition, systemic- and neuro-inflammation. These changes were differentially impacted by dietary supplementation with FS and FO, with FS inducing much stronger effects than FO across the GBA, suggesting that the effects of FS may not be based on the oil content, or that the FO interacts with the other bio-actives to induce these changes.

Similar to Study 1, LPS induced sickness, highlighted by a decrease in BW, lower DI, worse nest building, and increased sickness behaviours in the 24-hours post-injection. None of these changes were attenuated by dietary supplementation with FS or FO likely suggesting that the acute induction of sickness by LPS is too strong of a response to be attenuated by dietary supplementation by FS and/or FO.

LPS altered the cecal microbiota across all diets (Figure 5-8, Table S4), specifically, the family *S24-7*, established as *Muribaculaceae*[172], was decreased by LPS in all diets, an effect which has been previously identified in a chronic stress model of mental illness in mice[173]. The role of *Muribaculaceae*, a dominant microbe in the gut microbiota of mice[174], is still generally unknown, however one study in pathogen free Balb/c mice indicated that *Muribaculaceae* was associated with remission of colitis, suggesting a potential homeostatic function[175]. There were no consistently changed taxa 24-hours (Study 2), and 48-hours (Study 1) post-injection, however FO-LPS uniquely had decreased *rc4-4*, which was also evident 24-hours post-injection in Study 1. As *rc4-4* is potentially related to an obesogenic phenotype[156,157], the decrease may be highlighted by

the decreased BW induced by LPS, therefore this taxonomic change may be longer lasting than others based on the presence at both time points, however further studies would be required to verify this. Despite some overlap between diets regarding the cecal microbiota response to LPS, both FS and FO induced unique changes to the microbiota, highlighting that the effects of LPS may be dependent on the background diet. Critically, as the mice from Study 1 vs Study 2 were different subsets of animals, they may have had different baseline microbiota, potentially explaining the limited similarities between time points. Therefore, LPS impacted the cecal microbiota 24-hours post-injection, albeit providing a different response compared to 48-hours post-injection (Study 1), indicating a potential time-dependent effect of i.p LPS on the microbiota.

The cecal microbiota was also examined between diets (within SAL groups) to determine the effects of FS and FO on the microbiota composition. FS induces increased *Lachnospiraceae*, which is the same effect seen in female C57Bl/6 mice supplemented with 10% FS for three weeks indicating no sex difference[94]. Members of the *Lachnospiraceae* family are producers of SCFAs indicating a potentially beneficial role[176], but other members have controversial negative impacts on health including depression[177,178], suggesting the function is genus/species specific. One member of this family, *Dorea* (decreased by FS) may be proinflammatory[179], an effect that is dependent on surrounding bacteria and nutrient availability[180], potentially suggesting that FS is resulting in a general improvement of the microbiota, thus limiting growth of *Dorea*. The limited impact of FO on the healthy cecal microbiota suggests that the effects of FS are mostly independent of the oil content or based on a combination of all components.

The fecal microbiota was examined prior to SAL/LPS injection to determine if FS or FO could beneficially alter the baseline microbial community structure prior to LPS challenge. There is limited overlap between specific taxonomic changes with FS compared to a study in female

C57Bl/6 mice supplemented with 10% FS or 3.7% FO for 3 weeks[94], such that only *Actinobacteria,* and *Coriobacteriaceae* were consistently increased in the feces of FS-fed mice across studies (Table 5-6). Interestingly, *Coriobacteriaceae* are known SDG metabolizers, but the FS-induced increase is not sex dependant when comparing between studies[94]. Specific FS-increased genera, such as *Lachnospiraceae* and *Bifidobacterium* may suggest an anti-depressive role of FS. Decreased *Lachnospira*ceae has been linked to individuals living with depression[181], thus, an increase may be beneficial. Similarly, *Bifidobacterium,* (some of which are used as probiotics), have been shown to have anti-depressive/anxiolytic effect[182,184], again suggesting a potentially beneficial effect of FS. Furthermore, *Bifidobacterium* is also capable of metabolizing ALA[185], which could suggest the limited effects of FO systemically and in the brain based on the lack of *Bifidobacterium* present. Thus, FS but not FO impacts the gut microbiota composition, and the anti-inflammatory/anti-depressant role of FO highlighted previously in the literature[98] could be dependent on the presence of an ALA metabolizer such as *Bifidobacterium,* however additional studies are required to determine this possible relationship.

When comparing the fecal microbiota pre and post LPS injection, there was increased *Enterobacteriaceae* across all diets (Table S5), similar to a study in piglets where an increased in *E. coli* (family *Enterobacteriaceae*) was shown[49]. Increased *Enterobacteriaceae* has been described as a marker of microbial dysbiosis driven by increased reactive oxygen species (ROS) in the gut, causing a bloom in facultative anaerobes (can live with/without oxygen) (e.g. *Enterobacteriaceae*) and a decrease in obligate aerobes (cannot live in oxygen-rich environments)[186]. Thus, the LPS-induced increase in *Enterobacteriaceae* may be an indication of both microbial dysbiosis and ROS in the gut. FS and FO differentially impacted the LPS-induced changes to the fecal microbiota, notably, the FO-post-LPS mice uniquely showed decreased *rc4-*

4, similar to the same mice in the cecum, highlighting similarities across sampling location, and again a potential association to the LPS-induced BW loss in these mice. Therefore, LPS has an impact on the fecal microbiota in both a diet independent and dependent manner.

Taken together, in both the cecum and feces, LPS induced some level of dysbiosis of the microbiota, highlighted by taxonomic changes, which was differentially impacted FS or FO. Prior to LPS administration, FS has a greater effect on the microbiota compared to FO, but the microbiota response to LPS is not nearly as distinct between diets.

Although there were no significant effects of diet on neuroinflammation regardless of brain region and gene, it is important to highlight consistent trends. Throughout all three genes and both the HIP and mPFC, the FS-LPS mice show slightly lower gene expression compared to the BD-LPS and FO-LPS mice. This matches the effects of FS on the serum cytokines, specifically TNF-α and IL-10 (section 5.3.7). This is suggesting that FS is inducing anti-inflammatory responses, however they may not be a robust enough effect to completely attenuate the strong LPS inflammatory cascade. The decrease in gene expression of these markers may be related to a downregulation of the NF-κB pathway, potentially through oxidative stress pathways[187], or through increased production of SCFAs induced by FS. To elaborate, when reactive oxygen species (ROS) production is greater than the antioxidant state, then the oxidative stress can activate the NF-κB pathway[187]. With FS-supplementation, there is potential antioxidant effects through the poly-phenolic compound SDG, which may be contributing to the anti-oxidant state, thus lowering the impact of oxidative stress. Importantly, a study examining the effects of caffeine (an anti-oxidant polyphenolic compound) on LPS-induced oxidative stress found that caffeine reduced ROS production and other ROS regulators in the brain[188]. Although this study did not look at IL-1β, TNF-α and IL-10 specifically, they did show a decrease in NF-κB expression in the brain which

is suggestive of similar downstream effects. The increase in fecal SCFAs with FS supplementation corroborates a previous study in male C57Bl/6 mice fed 10% FS for 3 weeks, confirming the SCFA-promoting impact of dietary supplementation with FS, which may translate to anti-inflammatory effects systemically and in the brain such as reduced inflammatory signaling by glial cells and increased BBB integrity[104]. Taken together, LPS effectively induced neuroinflammation in both the HIP and mPFC, which was partially attenuated by FS but not FO, suggesting potential implication of SDG, SCFAs and oxidative stress pathways.

In conclusion this study demonstrated key effects of FS and FO on the LPS-induced changes across the GBA 24-hours post-injection. Importantly, FS dietary supplementation resulted in more beneficial changes than FO, highlighted by reduction of inflammation both in the serum, and in the HIP/mPFC. The beneficial effects of FS were also seen in the gut, with increased diversity and SCFA production, indication of a healthier microbiota. Therefore, the potential benefits of FS dietary supplementation are not only a result of the increased content of ALA found in FO, as there was a limited effect of FO across the GBA in this study. The resultant effects of FS could be primarily based on the fibre content, SDG, or an interaction between all of the bioactive components, but further studies will elaborate on specific interactions between bioactives.

Chapter 6. General Discussion and Future Directions

6.1 General Discussion

In this MSc thesis, I had hypothesized that mice injected with LPS will show symptoms similar to individuals living with depression and/or anxiety, specifically through microbial dysbiosis[42], systemic- and neuro-inflammation. This hypothesis was mostly supported in Study 1, as LPS induced significant changes in the cecal microbiota, systemic inflammation (spleen weight), and neuroinflammation in the HIP and PFC, however aside from total rearing time, there was no evidence of LPS inducing depressive- or anxiety-like behaviours. This hypothesis was again mostly supported in Study 2, as there were significant effects of LPS on the cecal and fecal microbiota, significantly increased systemic inflammation (serum cytokines), and significantly increased neuroinflammation in the HIP and mPFC. Secondly, I had hypothesized that compared to mice consuming FO alone, mice consuming diets supplemented with whole ground FS will further attenuate the microbial dysbiosis, systemic- and neuro-inflammation due to the combined beneficial effects of dietary fibre, lignans, and ALA-rich FO. This hypothesis was supported by Study 2, as FS had far greater effects across the GBA than FO, specifically, FS-dietary supplementation significantly altered the fecal and cecal microbiota composition and function, FS attenuated the LPS-induced increase in serum inflammation, and FS showed a decreasing trend in neuroinflammation. Although there was no complete attenuation of the LPS response in any diet, it is important to note that diet is only one factor in improving mental health, but it could highlight a potential role for FS in adjuvant therapies for depression and/or anxiety.

Across both studies, LPS significantly increased sickness behaviours, BW loss and decreased DI, especially within the first 24- hours post-injection. When comparing between studies here, it is important to note that the mice in Study 1 were euthanized at 7-8-weeks old, and the

mice in Study 2 were euthanized at 9-10- weeks old, which could induce some discrepancies in the response to LPS. The ambiguity/subjectivity of the sickness scores highlighted in Study 1 was not replicated in Study 2, indicating a vast improvement in the scoring system. The BW and DI 24-hours post-injection were the same across the two studies, validating the reproducibility of this aspect of the LPS response. The nesting scores were however different across studies, as in Study 1, both the SAL and LPS mice had a nest score of ~3 after 24-hours, whereas in Study 2, the SAL mice on average had nest scores of ~4 and the LPS mice ~2, suggesting some level of variation in the reproducibility of this metric. Despite the mice in Study 2 being in our facility for 2 weeks more than the mice in Study 1, by the time of injection there were no differences in time from last cage change (2- weeks), implying the differences were likely related to the differences between mice as highlighted by the large spread of data.

The cecal microbiota was examined in both studies, with the Study 1 cecum content collected at 48-hours post-injection, and the Study 2 cecum content collected at 24-hours post-injection. There were no changes in α-diversity 24-hours post-injection, but there was an increase in α-diversity (Pielou's evenness) 48-hours post-injection. LPS altered the β-diversity at both time points, and altered specific taxonomic groups, but there was no overlap between studies with significant bacteria within the BD group, potentially highlighting a time dependant microbial response to LPS. Despite the different time-points, there were seven taxonomic changes at both 24- and 48- hours post injection (again no overlap), which could indicate the effects of LPS on the gut microbiota are not as transient, as they could last until the 48-hour mark, but a more longitudinal study with fecal collections spanning a week could highlight the longer-term effects of i.p LPS on the gut microbiota.

Systemic inflammation was measured 24- and 48-hours post injection, specifically by measurement of cytokines in the serum and relative spleen weight. The relative spleen weight was slightly heavier 48- hours post-injection than at 24-hours post injection, which could be due to the age of the mice, or that splenomegaly had not fully developed at 24-hours post-injection. Serum IL-l0 and TNF-α were much more abundant at 24-hours post-injection compared to 48-hours post-injection, which follows the previously identified time course of TNF-α in the serum[65]. Neuroinflammation was also measured at 24- and 48-hours post-injection in the HIP and PFC (Study 1) and HIP and mPFC (Study 2). In Study 1, there was seemingly a greater impact of LPS on the HIP than the PFC, whereas in Study 2, there was no visible differences between brain regions, potentially validating that the mPFC provided a more refined response to i.p LPS than the whole PFC.

To rationalize this study, the role of ALA metabolism was highlighted as it would result in production of EPA and DHA; fish oils fatty acids shown to have anti-inflammatory/anti-depressive/anxiolytic effects[31,74]. In Study 2, we examined the effects of FO on the GBA in LPS-challenged mice, predicting that despite the low conversion rate of ALA to EPA/DHA, there would still be beneficially effects across the GBA. This was generally not the case, as there were limited effects throughout the mice, except for altered microbiota (cecal and fecal) in LPS-challenged mice. These findings suggest that the low conversion rate is not sufficient to induce these beneficial changes with this level of FO supplementation (3.97%), thus in order to see benefits, potentially a less physiologically relevant dose would be required, which could be investigated in a future study. As highlighted in section 5.4, the lack of response may be related to a lack of ALA metabolizers such as *Bidifobacterium* which may suggest that supplementation with both FO and *Bifidobacterium* may result in beneficial effects across the GBA.

In conclusion, in line with the hypotheses, LPS significantly impacted the GBA in male C57Bl/6 mice, the effects of which were partially attenuated/improved by previous FS dietary supplementation, but not with FO, suggesting that the beneficial effects of FS are largely based on the presence of fibre and SDG, or synergistic effects of all relevant bioactives.

6.2 Limitations and future directions

The limitations of this study include the lack of direct measures of systemic inflammation during the 24- hour period following i.p LPS, specifically the first few hours, as the strongest systemic response is visible during this time frame[65]. Thus, future studies would also include measurement of serum cytokines throughout the first few hours by collecting tail vein blood. In addition, male mice were used as a proof of concept to examine the effects of LPS on the GBA, however future studies could examine these changes in female mice, as there are known sex differences in the LPS immune response[189]. Similarly, dietary supplementation with FS prior to an LPS challenge in female mice would likely show different effects based on the metabolism of SDG into the phytoestrogens END and ENL which have beneficial impacts on health[116]. In Study 1 we examined the neuroinflammation in the HIP and PFC, and in Study 2, the HIP and mPFC. It is important to note that other brain regions are also relevant to mental illnesses (e.g. amygdala and hypothalamus)[190,191] which could be isolated in future studies. As this study was focused on the acute effects of LPS on the GBA, the effects of chronic LPS on the GBA have not yet been identified, highlighting another potential future study. We collected many tissues for histological purposes, and so future directions of this study include examining histological measures of health within the gut such as the intestinal crypts and goblet cells, known to be impacted by FS dietary supplementation[119]. Similarly, integrity of intestinal tight junctions could be measured indirectly

by qPCR analysis of tight junction mRNA (e.g. occludin, ZO-1, and claudin). Future studies could also examine specific cell types in the HIP and mPFC by flow cytometry, to determine the potential source of inflammatory cytokines (e.g microglia or astrocytes). Finally, to determine if the effects of FS on the GBA are truly independent of the presence of FO, a similar study would have to be performed with de-fatted FS, leaving only the fibre and SDG as the major bioactives.

Contributions of collaborators

Nutrition and Mental Health Master's Scholarship; funding

NSERC; funding

Animal Behaviour and Physiology Core; support during behaviour tests

Natunola; donation of flaxseeds

Dr. Krista A. Power; supervision, support during animal trials

Dr. Farah Hosseinian's lab; extraction of flaxseed oil

Dr. Lalit Kishore; training for SCFA extractions and GC, and support during animal trials

Alexane Rodrigue; training for a variety of methods, and support during animal trials

Allison Sweet; support during animal trials and analysis of intestinal health

Julia Loftus; supported the analysis of mPFC gene expression by qPCR

Farida Ghali; supported SCFA extractions

Salma Mahmoodianfard; support during sacrifice

Giorgio Freije; support during animal trial

References

1. Lim, K.-L., Jacobs, P., Ohinmaa, A., Schopflocher, D., and Dewa, C.S. (2008). A new population-based measure of the economic burden of mental illness in Canada. Chronic Dis Can *28*, 92–98.
2. Smetanin, P., Stiff, D., Briante, C., Adair, C.E., Ahmad, S., and Khan, M. (2011). The life and economic impact of major mental illnesses in Canada: 2011 to 2041. RiskAnalytica, behalf Ment. Heal. Comm. Canada.
3. Findlay, L. (2017). Depression and suicidal ideation among Canadians aged 15 to 24.
4. Pelletier, L., O'Donnell, S., McRae, L., and Grenier, J. (2017). The burden of generalized anxiety disorder in Canada TT - Le fardeau du trouble d'anxiété généralisée au Canada. Heal. Promot. chronic Dis. Prev. Canada Res. policy Pract. *37*, 54–62.
5. Jenkins, E.K., McAuliffe, C., Hirani, S., Richardson, C., Thomson, K.C., McGuinness, L., Morris, J., Kousoulis, A., and Gadermann, A. (2021). A portrait of the early and differential mental health impacts of the COVID-19 pandemic in Canada: Findings from the first wave of a nationally representative cross-sectional survey. Prev. Med. (Baltim). *145*, 106333.
6. Groen, R.N., Ryan, O., Wigman, J.T.W., Riese, H., Penninx, B.W.J.H., Giltay, E.J., Wichers, M., and Hartman, C.A. (2020). Comorbidity between depression and anxiety: assessing the role of bridge mental states in dynamic psychological networks. BMC Med. *18*, 308.
7. Enache, D., Pariante, C.M., and Mondelli, V. (2019). Markers of central inflammation in major depressive disorder: A systematic review and meta-analysis of studies examining cerebrospinal fluid, positron emission tomography and post-mortem brain tissue. Brain. Behav. Immun. *81*, 24–40.
8. Kim, Y.-K., and Won, E. (2017). The influence of stress on neuroinflammation and alterations in brain structure and function in major depressive disorder. Behav. Brain Res. *329*, 6–11.
9. Won, E., and Kim, Y.-K. (2020). Neuroinflammation-Associated Alterations of the Brain as Potential Neural Biomarkers in Anxiety Disorders. Int. J. Mol. Sci. *21*.
10. Lurie, D.I. (2018). An Integrative Approach to Neuroinflammation in Psychiatric disorders and Neuropathic Pain. J. Exp. Neurosci. *12*, 1179069518793639.
11. Liu, L., and Zhu, G. (2018). Gut-brain axis and mood disorder. Front. psychiatry *9*, 223.
12. Foster, J.A., and Mcvey Neufeld, K.-A. (2013). Gut–brain axis: how the microbiome influences anxiety and depression. Trends Neurosci. *36*, 305–312.
13. Walker, E.A., Katon, W.J., Jemelka, R.P., and Roy-Byrne, P.P. (1992). Comorbidity of gastrointestinal complaints, depression, and anxiety in the epidemiologic catchment area (ECA) study. Am. J. Med. *92*, S26–S30.
14. Shah, E., Rezaie, A., Riddle, M., and Pimentel, M. (2014). Psychological disorders in gastrointestinal disease: epiphenomenon, cause or consequence? Ann. Gastroenterol. *27*, 224–230.
15. Clapp, M., Aurora, N., Herrera, L., Bhatia, M., Wilen, E., and Wakefield, S. (2017). Gut microbiota's effect on mental health: The gut-brain axis. Clin. Pract. *7*, 987.
16. Cryan, J.F., O'Riordan, K.J., Cowan, C.S.M., Sandhu, K. V, Bastiaanssen, T.F.S., Boehme, M., Codagnone, M.G., Cussotto, S., Fulling, C., Golubeva, A. V, et al. (2019). The Microbiota-Gut-Brain Axis. Physiol. Rev. *99*, 1877–2013.

17. Carabotti, M., Scirocco, A., Maselli, M.A., and Severi, C. (2015). The gut-brain axis: interactions between enteric microbiota, central and enteric nervous systems. Ann. Gastroenterol. *28*, 203–209.
18. Stevens, B.R., Goel, R., Seungbum, K., Richards, E.M., Holbert, R.C., Pepine, C.J., and Raizada, M.K. (2018). Increased human intestinal barrier permeability plasma biomarkers zonulin and FABP2 correlated with plasma LPS and altered gut microbiome in anxiety or depression. Gut *67*, 1555–1557.
19. DellaGioia, N., and Hannestad, J. (2010). A critical review of human endotoxin administration as an experimental paradigm of depression. Neurosci. Biobehav. Rev. *34*, 130–143.
20. Reichenberg, A., Yirmiya, R., Schuld, A., Kraus, T., Haack, M., Morag, A., and Pollmächer, T. (2001). Cytokine-Associated Emotional and Cognitive Disturbances in Humans. Arch. Gen. Psychiatry *58*, 445–452.
21. Zhao, Y., Jaber, V., and Lukiw, W.J. (2017). Secretory products of the human GI tract microbiome and their potential impact on Alzheimer's disease (AD): detection of lipopolysaccharide (LPS) in AD hippocampus. Front. Cell. Infect. Microbiol. *7*, 318.
22. Pal, G.D., Shaikh, M., Forsyth, C.B., Ouyang, B., Keshavarzian, A., and Shannon, K.M. (2015). Abnormal lipopolysaccharide binding protein as marker of gastrointestinal inflammation in Parkinson disease. Front. Neurosci. *9*, 306.
23. Hasegawa, S., Goto, S., Tsuji, H., Okuno, T., Asahara, T., Nomoto, K., Shibata, A., Fujisawa, Y., Minato, T., and Okamoto, A. (2015). Intestinal dysbiosis and lowered serum lipopolysaccharide-binding protein in Parkinson's disease. PLoS One *10*, e0142164.
24. Rao, T.S.S., Asha, M.R., Ramesh, B.N., and Rao, K.S.J. (2008). Understanding nutrition, depression and mental illnesses. Indian J. Psychiatry *50*.
25. Firth, J., Stubbs, B., Teasdale, S.B., Ward, P.B., Veronese, N., Shivappa, N., Hebert, J.R., Berk, M., Yung, A.R., and Sarris, J. (2018). Diet as a hot topic in psychiatry: a population-scale study of nutritional intake and inflammatory potential in severe mental illness. World Psychiatry *17*, 365.
26. Nikaido, H. (1996). Outer membrane. Escherichia coli Salmonella. Cell. Mol. Biol., 29–47.
27. Migale, R., Herbert, B.R., Lee, Y.S., Sykes, L., Waddington, S.N., Peebles, D., Hagberg, H., Johnson, M.R., Bennett, P.R., and MacIntyre, D.A. (2015). Specific Lipopolysaccharide Serotypes Induce Differential Maternal and Neonatal Inflammatory Responses in a Murine Model of Preterm Labor. Am. J. Pathol. *185*, 2390–2401.
28. Lu, Y.-C., Yeh, W.-C., and Ohashi, P.S. (2008). LPS/TLR4 signal transduction pathway. Cytokine *42*, 145–151.
29. Alexander, A.F., Kelsey, I., Forbes, H., and Miller-Jensen, K. (2021). Single-cell secretion analysis reveals a dual role for IL-10 in restraining and resolving the TLR4-induced inflammatory response. Cell Rep. *36*, 109728.
30. Iyer, S.S., Ghaffari, A.A., and Cheng, G. (2010). Lipopolysaccharide-Mediated IL-10 Transcriptional Regulation Requires Sequential Induction of Type I IFNs and IL-27 in Macrophages. J. Immunol. *185*, 6599 LP – 6607.
31. de Gomes, M.G., Souza, L.C., Goes, A.R., Del Fabbro, L., Filho, C.B., Donato, F., Prigol, M., Luchese, C., Roman, S.S., Puntel, R.L., et al. (2018). Fish oil ameliorates sickness behavior induced by lipopolysaccharide in aged mice through the modulation of kynurenine pathway. J. Nutr. Biochem. *58*, 37–48.

32. Savignac, H.M., Couch, Y., Stratford, M., Bannerman, D.M., Tzortzis, G., Anthony, D.C., and Burnet, P.W.J. (2016). Prebiotic administration normalizes lipopolysaccharide (LPS)-induced anxiety and cortical 5-HT2A receptor and IL1-β levels in male mice. Brain. Behav. Immun. *52*, 120–131.
33. Qin, L., Wu, X., Block, M.L., Liu, Y., Breese, G.R., Hong, J., Knapp, D.J., and Crews, F.T. (2007). Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. Glia *55*, 453–462.
34. Banks, W.A., Gray, A.M., Erickson, M.A., Salameh, T.S., Damodarasamy, M., Sheibani, N., Meabon, J.S., Wing, E.E., Morofuji, Y., Cook, D.G., et al. (2015). Lipopolysaccharide-induced blood-brain barrier disruption: roles of cyclooxygenase, oxidative stress, neuroinflammation, and elements of the neurovascular unit. J. Neuroinflammation *12*, 223.
35. Rom, S., Zuluaga-Ramirez, V., Reichenbach, N.L., Erickson, M.A., Winfield, M., Gajghate, S., Christofidou-Solomidou, M., Jordan-Sciutto, K.L., and Persidsky, Y. (2018). Secoisolariciresinol diglucoside is a blood-brain barrier protective and anti-inflammatory agent: implications for neuroinflammation. J. Neuroinflammation *15*, 25.
36. Biesmans, S., Meert, T.F., Bouwknecht, J.A., Acton, P.D., Davoodi, N., De Haes, P., Kuijlaars, J., Langlois, X., Matthews, L.J.R., Ver Donck, L., et al. (2013). Systemic Immune Activation Leads to Neuroinflammation and Sickness Behavior in Mice. Mediators Inflamm. *2013*, 271359.
37. Saia, R.S., Ribeiro, A.B., and Giusti, H. (2020). Cholecystokinin Modulates the Mucosal Inflammatory Response and Prevents the Lipopolysaccharide-Induced Intestinal Epithelial Barrier Dysfunction. Shock *53*.
38. Zhuang, S., Zhong, J., Bian, Y., Fan, Y., Chen, Q., Liu, P., and Liu, Z. (2019). Rhein ameliorates lipopolysaccharide-induced intestinal barrier injury via modulation of Nrf2 and MAPKs. Life Sci. *216*, 168–175.
39. He, C., Hu, X., Xiao, D., Wu, J., Zhou, S., Deng, J., Xu, S., Huang, Y., Peng, M., and Yang, X. (2020). Vitamin A prevents lipopolysaccharide-induced injury on tight junctions in mice. Food Sci. Nutr. *8*, 1942–1948.
40. Sylvia, K.E., and Demas, G.E. (2018). Acute intraperitoneal lipopolysaccharide influences the immune system in the absence of gut dysbiosis. Physiol. Rep. *6*, e13639.
41. Huang, N., Hua, D., Zhan, G., Li, S., Zhu, B., Jiang, R., Yang, L., Bi, J., Xu, H., Hashimoto, K., et al. (2019). Role of Actinobacteria and Coriobacteriia in the antidepressant effects of ketamine in an inflammation model of depression. Pharmacol. Biochem. Behav. *176*, 93–100.
42. Barandouzi, Z.A., Starkweather, A.R., Henderson, W.A., Gyamfi, A., and Cong, X.S. (2020). Altered composition of gut microbiota in depression: A systematic review. Front. Psychiatry *11*, 541.
43. Kelly, J.R., Borre, Y., O'Brien, C., Patterson, E., El Aidy, S., Deane, J., Kennedy, P.J., Beers, S., Scott, K., and Moloney, G. (2016). Transferring the blues: depression-associated gut microbiota induces neurobehavioural changes in the rat. J. Psychiatr. Res. *82*, 109–118.
44. Weiss, G.A., and Hennet, T. (2017). Mechanisms and consequences of intestinal dysbiosis. Cell. Mol. Life Sci. *74*, 2959–2977.
45. Wagner, B.D., Grunwald, G.K., Zerbe, G.O., Mikulich-Gilbertson, S.K., Robertson, C.E., Zemanick, E.T., and Harris, J.K. (2018). On the use of diversity measures in longitudinal

sequencing studies of microbial communities. Front. Microbiol. *9*, 1037.
46. Lupp, C., Robertson, M.L., Wickham, M.E., Sekirov, I., Champion, O.L., Gaynor, E.C., and Finlay, B.B. (2007). Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. Cell Host Microbe *2*, 119–129.
47. Stecher, B., Robbiani, R., Walker, A.W., Westendorf, A.M., Barthel, M., Kremer, M., Chaffron, S., Macpherson, A.J., Buer, J., Parkhill, J., et al. (2007). Salmonella enterica Serovar Typhimurium Exploits Inflammation to Compete with the Intestinal Microbiota. PLOS Biol. *5*, e244.
48. Candelli, M., Franza, L., Pignataro, G., Ojetti, V., Covino, M., Piccioni, A., Gasbarrini, A., and Franceschi, F. (2021). Interaction between Lipopolysaccharide and Gut Microbiota in Inflammatory Bowel Diseases. Int. J. Mol. Sci. *22*.
49. Xu, B., Yan, Y., Yin, B., Zhang, L., Qin, W., Niu, Y., Tang, Y., Zhou, S., Yan, X., and Ma, L. (2021). Dietary glycyl-glutamine supplementation ameliorates intestinal integrity, inflammatory response, and oxidative status in association with the gut microbiota in LPS-challenged piglets. Food Funct. *12*, 3539–3551.
50. Ghosh, S.S., Wang, J., Yannie, P.J., and Ghosh, S. (2020). Intestinal Barrier Dysfunction, LPS Translocation, and Disease Development. J. Endocr. Soc. *4*, bvz039–bvz039.
51. Zhou, X., Zhang, Y., He, L., Wan, D., Liu, G., Wu, X., and Yin, Y. (2017). Serine prevents LPS-induced intestinal inflammation and barrier damage via p53-dependent glutathione synthesis and AMPK activation. J. Funct. Foods *39*, 225–232.
52. Gu, L., Li, N., Gong, J., Li, Q., Zhu, W., and Li, J. (2011). Berberine Ameliorates Intestinal Epithelial Tight-Junction Damage and Down-regulates Myosin Light Chain Kinase Pathways in a Mouse Model of Endotoxinemia. J. Infect. Dis. *203*, 1602–1612.
53. Xu, X., Hua, H., Wang, L., He, P., Zhang, L., Qin, Q., Yu, C., Wang, X., Zhang, G., and Liu, Y. (2020). Holly polyphenols alleviate intestinal inflammation and alter microbiota composition in lipopolysaccharide-challenged pigs. Br. J. Nutr. *123*, 881–891.
54. Vamadevan, A.S., Fukata, M., Arnold, E.T., Thomas, L.S., Hsu, D., and Abreu, M.T. (2010). Regulation of Toll-like receptor 4-associated MD-2 in intestinal epithelial cells: a comprehensive analysis. Innate Immun. *16*, 93–103.
55. Guo, S., Nighot, M., Al-Sadi, R., Alhmoud, T., Nighot, P., and Ma, T.Y. (2015). Lipopolysaccharide Regulation of Intestinal Tight Junction Permeability Is Mediated by TLR4 Signal Transduction Pathway Activation of FAK and MyD88. J. Immunol. *195*, 4999 LP – 5010.
56. Zhang, L., Wei, X., Zhang, R., Si, D., Petitte, J.N., Ahmad, B., and Zhang, M. (2019). A Novel Peptide Ameliorates LPS-Induced Intestinal Inflammation and Mucosal Barrier Damage via Its Antioxidant and Antiendotoxin Effects. Int. J. Mol. Sci. *20*, 3974.
57. Liu, Y., Huang, J., Hou, Y., Zhu, H., Zhao, S., Ding, B., Yin, Y., Yi, G., Shi, J., and Fan, W. (2008). Dietary arginine supplementation alleviates intestinal mucosal disruption induced by Escherichia coli lipopolysaccharide in weaned pigs. Br. J. Nutr. *100*, 552–560.
58. Liu, Y., Xu, Q., Wang, Y., Liang, T., Li, X., Wang, D., Wang, X., Zhu, H., and Xiao, K. (2021). Necroptosis is active and contributes to intestinal injury in a piglet model with lipopolysaccharide challenge. Cell Death Dis. *12*, 62.
59. Lee, C.-H., and Giuliani, F. (2019). The Role of Inflammation in Depression and Fatigue . Front. Immunol. *10*.
60. Felger, C.J. (2018). Imaging the Role of Inflammation in Mood and Anxiety-related Disorders. Curr. Neuropharmacol. *16*, 533–558.

61. Frank, P., Jokela, M., Batty, G.D., Cadar, D., Steptoe, A., and Kivimäki, M. (2021). Association Between Systemic Inflammation and Individual Symptoms of Depression: A Pooled Analysis of 15 Population-Based Cohort Studies. Am. J. Psychiatry *178*, 1107–1118.
62. Juskewitch, J.E., Knudsen, B.E., Platt, J.L., Nath, K.A., Knutson, K.L., Brunn, G.J., and Grande, J.P. (2012). LPS-induced murine systemic inflammation is driven by parenchymal cell activation and exclusively predicted by early MCP-1 plasma levels. Am. J. Pathol. *180*, 32–40.
63. Meneses, G., Rosetti, M., Espinosa, A., Florentino, A., Bautista, M., Díaz, G., Olvera, G., Bárcena, B., Fleury, A., Adalid-Peralta, L., et al. (2018). Recovery from an acute systemic and central LPS-inflammation challenge is affected by mouse sex and genetic background. PLoS One *13*, e0201375–e0201375.
64. Erickson, M.A., and Banks, W.A. (2011). Cytokine and chemokine responses in serum and brain after single and repeated injections of lipopolysaccharide: multiplex quantification with path analysis. Brain. Behav. Immun. *25*, 1637–1648.
65. Qin, L., He, J., Hanes, R.N., Pluzarev, O., Hong, J.-S., and Crews, F.T. (2008). Increased systemic and brain cytokine production and neuroinflammation by endotoxin following ethanol treatment. J. Neuroinflammation *5*, 10.
66. Alves-Rosa, F., Vulcano, M., Beigier-Bompadre, M., Fernández, G., Palermo, M., and Isturiz, M.A. (2002). Interleukin-1beta induces in vivo tolerance to lipopolysaccharide in mice. Clin. Exp. Immunol. *128*, 221–228.
67. Laflamme, N., and Rivest, S. (2001). Toll-like receptor 4: the missing link of the cerebral innate immune response triggered by circulating gram-negative bacterial cell wall components. FASEB J. *15*, 155–163.
68. Banks, W.A., and Robinson, S.M. (2010). Minimal penetration of lipopolysaccharide across the murine blood-brain barrier. Brain. Behav. Immun. *24*, 102–109.
69. Campbell, S., and MacQueen, G. (2004). The role of the hippocampus in the pathophysiology of major depression. J. Psychiatry Neurosci. *29*, 417–426.
70. Pizzagalli, D.A., and Roberts, A.C. (2022). Prefrontal cortex and depression. Neuropsychopharmacology *47*, 225–246.
71. Cominski, T.P., Jiao, X., Catuzzi, J.E., Stewart, A.L., and Pang, K.C.H. (2014). The role of the hippocampus in avoidance learning and anxiety vulnerability. Front. Behav. Neurosci. *8*, 273.
72. Kenwood, M.M., Kalin, N.H., and Barbas, H. (2022). The prefrontal cortex, pathological anxiety, and anxiety disorders. Neuropsychopharmacology *47*, 260–275.
73. Shi, Z., Ren, H., Huang, Z., Peng, Y., He, B., Yao, X., Yuan, T.-F., and Su, H. (2017). Fish oil prevents lipopolysaccharide-Induced depressive-Like behavior by inhibiting neuroinflammation. Mol. Neurobiol. *54*, 7327–7334.
74. Dang, R., Zhou, X., Tang, M., Xu, P., Gong, X., Liu, Y., Jiao, H., and Jiang, P. (2018). Fish oil supplementation attenuates neuroinflammation and alleviates depressive-like behavior in rats submitted to repeated lipopolysaccharide. Eur. J. Nutr. *57*, 893–906.
75. DiSabato, D.J., Quan, N., and Godbout, J.P. (2016). Neuroinflammation: the devil is in the details. J. Neurochem. *139 Suppl*, 136–153.
76. McCarthy, G.M., Bridges, C.R., Blednov, Y.A., and Harris, R.A. (2017). CNS cell-type localization and LPS response of TLR signaling pathways. F1000Research *6*, 1144.
77. Bsibsi, M., Ravid, R., Gveric, D., and van Noort, J.M. (2002). Broad Expression of Toll-

Like Receptors in the Human Central Nervous System. J. Neuropathol. Exp. Neurol. *61*, 1013–1021.
78. Ghosh, C., and Bishayi, B. (2015). Characterization of Toll-like receptor-4 (TLR-4) in the spleen and thymus of Swiss albino mice and its modulation in experimental endotoxemia. J. Immunol. Res. *2015*, 137981.
79. Wohleb, E.S. (2016). Neuron–Microglia Interactions in Mental Health Disorders: "For Better, and For Worse" . Front. Immunol. *7*.
80. Troubat, R., Barone, P., Leman, S., Desmidt, T., Cressant, A., Atanasova, B., Brizard, B., El Hage, W., Surget, A., Belzung, C., et al. (2020). Neuroinflammation and depression: A review. Eur. J. Neurosci. *n/a*.
81. Calder, P.C. (2010). Omega-3 fatty acids and inflammatory processes. Nutrients *2*, 355–374.
82. Hall, C., Tulbek, M.C., and Xu, Y.B.T.-A. in F. and N.R. (2006). Flaxseed. In (Academic Press), pp. 1–97.
83. Swanson, D., Block, R., and Mousa, S.A. (2012). Omega-3 fatty acids EPA and DHA: health benefits throughout life. Adv. Nutr. *3*, 1–7.
84. Zhuang, P., Zhang, Y., Shou, Q., Li, H., Zhu, Y., He, L., Chen, J., and Jiao, J. (2020). Eicosapentaenoic and Docosahexaenoic Acids Differentially Alter Gut Microbiome and Reverse High-Fat Diet–Induced Insulin Resistance. Mol. Nutr. Food Res. *64*, 1900946.
85. Zhu, X., Bi, Z., Yang, C., Guo, Y., Yuan, J., Li, L., and Guo, Y. (2021). Effects of different doses of omega-3 polyunsaturated fatty acids on gut microbiota and immunity. Food Nutr. Res. *65*, 10.29219/fnr.v65.6263.
86. Zhang, J., Yi, C., Han, J., Ming, T., Zhou, J., Lu, C., Li, Y., and Su, X. (2020). Novel high-docosahexaenoic-acid tuna oil supplementation modulates gut microbiota and alleviates obesity in high-fat diet mice. Food Sci. Nutr. *8*, 6513–6527.
87. Tung, T.-H., Tung, Y.-T., Lin, I.-H., Shih, C.-K., Nguyen, N.T.K., Shabrina, A., and Huang, S.-Y. (2019). Fish Oil, but Not Olive Oil, Ameliorates Depressive-Like Behavior and Gut Microbiota Dysbiosis in Rats under Chronic Mild Stress. Biomolecules *9*, 516.
88. Egerton, S., Donoso, F., Fitzgerald, P., Gite, S., Fouhy, F., Whooley, J., Dinan, T.G., Cryan, J.F., Culloty, S.C., Ross, R.P., et al. (2020). Investigating the potential of fish oil as a nutraceutical in an animal model of early life stress. Nutr. Neurosci., 1–23.
89. Lee, H.-C., Lo, Y.-C., Yu, S.-C., Tung, T.-H., Lin, I.-H., and Huang, S.-Y. (2019). Degree of lipid saturation affects depressive-like behaviour and gut microbiota in mice. Int. J. Food Sci. Nutr. *71*, 440–452.
90. Plourde, M., and Cunnane, S.C. (2007). Extremely limited synthesis of long chain polyunsaturates in adults: implications for their dietary essentiality and use as supplements. Appl. Physiol. Nutr. Metab. *32*, 619–634.
91. Goyens, P.L.L., Spilker, M.E., Zock, P.L., Katan, M.B., and Mensink, R.P. (2006). Conversion of α-linolenic acid in humans is influenced by the absolute amounts of α-linolenic acid and linoleic acid in the diet and not by their ratio. Am. J. Clin. Nutr. *84*, 44–53.
92. Goyens, P.L.L., Spilker, M.E., Zock, P.L., Katan, M.B., and Mensink, R.P. (2005). Compartmental modeling to quantify α-linolenic acid conversion after longer term intake of multiple tracer boluses. J. Lipid Res. *46*, 1474–1483.
93. Pawlosky, R.J., Hibbeln, J.R., Novotny, J.A., and Salem, N. (2001). Physiological compartmental analysis of α-linolenic acid metabolism in adult humans. J. Lipid Res. *42*,

1257–1265.
94. Taibi, A., Ku, M., Lin, Z., Gargari, G., Kubant, A., Lepp, D., Power, K.A., Guglielmetti, S., Thompson, L.U., and Comelli, E.M. (2021). Discriminatory and cooperative effects within the mouse gut microbiota in response to flaxseed and its oil and lignan components. J. Nutr. Biochem. *98*, 108818.
95. Millman, J., Okamoto, S., Kimura, A., Uema, T., Higa, M., Yonamine, M., Namba, T., Ogata, E., Yamazaki, S., and Shimabukuro, M. (2019). Metabolically and immunologically beneficial impact of extra virgin olive and flaxseed oils on composition of gut microbiota in mice. Eur. J. Nutr., 1–15.
96. Zhu, L., Sha, L., Li, K., Wang, Z., Wang, T., Li, Y., Liu, P., Dong, X., Dong, Y., Zhang, X., et al. (2020). Dietary flaxseed oil rich in omega-3 suppresses severity of type 2 diabetes mellitus via anti-inflammation and modulating gut microbiota in rats. Lipids Health Dis. *19*, 20.
97. Han, Y., Deng, X., Zhang, Y., Wang, X., Zhu, X., Mei, S., and Chen, A. (2020). Antidepressant-like effect of flaxseed in rats exposed to chronic unpredictable stress. Brain Behav. *10*, e01626.
98. El Tanbouly, N., El Sayed, A.M., Ali, Z.Y., Abdel Wahab, S., El Gayed, S.H., Ezzat, S.M., El Senousy, A.S., Choucry, M.A., and Abdel-Sattar, E. (2017). Antidepressant-like effect of selected Egyptian cultivars of flaxseed oil on a rodent model of postpartum depression. Evidence-Based Complement. Altern. Med. *2017*, 6405789.
99. Peng, Y., Shi, Z., Kumaran Satyanarayanan, S., He, C., Li, P., Wan, J.-B., and Su, H. (2020). Fish oil alleviates LPS-induced inflammation and depressive-like behavior in mice via restoration of metabolic impairments. Brain. Behav. Immun. *90*, 393–402.
100. Kajla, P., Sharma, A., and Sood, D.R. (2015). Flaxseed-a potential functional food source. J. Food Sci. Technol. *52*, 1857–1871.
101. Lattimer, J.M., and Haub, M.D. (2010). Effects of dietary fiber and its components on metabolic health. Nutrients *2*, 1266–1289.
102. Wang, M., Wichienchot, S., He, X., Fu, X., Huang, Q., and Zhang, B. (2019). In vitro colonic fermentation of dietary fibers: Fermentation rate, short-chain fatty acid production and changes in microbiota. Trends Food Sci. Technol. *88*, 1–9.
103. Oliphant, K., and Allen-Vercoe, E. (2019). Macronutrient metabolism by the human gut microbiome: major fermentation by-products and their impact on host health. Microbiome *7*, 91.
104. Silva, Y.P., Bernardi, A., and Frozza, R.L. (2020). The Role of Short-Chain Fatty Acids From Gut Microbiota in Gut-Brain Communication . Front. Endocrinol. *11*, 25.
105. Kim, M.H., Kang, S.G., Park, J.H., Yanagisawa, M., and Kim, C.H. (2013). Short-Chain Fatty Acids Activate GPR41 and GPR43 on Intestinal Epithelial Cells to Promote Inflammatory Responses in Mice. Gastroenterology *145*, 396-406.e10.
106. Arora, T., Rudenko, O., Egerod, K.L., Husted, A.S., Kovatcheva-Datchary, P., Akrami, R., Kristensen, M., Schwartz, T.W., and Bäckhed, F. (2018). Microbial fermentation of flaxseed fibers modulates the transcriptome of GPR41-expressing enteroendocrine cells and protects mice against diet-induced obesity. Am. J. Physiol. Metab. *316*, E453–E463.
107. Lin, B., Gong, J., Wang, Q., Cui, S., Yu, H., and Huang, B. (2011). In-vitro assessment of the effects of dietary fibers on microbial fermentation and communities from large intestinal digesta of pigs. Food Hydrocoll. *25*, 180–188.
108. Fodje, A.M.L., Chang, P.R., and Leterme, P. (2009). In vitro bile acid binding and short-

chain fatty acid profile of flax fiber and ethanol co-products. J. Med. Food *12*, 1065–1073.
109. Dalile, B., Van Oudenhove, L., Vervliet, B., and Verbeke, K. (2019). The role of short-chain fatty acids in microbiota–gut–brain communication. Nat. Rev. Gastroenterol. Hepatol. *16*, 461–478.
110. Vinolo, M.A.R., Rodrigues, H.G., Nachbar, R.T., and Curi, R. (2011). Regulation of inflammation by short chain fatty acids. Nutrients *3*, 858–876.
111. Corrêa-Oliveira, R., Fachi, J.L., Vieira, A., Sato, F.T., and Vinolo, M.A.R. (2016). Regulation of immune cell function by short-chain fatty acids. Clin. Transl. Immunol. *5*, e73.
112. Huuskonen, J., Suuronen, T., Nuutinen, T., Kyrylenko, S., and Salminen, A. (2004). Regulation of microglial inflammatory response by sodium butyrate and short-chain fatty acids. Br. J. Pharmacol. *141*, 874–880.
113. Matt, S.M., Allen, J.M., Lawson, M.A., Mailing, L.J., Woods, J.A., and Johnson, R.W. (2018). Butyrate and dietary soluble fiber improve neuroinflammation associated with aging in mice. Front. Immunol. *9*, 1832.
114. Amira, S., Senousy, E., Sabah, H., Gayed, E., Saber, H.A., Saeed, Z.A., Mohamad Louai M, H., Nebal, D., Tanbouly, E., and Abdel-Sattar, E. (2019). Optimization of linseed cultivation, a promising way to enhance its secoisolariciresinol diglucoside lignan content.
115. Badger, R., Aho, K., and Serve, K. (2021). Short-term exposure to synthetic flaxseed lignan LGM2605 alters gut microbiota in mice. Microbiologyopen *10*, e1185–e1185.
116. Setchell, K.D.R., Brown, N.M., Zimmer-Nechemias, L., Wolfe, B., Jha, P., and Heubi, J.E. (2014). Metabolism of secoisolariciresinol-diglycoside the dietary precursor to the intestinally derived lignan enterolactone in humans. Food Funct. *5*, 491–501.
117. Wang, L.-Q. (2002). Mammalian phytoestrogens: enterodiol and enterolactone. J. Chromatogr. B *777*, 289–309.
118. Ma, X., Wang, R., Zhao, X., Zhang, C., Sun, J., Li, J., Zhang, L., Shao, T., Ruan, L., Chen, L., et al. (2013). Antidepressant-like effect of flaxseed secoisolariciresinol diglycoside in ovariectomized mice subjected to unpredictable chronic stress. Metab. Brain Dis. *28*, 77–84.
119. Power, K.A., Lepp, D., Zarepoor, L., Monk, J.M., Wu, W., Tsao, R., and Liu, R. (2016). Dietary flaxseed modulates the colonic microenvironment in healthy C57Bl/6 male mice which may alter susceptibility to gut-associated diseases. J. Nutr. Biochem. *28*, 61–69.
120. Xu, Y., Wang, N., Tan, H.-Y., Li, S., Zhang, C., and Feng, Y. (2020). Function of Akkermansia muciniphila in Obesity: Interactions With Lipid Metabolism, Immune Response and Gut Systems . Front. Microbiol. *11*.
121. Corsini, E., Dell'Agli, M., Facchi, A., De Fabiani, E., Lucchi, L., Boraso, M.S., Marinovich, M., and Galli, C.L. (2010). Enterodiol and Enterolactone Modulate the Immune Response by Acting on Nuclear Factor-κB (NF-κB) Signaling. J. Agric. Food Chem. *58*, 6678–6684.
122. Painsipp, E., Köfer, M.J., Sinner, F., and Holzer, P. (2011). Prolonged Depression-Like Behavior Caused by Immune Challenge: Influence of Mouse Strain and Social Environment. PLoS One *6*, e20719.
123. O'Connor, J.C., Lawson, M.A., André, C., Moreau, M., Lestage, J., Castanon, N., Kelley, K.W., and Dantzer, R. (2009). Lipopolysaccharide-induced depressive-like behavior is mediated by indoleamine 2,3-dioxygenase activation in mice. Mol. Psychiatry *14*, 511–522.

124. O'neil, A., Quirk, S.E., Housden, S., Brennan, S.L., Williams, L.J., Pasco, J.A., Berk, M., and Jacka, F.N. (2014). Relationship between diet and mental health in children and adolescents: a systematic review. Am. J. Public Health *104*, e31–e42.
125. Pearson, C., Janz, T., and Ali, J. (2013). Mental and substance use disorders in Canada.
126. Bassi, G.S., Kanashiro, A., Santin, F.M., de Souza, G.E.P., Nobre, M.J., and Coimbra, N.C. (2012). Lipopolysaccharide-Induced Sickness Behaviour Evaluated in Different Models of Anxiety and Innate Fear in Rats. Basic Clin. Pharmacol. Toxicol. *110*, 359–369.
127. Kolmogorova, D., Murray, E., and Ismail, N. (2017). Monitoring pathogen-induced sickness in mice and rats. Curr. Protoc. Mouse Biol. *7*, 65–76.
128. Chlodzinska, N., Gajerska, M., Bartkowska, K., Turlejski, K., and Djavadian, R.L. (2011). Lipopolysaccharide injected to pregnant mice affects behavior of their offspring in adulthood. Acta Neurobiol. Exp. (Wars).
129. Gaskill, B.N., and Pritchett-Corning, K.R. (2016). Nest building as an indicator of illness in laboratory mice. Appl. Anim. Behav. Sci. *180*, 140–146.
130. Seibenhener, M.L., and Wooten, M.C. (2015). Use of the Open Field Maze to Measure Locomotor and Anxiety-like Behavior in Mice. JoVE, e52434.
131. Walf, A.A., and Frye, C.A. (2007). The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. Nat. Protoc. *2*, 322–328.
132. Hu, C., Luo, Y., Wang, H., Kuang, S., Liang, G., Yang, Y., Mai, S., and Yang, J. (2017). Re-evaluation of the interrelationships among the behavioral tests in rats exposed to chronic unpredictable mild stress. PLoS One *12*, e0185129–e0185129.
133. Can, A., Dao, D.T., Terrillion, C.E., Piantadosi, S.C., Bhat, S., and Gould, T.D. (2012). The tail suspension test. J. Vis. Exp., e3769–e3769.
134. Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., et al. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat. Biotechnol. *37*, 852–857.
135. Walker, A.K., Budac, D.P., Bisulco, S., Lee, A.W., Smith, R.A., Beenders, B., Kelley, K.W., and Dantzer, R. (2013). NMDA receptor blockade by ketamine abrogates lipopolysaccharide-induced depressive-like behavior in C57BL/6J mice. Neuropsychopharmacology *38*, 1609–1616.
136. Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., and Holmes, S.P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. Nat. Methods *13*, 581–583.
137. Sharma, R., Rooke, J., Kolmogorova, D., Melanson, B., Mallet, J.-F., Matar, C., Schwarz, J., and Ismail, N. (2018). Sex differences in the peripheral and central immune responses following lipopolysaccharide treatment in pubertal and adult CD-1 mice. Int. J. Dev. Neurosci. *71*, 94–104.
138. Price, M.N., Dehal, P.S., and Arkin, A.P. (2010). FastTree 2 – Approximately Maximum-Likelihood Trees for Large Alignments. PLoS One *5*, e9490.
139. Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W.S., and Huttenhower, C. (2011). Metagenomic biomarker discovery and explanation. Genome Biol. *12*, R60.
140. Ma, M., Ren, Q., Yang, C., Zhang, J., Yao, W., Dong, C., Ohgi, Y., Futamura, T., and Hashimoto, K. (2017). Antidepressant effects of combination of brexpiprazole and

fluoxetine on depression-like behavior and dendritic changes in mice after inflammation. Psychopharmacology (Berl). *234*, 525–533.
141. Katoh, K., and Standley, D.M. (2013). MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability. Mol. Biol. Evol. *30*, 772–780.
142. Shoji, H., Takao, K., Hattori, S., and Miyakawa, T. (2016). Age-related changes in behavior in C57BL/6J mice from young adulthood to middle age. Mol. Brain *9*, 11.
143. McDonald, D., Price, M.N., Goodrich, J., Nawrocki, E.P., DeSantis, T.Z., Probst, A., Andersen, G.L., Knight, R., and Hugenholtz, P. (2012). An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. ISME J. *6*, 610–618.
144. Grieco, F., Bernstein, B.J., Biemans, B., Bikovski, L., Burnett, C.J., Cushman, J.D., van Dam, E.A., Fry, S.A., Richmond-Hacham, B., Homberg, J.R., et al. (2021). Measuring Behavior in the Home Cage: Study Design, Applications, Challenges, and Perspectives . Front. Behav. Neurosci. *15*.
145. Pedregosa, F., Varoquaux, G., Gramfort, A., Michel, V., Thirion, B., Grisel, O., Blondel, M., Prettenhofer, P., Weiss, R., and Dubourg, V. (2011). Scikit-learn: Machine learning in Python. J. Mach. Learn. Res. *12*, 2825–2830.
146. Rosso, M., Wirz, R., Loretan, A.V., Sutter, N.A., Pereira da Cunha, C.T., Jaric, I., Würbel, H., and Voelkl, B. (2021). Reliability of Mouse Behavioural Tests of Anxiety: a Systematic Review and Meta-Analysis on the Effects of Anxiolytics.
147. Bokulich, N.A., Kaehler, B.D., Rideout, J.R., Dillon, M., Bolyen, E., Knight, R., Huttley, G.A., and Gregory Caporaso, J. (2018). Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. Microbiome *6*, 1–17.
148. Gulinello, M., Mitchell, H.A., Chang, Q., Timothy O'Brien, W., Zhou, Z., Abel, T., Wang, L., Corbin, J.G., Veeraragavan, S., Samaco, R.C., et al. (2019). Rigor and reproducibility in rodent behavioral research. Neurobiol. Learn. Mem. *165*, 106780.
149. Shannon, C.E. (1948). A mathematical theory of communication. Bell Syst. Tech. J. *27*, 379–423.
150. Faith, D.P. (1992). Conservation evaluation and phylogenetic diversity. Biol. Conserv. *61*, 1–10.
151. Tamanai-Shacoori, Z., Smida, I., Bousarghin, L., Loreal, O., Meuric, V., Fong, S.B., Bonnaure-Mallet, M., and Jolivet-Gougeon, A. (2017). Roseburia spp.: a marker of health? Future Microbiol. *12*, 157–170.
152. Bray, J.R., and Curtis, J.T. (1957). An ordination of the upland forest communities of southern Wisconsin. Ecol. Monogr. *27*, 326–349.
153. Anderson, M.J. (2014). Permutational multivariate analysis of variance (PERMANOVA). Wiley statsref Stat. Ref. online, 1–15.
154. Nie, K., Ma, K., Luo, W., Shen, Z., Yang, Z., Xiao, M., Tong, T., Yang, Y., and Wang, X. (2021). Roseburia intestinalis: A Beneficial Gut Organism From the Discoveries in Genus and Species . Front. Cell. Infect. Microbiol. *11*.
155. Gandy, K.A.O., Zhang, J., Nagarkatti, P., and Nagarkatti, M. (2019). The role of gut microbiota in shaping the relapse-remitting and chronic-progressive forms of multiple sclerosis in mouse models. Sci. Rep. *9*, 6923.
156. Ziętak, M., Kovatcheva-Datchary, P., Markiewicz, L.H., Ståhlman, M., Kozak, L.P., and Bäckhed, F. (2016). Altered Microbiota Contributes to Reduced Diet-Induced Obesity

upon Cold Exposure. Cell Metab. *23*, 1216–1223.
157. Hwang, N., Eom, T., Gupta, S.K., Jeong, S.-Y., Jeong, D.-Y., Kim, Y.S., Lee, J.-H., Sadowsky, M.J., and Unno, T. (2017). Genes and Gut Bacteria Involved in Luminal Butyrate Reduction Caused by Diet and Loperamide. Genes (Basel). *8*, 350.
158. Dantzer, R. (2009). Cytokine, Sickness Behavior, and Depression. Immunol. Allergy Clin. North Am. *29*, 247–264.
159. Bluthé, R.-M., Layé, S., Michaud, B., Combe, C., Dantzer, R., and Parnet, P. (2000). Role of interleukin-1β and tumour necrosis factor-α in lipopolysaccharide-induced sickness behaviour: a study with interleukin-1 type I receptor-deficient mice. Eur. J. Neurosci. *12*, 4447–4456.
160. Palin, K., McCusker, R.H., Strle, K., Moos, F., Dantzer, R., and Kelley, K.W. (2008). Tumor necrosis factor-alpha-induced sickness behavior is impaired by central administration of an inhibitor of c-jun N-terminal kinase. Psychopharmacology (Berl). *197*, 629–635.
161. Chapman, J., Bansal, P., Goyal, A., and Azevedo, A.M. (2017). Splenomegaly (StatPearls Publishing, Treasure Island (FL)).
162. Silverman, H.A., Dancho, M., Regnier-Golanov, A., Nasim, M., Ochani, M., Olofsson, P.S., Ahmed, M., Miller, E.J., Chavan, S.S., Golanov, E., et al. (2014). Brain Region-Specific Alterations in the Gene Expression of Cytokines, Immune Cell Markers and Cholinergic System Components during Peripheral Endotoxin-Induced Inflammation. Mol. Med. *20*, 601–611.
163. McCoy, M.K., and Tansey, M.G. (2008). TNF signaling inhibition in the CNS: implications for normal brain function and neurodegenerative disease. J. Neuroinflammation *5*, 1–13.
164. Lambertsen, K.L., Finsen, B., and Clausen, B.H. (2019). Post-stroke inflammation—target or tool for therapy? Acta Neuropathol. *137*, 693–714.
165. Cryan, J.F., and O'Mahony, S.M. (2011). The microbiome-gut-brain axis: from bowel to behavior. Neurogastroenterol. Motil. *23*, 187–192.
166. Jobson, D.D., Hase, Y., Clarkson, A.N., and Kalaria, R.N. (2021). The role of the medial prefrontal cortex in cognition, ageing and dementia. Brain Commun. *3*, fcab125.
167. Bittar, T.P., and Labonté, B. (2021). Functional contribution of the medial prefrontal circuitry in major depressive disorder and stress-induced depressive-like behaviors. Front. Behav. Neurosci., 128.
168. Jiao, X., Beck, K., Myers, C., Servatius, R., and Pang, K. (2015). Altered activity of the medial prefrontal cortex and amygdala during acquisition and extinction of an active avoidance task . Front. Behav. Neurosci. *9*.
169. Carrillo, W., Carpio, C., Morales, D., Vilcacundo, E., Álvarez, M., and Silva, M. (2017). Content of fatty acids in corn (Zea mays L.) oil from Ecuador. Asian J. Pharm. Clin. Res. *10*, 150–153.
170. Langille, M.G.I., Zaneveld, J., Caporaso, J.G., McDonald, D., Knights, D., Reyes, J.A., Clemente, J.C., Burkepile, D.E., Vega Thurber, R.L., Knight, R., et al. (2013). Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat. Biotechnol. *31*, 814–821.
171. Parks, D.H., Tyson, G.W., Hugenholtz, P., and Beiko, R.G. (2014). STAMP: statistical analysis of taxonomic and functional profiles. Bioinformatics *30*, 3123–3124.
172. Lagkouvardos, I., Lesker, T.R., Hitch, T.C.A., Gálvez, E.J.C., Smit, N., Neuhaus, K.,

Wang, J., Baines, J.F., Abt, B., Stecher, B., et al. (2019). Sequence and cultivation study of Muribaculaceae reveals novel species, host preference, and functional potential of this yet undescribed family. Microbiome *7*, 28.
173. Wong, M.-L., Inserra, A., Lewis, M.D., Mastronardi, C.A., Leong, L., Choo, J., Kentish, S., Xie, P., Morrison, M., Wesselingh, S.L., et al. (2016). Inflammasome signaling affects anxiety- and depressive-like behavior and gut microbiome composition. Mol. Psychiatry *21*, 797–805.
174. Tropini, C., Moss, E.L., Merrill, B.D., Ng, K.M., Higginbottom, S.K., Casavant, E.P., Gonzalez, C.G., Fremin, B., Bouley, D.M., and Elias, J.E. (2018). Transient osmotic perturbation causes long-term alteration to the gut microbiota. Cell *173*, 1742–1754.
175. Rooks, M.G., Veiga, P., Wardwell-Scott, L.H., Tickle, T., Segata, N., Michaud, M., Gallini, C.A., Beal, C., van Hylckama-Vlieg, J.E.T., and Ballal, S.A. (2014). Gut microbiome composition and function in experimental colitis during active disease and treatment-induced remission. ISME J. *8*, 1403–1417.
176. Vacca, M., Celano, G., Calabrese, F.M., Portincasa, P., Gobbetti, M., and De Angelis, M. (2020). The Controversial Role of Human Gut Lachnospiraceae. Microorg. *8*.
177. Jiang, H., Ling, Z., Zhang, Y., Mao, H., Ma, Z., Yin, Y., Wang, W., Tang, W., Tan, Z., Shi, J., et al. (2015). Altered fecal microbiota composition in patients with major depressive disorder. Brain. Behav. Immun. *48*, 186–194.
178. Zheng, P., Zeng, B., Zhou, C., Liu, M., Fang, Z., Xu, X., Zeng, L., Chen, J., Fan, S., and Du, X. (2016). Gut microbiome remodeling induces depressive-like behaviors through a pathway mediated by the host's metabolism. Mol. Psychiatry *21*, 786–796.
179. Schirmer, M., Smeekens, S.P., Vlamakis, H., Jaeger, M., Oosting, M., Franzosa, E.A., Ter Horst, R., Jansen, T., Jacobs, L., and Bonder, M.J. (2016). Joosten LAB, Zhernakova A, Huttenhower C, Wijmenga C, Netea MG, Xavier RJ. 2016. Linking the human gut microbiome to inflammatory cytokine production capacity. Cell *167*.
180. Shahi, S.K., Freedman, S.N., and Mangalam, A.K. (2017). Gut microbiome in multiple sclerosis: The players involved and the roles they play. Gut Microbes *8*, 607–615.
181. Huang, T.-T., Lai, J.-B., Du, Y.-L., Xu, Y., Ruan, L.-M., and Hu, S.-H. (2019). Current Understanding of Gut Microbiota in Mood Disorders: An Update of Human Studies . Front. Genet. *10*.
182. Cheng, L.-H., Liu, Y.-W., Wu, C.-C., Wang, S., and Tsai, Y.-C. (2019). Psychobiotics in mental health, neurodegenerative and neurodevelopmental disorders. J. Food Drug Anal. *27*, 632–648.
183. Kanehisa, M., and Goto, S. (2000). KEGG: Kyoto Encyclopedia of Genes and Genomes. Nucleic Acids Res. *28*, 27–30.
184. Yang, C., Fujita, Y., Ren, Q., Ma, M., Dong, C., and Hashimoto, K. (2017). Bifidobacterium in the gut microbiota confer resilience to chronic social defeat stress in mice. Sci. Rep. *7*, 45942.
185. Gorissen, L., Raes, K., Weckx, S., Dannenberger, D., Leroy, F., De Vuyst, L., and De Smet, S. (2010). Production of conjugated linoleic acid and conjugated linolenic acid isomers by Bifidobacterium species. Appl. Microbiol. Biotechnol. *87*, 2257–2266.
186. Gresse, R., Chaucheyras-Durand, F., Fleury, M.A., Van de Wiele, T., Forano, E., and Blanquet-Diot, S. (2017). Gut Microbiota Dysbiosis in Postweaning Piglets: Understanding the Keys to Health. Trends Microbiol. *25*, 851–873.
187. Lingappan, K. (2018). NF-κB in oxidative stress. Curr. Opin. Toxicol. *7*, 81–86.

188. Badshah, H., Ikram, M., Ali, W., Ahmad, S., Hahm, J.R., and Kim, M.O. (2019). Caffeine May Abrogate LPS-Induced Oxidative Stress and Neuroinflammation by Regulating Nrf2/TLR4 in Adult Mouse Brains. Biomol. *9*.
189. Cai, K.C., van Mil, S., Murray, E., Mallet, J.-F., Matar, C., and Ismail, N. (2016). Age and sex differences in immune response following LPS treatment in mice. Brain. Behav. Immun. *58*, 327–337.
190. Bao, A.-M., Meynen, G., and Swaab, D.F. (2008). The stress system in depression and neurodegeneration: focus on the human hypothalamus. Brain Res. Rev. *57*, 531–553.
191. Van Eijndhoven, P., van Wingen, G., van Oijen, K., Rijpkema, M., Goraj, B., Verkes, R.J., Voshaar, R.O., Fernández, G., Buitelaar, J., and Tendolkar, I. (2009). Amygdala volume marks the acute state in the early course of depression. Biol. Psychiatry *65*, 812–818.

Appendix

Table S1. Nest building scoring criteria. Highest possible score=6.5

Criteria	Scoring details

Shredding	• 0 = nestlets are unshredded
	• 0.5 = 1-25% shredded. 1 full nestlet square is left unshredded & 1 nestlet has been partially shredded, but is still less than half-shredded.
	• 1 = 26-50% shredded. One nestlet square remains unshredded. Other square is half- to fully-shredded.
	• 1.5 = 51-75% - Both nestlets have been shredded/touched (at least a little). Sum of the chunks are approximately ¾ to ½ of one nestlet square
	• 2 = 76-95% >1 pieces of nestlet material noticeable
	  o Chunky texture
	• 2.5 = >/= 96% - at most one small piece of unshredded material
	  o Fluffy texture

Shape	• 0 = Scattered
	  o Pieces of nest material are not all connected
	  o Very dispersed within the cage
	  o Scores of 0-1 for nest shredding
	• 0.5 = Somewhat defined shape
	  o Most of the material connects
	  o Both of the following:
	  ▪ > 2 edges are sticking out
	  ▪ Very dispersed within the cage
	1 = Mostly defined
	• All of the material connects
	• Mostly rounded shape
	• One of the following:
	  ▪ > 2 edges are sticking out
	  ▪ Very dispersed within the cage
	1.5 = Defined shape
	• Round shape
	• Material is gathered *tightly* to ensure snug fit around the mouse

Location	• 0 = Front: touching front wall • 0.5 = Middle • 1 = Back: touching back wall
Nest walls	• 0 = < 25% • 0.5 = 25-49% • 1 = 50-75% • 1.5 = > 75%

Table S2: Complete spearman correlation matrix from Study 1 (Figure 4-9)
Link

Table S3: Complete fatty acid content of FO

Common Name	Systematic Name	Lipid numbers	Fatty acid content	
			%	mg/g
Butyric acid	Butanoic acid	**C4:0**	0.00	0.00
Caproic acid	Hexanoic acid	**C6:0**	0.00	0.00
Caprylic acid	Octanoic acid	**C8:0**	0.47	4.52
Capric acid	Decanoic acid	**C10:0**	0.01	0.06
Lauric acid	Dodecanoic acid	**C12:0**	0.01	0.14
Myristic acid	Tetradecanoic acid	**C14:0**	0.19	1.78
Myristoleic acid	Tetradecenoic acid	**C14:1**	0.00	0.00
Pentadecylic acid	Pentadecanoic acid	**C15:0**	0.01	0.13
Palmitic acid	Hexadecanoic acid	**C16:0**	5.46	52.63
Palmitoleic acid	Hexadecenoic acid	**C16:1**	0.07	0.63
Stearic acid	Octadecanoic acid	**C18:0**	3.98	38.30
Oleic acid	Octadecenoic acid	**C18:1 (C9)**	16.64	160.30
Vaccenic acid	Octadecenoic acid	**C18:1 (C11)**	0.83	7.99
Linoleic acid	Octadecadienoic	**C18:2N6**	15.97	153.82
γ-Linolenic acid	Octadecatrienoic acid	**C18:3N6**	0.00	0.00
α-linolenic acid (ALA)	Octadecatrienoic acid	**C18:3N3**	55.03	530.17
Stearidonic acid	Octadecatetraenoic acid	**C18:4N3**	0.00	0.00
Arachidic acid	Icosanoic acid	**C20:0**	0.16	1.51
Gadoleic acid	Eicosenoic acid	**C20:1 (C7)**	0.04	0.39
Gondoic acid	Eicosenoic acid	**C20:1 (C9)**	0.14	1.36
Paullinic acid	Eicosenoic acid	**C20:1 (C11)**	0.02	0.19
Eicosadienoic acid	Eicosadienoic acid	**C20:2N6**	0.08	0.75
Dihomo-γ-linolenic acid (DGLA)	Eicosatrienoic acid	**C20:3N6**	0.00	0.00
Arachidonic acid	Eicosatetraenoic acid	**C20:4N6**	0.00	0.00
ETrA	Eicosatrienoic acid	**C20:3N3**	0.13	1.22
ETA	Eicosatetraenoic acid	**C20:4N3**	0.34	3.26
EPA	Eicosapentaenoic acid	**C20:5N3**	0.03	0.32
Behenic acid	Docosanoic acid	**C22:0**	0.14	1.36
Erucic acid	Docosenoic acid	**C22:1**	0.06	0.61
Docosadienoate	Docosadienoic acid	**C22:2N6**	0.00	0.00
Adrenic acid	Docosatetraenoic acid	**C22:4N6**	0.11	1.08
Osbond acid	Docosapentaenoic acid	**C22:5N6**	0.00	0.00
Clupanodonic acid	Docosapentaenoic acid	**C22:5N3**	0.00	0.00
DHA	Docosahexaenoic acid	**C22:6N3**	0.09	0.84
Lignoceric acid	Tetracosanoic acid	**C24:0**	0.00	0.00

Table S4. The relative abundance of the cecal microbiota 24-hours post-injection

Taxonomy	Relative abundance (% ±SEM)											
	BD-SAL		BD-LPS		FS-SAL		FS-LPS		FO-SAL		FO-LPS	
p__Firmicutes	41.125	± 1.295	40.755	± 2.749	47.798	± 2.222	43.857	± 3.14	40.229	± 1.496	34.291	± 2.031
f__Ruminococcaceae	7.681	± 0.287	9.299	± 0.871	7.286	± 0.761	10.01	± 0.784	7.584	± 0.582	9.587	± 0.903
f__Lachnospiraceae	6.658	± 0.77	4.277	± 0.542	10.649	± 1.344	7.87	± 1.337	5.249	± 0.691	2.568	± 0.444
f__Ruminococcaceae; g__Clostridium	3.624	± 0.457	4.08	± 0.613	2.691	± 0.367	3.052	± 0.403	4.409	± 0.427	3.639	± 0.407
g__Sporobacter	2.873	± 0.662	2.49	± 0.502	2.043	± 0.296	3.151	± 0.484	1.478	± 0.369	1.601	± 0.418
f__Lachnospiraceae; g__Clostridium	2.028	± 0.314	1.541	± 0.263	4.761	± 0.44	3.428	± 0.358	1.788	± 0.141	0.779	± 0.102
o__Clostridiales	13.65	± 1.129	14.898	± 1.556	16.73	± 1.164	13.365	± 1.888	14.96	± 0.761	12.751	± 1.697
g__rc4-4	1.003	± 0.167	0.788	± 0.104	0.918	± 0.254	0.772	± 0.143	1.475	± 0.243	0.826	± 0.198
g__Dorea	0.794	± 0.103	0.708	± 0.069	0.36	± 0.043	0.249	± 0.031	0.821	± 0.061	0.439	± 0.057
g__Oscillospira	0.743	± 0.143	0.878	± 0.114	0.377	± 0.042	0.971	± 0.257	0.797	± 0.107	0.853	± 0.102
g__Defluviitalea	0.641	± 0.081	0.753	± 0.132	0.362	± 0.056	0.522	± 0.057	0.607	± 0.103	0.638	± 0.105
f__Peptostreptococcaceae	0.471	± 0.246	0.347	± 0.223	0.062	± 0.048	0.012	± 0.008	0.305	± 0.245	0	± 0
f__Clostridiaceae; g__Clostridium	0.237	± 0.06	0.183	± 0.054	0.122	± 0.035	0.053	± 0.019	0.16	± 0.053	0.077	± 0.02
f__Bacillaceae	0.223	± 0.079	0.037	± 0.017	0.789	± 0.208	0.054	± 0.017	0.083	± 0.037	0.049	± 0.024
f__Streptococcaceae	0.216	± 0.041	0.101	± 0.028	0.173	± 0.03	0.068	± 0.017	0.212	± 0.038	0.082	± 0.014
g__Ruminococcus	0	± 0	0	± 0	0.256	± 0.121	0.099	± 0.04	0	± 0	0	± 0
p__Bacteroidetes	44.771	± 1.477	39.898	± 2.087	46.964	± 2.052	41.211	± 2.386	45.777	± 1.333	44.891	± 2.028
g__Bacteroides	7.037	± 0.533	10.943	± 1.007	8.148	± 0.912	11.477	± 0.975	7.278	± 1.008	14.041	± 1.079
g__Parabacteroides	6.734	± 0.691	6.783	± 0.637	3.716	± 0.478	6.725	± 0.784	8.705	± 1.239	10.251	± 0.893
f__S24-7	30.999	± 0.838	22.172	± 1.324	35.101	± 1.409	23.01	± 1.615	29.794	± 1.584	20.6	± 0.881
p__Tenericutes	0.859	± 0.393	0.568	± 0.314	0.09	± 0.047	0.109	± 0.062	0.731	± 0.233	0.197	± 0.08
g__Anaeroplasma	0.859	± 0.393	0.568	± 0.314	0.09	± 0.047	0.109	± 0.062	0.731	± 0.233	0.197	± 0.08
p__Actinobacteria	0.005	± 0.005	0	± 0	0.299	± 0.197	0.001	± 0.001	0.043	± 0.043	0	± 0
g__Bifidobacterium	0.005	± 0.005	0	± 0	0.299	± 0.197	0.001	± 0.001	0.043	± 0.043	0	± 0

p__Proteobacteria	1.143 ± 0.841	±	2.521 ± 1.794	±	0.162 ± 0.048	±	1.562 ± 0.349	±	0.495 ± 0.321	±	1.391 ± 0.504	±
o__RF32	1.076 ± 0.837	±	0.353 ± 0.316	±	0.056 ± 0.032	±	0.02 ± 0.014	±	0.4 ± 0.313	±	0.092 ± 0.084	±
f__Enterobacteriaceae	0.067 ± 0.024	±	2.168 ± 1.791	±	0.105 ± 0.025	±	1.542 ± 0.351	±	0.095 ± 0.022	±	1.299 ± 0.516	±
p__Verrucomicrobia	9.202 ± 2.027	±	12.874 ± 1.877	±	3.35 ± 0.718	±	11.596 ± 2.371	±	9.551 ± 1.122	±	15.97 ± 2.365	±
g__Akkermansia	9.202 ± 2.027	±	12.874 ± 1.877	±	3.35 ± 0.718	±	11.596 ± 2.371	±	9.551 ± 1.122	±	15.97 ± 2.365	±
p__Deferribacteres	2.863 ± 0.438	±	3.371 ± 0.442	±	1.27 ± 0.12	±	1.664 ± 0.187	±	2.983 ± 0.419	±	3.252 ± 0.373	±
g__Mucispirillum	2.863 ± 0.438	±	3.371 ± 0.442	±	1.27 ± 0.12	±	1.664 ± 0.187	±	2.983 ± 0.419	±	3.252 ± 0.373	±

Mean relative abundance (% ± SEM) of taxonomic groups in the fecal microbiota pre-injection at the Phylum level (grey), or the order, family, or genus level (white) with mean abundance greater than 0.2% in any group (n=12/group).

Table S5. The relative abundance of the fecal microbiota before and after LPS injection

Taxonomy	Relative abundance (% ± SEM)					
	BD-pre-LPS	BD-post-LPS	FS-pre-LPS	FS-post-LPS	FO-pre-LPS	FO-post-LPS
p__Firmicutes	27.661 ± 1.963	22.983 ± 1.942	39.540 ± 1.838	32.032 ± 2.164	28.977 ± 2.016	24.517 ± 1.927
f__Ruminococcaceae;__	5.346 ± 0.702	5.362 ± 0.495	7.2 ± 0.749	8.034 ± 0.768	6.223 ± 0.755	6.688 ± 0.746
f__Lachnospiraceae;__	4.78 ± 0.732	2.537 ± 0.405	9.325 ± 1.05	5.656 ± 0.708	3.97 ± 0.761	2.287 ± 0.415
o__Clostridiales	6.365 ± 0.691	6.942 ± 0.644	8.586 ± 1.219	7.558 ± 0.853	7.242 ± 0.727	7.347 ± 0.682
g__rc4-4	2.353 ± 0.292	2.083 ± 0.312	1.349 ± 0.296	0.99 ± 0.199	2.717 ± 0.254	1.566 ± 0.282
g__Clostridium (f__Lachnospiraceae)	1.186 ± 0.141	0.753 ± 0.113	3.307 ± 0.259	3.075 ± 0.37	1.21 ± 0.204	0.56 ± 0.081
g__Sporobacter	1.423 ± 0.495	1.115 ± 0.385	1.422 ± 0.458	1.381 ± 0.24	0.856 ± 0.152	0.99 ± 0.387
g__Clostridium (f__Ruminococcaceae)	1.517 ± 0.216	1.42 ± 0.303	2.433 ± 0.227	2.273 ± 0.234	1.996 ± 0.343	1.723 ± 0.27
g__Clostridium (f__Peptostreptococcaceae)	0.363 ± 0.116	0.082 ± 0.03	0.03 ± 0.015	0.027 ± 0.015	0.228 ± 0.113	0.035 ± 0.01
g__Lactobacillus	0.671 ± 0.338	0.28 ± 0.096	0.498 ± 0.418	0.116 ± 0.026	0.38 ± 0.174	0.314 ± 0.122
f__Bacillaceae;__	0.475 ± 0.211	0.107 ± 0.082	2.756 ± 0.738	0.522 ± 0.169	0.876 ± 0.319	0.068 ± 0.028
g__Dorea	0.392 ± 0.053	0.363 ± 0.043	0.163 ± 0.024	0.287 ± 0.055	0.51 ± 0.068	0.411 ± 0.069
g__Clostridium (f__Clostridiaceae)	1.137 ± 0.19	0.298 ± 0.094	0.571 ± 0.12	0.107 ± 0.049	0.765 ± 0.219	0.118 ± 0.036
g__Oscillospira	0.369 ± 0.076	0.405 ± 0.122	0.387 ± 0.094	0.31 ± 0.031	0.413 ± 0.11	0.539 ± 0.105

g__Defluviitalea	0.294 ± 0.045	0.203 ± 0.024	0.191 ± 0.041	0.249 ± 0.048	0.375 ± 0.045	0.387 ± 0.089
o__Lactobacillales	0.203 ± 0.022	0.127 ± 0.026	0.141 ± 0.019	0.091 ± 0.018	0.223 ± 0.031	0.106 ± 0.012
g__Allobaculum	0.186 ± 0.103	0.027 ± 0.016	0.424 ± 0.13	0.14 ± 0.053	0.226 ± 0.095	0.089 ± 0.063
f_Peptostreptococcaceae	0.094 ± 0.094	0.072 ± 0.064	0.039 ± 0.032	0.002 ± 0.002	0 ± 0	0 ± 0
g__Clostridium (f__Erysipelotrichaceae)	0.1 ± 0.022	0.21 ± 0.072	0.076 ± 0.026	0.374 ± 0.144	0.136 ± 0.031	0.338 ± 0.065
g__Ruminococcus (f__Lachnospiraceae)	0.083 ± 0.017	0.119 ± 0.054	0.28 ± 0.051	0.352 ± 0.068	0.11 ± 0.015	0.217 ± 0.066
g__Papillibacter	0.034 ± 0.01	0.116 ± 0.056	0.065 ± 0.013	0.109 ± 0.03	0.162 ± 0.085	0.21 ± 0.044
g__Anaerotruncus	0.03 ± 0.009	0.057 ± 0.015	0.019 ± 0.007	0.062 ± 0.03	0.036 ± 0.009	0.112 ± 0.033
p__Bacteroidetes	48.878 ± 1.958	51.488 ± 1.955	50.256 ± 1.694	43.421 ± 2.289	53.851 ± 1.765	50.558 ± 3.234
f__S24-7;g__	33.658 ± 1.434	31.208 ± 1.297	35.958 ± 1.326	29.822 ± 1.763	36.607 ± 2.153	26.647 ± 1.23
g__Bacteroides	9.593 ± 1.018	14.354 ± 1.375	9.907 ± 1.254	9.47 ± 0.933	10.506 ± 1.063	15.302 ± 1.848
g__Parabacteroides	5.627 ± 0.555	5.926 ± 0.537	4.391 ± 0.758	4.128 ± 0.454	6.737 ± 0.495	8.609 ± 0.975
p__Deferribacteres	0.512 ± 0.127	0.613 ± 0.127	0.359 ± 0.173	0.475 ± 0.178	0.412 ± 0.121	0.382 ± 0.099
g__Mucispirillum	0.512 ± 0.127	0.613 ± 0.127	0.359 ± 0.173	0.475 ± 0.178	0.412 ± 0.121	0.382 ± 0.099
p__Tenericutes	0.481 ± 0.165	0.326 ± 0.193	0.146 ± 0.083	0.073 ± 0.06	0.465 ± 0.191	0.062 ± 0.03
g__Anaeroplasma	0.481 ± 0.165	0.326 ± 0.193	0.146 ± 0.083	0.073 ± 0.06	0.465 ± 0.191	0.062 ± 0.03
p__Verrucomicrobia	19.786 ± 1.503	21.225 ± 1.745	7.891 ± 1.571	16.579 ± 2.01	15.82 ± 2.000	21.164 ± 2.663
g__Akkermansia	19.786 ± 1.503	21.225 ± 1.745	7.891 ± 1.571	16.579 ± 2.01	15.82 ± 2.000	21.164 ± 2.663
p__Actinobacteria	0.066 ± 0.014	0.042 ± 0.012	0.817 ± 0.370	0.198 ± 0.043	0.068 ± 0.018	0.123 ± 0.03
g__Bifidobacterium	0 ± 0	0 ± 0	0.545 ± 0.363	0.043 ± 0.038	0.001 ± 0.001	0 ± 0
p__Proteobacteria	2.610 ± 1.264	3.313 ± 1.868	0.833 ± 0.256	7.113 ± 2.152	0.346 ± 0.221	3.191 ± 1.243
f__Enterobacteriaceae	0.105 ± 0.035	3.148 ± 1.879	0.206 ± 0.047	7.071 ± 2.153	0.177 ± 0.086	3.124 ± 1.256
o__RF32	2.505 ± 1.243	0.165 ± 0.157	0.580 ± 0.240	0.012 ± 0.008	0.168 ± 0.138	0.067 ± 0.065

Mean relative abundance (% ± SEM) of taxonomic groups in the fecal microbiota pre and post LPS injection at the Phylum level (grey), or the class, order, family, or genus level (white) with mean abundance greater than 0.2% in any group (n=12/group).

Table S6. Effects of SAL injection on the fecal microbiota

Taxonomy	Relative abundance (% ± SEM)					
	BD+SAL-before	BD+SAL-after	FS+SAL-before	FS+SAL-after	FO+SAL-before	FO-SAL-after
p__Bacteroidetes	53.159 ± 1.978	53.957 ± 1.76	50.301 ± 2.378	52.233 ± 1.727	52.265 ± 0.983	52.881 ± 1.381
f__S24-7;g__	36.718 ± 1.681	37.901 ± 1.641	37.746 ± 1.132	40.949 ± 1.65	37.081 ± 1.891	38.244 ± 2.141
g__Bacteroides	10.245 ± 1.019	9.699 ± 0.98	9.426 ± 1.201	8.372 ± 1.076	8.778 ± 0.913	8.414 ± 1.102
g__Parabacteroides	6.196 ± 0.636	6.357 ± 0.632	3.129 ± 0.415	2.912 ± 0.443	6.406 ± 1.081	6.223 ± 0.845
p__Firmicutes	31.52 ± 1.956	29.225 ± 1.679	35.872 ± 1.577	38.455 ± 1.851	24.31 ± 1.656	25.865 ± 1.414
f__Lachnospiraceae;__	5.749 ± 0.998	5.569 ± 0.881	7.175 ± 0.544	8.868 ± 0.757	3.876 ± 0.599	3.407 ± 0.456
f__Ruminococcaceae;__	5.994 ± 0.473	5.674 ± 0.447	6.027 ± 0.68	6.13 ± 0.459	4.557 ± 0.477	5.597 ± 0.348
o__Clostridiales; f__	6.668 ± 0.707	7.606 ± 0.869	8.153 ± 0.678	8.377 ± 0.663	6.167 ± 0.426	7.761 ± 0.841
g__rc4-4	3.228 ± 0.623	2.334 ± 0.482	1.375 ± 0.303	1.075 ± 0.209	2.928 ± 0.289	2.383 ± 0.243
g__Clostridium (f__Lachnospiraceae)	1.347 ± 0.257	1.383 ± 0.207	2.976 ± 0.302	4.002 ± 0.396	0.834 ± 0.097	1.07 ± 0.126
g__Sporobacter	1.449 ± 0.326	1.659 ± 0.309	1.159 ± 0.216	1.779 ± 0.399	0.994 ± 0.23	1.07 ± 0.224
g__Clostridium (f__Ruminococcaceae)	1.976 ± 0.349	1.695 ± 0.183	2.311 ± 0.241	2.824 ± 0.3	1.405 ± 0.169	1.615 ± 0.074
f__Bacillaceae;__	1.06 ± 0.33	0.32 ± 0.108	3.913 ± 0.802	2.735 ± 0.564	0.536 ± 0.265	0.195 ± 0.095
g__Clostridium (f__Clostridiaceae)	0.888 ± 0.235	0.224 ± 0.068	0.535 ± 0.193	0.305 ± 0.115	0.667 ± 0.229	0.334 ± 0.117
g__Oscillospira	0.716 ± 0.174	0.505 ± 0.058	0.357 ± 0.104	0.338 ± 0.071	0.349 ± 0.051	0.493 ± 0.068
g__Defluviitalea	0.467 ± 0.074	0.36 ± 0.036	0.178 ± 0.023	0.271 ± 0.046	0.223 ± 0.032	0.319 ± 0.045
g__Dorea	0.41 ± 0.073	0.384 ± 0.049	0.208 ± 0.027	0.274 ± 0.043	0.261 ± 0.021	0.348 ± 0.043
g__Lactobacillus	0.327 ± 0.109	0.44 ± 0.247	0.311 ± 0.133	0.176 ± 0.047	0.363 ± 0.137	0.283 ± 0.116
o__Lactobacillales; f__	0.188 ± 0.025	0.231 ± 0.046	0.17 ± 0.035	0.219 ± 0.024	0.208 ± 0.032	0.211 ± 0.017
p__Actinobacteria	0.082 ± 0.042	0.033 ± 0.006	1.139 ± 0.519	0.53 ± 0.112	0.46 ± 0.42	0.252 ± 0.178
g__Bifidobacterium	0.036 ± 0.036	0.004 ± 0.004	0.74 ± 0.505	0.214 ± 0.108	0.414 ± 0.414	0.175 ± 0.175
g__Coriobacteriaceae	0.002 ± 0.002	0 ± 0	0.252 ± 0.049	0.21 ± 0.049	0.001 ± 0.001	0.002 ± 0.001

p__Deferribacteres	0.443 ± 0.163	0.475 ± 0.154	0.239 ± 0.067	0.21 ± 0.048	0.202 ± 0.04	0.149 ± 0.025
g__Mucispirillum	0.443 ± 0.163	0.475 ± 0.154	0.239 ± 0.067	0.21 ± 0.048	0.202 ± 0.04	0.149 ± 0.025
p__Proteobacteria	1.052 ± 0.688	1.815 ± 1.317	1.214 ± 0.752	0.743 ± 0.242	1.424 ± 0.871	0.782 ± 0.282
f__Enterobacteriaceae	0.071 ± 0.019	0.4 ± 0.11	0.203 ± 0.091	0.603 ± 0.195	0.137 ± 0.027	0.429 ± 0.136
o__RF32 ;f__	0.981 ± 0.697	1.415 ± 1.274	0.931 ± 0.758	0.039 ± 0.031	1.287 ± 0.88	0.353 ± 0.248
p__Tenericutes	0.736 ± 0.222	0.395 ± 0.147	0.149 ± 0.105	0.058 ± 0.03	0.415 ± 0.138	0.269 ± 0.09
g__Anaeroplasma	0.736 ± 0.222	0.395 ± 0.147	0.149 ± 0.105	0.058 ± 0.03	0.415 ± 0.138	0.269 ± 0.09
p__Verrucomicrobia	12.975 ± 2.452	13.974 ± 2.587	10.147 ± 1.874	7.368 ± 1.466	20.857 ± 1.126	19.603 ± 1.414
g__Akkermansia	12.975 ± 2.452	13.974 ± 2.587	10.147 ± 1.874	7.368 ± 1.466	20.857 ± 1.126	19.603 ± 1.414
p__Cyanobacteria	0.033 ± 0.031	0.126 ± 0.126	0.938 ± 0.535	0.403 ± 0.087	0.066 ± 0.065	0.2 ± 0.199
o__Streptophyta	0 ± 0	0 ± 0	0.271 ± 0.096	0.31 ± 0.075	0 ± 0	0 ± 0
o__YS2	0.033 ± 0.031	0.126 ± 0.126	0.667 ± 0.493	0.093 ± 0.076	0.066 ± 0.065	0.2 ± 0.199

Mean relative abundance (% ± SEM) of taxonomic groups in the fecal microbiota pre and post SAL injection at the Phylum level (grey), or the order, family, or genus level (white) with mean abundance greater than 0.2% in any group (n=12/group).

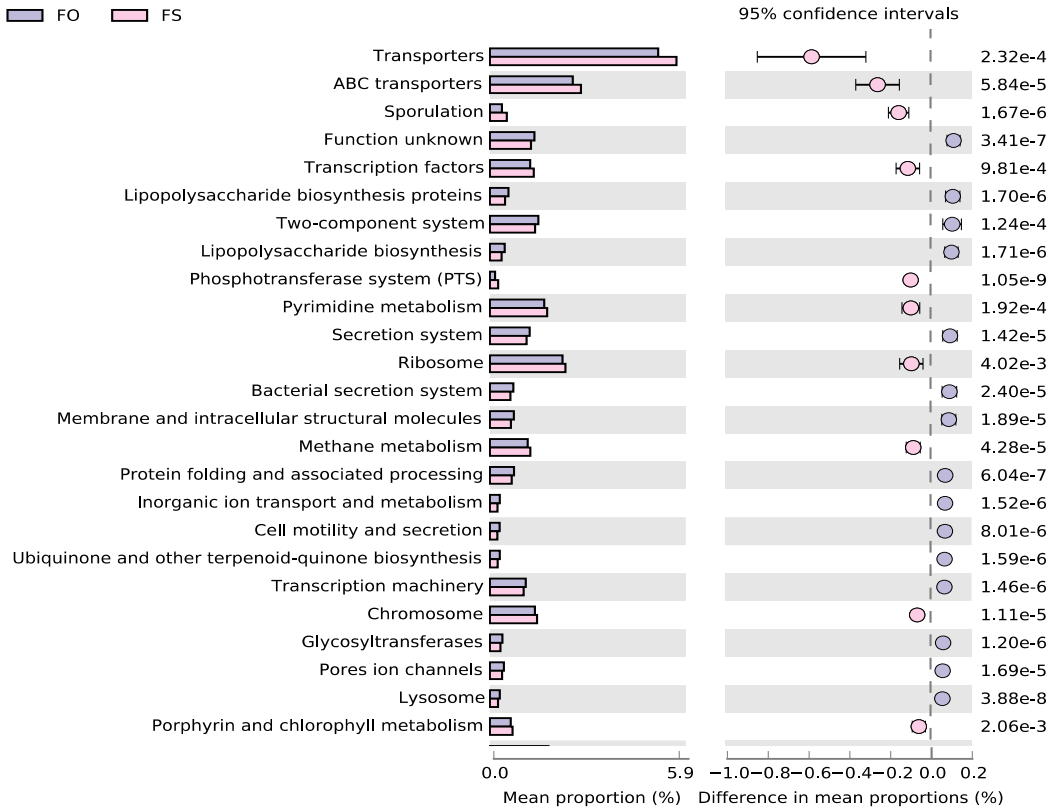

Figure S1. The inferred function of the fecal microbiota before injection comparing FS and FO.
The inferred function was analyzed in STAMP using Welch's two-sided t-test with Benjamini-Hochberg multiple test correction (q<0.1)

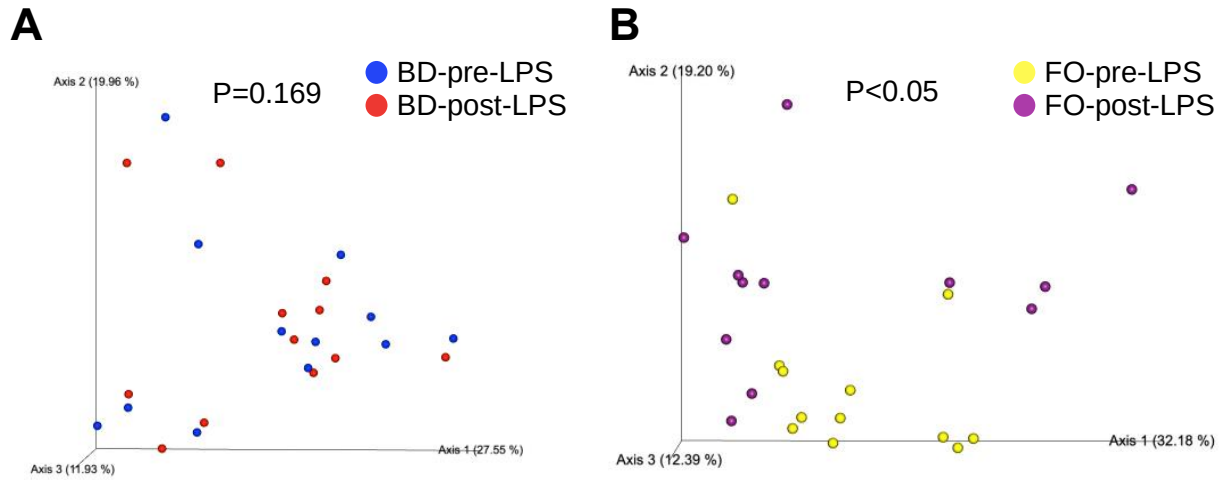

Figure S2. The effects of LPS injection on BD and FO Bray-Curtis β-diversity
A. BD pre vs. post LPS injection. **B.** FO pre vs post LPS injection. β-diversity was visualized by PCoA and analyzed by PERMANOVA (n=12/group).

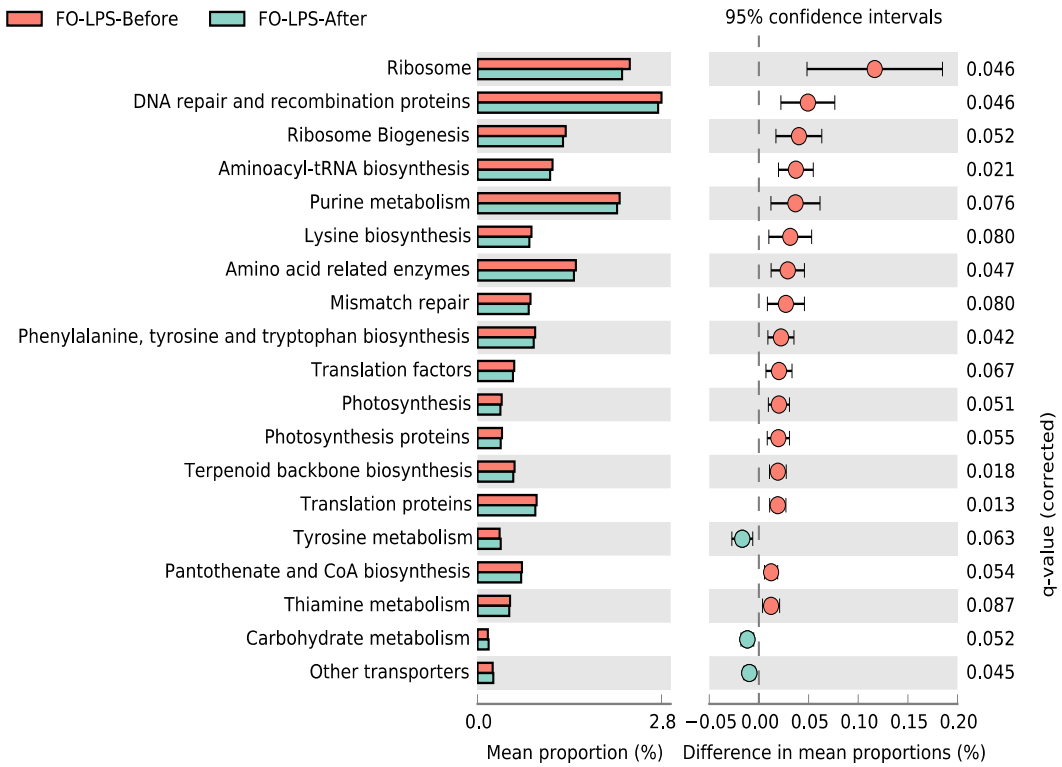

Figure S3. The inferred function of the fecal microbiota pre vs. post LPS in FO-fed mice
The inferred function was analyzed in STAMP using Welch's two-sided t-test with Benjamini-Hochberg multiple test correction ($q<0.1$).