Mom's diet matters: Maternal prebiotic intake in mice reduces anxiety and alters brain gene expression and the fecal microbiome in offspring.

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Abstract

Compelling evidence links enteric microbes to brain function and behavior. Galacto-oligosaccharide prebiotics have been shown to modulate the composition of gut flora and induce metabolic, neurochemical, and behavioral changes in adult rodents. Despite the brain being most susceptible to environmental factors, such as nutrients and toxins, during the earliest stages of development, it is unknown whether maternal prebiotic supplementation during gestation and lactation influences the offspring gut microbiome, brain, or behavior. The aim of this study was to test whether maternal galacto-oligosaccharide intake during pregnancy and lactation alters the brain and behavior in naïve and endotoxin-challenged offspring.

CD1 female mice received either normal drinking water or water supplemented with Bimuno[®] galacto-oligosaccharides (B-GOS) during gestation and suckling. Offspring behavior was tested at weaning age or adulthood, and a cross-foster design was employed in a separate cohort to differentiate between effects of prenatal and postnatal maternal B-GOS intake. Lipopolysaccharide was also administered to pups at postnatal day 9 to determine whether maternal B-GOS influences the neurobiological and behavioral effects of a neonatal pro-inflammatory challenge in adulthood. Fecal microbiome composition and metabolites were analyzed to explore potential relationships between the maternal microbiome, the offspring gut microbiome, and the offspring brain and behavior.

Maternal B-GOS supplementation increased exploratory behavior and reduced expression of hippocampal glutamate receptor genes in young, weaning-age offspring. In addition, postnatal, but not prenatal, B-GOS supplementation increased fecal butyrate and propionate levels. Finally, in adult offspring, perinatal B-GOS intake increased cortical glutamate receptor subunits in females, increased social preference, and reduced anxiety. We provide novel and comprehensive

evidence for the influence of maternal prebiotic intake on offspring behavior, brain gene expression, and gut microbiome composition in mice.

1 Introduction

The gut microbiome is an important modulator of brain development and function (Heijtz et al., 2011; Kelly et al., 2016; Neufeld et al., 2011; Sudo et al., 2004). There is growing evidence for a relationship between the gut microbiome and the brain, via circulation of microbial metabolites, modulation of the immune system, and the direct gut-brain connection through the vagus nerve (Bercik et al., 2011; Rea et al., 2016; Stilling et al., 2016). Intake of prebiotic galactooligosaccharides (GOS) has been shown to alter expression of N-methyl-D-aspartate receptor (NMDAR) subunits, brain-derived neurotrophic factor (BDNF), and serotonin receptors in adult mice, with corresponding changes in glutamatergic transmission and reduced anxiety- and passive stress-coping behavior (Burokas et al., 2017; Gronier et al., 2017; Mika et al., 2018; Savignac et al., 2013; Williams et al., 2016). In addition, GOS may have anti-inflammatory effects (Lecerf et al., 2012; Liu et al., 2016; Savignac et al., 2016; Vulevic et al., 2015, 2013). Bimuno[®] galactooligosaccharide (B-GOS) fermentation produces metabolites including short-chain fatty acids (SCFAs) (Hopkins and Macfarlane, 2003) that likely contribute to beneficial effects on host health.

Despite evidence demonstrating the advantages of prebiotic supplementation for host health, the relationship between maternal prebiotic intake and offspring brain biology and behavior remains unclear. Maternal diet or antimicrobial treatment has been shown to influence the brain and gut microbiome in progeny (Buffington et al., 2016; Tochitani et al., 2016). Therefore, prebiotic-induced changes in maternal microbiome composition, SCFA levels (Liu et al., 2016), or breastmilk composition (Kubota et al., 2014; Nikniaz et al., 2013) may also influence offspring

neurobehavioral and microbiome development (Ding and Schloss, 2014; Jašarević et al., 2017; Rogier et al., 2014; Thum et al., 2016).

The aim of the current study was to test whether maternal B-GOS intake during pregnancy and lactation alters the brain and behavior in naïve and endotoxin-challenged offspring. Pregnant mice received B-GOS until pups reached weaning age. Offspring were subjected to tests of anxiety-like, passive stress-coping, and social behaviors. Some animals were administered lipopolysaccharide either 24 hours before behavioral tasks or neonatally to investigate if maternal prebiotic intake conveys anti-inflammatory effects to offspring. We also included a cross-fostering paradigm to differentiate between influences of prenatal and postnatal maternal prebiotic intake on offspring behavior. Brain tissues from offspring were collected for gene expression analysis, and fecal samples were processed to measure differences in gut bacterial communities and metabolic profiles.

2 Materials and methods

Detailed methods can be found in the **Supplementary material**.

2.1 Animals

All experiments were carried out with local ethical approval and a UK Home Office license granted under the Animals (Scientific Procedures) Act (1986). Female (28–30 g) and male (30–32 g) CD1 mice (Charles River, UK) were housed under normal laboratory conditions (12-h light-dark cycle, lights on at 7 a.m., 21 ± 1 °C, humidity $50 \pm 5\%$). The experimental unit was one mouse offspring, although litter effects were accounted for in the linear mixed-effects analysis. Group assignment and the order of testing and sample processing were randomized using the “Randomize range” function in Google Sheets. Experimental design and schematic diagrams were created with BioRender.com.

2.2 Experimental design

This study explored the effects of maternal B-GOS supplementation on behavior, brain gene expression, and the gut microbiome in young, weaning-age offspring and adult progeny.

2.2.1 Cohort A: young offspring

The experimental design for Cohort A is summarized in **Fig. 1A**. At E1, dams were randomly assigned to receive either standard drinking water provided by the animal facility (H₂O, five dams) or water supplemented with 1.5 mg/mL prebiotic (B-GOS, three dams). Prebiotic supplementation continued through gestation and suckling. Between P22–P27, pups received one intraperitoneal (I.P.) injection of saline or 1 mg/kg lipopolysaccharide (LPS). The open field test (OFT), light-dark box (LDB), and forced swim test (FST) were performed 24 hours later in that order. All tests were performed on the same day. After the FST, mice were culled, and the prefrontal cortex (PFC) and hippocampus were dissected from brains for qPCR (tumor necrosis factor [TNF]- α , GluNR1, GluNR2A, GluNR2B, BDNF, and 5-hydroxytryptamine [5-HT_{2A}]). These genes were selected based on previous studies that reported expression changes in adult rodents after GOS intake (Burokas et al., 2017; Gronier et al., 2017; Mika et al., 2018; Savignac et al., 2013; Williams et al., 2016). Fecal DNA was extracted for 16S ribosomal RNA (rRNA) sequencing from offspring (N = 10–11 males and N = 10–11 females; N = 20–22 total/group).

2.2.2 Cohort B: young cross-fostered offspring

The experimental design for Cohort B is summarized in **Fig. 1B**. A cross-fostering design was employed to differentiate between effects of prenatal and postnatal maternal B-GOS intake on offspring behavior and fecal metabolites. Maternal B-GOS feeding continued through gestation and pregnancy as in Cohort A. Shortly after birth, however, half of the litters were cross-fostered to a dam of the opposite treatment. All pups in each litter were cross-fostered to the new dam. This

resulted in four groups: pups that were born to and suckled by an H₂O control dam (pre H₂O/post H₂O); pups born to an H₂O dam and suckled by a B-GOS-fed dam (pre H₂O/post B-GOS); pups born to a B-GOS dam and suckled by an H₂O dam (pre B-GOS/post H₂O); and pups born to and suckled by a B-GOS dam (pre B-GOS/post B-GOS). Each group comprised three litters. The OFT and LDB were then performed between P22–P23 (N = 16 males and N = 16 females; N = 32 total/group), and plasma and fecal samples were collected for ¹H NMR metabolomics after the LDB. The ¹H NMR spectrum peaks of the most abundant SCFAS in the gut – acetate, butyrate, and propionate – were also identified and measured. Data from both sexes were combined for all metabolite analysis (N = 6 males and N = 6–8 females; N = 12–14 total/group). Fecal DNA was extracted for 16S sequencing and metabolite analysis from dams.

2.2.3 Cohort C: adult offspring

The experimental design for Cohort C is summarized in **Fig. 1C**. Five dams were randomly assigned to receive B-GOS during gestation, and six dams received standard drinking water. At postnatal day 9 (P9), pups received subcutaneous injection of saline or 3 mg/kg LPS based on previous studies showing brain and behavioral deficits in adulthood after P9 LPS (Li et al., 2018; Missig et al., 2018). Because maternal B-GOS was not expected to produce measurable changes in healthy adult offspring, the purpose of this challenge was to induce brain, behavioral, and metabolic deficits and determine whether perinatal B-GOS ameliorates these changes.

At ~P22, pups were weaned onto normal drinking water and housed with same-sex littermates (2–5/cage). At ~P42, offspring were tested in the social interaction test (SIT). At ~P56, the OFT, LDB, and FST were performed. After the FST, the PFC and hippocampi were collected for qPCR analysis, and plasma and fecal samples were processed for ¹H NMR. Fecal acetate, butyrate, and propionate concentrations were also measured by quantifying signal intensity at their respective ¹H NMR spectral peaks. Data from both sexes were combined for all metabolite

1 analyses (N = 10 males, N = 10 females; N = 20 total/group). Fecal samples from dams were
2 collected for 16S sequencing and metabolite analysis. Maternal care behavior, including latency
3 to retrieve pups and home cage observations (Meaney, 2001), was assessed when pups were P2–
4 P4.

5

Fig. 1. Experimental design. Mating procedure and outcomes for each cohort: **A)** Cohort A included young offspring of B-GOS-fed or H₂O control dams. Weaning-age pups were treated with saline or LPS and underwent behavioral tests, brain qPCR analysis, and 16S sequencing of the fecal samples. **B)** Cohort B included young cross-fostered offspring of B-GOS-fed or H₂O control dams. Pups underwent behavioral testing and fecal/plasma ¹H NMR metabolic profiling. **C)** Cohort C included adult offspring of B-GOS-fed or H₂O control dams. Pups were treated with saline or LPS at P9 and underwent behavioral testing, brain qPCR analysis, and fecal/plasma ¹H NMR metabolic profiling in adulthood (P42–P56).

2.3 Statistical analysis

Statistical tests were run in R 3.6.1 (R Foundation for Statistical Computing), and graphs were built in Prism 8.0.0 (Graphpad) and R. Grubbs' test for outliers was applied before statistical analysis, and statistically significant outliers were excluded. Group numbers in figure captions signify the final numbers after removal of outliers. Linear mixed-effects analysis was used to assess effects of maternal diet, LPS, and sex in offspring. Litter effects were accounted for in the analysis as a random effect. If two- or three-way interactions with sex were found, two two-way tests for males and females separately were performed to interpret sex-specific effects of maternal diet or LPS treatment. Otherwise, if no significant interactions of any variable with sex were found, male and female data were consolidated to minimize the number of comparisons and simplify the interpretation. Tukey tests were used for post hoc group comparisons when a significant interaction was detected.

Orthogonal partial-least squares discriminant analysis (OPLS-DA) was used to identify differences in "metabolic fingerprints" between groups. Ten-fold external cross validation with repetition coupled with permutation testing was used to validate the models as previously described (Jurynczyk et al., 2017). The accuracy of the OPLS-DA models was determined on independent test data (10% of dataset) and compared to the models' performance in classifying randomly permuted data (accuracy $\approx$ 50%). Specific metabolites driving differences in metabolic profiles were identified using average variable importance in projection (VIP) scores.

2.4 16S sequencing

Fecal DNA was sequenced on Illumina MiSeq (V3 chemistry). Downstream processing was performed using *dada2* (Callahan et al., 2016). We assessed the effects of maternal diet on the Chao1 measure of alpha diversity (number and distribution of amplicon sequence variants

[ASVs] in each sample) and the Bray-Curtis measure of beta diversity (how different each sample is from every other sample based on relative ASV abundances) using *phyloseq* in R (McMurdie and Holmes, 2013). Permutational multivariate analysis of variance (PERMANOVA) was used to explore differences in microbiome composition between groups. Finally, differentially abundant taxa were identified using DESeq in the R package *deseq2* (Love et al., 2014) and mixed-effects analysis.

3 Results

3.1 Maternal B-GOS increases rearing in the OFT in all offspring and reduces anxiety-like behavior in males

In the OFT, there were no two- or three-way interactions between sex and F0 diet or acute treatment in young offspring (Cohort A). Therefore, male and female data were consolidated for mixed-effects analysis. There was a main effect of maternal diet on the number of rearing events, indicating that maternal B-GOS increased rearing in the OFT in offspring compared to maternal H₂O, $F(1, 5.04) = 7.82, p = 0.04$, but there was no effect of LPS injection (**Fig. 2A**). There was a main effect of LPS on distance traveled, however, indicating that the endotoxin reduced distance traveled in the OFT, $F(1, 5.04) = 76.97, p = 0.002$. F0 diet did not affect distance traveled (**Fig. 2B**). Finally, there was an F0 diet x LPS effect on time in the center, $F(1, 76.00) = 8.30, p = 0.005$. Tukey post hoc tests, however, did not identify significant differences between groups to explain the interaction, but it was likely due to the slight increase in time in center in B-GOS/Saline pups that was blunted in B-GOS/LPS pups (predominantly in male offspring) (**Fig. 2C**).

In the LDB, there were significant interaction effects with sex (LPS x sex, $F[1, 74.82] = 5.51, p = 0.02$, F0 diet x LPS x sex, $F[1, 74.82] = 12.12, p = 0.0008$) as well as an effect of F0 diet,

$F(1, 5.01] = 13.31, p = 0.01$, on time spent in the light. Male and female data were then analyzed in separate two-way tests to interpret the differential effects of maternal diet and acute LPS on time spent in the light. In males, there was a significant F0 diet x LPS effect on time in light, $F(1, 33.28) = 5.61, p = 0.02$, and a main effect of LPS, $F(1, 33.28) = 8.10, p = 0.008$. Tukey post hoc tests revealed that both B-GOS/Saline male offspring, $t(7.84) = 3.35, p = 0.04$, and H₂O/LPS males, $t(33.57) = 3.72, p = 0.004$, spent significantly more time in the light than H₂O/Saline males. There was also a significant F0 diet x LPS effect on time spent in the light in females, $F(1, 37.80) = 7.29, p = 0.01$. Although no post hoc comparisons were significant, LPS tended to reduce time in the light in female offspring of H₂O dams and increase time in light in female offspring of B-GOS-fed dams (**Fig. 2D**). Number of rears in the LDB followed a similar pattern to time spent in the light. There were LPS x sex, $F(1, 75.00) = 7.20, p = 0.009$, and F0 diet x treatment x sex, $F(1, 75.00) = 16.17, p = 0.0001$, effects on rearing. In males, there was an F0 diet x acute treatment effect on rearing, $F(1, 33.08) = 9.60, p = 0.004$, and a main effect of acute treatment, $F(1, 33.08) = 4.78, p = 0.04$. According to post hoc tests, both B-GOS/Saline male offspring, $t(7.54) = 4.11, p = 0.02$, and H₂O/LPS males, $t(33.40) = 3.77, p = 0.003$, exhibited more rearing behavior than H₂O/Saline male offspring. In females, there was an F0 diet x LPS interaction, $F(1, 36.56) = 7.59, p = 0.01$. Tukey tests showed that H₂O/LPS females reared less than H₂O/Saline females, $t(75) = -3.38, p = 0.02$ (**Fig. 2E**). Overall, maternal B-GOS increased time in light and rears in male offspring but not in females. In addition, LPS reduced anxiety-like behavior in male offspring 24 hours post injection, while it increased anxious behavior in females.

There was an F0 diet x LPS x sex effect on entries into the light in the LDB, $F(1, 73.62) = 7.90, p = 0.006$. This three-way interaction was driven by an F0 diet x acute treatment effect in male offspring, $F(1, 33.07) = 5.88, p = 0.02$, that was not present in females. Post hoc tests did not

1 identify significant group differences, but the interaction was due to differential effects of LPS on
2 H₂O male offspring compared to B-GOS male offspring (**Fig. 2F**). Similarly, there was an F0 diet
3 x LPS x sex effect on latency to enter the light, $F(1, 74.74) = 7.36, p = 0.008$, as well as a main
4 effect of sex, $F(1, 74.99) = 5.10, p = 0.03$. This was driven by an F0 diet x treatment interaction in
5 males, $F(1, 33.23) = 5.34, p = 0.03$, but not in female offspring. Post hoc tests showed that acute
6 LPS reduced latency to enter the light in male H₂O offspring, $t(33.61) = -2.74, p = 0.04$, but had
7 no effect in male B-GOS offspring (**Fig. 2G**).

8 In the FST, there was a significant main effect of LPS on increasing the time spent floating,
9 $F(1, 77.74) = 4.41, p = 0.04$ (**Fig. 2H**). There were no effects of F0 diet, LPS, or sex on latency to
10 float and no significant interactions (**Fig. 2I**).

11 Analysis of maternal care behavior (**Fig. S5**) showed that offspring behavioral changes
12 were unlikely to be due the behavior of dams towards their pups, which was not affected by B-
13 GOS intake.

Fig. 2. Effects of maternal B-GOS and acute LPS on behavior in young offspring. There were main effects of maternal diet and acute treatment on OFT rears: **A)** perinatal prebiotic intake increased rearing in offspring, while **B)** acute LPS reduced distance traveled (significance not indicated in graph). **C)** There was an F0 diet x acute treatment effect on time spent in the center of the open field, but no post hoc tests were significant. **D)** In the LDB, there was an F0 diet x LPS effect on time in light. Post hoc tests revealed that both B-GOS/Saline male offspring and H₂O/LPS males spent significantly more time in the light than H₂O/Saline males. There was also a significant F0 diet x LPS effect on time

spent in the light in females, but no post hoc tests were significant. **E)** There was an F0 diet x LPS effect on rearing in the light in males. According to post hoc tests, both B-GOS/Saline male offspring and H₂O/LPS males exhibited more rearing behavior than H₂O/Saline male offspring. In females, there was an F0 diet x LPS interaction. Tukey tests showed that H₂O/LPS females reared less than H₂O/Saline females. **F)** There was an F0 diet x LPS effect in male but not female offspring on entries into the light. No post hoc tests were significant. **G)** There was an F0 diet x LPS effect latency to enter the light in male but not female offspring. Post hoc tests showed that acute LPS reduced latency to enter the light in male H₂O offspring. **H)** There was a main effect of LPS on time floating: LPS increased time floating, while maternal B-GOS had no effect. **I)** There were no changes in latency to float in the FST. Mixed-effects analysis, $*p < 0.05$, $**p < 0.01$. N = 10–11 males/females; N = 20–22 total/group.

3.2 Prenatal and postnatal maternal B-GOS increase exploratory rearing behavior in offspring

In the OFT, analysis of main effects showed that both prenatal, $F(1, 7.81) = 8.79, p = 0.02$, and postnatal maternal B-GOS, $F(1, 7.81) = 12.91, p = 0.007$, increased rearing in Cohort B cross-fostered offspring (**Fig. 3A**). There were no effects of prenatal or postnatal treatment on distance traveled (**Fig. 3B**) or time spent in the center (**Fig. 3C**).

In the LDB, there were no significant main effects or prenatal x postnatal diet interactions. The effects of postnatal maternal B-GOS on anxiety-like behavior in the LDB did not reach significance, including time in light, $F(1, 7.91) = 4.46, p = 0.07$ (**Fig. 3D**), and rearing, $F(1, 7.76) = 3.92, p = 0.08$ (**Fig. 3E**). Neither prenatal nor postnatal B-GOS affected entries into the light (**Fig. 3F**) or latency to enter the light side (**Fig. 3G**) in offspring.

Fig. 3. Effects of prenatal and postnatal maternal B-GOS on behavior in cross-fostered young offspring. A) There were main effects of prenatal and postnatal diet on rearing, indicating that both prenatal and postnatal maternal B-GOS increased rearing in the OFT. There were no effects on B) distance traveled or C) time in center. There were trends towards main effects of

maternal diet in the LDB: **D)** maternal B-GOS tended to reduce time in light and **E)** increase rearing in the LDB in young offspring nursed by B-GOS-fed dams. There were no changes, however, in **F)** entries into the light or **G)** latency to enter the light side. Mixed-effects analysis, $**p < 0.01$. N = 15–16 males/females; N = 31–32 total/group.

3.3 Perinatal B-GOS reduces hippocampal NMDAR subunit expression in young offspring

In Cohort A, there were main effects of maternal diet and LPS on offspring NMDAR subunit expression. Maternal B-GOS reduced expression of the hippocampal NMDAR subunits GluNR1, $F(1, 4.82) = 10.59, p = 0.02$ (**Fig. 4A**), GluNR2A, $F(1, 4.16) = 8.46, p = 0.04$ (**Fig. 4B**), and GluNR2B, $F(1, 5.02) = 8.37, p = 0.03$ (**Fig. 4C**). Acute LPS also reduced expression of these genes (GluNR1, $F[1, 75.59] = 7.71, p = 0.007$, GluNR2A, $F[1, 75.28] = 6.92, p = 0.01$, and GluNR2B, $F[1, 76.34] = 9.04, p = 0.004$). In addition, LPS reduced expression of hippocampal BDNF in offspring, $F(1, 76.52) = 17.70, p < 0.0001$ (**Fig. 4D**). There were no effects of maternal B-GOS or acute LPS treatment on hippocampal 5-HT_{2A} (**Fig. 4E**). There was an F0 diet x sex effect on hippocampal TNF- α expression, $F(1, 70.99) = 4.28, p = 0.04$, as well as a main effect of sex, $F(1, 70.99) = 4.44, p = 0.04$ (**Fig. 4F**). When male and female TNF- α expression data were analyzed separately, however, there were no interactions or effects of F0 diet or LPS. In addition, there were no changes in gene expression in the PFC (**Fig. S6**).

Fig. 4. Effects of maternal B-GOS and acute LPS on behavior in young offspring. There were main effects of maternal diet and acute treatment on NMDAR expression: both maternal B-GOS and acute LPS (significance not denoted in graph) reduced expression of **A)** GluNR1, **B)** GluNR2A, and **C)** GluNR2B. **D)** There was also a main effect of acute treatment on BDNF

expression, indicating that LPS reduced BDNF expression (significance not denoted in graph). There were no changes in **E**) 5-HT_{2A} or **F**) TNF- α . Mixed-effects analysis, $*p < 0.05$. N = 10–11 males/females; N = 20–22 total/group.

3.4 Microbiome composition in offspring of B-GOS-fed dams differs significantly from control offspring

Fecal DNA was extracted for 16S sequencing from F0 animals from Cohort B and C and F1 animals from Cohort A (**Fig. S7**). There were no differences in alpha diversity between B-GOS-fed and control dams (**Fig. S8A**), and there was no effect of maternal diet on alpha diversity in offspring (**Fig. S8B**).

Because there were no differences in beta diversity between male and female offspring, $F(1, 38) = 1.49$, $R^2 = 0.04$, $p = 0.12$, offspring of both sexes were combined for analysis. The clearest separation in the principal coordinate analysis (PCoA) plots was between F0 dams and F1 offspring, $F(1, 60) = 8.29$, $R^2 = 0.12$, $p = 0.001$ (**Fig. 5A**). While there were no differences in beta diversity between H₂O control and B-GOS-fed dams, $F(1, 20) = 1.00$, $R^2 = 0.05$, $p = 0.42$, pups clustered by maternal diet, $F(1, 38) = 3.80$, $R^2 = 0.09$, $p = 0.001$ (**Fig. 5B**), and litter, $F(6, 33) = 5.59$, $R^2 = 0.50$, $p = 0.001$ (**Fig. 5C**). Because of the nested design (litters nested within groups), R^2 values in these tests represent the maximum variance explained by maternal diet or litter rather than the actual variance explained.

3.5 Relative abundances of taxa in the Bacteroidetes and Actinobacteria phyla differ between control and B-GOS-fed mothers and their pups

DEseq analysis resulted in 5 differentially abundant ASVs in dams (**Fig. S9**) and 20 ASVs in offspring. Mixed-effects analysis was then used to account for litter effects, and three significant ASVs associated with the following taxa were reduced in B-GOS offspring: the species *Bacteroides caecimuris* (**Fig. 5D**), the *Barnesiella* genus (*Porphyromonadaceae* family, **Fig. 5E**), and the *Bifidobacterium* genus (**Fig. 5F**). Because specific species of *Bifidobacterium* and *Barnesiella* could not be identified, relative abundances of total *Bifidobacterium* and *Barnesiella*

were compared between B-GOS and H₂O pups; however, there were no significant differences between groups (**Fig. S10**).

Fig. 5. Effects of maternal B-GOS on beta diversity and relative abundances. PCoA plots based on Bray-Curtis dissimilarity matrices showed that the following groups clustered based on microbiome composition: **A)** dams vs offspring, **B)** B-GOS vs H₂O offspring, and **C)** litters. Three differentially abundant ASVs in B-GOS offspring compared to H₂O pups were identified that were associated with **D)** the species *Bacteroides caecimuris*, **E)** the *Barnesiella* genus, and **F)** the *Bifidobacterium* genus. Each boxplot represents one ASV, and the lowest taxonomic level assigned by *dada2* is indicated in the boxplot titles. Data points are colored by litter. F0.H2O = control H₂O dams; F0.B-GOS = B-GOS-fed dams; F1.H2O = H₂O/Saline pups from Cohort A; F1.B-GOS = B-GOS/Saline pups from Cohort A. LPS-treated offspring were not included in 16S analysis. DESeq and mixed-effects analysis, * $p < 0.05$, N = 9–10 male/female offspring, N = 19–20 total/group.

3.6 B-GOS has no effects on maternal fecal metabolite profiles

B-GOS had no effect on fecal metabolite profiles in dams (**Fig. S11A**). In addition, there were no effects of B-GOS intake on SCFA concentrations based on ^1H NMR signal intensity: fecal acetate, $t(19) = 1.86$, $p = 0.08$ (**Fig. S11B**), butyrate, $t(20) = 1.57$, $p = 0.13$ (**Fig. S11C**), or propionate, $t(20) = 1.09$, $p = 0.29$ (**Fig. S11D**).

3.7 Postnatal, but not prenatal, maternal prebiotic feeding alters offspring fecal metabolites

According to the OPLS-DA analysis, the pre H₂O/post H₂O vs. pre B-GOS/post B-GOS (**Fig. 6A**) and pre H₂O/post H₂O vs. pre H₂O/post B-GOS (**Fig. 6B**) models performed significantly better than chance, indicating an effect of postnatal maternal B-GOS intake on fecal metabolites in Cohort B offspring. The pre H₂O/post H₂O vs. pre B-GOS/post H₂O model was not significant, however, suggesting that prenatal maternal B-GOS did not change offspring fecal metabolite profiles (**Fig. 6C**). Pre B-GOS/post H₂O mice also had significantly different fecal metabolite profiles than pre H₂O/post B-GOS mice (**Fig. 6D**). The detailed results of the OPLS-DA analysis, including $R^2\text{X}$, $R^2\text{Y}$, and Q^2 values, are summarized in **Table S2**.

A heat map was used to visualize significantly different spectral bins and the corresponding metabolites (**Fig. 7A**). Fecal butyrate was elevated in pups that were suckled by a B-GOS-fed dam (pre H₂O/post B-GOS and pre B-GOS/post B-GOS groups), while glucose and leucine/isoleucine signals were reduced in these animals compared to pre H₂O/post H₂O pups. Succinate levels were highest in pre B-GOS/post H₂O pups.

Based on our *a priori* hypothesis that maternal B-GOS would increase fecal SCFAs, we also performed mixed-effects analysis to determine the effects of prenatal and postnatal B-GOS on acetate, butyrate, and propionate signals in offspring fecal extracts. There was a main effect of

1 postnatal diet on the average integral of the butyrate region, $F(1, 7.66) = 5.69$, $p = 0.04$, indicating
2 higher fecal butyrate concentrations in pups suckled by B-GOS-fed dams compared to pups nursed
3 by controls (**Fig. 7B**). Similarly, postnatal maternal B-GOS increased fecal propionate levels, $F(1,$
4 $6.57) = 11.85$, $p = 0.01$ (**Fig. 7C**). Neither butyrate nor propionate were affected by prenatal
5 maternal B-GOS. In addition, there were no changes in fecal acetate (**Fig. 7D**).

6 Average Nuclear Overhauser Effect Spectroscopy (NOESY) spectra for fecal samples from
7 the pre H₂O/post H₂O and pre B-GOS/post B-GOS groups are shown in **Fig. 7E**, and butyrate,
8 propionate, and glucose signals are labeled. Higher intensity signals (which correspond to higher
9 metabolite concentrations) in the butyrate and propionate regions can be seen in the pre B-
10 GOS/post B-GOS group. Note that each metabolite has multiple peaks because the hydrogen
11 protons within each molecule have different chemical environments, leading to energy absorption
12 at multiple different frequencies in a given compound.

Fig. 6. Postnatal B-GOS feeding results in significant changes in offspring fecal metabolite profiles. Fecal samples were collected from Cohort B cross-fostered offspring for metabolite analysis. According to OPLS-DA, **A)** pre B-GOS/post B-GOS pups and **B)** pre H_2O /post B-GOS offspring had significantly different fecal metabolic profiles than pre H_2O /post H_2O animals, suggesting an effect of postnatal maternal B-GOS intake on offspring fecal metabolite levels. **C)** Prenatal B-GOS alone (represented by pre B-GOS/post H_2O group), however, had no effect. **D)** In addition, the fecal metabolite profile of pre H_2O /post B-GOS pups was distinct from that of pre B-GOS/post H_2O animals. Kolmogorov-Smirnov, *** $p < 0.001$.

Fig. 7. Postnatal B-GOS significantly increases fecal butyrate and acetate in young offspring. **A)** To visualize group differences in metabolite levels, spectral bins with top VIP scores in at least one of the three significant OPLS-DA models were used to construct a heat map. Colors represent integral values for each bin normalized to the average integral in the pre H₂O/post H₂O group. Bins are labeled with the corresponding metabolite for unambiguous signals. There were also significant effects of postnatal diet on butyrate and propionate: postnatal B-GOS intake was associated with elevated fecal **B)** butyrate and **C)** propionate compared to offspring suckled by H₂O control dams. **D)** There was no change in fecal acetate. Mixed-effects analysis, $*p < 0.05$. **E)** Average 1D ¹H NMR spectra for pre B-GOS/post B-GOS and pre H₂O/post H₂O offspring fecal samples. Pre B-

- 1 GOS/post B-GOS pups have more intense signals in the butyrate and propionate regions than pre H₂O/post H₂O pups. N = 6–8
- 2 males/females; N = 12–14 total/group.
- 3

3.8 Maternal B-GOS alters plasma metabolite profiles and reduces glucose levels in young offspring

The combination of prenatal and postnatal maternal B-GOS intake significantly modified plasma metabolite profiles in young offspring, and this difference was driven largely by reduced plasma glucose levels (**Fig. S12**).

3.9 Maternal B-GOS increases social preference and reduces passive stress-coping behavior in adult progeny

Adult offspring in Cohort C underwent the SIT, and male and female data were consolidated for mixed-effects analysis. Both maternal diet and P9 treatment had significant main effects: maternal B-GOS increased social preference, $F(1, 76.00) = 4.46, p = 0.04$, while P9 LPS reduced sociability, $F(1, 76.00) = 5.54, p = 0.02$ (**Fig. 8A**).

In the FST, male and female adult data were also consolidated, and there was an F0 diet x postnatal day 9 LPS injection (LPS) effect on time floating, $F(1, 69.12) = 4.56, p = 0.04$. Although post hoc comparisons were not significant, there was a trend toward increased floating time in B-GOS/Saline adult offspring compared to H₂O/Saline offspring, $t(14.60) = -2.76, p = 0.06$ (**Fig. 8B**). There were no effects on latency to float (**Fig. 8C**).

In the LDB, there was a three-way effect on latency to enter the light, $F(1, 74.74) = 7.37, p = 0.008$, and a main effect of sex, $F(1, 74.99) = 5.15, p = 0.03$. According to separate male and female analyses, there was a main effect of maternal diet in females: maternal B-GOS reduced the latency to enter the light in females, $F(1, 74.99) = 5.15, p = 0.03$, but not in males (**Fig. 8D**).

There was an F0 diet x sex effect on time in light, $F(1, 65.05) = 13.39, p = 0.0005$, and a main effect of sex, $F(1, 65.06) = 8.00, p = 0.006$. (**Fig. 8E**). Similarly, there were significant F0 diet x sex, $F(1, 66.12) = 4.19, p = 0.04$, and F0 diet x LPS x sex, $F(1, 65.48) = 5.37, p = 0.02$,

effects on rears in the LDB (**Fig. 8F**). Finally, there was an F0 diet x LPS x sex effect on entries into the light, $F(1, 73.62) = 7.90, p = 0.006$ (**Fig. 8G**). When adult male and female data were analyzed separately, however, no significant effects of F0 diet or LPS on time in the light, rearing, or entries were found.

There was a P9 LPS x sex effect on rearing in the OFT, $F(1, 66.34) = 4.09, p = 0.04$. Separate analysis of male and female data showed a main effect of LPS in females: LPS reduced rearing in adult female offspring, $F(1, 30.38) = 8.63, p = 0.006$, but not in males (**Fig. 8H**). There were no effects of maternal diet or LPS on distance traveled (**Fig. 8I**) or time in the center of the OFT (**Fig. 8J**) in adult offspring.

Fig. 8. Maternal B-GOS increases sociability in all adult offspring and reduces anxious behavior in female progeny. **A)** There were main effects of maternal diet and acute treatment on social preference, indicating that maternal B-GOS increased social preference while postnatal day 9 LPS injection (LPS) reduced sociability in adult offspring. **B)** There was also an F0 diet x LPS effect on time floating. Although post hoc comparisons were not significant, there was a trend toward increased floating time in B-GOS/Saline

adult offspring compared to H₂O/Saline offspring. **C)** There were no changes in latency to float. **D)** There was a main effect of maternal diet on latency to enter the light in females: perinatal B-GOS reduced latency to enter the light in adult female offspring but not in males. Although there was an F0 diet x sex effect on **E)** time in light and an F0 diet x LPS x sex effect on **F)** rears and **G)** entries into the light in the LDB, separate analyses of male and female to interpret the interactions did not indicate any significant differences. **H)** A significant main effect of LPS indicated that the endotoxin injection reduced rearing in adult female offspring but not in males in the OFT. There were no effects of maternal diet, however, on rears. There were also no changes in **I)** distance traveled, or **J)** time in the center of the OFT in adult offspring. Mixed-effects analysis, $*p < 0.05$. N = 9–10 males/females; N = 19–20 total/group.

3.10 Maternal B-GOS intake increases NMDAR expression in the hippocampus in adult offspring

In contrast to young pups, maternal B-GOS supplementation, as well as early-life LPS, influenced expression of NMDAR subunits in the PFC in adult offspring but had no effect in the hippocampus (**Fig. S13**). In the PFC, there was an F0 diet x LPS x sex effect, $F(1, 72.00) = 10.76$, $p = 0.002$, and an overall F0 diet x LPS effect, $F(1, 72.00) = 6.99$, $p = 0.01$, on GluNR1 expression. This three-way interaction was driven by an F0 diet x LPS effect on GluNR1 expression in females, $F(1, 36) = 21.28$, $p < 0.0001$, but not in males. According to Tukey post hoc tests, B-GOS increased GluNR1 expression in females, $t(36) = 4.23$, $p = 0.0008$, while LPS prevented this increase, $t(36) = -4.04$, $p = 0.002$ (**Fig. 9A**). Sex did not influence expression of GluNR2A, and there was an overall F0 diet x LPS effect, $F(1, 75) = 6.23$, $p = 0.01$. Post hoc tests did not identify significant group differences, but GluNR2A followed a similar pattern to GluNR1 – maternal B-GOS tended to increase GluNR2A in offspring, while GluNR2A levels returned to baseline with LPS (**Fig. 9B**). Finally, there was an F0 diet x LPS x sex effect on PFC GluNR2B expression, $F(1, 65.72) = 4.30$, $p = 0.04$, as well as an overall F0 diet x LPS effect, $F(1, 65.72) = 5.97$, $p = 0.02$. Similar to GluNR1, this three-way interaction was driven by an F0 diet x LPS interaction in females only, $F(1, 35) = 9.23$, $p = 0.004$. Tukey tests showed that B-GOS elevated GluNR2B, $t(36) = -3.43$, $p = 0.008$ in saline-treated animals, and LPS blunted this increase, $t(36) = 2.82$, $p = 0.04$ (**Fig. 9C**). There were no changes in 5-HT_{2A} (**Fig. 9D**) or BDNF (**Fig. 9E**).

Fig. 9. Perinatal B-GOS feeding alters PFC gene expression in adult offspring in a sex-specific manner. A) There was an F0 diet x P9 treatment effect on GluNR1 expression in females. Post hoc tests showed that B-GOS increased GluNR1 expression in saline-treated female offspring, while LPS prevented this increase. B) There was an overall F0 diet x LPS effect on GluNR2A expression. No post hoc tests were significant. C) There was an F0 diet x LPS effect on GluNR2B in females only. Post hoc tests

1 showed that B-GOS elevated GluNR2B in saline-treated animals, and LPS blunted this increase. There were no changes in **D**) 5-
2 HT_{2A} or **E**) BDNF expression. Mixed-effects analysis, * $p < 0.05$, ** $p < 0.01$. *** $p < 0.001$. N = 9–10 males/females; N = 19–20
3 total/group.

4

3.11 P9 LPS alters fecal metabolites in adult offspring, but perinatal B-GOS has no effect

According to the OPLS-DA analysis, maternal B-GOS had no effect on fecal metabolite profiles in adult offspring – there were no differences between the H₂O/Sal vs. B-GOS/Sal groups (**Fig. S14A**) or H₂O/LPS vs. B-GOS/LPS groups (**Fig. S14B**). The only significant model was H₂O/Sal vs. H₂O/LPS, indicating a significant effect of early-life LPS on adult fecal metabolites (**Fig. S14C**). The B-GOS/Sal vs. B-GOS/LPS model, however, was not significant (**Fig. S14D**).

Spectral bins with the highest VIP scores were identified, and metabolites were assigned to bins where possible. Glucose, succinate, and choline drove the separation between groups, and these metabolites were lower in the H₂O/LPS group compared to H₂O/Saline controls (**Fig. S14E**). Detailed OPLS-DA results are summarized in **Table S3**.

No significant effects of F0 diet or LPS on fecal acetate (**Fig. S14F**), butyrate (**Fig. S14G**), or propionate (**Fig. S14H**) were identified.

3.12 Effects of perinatal B-GOS and early-life LPS on body weights and plasma metabolite profiles in adult offspring

While there were no differences in body weights at P9 before the LPS injection, there was a significant main effect of P9 treatment in neonates, indicating that LPS administration significantly reduced body weights at P10 and P14 (**Fig. S15**). In adult offspring, maternal B-GOS significantly altered plasma lipid composition (**Fig. S16**).

4 Discussion

The impact of prebiotics on the brain and behavior in adult rodents has been described previously (Gronier et al., 2017; Savignac et al., 2016, 2013; Williams et al., 2016). Despite the importance of the gut microbiome in early neurodevelopment (Clarke et al., 2013; Heijtz et al.,

2011), the influence of galacto-oligosaccharide intake during gestation and nursing on pups has not been explored. We hypothesized that perinatal B-GOS intake in mice would reverse the effects of LPS on brain gene expression and behavior and that these changes would be associated with altered gut microbiome composition and metabolic fingerprints in dams and offspring. Overall, although LPS had mixed effects, increased exploratory behavior and social preference and reduced anxiety and passive stress-coping behavior in young and adult offspring following maternal B-GOS supplementation supported our hypothesis. Maternal prebiotic intake was also associated with changes in the expression of NMDAR subunits in the hippocampus of young offspring and the PFC of adult progeny. Finally, changes in fecal metabolite levels, including increased SCFAs, and bacterial taxa in young offspring were also observed following perinatal B-GOS supplementation.

4.1 Behavioral effects of maternal B-GOS and acute LPS in offspring

Exploratory behavior in the OFT was the most robust behavioral effect of maternal B-GOS intake – this increase was shown in both Cohort A and B. In addition, the LDB results showed that maternal B-GOS intake during lactation may reduce anxiety-like behavior in offspring. These findings are consistent with previous studies on the behavioral effects of prebiotic intake in adult rodents (Burokas et al., 2017; Savignac et al., 2016). LPS did not, however, increase anxiety-like and passive stress-coping behavior and reduce exploratory behavior as we had anticipated (Couch et al., 2015; O'Connor et al., 2009; Zhao et al., 2016), although some studies have reported minimal or no changes in behavior in the same paradigm (Biesmans et al., 2013; Wang et al., 2011). The lack of an effect of LPS on TNF- α expression 24 hours post injection in our study may explain the mixed effects of LPS on behavior in young offspring. Identifying reduced anxiety-like behavior

1 in healthy mice, however, provides strong evidence for F0 B-GOS-mediated changes in offspring
2 behavior.

3 4.2 Effects of maternal B-GOS and acute LPS on NMDAR expression in offspring

4 The reduction of NMDAR subunit expression in the hippocampus of young offspring
5 following maternal prebiotic feeding, accompanied by increases in exploratory and anxiety-like
6 behavior, suggests that this receptor may have mediated the behavioral changes. Indeed,
7 NMDAR antagonists, such as ketamine, reduce anxiety and behavioral despair in animal models
8 (Engin et al., 2009) and have antidepressant effects in humans (McGirr et al., 2015; Murrough et
9 al., 2013). Although the decrease in NMDAR subunits observed in the current investigation
10 contradicts data from a prebiotic study in rats (Savignac et al., 2013), it is consistent with other
11 studies reporting reduced NMDAR mRNA and protein after GOS intake in adult mice (Burokas
12 et al., 2017; Savignac et al., 2016). Our current observations, therefore, suggest that the
13 multigenerational effects of maternal B-GOS intake on central NMDARs may mediate its
14 anxiolytic effects in offspring.

15 In light of the changes in NDMAR subunit expression, it will be important to investigate
16 the effects of perinatal B-GOS on cognitive behaviors, such as the Morris water maze or the Y-
17 maze spatial recognition task. Indeed, B-GOS has been shown to improve attentional set-shifting
18 performance (Gronier et al., 2017) and novel object recognition in a rat model of postoperative
19 cognitive dysfunction (Yang et al., 2018) and may have similar pro-cognitive effects in offspring
20 following perinatal supplementation. In addition, given the importance of the PFC and
21 hippocampus in learning and memory, future studies should investigate whether offspring gene
22 expression changes occur in other brain regions that modulate emotional behaviors, such as the

hypothalamus, pituitary, amygdala, bed nucleus of the stria terminalis (BNST), and specific regions of the PFC, such as the medial PFC.

LPS was associated with decreased expression of NMDAR subunits in the hippocampus. It is interesting that both maternal B-GOS and LPS had the same effect on hippocampal gene expression. Because quinolinic acid, an NMDAR agonist, increases following LPS (Walker et al., 2013), there may be a compensatory decrease in NMDAR subunit expression. The relationships between inflammation, central NMDARs, and the gut microbiome should be investigated further. Acute LPS did, however, reduce hippocampal BDNF, which is consistent with the effects of systemic inflammation in previous studies (Guo et al., 2014; Li et al., 2017; Martin et al., 2013; Sriram et al., 2016; Zhao et al., 2017).

4.3 Maternal B-GOS and the offspring gut microbiome

Contrary to our hypothesis, B-GOS had no effects on maternal fecal metabolites or global microbiome composition after prebiotic intake. While DESeq did detect differentially abundant taxa in B-GOS-fed dams compared to controls, these differences were driven by two or three outliers. It is possible that large microbiome shifts that occur in the perinatal period, including increases in *Actinobacteria* (Koren et al., 2012), mask any prebiotic-induced changes in the maternal gut microbiome. Furthermore, the B-GOS dose was chosen because it was shown to increase *Bifidobacterium* levels in female mice in a small pilot study (**Fig. S1A**). However, group numbers in the pilot were low, and all animals in each group were housed in the same cage. In addition, these females were not pregnant/suckling. Although the dose of B-GOS that was ultimately used did influence offspring development and gut microbiome composition, future studies should test a range of doses.

1 Despite the lack of changes in the maternal microbiome, maternal B-GOS intake was
2 associated with global changes in gut microbiome composition in offspring. Differences in
3 microbiome composition after consumption of another GOS prebiotic has been reported in line
4 with this study (Cheng et al., 2018). Although *Bacteroides caecimuris*, a member of the gram-
5 negative, obligate anaerobic *Bacteroides* genus, has not been linked to brain gene expression or
6 behavior in previous studies, other *Bacteroides* species have been implicated in beneficial (Hsiao
7 et al., 2013; Strandwitz et al., 2019) as well as detrimental brain and behavioral traits (Rincel et
8 al., 2019). *Bacteroides caecimuris* may be an additional important member of the *Bacteroides*
9 genus in the microbiome-gut-brain axis. Increased *Barnesiella* has been associated with autism
10 spectrum disorder in humans (Liu et al., 2019). This supports our finding that this taxon is
11 associated with increased anxiety and behavioral despair. *Barnesiella* and *Bacteroides caecimuris*
12 may therefore be implicated in the brain and behavioral changes in offspring.

13 The decrease in a *Bifidobacterium* was surprising, given that B-GOS has previously been
14 shown to increase *Bifidobacterium in vitro* (Grimaldi et al., 2016; Liu et al., 2016) and in rats (Kao
15 et al., 2018; Savignac et al., 2013). When all ASVs in the *Bifidobacterium* genus were consolidated,
16 there was also a trend towards reduced relative abundance of total *Bifidobacterium* in B-GOS pups.
17 Partial sequencing of the 16S gene gives limited resolution, especially at the species level, and
18 cannot give information about the effects of B-GOS on relative abundances of specific species or
19 strains of *Bifidobacterium* (Johnson et al., 2019). Metagenomics should therefore be used in future
20 studies to determine which species of *Barnesiella* and *Bifidobacterium* are affected by perinatal
21 B-GOS and their associated functions.

22 Although we had anticipated effects of both prenatal and postnatal maternal B-GOS intake
23 on offspring fecal metabolite profiles, only postnatal maternal B-GOS changed fecal metabolite

1 levels, including increases in butyrate and propionate and reductions in glucose and
2 leucine/isoleucine. The robust effect of postnatal B-GOS on fecal metabolite profiles is consistent
3 with studies showing that the offspring gut microbiome is determined by the nursing dam rather
4 than the birth mother (Daft et al., 2015; Treichel et al., 2019), although more studies on the
5 differential contributions of the birth and nursing dam on the offspring gut microbiome are needed.

6 Given the minimal changes in maternal microbiome composition and metabolism after
7 consuming the prebiotic, as well as the influence of postnatal B-GOS on offspring behavior and
8 fecal metabolites, maternal B-GOS may exert its effects by altering breastmilk composition.
9 Elevated levels of SCFAs in B-GOS pups may come from breastmilk; breastmilk itself contains
10 high levels of SCFAs (Jiang et al., 2016), while compounds in breastmilk, such as milk
11 oligosaccharides, can also be fermented in the offspring gut to produce SCFAs (Xiao et al., 2018).
12 B-GOS supplementation in mothers may therefore increase SCFA levels in offspring through one
13 or both of these mechanisms. High-fiber diet, for example, increases oligosaccharide levels in
14 maternal milk in rats (Hallam et al., 2014). The influence of prebiotic intake on breastmilk
15 composition, however, needs to be investigated further.

16 SCFAs can also cross the blood-brain barrier (Frost et al., 2014; Minamiyama et al., 2004).
17 The most likely mechanism by which SCFAs modulate gene expression is via their histone
18 deacetylase (HDAC) inhibitor function. SCFAs have been shown to inhibit mRNA and protein
19 expression of HDAC3 and HDAC5 *in vitro* (Zheng et al., 2020), and oral administration of butyrate
20 increases acetylation at histone H3 in the brain in mice (Sharma et al., 2015; Yamawaki et al.,
21 2018). In young offspring from B-GOS-fed dams, increased SCFAs may increase histone
22 acetylation and, in turn, increase expression of genes/proteins that negatively regulate expression
23 of NMDAR subunits. Future studies will need to investigate brain HDAC activity in offspring

following maternal prebiotic feeding to help determine whether changes in histone acetylation contribute to the alterations in gene expression observed in the current study.

4.4 Long-lasting neurobehavioral effects of maternal B-GOS and early-life inflammation in adult offspring

Perinatal prebiotic intake had a variety of effects on behavior in adult offspring. The increase in social interaction in the SIT in B-GOS offspring was consistent with our original hypothesis and in agreement with studies of prebiotic supplementation in adult rodents (Burokas et al., 2017; Grimaldi et al., 2018). This is the first study, however, showing a multigenerational effect of prebiotic intake on social preference. Changes in the offspring microbiome after maternal prebiotic feeding may influence the development of social behavior (Sherwin et al., 2019). The decrease in social preference in LPS-treated animals was also expected based on previous studies (Li et al., 2018). These results indicate that maternal prebiotic intake and early-life inflammation have opposing effects on social behavior in adult offspring that may reflect disparate effects of the two treatments on the neonatal immune system. Furthermore, in the FST, maternal B-GOS reduced floating time as anticipated, and the significant F0 diet x P9 treatment interaction showed that LPS prevented the B-GOS-induced decrease in floating time. Therefore, maternal prebiotic may produce behavioral changes through modification of the offspring immune system, although more evidence is needed.

Other behavioral results in adult progeny included reduced rearing in LPS-treated animals in the OFT and reduced latency to enter the light in female offspring of B-GOS-fed dams. Reduced exploratory rearing after early-life inflammation was expected based on previous studies (Li et al., 2018), although the increase in rearing in young pups from B-GOS-fed dams did not last into adulthood. We also expected reduced anxious behavior in the LDB, including reduced latency to

enter the light, but in contrast to the young offspring, this change was only found in female progeny of B-GOS-fed dams. Similarly, maternal B-GOS tended to increase NMDAR subunit expression in female adult progeny. Sex-dependent effects of maternal diet and dietary supplements on adult offspring behavior and gene expression have been reported previously (Keleher et al., 2018; Mckee et al., 2018; Surzenko et al., 2019; Ye et al., 2018). The sex differences in anxiety-like behavior may be explained by an interaction between exposure to maternal B-GOS in early life and estrogen and estrogen receptors, which are involved in anxiety-like behavior (Eid et al., 2019). In summary, maternal B-GOS feeding may induce long-lasting changes in the neonatal brain that alter behavior, although the exact phenotype is not consistent across age and sex.

4.5 Maternal B-GOS and the gut microbiome in adult offspring

Although we had hypothesized that functional changes in the gut microbiome would last until adulthood, there were no maternal B-GOS-induced changes in fecal metabolites in adult offspring. This suggests that while perinatal prebiotic exposure exerts enduring changes on offspring brain and behavior through to adulthood, alterations to the microbiome are not long-lasting. Although it will be necessary to perform 16S sequencing on fecal samples from adult offspring, we would predict that there are no differences in microbiome composition in adults based on the metabolic data. Therefore, after offspring are separated from the mother and weaned to normal drinking water, any differences in microbiome composition and metabolic function are likely overshadowed by common environmental and dietary factors. Thus, changes in the gut microbiome in early life may produce changes in the brain and behavior that last into adulthood independent of the ensuing environment (Clarke et al., 2013; Heijtz et al., 2011; Stilling et al., 2015).

4.6 Conclusion

In summary, maternal prebiotic intake influences behavior and gene expression in young and adult offspring and reduces fecal levels of *Barnesiella* and *Bacteroides caecimuris*. This supports the role of the microbiome early in development on programming brain function and behavior and provides rationale for investigating a causal relationship between the offspring microbiome and the brain and behavior in future studies of maternal prebiotic feeding. Despite minimal changes to the postnatal maternal gut microbiome, postnatal B-GOS feeding strongly influences offspring fecal metabolites. These results suggest that direct exposure to maternal gut microbes through coprophagia and co-housing cannot explain the changes in the offspring brain and behavior. Rather, breastmilk composition may account for the differences in brain gene expression and behavior in B-GOS pups compared to controls and should be investigated in future studies. Maternal prebiotic intake, particularly during nursing, could represent an important opportunity to safely modulate the offspring gut microbiome. It may also mitigate against adverse behavioral adaptations to early-life inflammation.

5 Conflicts of interest

Clasado Biosciences Ltd, made a 10% financial contribution towards the study, as part of the BBSRC IPA scheme awarded to PWJB (see below). All other authors report no conflicts of interest in this work.

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7 7 References

- 8 Bercik, P., Park, A.J., Sinclair, D., Khoshdel, A., Lu, J., Huang, X., Deng, Y., Blennerhassett,
9 P.A., Fahnestock, M., Moine, D., Berger, B., Huizinga, J.D., Kunze, W., Mclean, P.G.,
10 Bergonzelli, G.E., Collins, S.M., Verdu, E.F., 2011. The anxiolytic effect of
11 *Bifidobacterium longum* NCC3001 involves vagal pathways for gut-brain communication.
12 *Neurogastroenterol. Motil.* 23, 1132–1139. [https://doi.org/10.1111/j.1365-](https://doi.org/10.1111/j.1365-2982.2011.01796.x)
13 [2982.2011.01796.x](https://doi.org/10.1111/j.1365-2982.2011.01796.x)
- 14 Biesmans, S., Meert, T.F., Bouwknecht, J.A., Acton, P.D., Davoodi, N., De Haes, P., Kuijlaars,
15 J., Langlois, X., Matthews, L.J.R., Ver Donck, L., Hellings, N., Nuydens, R., 2013.
16 Systemic immune activation leads to neuroinflammation and sickness behavior in mice.
17 *Mediators Inflamm.* 2013. <https://doi.org/10.1155/2013/271359>
- 18 Buffington, S.A., Di Prisco, G.V., Auchtung, T.A., Ajami, N.J., Petrosino, J.F., Costa-Mattioli,
19 M., 2016. Microbial Reconstitution Reverses Maternal Diet-Induced Social and Synaptic
20 Deficits in Offspring. *Cell* 165, 1762–1775. <https://doi.org/10.1016/j.cell.2016.06.001>
- 21 Burokas, A., Arboleya, S., Moloney, R.D., Peterson, V.L., Murphy, K., Clarke, G., Stanton, C.,
22 Dinan, T.G., Cryan, J.F., 2017. Targeting the Microbiota-Gut-Brain Axis: Prebiotics Have
23 Anxiolytic and Antidepressant- like Effects and Reverse the Impact of Chronic Stress in

Mice. *Biol. Psychiatry* 82, 472–487. <https://doi.org/10.1016/j.biopsych.2016.12.031>

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583. <https://doi.org/10.1038/nmeth.3869>

Cheng, W., Lu, J., Lin, W., Wei, X., Li, H., Zhao, X., Jiang, A., Yuan, J., 2018. Effects of a galacto-oligosaccharide-rich diet on fecal microbiota and metabolite profiles in mice. *Food Funct.* 9, 1612–1620. <https://doi.org/10.1039/c7fo01720k>

Clarke, G., Grenham, S., Scully, P., Fitzgerald, P., Moloney, R.D., Shanahan, F., Dinan, T.G., Cryan, J.F., 2013. The microbiome-gut-brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner. *Mol. Psychiatry* 18, 666–673. <https://doi.org/10.1038/mp.2012.77>

Couch, Y., Xie, Q., Lundberg, L., Sharp, T., Anthony, D.C., 2015. A Model of Post-Infection Fatigue Is Associated with Increased TNF and 5-HT_{2A} Receptor Expression in Mice. *PLoS One* 10. <https://doi.org/10.1371/journal.pone.0130643>

Daft, J.G., Ptacek, T., Kumar, R., Morrow, C., Lorenz, R.G., 2015. Cross-fostering immediately after birth induces a permanent microbiota shift that is shaped by the nursing mother. *Microbiome* 3, 1–10. <https://doi.org/10.1186/s40168-015-0080-y>

Ding, T., Schloss, P.D., 2014. Dynamics & Associations of Microbial Organism Types Across the Human Body. *Nature* 509, 357–360. <https://doi.org/10.1038/nature13178>

Eid, R.S., Lieblich, S.E., Duarte-Guterman, P., Chaiton, J.A., Mah, A.G., Wong, S.J., Wen, Y., Galea, L.A.M., 2019. Selective activation of estrogen receptors α and β : Implications for depressive-like phenotypes in female mice exposed to chronic unpredictable stress. *Horm. Behav.* 119, . <https://doi.org/10.1101/758862>

Engin, E., Treit, D., Dickson, C.T., 2009. Anxiolytic- and antidepressant-like properties of ketamine in behavioral and neurophysiological animal models. *Neuroscience* 161, 359–369. <https://doi.org/10.1016/j.neuroscience.2009.03.038>

Frost, G., Sleeth, M.L., Sahuri-Arisoylu, M., Lizarbe, B., Cerdan, S., Brody, L., Anastasovska, J., Ghourab, S., Hankir, M., Zhang, S., Carling, D., Swann, J.R., Gibson, G., Viardot, A., Morrison, D., Louise Thomas, E., Bell, J.D., 2014. The short-chain fatty acid acetate reduces appetite via a central homeostatic mechanism. *Nat Commun* 5, 3611. <https://doi.org/10.1038/ncomms4611>

Grimaldi, R., Gibson, G.R., Vulevic, J., Giallourou, N., Castro-Mejía, J.L., Hansen, L.H., Leigh Gibson, E., Nielsen, D.S., Costabile, A., 2018. A prebiotic intervention study in children with autism spectrum disorders (ASDs). *Microbiome* 6, 1–13. <https://doi.org/10.1186/s40168-018-0523-3>

Grimaldi, R., Swann, J.R., Vulevic, J., Gibson, G.R., Costabile, A., 2016. Fermentation properties and potential prebiotic activity of B-GOS (65% GOS content) on in vitro gut microbiota parameters. *Br. J. Nutr.* 1–7. <https://doi.org/10.1017/S0007114516002269>

Gronier, B., Savignac, H.M., Di Miceli, M., Idriss, S.M., Tzortzis, G., Anthony, D., Burnet, P.W.J., 2017. Increased cortical neuronal responses to NMDA and improved attentional set-shifting performance in rats following prebiotic (B-GOS®) ingestion. *Eur. Neuropsychopharmacol.* <https://doi.org/10.1016/j.euroneuro.2017.11.001>

Guo, J., Lin, P., Zhao, X., Zhang, J., Wei, X., Wang, Q., Wang, C., 2014. Etazolate abrogates the lipopolysaccharide (LPS)-induced downregulation of the cAMP/pCREB/BDNF signaling, neuroinflammatory response and depressive-like behavior in mice. *Neuroscience* 263, 1–14. <https://doi.org/10.1016/j.neuroscience.2014.01.008>

1 Hallam, M.C., Barile, D., Meyrand, M., Bruce German, J., Reimer, R.A., 2014. Maternal high-
2 protein or high-prebiotic-fiber diets affect maternal milk composition and gut microbiota in
3 rat dams and their offspring. *Obesity* 22, 2344–2351. <https://doi.org/10.1002/oby.20849>

4 Heijtz, R.D., Wang, S., Anuar, F., Qian, Y., Bjorkholm, B., Samuelsson, A., Hibberd, M.L.,
5 Forssberg, H., Pettersson, S., 2011. Normal gut microbiota modulates brain development
6 and behavior. *Proc. Natl. Acad. Sci.* 108, 3047–3052.
7 <https://doi.org/10.1073/pnas.1010529108>

8 Hopkins, M.J., Macfarlane, G.T., 2003. Nondigestible Oligosaccharides Enhance Bacterial
9 Colonization Resistance against. *Appl. Environ. Microbiol.* 69, 1920–1927.
10 <https://doi.org/10.1128/AEM.69.4.1920>

11 Hsiao, E.Y., McBride, S.W., Hsien, S., Sharon, G., Hyde, E.R., McCue, T., Codelli, J.A., Chow,
12 J., Reisman, S.E., Petrosino, J.F., Patterson, P.H., Mazmanian, S.K., 2013. Microbiota
13 modulate behavioral and physiological abnormalities associated with neurodevelopmental
14 disorders. *Cell* 155, 1451–1463. <https://doi.org/10.1016/j.cell.2013.11.024>

15 Jašarević, E., Howard, C.D., Misic, A.M., Beiting, D.P., Bale, T.L., 2017. Stress during
16 pregnancy alters temporal and spatial dynamics of the maternal and offspring microbiome
17 in a sex-specific manner. *Sci. Rep.* 7, 1–13. <https://doi.org/10.1038/srep44182>

18 Jiang, Z., Liu, Y., Zhu, Y., Yang, J., Sun, L., Chai, X., Wang, Y., 2016. Characteristic
19 chromatographic fingerprint study of short-chain fatty acids in human milk, infant formula,
20 pure milk and fermented milk by gas chromatography–mass spectrometry. *Int. J. Food Sci.*
21 *Nutr.* 67, 632–640. <https://doi.org/10.1080/09637486.2016.1195798>

22 Johnson, J.S., Spakowicz, D.J., Hong, B.Y., Petersen, L.M., Demkowicz, P., Chen, L., Leopold,
23 S.R., Hanson, B.M., Agresta, H.O., Gerstein, M., Sodergren, E., Weinstock, G.M., 2019.

Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis.
 Nat. Commun. 10, 1–11. <https://doi.org/10.1038/s41467-019-13036-1>

Jurynczyk, M., Probert, F., Yeo, T., Tackley, G., Claridge, T.D.W., Cavey, A., Woodhall, M.R.,
 Arora, S., Winkler, T., Schiffer, E., Vincent, A., DeLuca, G., Sibson, N.R., Isabel Leite, M.,
 Waters, P., Anthony, D.C., Palace, J., 2017. Metabolomics reveals distinct, antibody-
 independent, molecular signatures of MS, AQP4-antibody and MOG-antibody disease. *Acta*
Neuropathol. Commun. 5, 95. <https://doi.org/10.1186/s40478-017-0495-8>

Kao, A.C.C., Spitzer, S., Anthony, D.C., Lennox, B., Burnet, P.W.J., 2018. Prebiotic attenuation
 of olanzapine-induced weight gain in rats: Analysis of central and peripheral biomarkers
 and gut microbiota. *Transl. Psychiatry* 8. <https://doi.org/10.1038/s41398-018-0116-8>

Keleher, M.R., Zaidi, R., Shah, S., Oakley, M.E., Pavlatos, C., Idrissi, S. El, Xing, X., Li, D.,
 Wang, T., Cheverud, J.M., 2018. Maternal high-fat diet associated with altered gene
 expression, DNA methylation, and obesity risk in mouse offspring. *PLoS One* 13, 1–28.
<https://doi.org/10.1371/journal.pone.0192606>

Kelly, J.R., Borre, Y., O’ Brien, C., Patterson, E., El Aidy, S., Deane, J., Kennedy, P.J., Beers,
 S., Scott, K., Moloney, G., Hoban, A.E., Scott, L., Fitzgerald, P., Ross, P., Stanton, C.,
 Clarke, G., Cryan, J.F., Dinan, T.G., 2016. Transferring the blues: Depression-associated
 gut microbiota induces neurobehavioural changes in the rat. *J. Psychiatr. Res.* 82, 109–118.
<https://doi.org/10.1016/j.jpsychires.2016.07.019>

Koren, O., Goodrich, J.K., Cullender, T.C., Spor, A., Laitinen, K., Kling Bäckhed, H., Gonzalez,
 A., Werner, J.J., Angenent, L.T., Knight, R., Bäckhed, F., Isolauri, E., Salminen, S., Ley,
 R.E., 2012. Host remodeling of the gut microbiome and metabolic changes during
 pregnancy. *Cell* 150, 470–480. <https://doi.org/10.1016/j.cell.2012.07.008>

- Kubota, T., Shimojo, N., Nonaka, K., Yamashita, M., Ohara, O., Igoshi, Y., Ozawa, N., Nakano, T., Morita, Y., Inoue, Y., Arima, T., Chiba, K., Nakamura, Y., Ikegami, S., Masuda, K., Suzuki, S., Kohno, Y., 2014. Prebiotic consumption in pregnant and lactating women increases IL-27 expression in human milk. *Br. J. Nutr.* 111, 625–632.
<https://doi.org/10.1017/S0007114513003036>
- Lecerf, J.M., Dépeint, F., Clerc, E., Dugenet, Y., Niamba, C.N., Rhazi, L., Cayzeele, A., Abdelnour, G., Jaruga, A., Younes, H., Jacobs, H., Lambrey, G., Abdelnour, A.M., Pouillart, P.R., 2012. Xylo-oligosaccharide (XOS) in combination with inulin modulates both the intestinal environment and immune status in healthy subjects, while XOS alone only shows prebiotic properties. *Br. J. Nutr.* 108, 1847–1858.
<https://doi.org/10.1017/S0007114511007252>
- Li, M., Li, C., Yu, H., Cai, X., Shen, X., Sun, X., Wang, J., Zhang, Y., Wang, C., 2017. Lentivirus-mediated interleukin-1 β (IL-1 β) knock-down in the hippocampus alleviates lipopolysaccharide (LPS)-induced memory deficits and anxiety- and depression-like behaviors in mice. *J. Neuroinflammation* 14, 1–12. <https://doi.org/10.1186/s12974-017-0964-9>
- Li, Y., Missig, G., Finger, B.C., Landino, S.M., Alexander, A.J., Mokler, E.L., Robbins, J.O., Manasian, Y., Kim, W., Kim, K.-S., McDougale, C.J., Carlezon, W.A., Bolshakov, V.Y., 2018. Maternal and Early Postnatal Immune Activation Produce Dissociable Effects on Neurotransmission in mPFC–Amygdala Circuits. *J. Neurosci.* 38, 3358–3372.
<https://doi.org/10.1523/JNEUROSCI.3642-17.2018>
- Liu, S., Li, E., Sun, Z., Fu, D., Duan, G., Jiang, M., Yu, Y., Mei, L., Yang, P., Tang, Y., Zheng, P., 2019. Altered gut microbiota and short chain fatty acids in Chinese children with autism

spectrum disorder. *Sci. Rep.* 9, 1–9. <https://doi.org/10.1038/s41598-018-36430-z>

Liu, Y., Gibson, G.R., Walton, G.E., 2016. An in vitro approach to study effects of prebiotics and probiotics on the faecal microbiota and selected immune parameters relevant to the elderly. *PLoS One* 11, 1–18. <https://doi.org/10.1371/journal.pone.0162604>

Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15, 1–21. <https://doi.org/10.1186/s13059-014-0550-8>

Martin, S.A., Pence, B.D., Greene, R.M., Johnson, S.J., Dantzer, R., Kelley, K.W., Woods, J.A., 2013. Effects of voluntary wheel running on LPS-induced sickness behavior in aged mice. *Brain. Behav. Immun.* 29, 113–123. <https://doi.org/10.1016/j.bbi.2012.12.014>

McGirr, A., Berlim, M.T., Bond, D.J., Fleck, M.P., Yatham, L.N., Lam, R.W., 2015. A systematic review and meta-analysis of randomized, double-blind, placebo-controlled trials of ketamine in the rapid treatment of major depressive episodes. *Psychol. Med.* 45, 693–704. <https://doi.org/10.1017/S0033291714001603>

Mckee, S.E., Zhang, S., Chen, L., Rabinowitz, J.D., Reyes, T.M., 2018. Perinatal high fat diet and early life methyl donor supplementation alter one carbon metabolism and DNA methylation in the brain. *J. Neurochem.* 362–373. <https://doi.org/10.1111/jnc.14319>

McMurdie, P.J., Holmes, S., 2013. Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS One* 8. <https://doi.org/10.1371/journal.pone.0061217>

Meaney, M.J., 2001. Maternal care, gene expression, and the transmission of individual differences across generations. *Annu Rev Neurosci* 24, 1161–192. <https://doi.org/10.1002/9783527678679.dg04837>

1 Mika, A., Gaffney, M., Roller, R., Hills, A., Bouchet, C.A., Hulen, K.A., Thompson, R.S.,
2 Chichlowski, M., Berg, B.M., Fleshner, M., 2018. Feeding the developing brain: Juvenile
3 rats fed diet rich in prebiotics and bioactive milk fractions exhibit reduced anxiety-related
4 behavior and modified gene expression in emotion circuits. *Neurosci. Lett.* 677, 103–109.
5 <https://doi.org/10.1016/j.neulet.2018.01.052>

6 Minamiyama, M., Katsuno, M., Adachi, H., Waza, M., Sang, C., Kobayashi, Y., Tanaka, F.,
7 Doyu, M., Inukai, A., Sobue, G., 2004. Sodium butyrate ameliorates phenotypic expression
8 in a transgenic mouse model of spinal and bulbar muscular atrophy. *Hum. Mol. Genet.* 13,
9 1183–1192. <https://doi.org/10.1093/hmg/ddh131>

10 Missig, G., Mokler, E.L., Robbins, J.O., Alexander, A.J., McDougale, C.J., Carlezon, W.A., 2018.
11 Perinatal Immune Activation Produces Persistent Sleep Alterations and Epileptiform
12 Activity in Male Mice. *Neuropsychopharmacology* 43, 482–491.
13 <https://doi.org/10.1038/npp.2017.243>

14 Murrough, J.W., Iosifescu, D. V., Chang, L.C., Al Jurdi, R.K., Green, C.E., Perez, A.M., Iqbal,
15 S., Pillemer, S., Foulkes, A., Shah, A., Charney, D.S., Mathew, S.J., 2013. Antidepressant
16 efficacy of ketamine in treatment-resistant major depression: A two-site randomized
17 controlled trial. *Am. J. Psychiatry* 170, 1134–1142.
18 <https://doi.org/10.1176/appi.ajp.2013.13030392>

19 Neufeld, K.M., Kang, N., Bienenstock, J., Foster, J.A., 2011. Reduced anxiety-like behavior and
20 central neurochemical change in germ-free mice. *Neurogastroenterol. Motil.* 23, 255–265.
21 <https://doi.org/10.1111/j.1365-2982.2010.01620.x>

22 Nikniaz, L., Mahdavi, R., Ostadrahimi, A., Hejazi, M.A., Vatankhah, A.M., 2013. Effects of
23 Synbiotic Supplementation on Total Antioxidant Capacity of Human Breastmilk.

Breastfeed. Med. 8, 217–222. <https://doi.org/10.1089/bfm.2012.0078>

O'Connor, J.C., Lawson, M.A., André, C., Moreau, M., Lestage, J., Castanon, N., Kelley, K.W., Dantzer, R., 2009. Lipopolysaccharide-induced depressive-like behavior is mediated by indoleamine 2,3-dioxygenase activation in mice. *Mol. Psychiatry* 14, 511–522. <https://doi.org/10.1038/sj.mp.4002148>

Rea, K., Dinan, T.G., Cryan, J.F., 2016. The microbiome: A key regulator of stress and neuroinflammation. *Neurobiol. Stress* 4, 23–33. <https://doi.org/10.1016/j.ynstr.2016.03.001>

Rincel, M., Aubert, P., Chevalier, J., Grohard, P.A., Basso, L., Monchaux de Oliveira, C., Helbling, J.C., Lévy, É., Chevalier, G., Leboyer, M., Eberl, G., Layé, S., Capuron, L., Vergnolle, N., Neunlist, M., Boudin, H., Lepage, P., Darnaudéry, M., 2019. Multi-hit early life adversity affects gut microbiota, brain and behavior in a sex-dependent manner. *Brain. Behav. Immun.* 80, 179–192. <https://doi.org/10.1016/j.bbi.2019.03.006>

Rogier, E.W., Frantz, A.L., Bruno, M.E.C., Wedlund, L., Cohen, D.A., Stromberg, A.J., Kaetzel, C.S., 2014. Secretory antibodies in breast milk promote long-term intestinal homeostasis by regulating the gut microbiota and host gene expression. *Proc. Natl. Acad. Sci.* 111, 3074–3079. <https://doi.org/10.1073/pnas.1315792111>

Savignac, H.M., Corona, G., Mills, H., Chen, L., Spencer, J.P.E., Tzortzis, G., Burnet, P.W.J., 2013. Prebiotic feeding elevates central brain derived neurotrophic factor, N-methyl-d-aspartate receptor subunits and d-serine. *Neurochem. Int.* 63, 756–764. <https://doi.org/10.1016/j.neuint.2013.10.006>

Savignac, H.M., Couch, Y., Stratford, M., Bannerman, D.M., Tzortzis, G., Anthony, D.C., Burnet, P.W.J., 2016. Prebiotic administration normalizes lipopolysaccharide (LPS)-induced anxiety and cortical 5-HT_{2A} receptor and IL1- β levels in male mice. *Brain. Behav.*

Immun. 52, 120–131. <https://doi.org/10.1016/j.bbi.2015.10.007>

Sharma, S., Taliyan, R., Singh, S., 2015. Beneficial effects of sodium butyrate in 6-OHDA induced neurotoxicity and behavioral abnormalities: Modulation of histone deacetylase activity. *Behav. Brain Res.* 291, 306–314. <https://doi.org/10.1016/j.bbr.2015.05.052>

Sherwin, E., Bordenstein, S.R., Quinn, J.L., Dinan, T.G., Cryan, J.F., 2019. Microbiota and the social brain 2016. <https://doi.org/10.1126/science.aar2016>

Sriram, C.S., Jangra, A., Gurjar, S.S., Mohan, P., Bezbaruah, B.K., 2016. Edaravone abrogates LPS-induced behavioral anomalies, neuroinflammation and PARP-1. *Physiol. Behav.* 154, 135–144. <https://doi.org/10.1016/j.physbeh.2015.10.029>

Stilling, R.M., Ryan, F.J., Hoban, A.E., Shanahan, F., Clarke, G., Claesson, M.J., Dinan, T.G., Cryan, J.F., 2015. Microbes & neurodevelopment - Absence of microbiota during early life increases activity-related transcriptional pathways in the amygdala. *Brain. Behav. Immun.* 50, 209–220. <https://doi.org/10.1016/j.bbi.2015.07.009>

Stilling, R.M., van de Wouw, M., Clarke, G., Stanton, C., Dinan, T.G., Cryan, J.F., 2016. The neuropharmacology of butyrate: The bread and butter of the microbiota-gut-brain axis? *Neurochem. Int.* 99, 110–132. <https://doi.org/10.1016/j.neuint.2016.06.011>

Strandwitz, P., Kim, K.H., Terekhova, D., Liu, J.K., Sharma, A., Levering, J., McDonald, D., Dietrich, D., Ramadhar, T.R., Lekbua, A., Mroue, N., Liston, C., Stewart, E.J., Dubin, M.J., Zengler, K., Knight, R., Gilbert, J.A., Clardy, J., Lewis, K., 2019. GABA-modulating bacteria of the human gut microbiota. *Nat. Microbiol.* 4, 396–403. <https://doi.org/10.1038/s41564-018-0307-3>

Sudo, N., Chida, Y., Aiba, Y., Sonoda, J., Oyama, N., Yu, X.-N.X., Kubo, C., Koga, Y., 2004. Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for

stress response in mice. *J. Physiol.* 558, 263–75.
<https://doi.org/10.1113/jphysiol.2004.063388>

Surzenko, N., Pjetri, E., Munson, C.A., Friday, W.B., Hauser, J., Mitchell, E.S., 2019. Prenatal exposure to the probiotic *Lactococcus lactis* decreases anxiety-like behavior and modulates cortical cytoarchitecture in a sex specific manner. *bioRxiv*.
<https://doi.org/http://dx.doi.org/10.1101/780072>

Thum, C., McNabb, W.C., Young, W., Cookson, A.L., Roy, N.C., 2016. Prenatal caprine milk oligosaccharide consumption affects the development of mice offspring. *Mol. Nutr. Food Res.* 2076–2085. <https://doi.org/10.1002/mnfr.201600118>

Tochitani, S., Ikeno, T., Ito, T., Sakurai, A., Yamauchi, T., Matsuzaki, H., 2016. Administration of non-Absorbable antibiotics to pregnant mice to perturb the maternal gut microbiota is associated with alterations in offspring behavior. *PLoS One* 11, 1–22.
<https://doi.org/10.1371/journal.pone.0138293>

Treichel, N.S., Prevorsek, Z., Mrak, V., Kostrić, M., Vestergaard, G., Foessel, B., Pfeiffer, S., Stres, B., Schöler, A., Schlöter, M., 2019. Effect of the Nursing Mother on the Gut Microbiome of the Offspring During Early Mouse Development. *Microb. Ecol.* 78, 517–527. <https://doi.org/10.1007/s00248-019-01317-7>

Vulevic, J., Juric, A., Tzortzis, G., Gibson, G.R., 2013. A Mixture of trans - Galactooligosaccharides Reduces Markers of Metabolic Syndrome and Modulates the Fecal Microbiota and Immune Function of Overweight Adults 1 – 3. *J. Nutr.* 324–331.
<https://doi.org/10.3945/jn.112.166132.galactooligosaccharides>.

Vulevic, J., Juric, A., Walton, G.E., Claus, S.P., Tzortzis, G., Toward, R.E., Gibson, G.R., 2015. Influence of galacto-oligosaccharide mixture (B-GOS) on gut microbiota, immune

parameters and metabonomics in elderly persons. *Br. J. Nutr.* 114, 586–595.
<https://doi.org/10.1017/S0007114515001889>

Walker, A.K., Budac, D.P., Bisulco, S., Lee, A.W., Smith, R.A., Beenders, B., Kelley, K.W.,
 Dantzer, R., 2013. NMDA Receptor Blockade by Ketamine Abrogates Lipopolysaccharide-
 Induced Depressive-Like Behavior in C57BL/6J Mice. *Neuropsychopharmacology* 38,
 1609–1616. <https://doi.org/10.1038/npp.2013.71>

Wang, D., Lin, W., Pan, Y., Kuang, X., Qi, X., Sun, H., 2011. Chronic blockade of
 glucocorticoid receptors by RU486 enhances lipopolysaccharide-induced depressive-like
 behaviour and cytokine production in rats. *Brain. Behav. Immun.* 25, 706–714.
<https://doi.org/10.1016/j.bbi.2011.01.011>

Williams, S., Chen, L., Savignac, H.M., Tzortzis, G., Anthony, D.C., Burnet, P.W., 2016.
 Neonatal prebiotic (BGOS) supplementation increases the levels of synaptophysin,
 GluN2A-subunits and BDNF proteins in the adult rat hippocampus. *Synapse* 125, 121–125.
<https://doi.org/10.1002/syn.21880>

Xiao, L., Van't Land, B., Engen, P.A., Naqib, A., Green, S.J., Nato, A., Leusink-Muis, T.,
 Garssen, J., Keshavarzian, A., Stahl, B., Folkerts, G., 2018. Human milk oligosaccharides
 protect against the development of autoimmune diabetes in NOD-mice. *Sci. Rep.* 8, 1–15.
<https://doi.org/10.1038/s41598-018-22052-y>

Yamawaki, Y., Yoshioka, N., Nozaki, K., Ito, H., Oda, K., Harada, K., Shirawachi, S., Asano, S.,
 Aizawa, H., Yamawaki, S., Kanematsu, T., Akagi, H., 2018. Sodium butyrate abolishes
 lipopolysaccharide-induced depression-like behaviors and hippocampal microglial
 activation in mice. *Brain Res.* 1680, 13–38. <https://doi.org/10.1016/j.brainres.2017.12.004>

Yang, X.D., Wang, L.K., Wu, H.Y., Jiao, L., 2018. Effects of prebiotic galacto-oligosaccharide

on postoperative cognitive dysfunction and neuroinflammation through targeting of the gut-brain axis. *BMC Anesthesiol.* 18, 1–11. <https://doi.org/10.1186/s12871-018-0642-1>

Ye, W., Pitlock, M.D., Javors, M.A., Thompson, B.J., Lechleiter, J.D., Hensler, J.G., 2018. The long-term effect of maternal dietary protein restriction on 5-HT1A receptor function and behavioral responses to stress in adulthood. *Behav. Brain Res.* 349, 116–124. <https://doi.org/10.1016/j.bbr.2018.03.038>

Zhao, W.X., Zhang, J.H., Cao, J.B., Wang, W., Wang, D.X., Zhang, X.Y., Yu, J., Zhang, Y.Y., Zhang, Y.Z., Mi, W.D., 2017. Acetaminophen attenuates lipopolysaccharide-induced cognitive impairment through antioxidant activity. *J. Neuroinflammation* 14, 1–15. <https://doi.org/10.1186/s12974-016-0781-6>

Zhao, X., Cao, F., Liu, Q., Li, X., Xu, G., Liu, G., Zhang, Y., Yang, X., Yi, S., Xu, F., Fan, K., Ma, J., 2016. Behavioral, inflammatory and neurochemical disturbances in LPS and UCMS-induced mouse models of depression. *Behav. Brain Res.* 1–9. <https://doi.org/10.1016/j.bbr.2017.05.064>

Zheng, N., Gao, Y., Zhu, W., Meng, D., Allan Walker, W., 2020. Short chain fatty acids produced by colonizing intestinal commensal bacterial interaction with expressed breast milk are anti-inflammatory in human immature enterocytes. *PLoS One* 15, 1–15. <https://doi.org/10.1371/journal.pone.0229283>