1   **Title: Antidepressant treatment modulates the gut microbiome and metabolome**
2   **during pregnancy and lactation in rats with a depressive-like phenotype**

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Abstract

Background: Up to 10% of women use selective serotonin reuptake inhibitor (SSRI) antidepressants during and after pregnancy to manage mood disorders, with possible implications for the developing offspring. The microbiota within the gastrointestinal tract contributes to the regulation of serotonin synthesis. However, the interaction between maternal depression, SSRI use, bacterial community composition, and availability of microbiota-derived metabolites during pregnancy and lactation is not clear and may be consequential to the long-term health of mother and offspring. To determine the impact of SSRI treatment on maternal microbial community dynamics, we conducted these studies in a rat model of maternal vulnerability (MV). All MV females are on a background of genetic vulnerability, where rats exposed to early life stress (sMV) develop a depressive-like phenotype. In adulthood, sMV- and control (cMV) females were treated with either the SSRI fluoxetine (FLX) or the vehicle (Veh) throughout pregnancy and lactation. High-resolution 16S ribosomal RNA gene sequencing and targeted metabolomic analysis were used to assess the fecal microbiome and metabolite availability, respectively.

Results: The diversity, structure, and composition of the fecal bacterial community differed between pregnancy and lactation. Shifts in microbiota composition were accompanied by changes in fecal metabolite availability. FLX altered some key features of the transition from pregnancy to lactation, but only in females exposed to early life stress (sMV-Veh vs sMV-FLX). For instance, sMV-FLX females had lower fecal availability of the amino acids serine, proline, and aspartic acid than sMV-Veh females. These metabolite concentrations correlated negatively with the relative abundance of bacterial taxa *Prevotella*, *Ruminococcus*, and *Oscillospira*.

Conclusions: Our studies demonstrate an important relationship between antidepressant use during the perinatal period and maternal fecal metabolite availability in sMV rats, possibly through parallel changes in the maternal gut microbiome. Since maternal microbial metabolites contribute to health outcomes in offspring, insults to the maternal microbiome by SSRIs might have inter-generational consequences.

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51   **Keywords:** Fecal microbiome, fecal metabolome, pregnancy, lactation, depression, SSRI antidepressants,

52   fluoxetine, rat, serotonin transporter, 16s rRNA

Background

54   Dysregulation of the neurotransmitter serotonin in the brain is a widely recognized hallmark of major

55   depressive disorder [1, 2]. Selective serotonin reuptake inhibitor (SSRI) antidepressants target the

56   serotonin transporter (SERT), thereby influencing the serotonergic tone [3]. Serotonin (also referred to as

57   5-hydroxytryptamine or 5-HT) and SERT are particularly well-known for playing crucial regulatory roles

58   in the brain, but they also act in many peripheral tissues. Nearly 95% of the body's serotonin resides

59   within the gastrointestinal tract, where it is mainly produced by enterochromaffin cells [4]. Interestingly,

60   germ-free and antibiotic-treated rodents show lower levels of colonic and serum serotonin. This is

61   reversible by microbial colonization, suggesting an important role for specific members of the gut

62   microbiome in modulating serotonin availability [5–11]. Indeed, microbiota synthesize tryptophan, the

63   precursor of serotonin, and promote serotonin biosynthesis in the host organism [8, 9]. Conversely, host

64   serotonin signaling impacts the gut microbiota composition, as we have shown using rats with reduced or

65   no SERT gene expression [12]. Several studies have found alterations in the gut microbiota composition

66   of patients with major depression versus healthy controls [13, 14]. The same has been observed in rodents

67   with depressive-like symptoms [15]. Moreover, a recent *in vivo* study showed that SSRIs modulate the gut

68   microbiota composition, an effect that may be related to *in vitro* antimicrobial properties of SSRIs [16–

69   20].

70   The maternal gut microbiota synthesizes a variety of metabolites that reach the systemic

71   circulation during pregnancy and lactation, and influence offspring innate immune development [21, 22].

72   Environmental and pharmacological insults disrupt the compositional and functional states of the maternal

73   gut microbiota, as recently demonstrated by several animal studies focusing on stress, diet, and antibiotic

74   use during pregnancy [23–27]. Despite clear evidence that the microbiome interacts with serotonin

75   homeostasis, it is currently unknown whether alterations in serotonin homeostasis, such as those often co-

76   occurring with depression or resulting from SSRI use, affect the maternal gut microbiome during the

77   perinatal period [5, 8–12]. An estimated 7-13% of women suffer from a major depressive disorder in the

78   perinatal period and 1-10% of pregnant women take SSRI antidepressants, further highlighting the

79   necessity to better understand the effect of SSRI medication during this period on the gut microbiota [28–

80   31].

81   We aimed to investigate whether a depressive-like phenotype, SSRI antidepressant treatment, and

82   their combination affect the microbial community composition and function during pregnancy and the

83   postpartum period. To this end, we used a rat model of maternal vulnerability (MV) in combination with

84   early life stress by maternal separation (sMV) or control handling (cMV). In adulthood, sMV females

85   show a lower sucrose preference and lower neural growth factor gene expression in the basolateral

86   amygdala and paraventricular nucleus of the brain than cMV females [32]. Thus, sMV females show

87   anhedonia, which is an endophenotype associated with depressive symptoms. We use sMV rats as a model

88   for depression, allowing us to study the separate effects of the maternal depressive-like phenotype and

89   SSRI use during pregnancy, as well as the combination of both.

90   In the current study, adult female sMV and cMV rats were treated daily with fluoxetine (FLX), a

91   commonly used SSRI, or vehicle (Veh) throughout pregnancy and lactation. There were 4 groups of MV

92   females; the control group that was treated with vehicle (cMV-Veh), the maternally separated group that

93   was treated with vehicle (sMV-Veh), the control group that was treated with fluoxetine (cMV-FLX), and

94   the maternally separated group that was treated with fluoxetine (sMV-FLX). Weekly fecal samples were

95   collected for 16S rRNA gene sequencing. In addition, targeted metabolomic analysis of amino acids,

96   short-chain fatty acids and bile acids was done on a subset of samples. We examined the hypothesis that

97   depressive-like symptoms and antidepressant treatment during pregnancy and lactation affect the maternal

98   fecal microbiome and its functional capacity. Specifically, we hypothesized that (1) pregnancy and

99   lactation have distinct fecal microbial signatures; (2) early life stress and FLX alter the fecal microbial

100   signatures of pregnancy and lactation, and the combination of early life stress and FLX has the most

101   pronounced effect; (3) pregnancy and lactation have distinct fecal metabolic signatures; (4) early life

102   stress and FLX treatment alter the fecal metabolic signatures of pregnancy and lactation, and the

103   combination of early life stress and FLX has the most pronounced effect; and (5) the abundances of

104   microbial taxa correlate with the concentrations of fecal metabolites.

Methods

Experimental animals

107   The animals came from our colony of serotonin transporter knockout (SERT$^{-/-}$, Slc6a41^{Hubr}) Wistar rats at

108   the University of Groningen (Groningen, the Netherlands), and were derived from outcrossing SERT$^{+/-}$

109   rats [33]. Animals were supplied *ad libitum* with standard lab chow (RMH-B, AB Diets; Woerden, the

110   Netherlands) and water and were kept on a 12:12 h light-dark cycle (lights off at 11:00 h), with an ambient

111   temperature of 21 ± 2 °C and humidity of 50 ± 5%. Cages were cleaned weekly, and animals were

112   provided with a wooden stick for gnawing (10x2x2cm) and nesting material (Enviro-dri™, Shepherd

113   Specialty Papers, Richland, MI, USA). During pregnancy, females were housed individually in type III

114   Makrolon cages (38.2x22.0x15.0cm) and stayed with their pups until postnatal day (PND)21. Animals

115   were weaned at PND21 and group housed in same-sex cages of 3-5 animals in type IV

116   (55.6x33.4x19.5cm) Makrolon cages. Genotyping of the animals was performed as described previously

117   [12]. All experimental procedures were approved by the Institutional Animal Care and Use Committee of

118   The University of Groningen and were conducted in agreement with the Law on Animal Experiments of

119   The Netherlands.

Early life stress by maternal separation in a rat model of maternal vulnerability

121   Our rat model of maternal vulnerability (MV) consists of heterozygous SERT knockout (SERT$^{+/-}$) female

122   rats. MV rats show 40-50% lower SERT gene expression levels than wildtypes [34]. These levels are

123   similar to those of a considerable portion of the human population, who carry at least one short allele of

124   the SERT gene [35]. When human carriers of this allele experience severe stressful life events, they are at

125   higher risk of developing symptoms of depression [36]. We mimic this gene-by-environment interaction

126   contributing to depression in MV rats by exposing them to early life stress [37]. The early life stress

127   protocol was conducted as previously described [12]. In short, SERT$^{+/-}$ females were mated with SERT$^{+/-}$

128   males (F0). Females were left undisturbed until delivery, which was defined as PND0. From PND2 to

129   PND15, pups were either separated from the dam for 6 hours per day, or handled 15 minutes per day

130   (Figure 1a). The SERT$^{+/-}$ female pups from these nests (F1) then matured to become the sMV- and cMV

131   females.

Breeding and fluoxetine treatment

133   sMV and cMV females were mated with wildtype males. Females were between the age of 3 and 6

134   months, and mated when in estrus (checked with a disinfected impedance meter, model MK-11,

135   Muromachi, Tokyo, Japan). This was termed gestational day (GD)0. The males were removed after 24

136   hours, and the females stayed isolated in a type III cage. Throughout pregnancy and lactation, from GD1

137   until PND21, the dams were weighed and received an oral gavage of either 10 mg/kg fluoxetine (FLX,

138   Fluoxetine 20 PCH, Pharmachemie BV, the Netherlands) or vehicle daily at 11:00AM (Figure 1b).

139   Methylcellulose (MC; Sigma Aldrich Chemie BV, Zwijndrecht, the Netherlands) was used for vehicle

140   injections since it is the constituent of the fluoxetine capsule. FLX (5 mg/mL) and MC (1%) solutions

141   were prepared with autoclaved water. The gavage volume ranged from 0.9 mL to 2.0 mL (depending on

142   body weight). Animals were treated orally by gently picking up the animal without restraint, and using

143   flexible PVC feeding tubes (40 cm length, Vygon, Valkenswaard, the Netherlands) in order to minimize

144   discomfort and stress in the animals. In total, 4 groups of MV females were sampled for this study: cMV-

145   Veh (N=20), sMV-Veh (N=13), cMV-FLX (N=34) and sMV-FLX (N=25). However, the final number of

146   animals selected for 16S rRNA gene sequencing for this study were 11 cMV-Veh, 8 sMV-Veh, 7 cMV-

147   FLX and 6 sMV-FLX. Because littermates can share microbiotas, no females from the same litter were

148   used within each group.

Fecal sample collection

150   Fecal samples to be sequenced were collected at GD0 (before conception), GD7, GD14, PND2, PND7,

151   PND14 and PND21 (Figure 1b). We chose not to collect samples closer to the day of giving birth to not

152   induce unnecessary stress. The samples from GD7 and GD14 were grouped as pregnancy and the samples

153   from PND7, PND14 and PND21 as lactation, since we observed no significant differences within these

154   periods in terms of alpha diversity, beta diversity, and composition of the microbiome (data not shown), as

155   was also shown in a previous human study [38]. Conversely, the samples from PND2 were left out of the

156   analysis, since they did not fit either category (data not shown). The samples were freshly collected

157   directly from the animal between 10:00AM and 11:00AM, and precautions were taken to minimize

158   sample contamination, such as the use of gloves and disinfection of work surfaces. The samples were

159   placed in clean 2.0 mL Safe-Lock Eppendorf tubes (Nijmegen, The Netherlands), snap frozen in liquid

160   nitrogen, and stored at -80 °C.

16S rRNA gene sequencing and analysis

162   Genomic DNA was isolated from fecal pellets using the PSP® Spin Stool DNA Kit (STRATEC

163   Molecular GmbH, Berlin, Germany), according to the manufacturer's instructions for difficult to lyse

164   bacteria. About 100-200 mg fecal matter per sample was used for the DNA isolation; the remainder of the

165   sample was stored for metabolite measurements. A dual-index sequencing strategy targeting the V4 region

166   of the 16S rRNA gene was employed, using barcoded primers for the Illumina platform, as described

167   previously [39]. Library concentration was quantified using Qubit and an additional primer dimer clean-up

168   step was conducted using the AMPure XP beads according to the manufacturer's protocol (Beckman

169   Coulter, Brea, CA, USA). Sequencing was executed on a MiSeq instrument (Illumina, San Diego, CA)

170   using 250 base paired-end chemistry at the University of Pennsylvania Next Generation Sequencing Core.

171   For quality control purposes, a sample of the Human Microbiome Project Mock Community was included

172   as a positive control, and water as a negative control. Quality filtering and chimera checking yielded

173   7,867,454 quality-filtered sequences with a mean ± SD depth of ± 2560 reads per sample. 16S rRNA gene

174   analysis was performed using mothur [40] and QIIME version 1.9.1 (Quantitative Insights Into Microbial

175   Ecology [41]) as described previously [23]. In short, Operational Taxonomic Units (OTUs) were defined

176   with 97% sequence similarity using clustering method CD-HIT. The samples were rarified to 1,000

177   sequences per sample before calculating diversity metrics. The Shannon diversity index was used to

178   calculate alpha diversity, and weighted UniFrac distances were used to calculate beta diversity.

Statistical analysis

180   To identify the fecal microbial OTUs that differentiate between pregnancy and lactation, the machine-

181   based learning algorithm Random Forests was used in R version 3.4.1 as previously described [23, 42–

182   44]. OTU importance was ranked by the percent increase in prediction error of the model as a result of

183   removal of that particular OTU from the model. The resulting model, derived from cMV-Veh samples,

184   was then applied to OTU tables from the other groups, to see how early life stress, FLX and their

185   combination alter the microbial signature of pregnancy and lactation. The R package "gplots" was used to

186   plot OTU relative abundances as heat maps [45]. To predict the metabolic capacity of the gut microbiome

187   during pregnancy and lactation, a computational approach known as Phylogenetic Investigation of

188   Communities by Reconstruction of Unobserved States (PICRUSt [46]) was used. This algorithm uses the

189   16S rRNA data to predict the total metagenomic content, and these metagenomes are then mapped onto

190   functional Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in order to predict the full

191   functional capacity of the microbial community, as previously described [23]. Random Forests were

192   applied to the dataset of predicted metabolic pathways to identify those that shift from pregnancy to

193   lactation in the cMV-Veh group. Then, the impact of early life stress, FLX, and their combination on the

194   proportional representation of these predicted microbial metabolic pathways was plotted in a heat map.

Targeted metabolomics

To draw associations between bacterial community composition, predicted gut microbial metabolic capacity, and gut metabolite output, fecal metabolites were quantified in a subset of samples. GD7 was chosen as a representative time point for pregnancy, and PND7 for lactation. Fecal samples weighing about 300 mg from 5 randomly selected animals per treatment group were used (N=36 in total). Targeted metabolomics was performed by the Microbial Culture and Metabolomics Core as part of the PennCHOP Microbiome Program at the University of Pennsylvania via ultra-performance liquid chromatography (Acquity UPLC system, Waters Corporation, Milford, MA). Amino acid concentrations were quantified with an AccQ-Tag Ultra C18 1.7 μm 2.1x100mm column and a photodiode array detector. Analysis was performed using the UPLC AAA H-Class Application Kit (Waters Corporation, Milford, MA) according to manufacturer's instructions. The limit of detection was 1 nmol/g stool. Bile acid concentrations were measured using a Cortecs UPLC C-18+ 1.6 mm 2.1 x 50 mm column, a QDa single quadrupole mass detector and an autosampler (192 sample capacity). The flow rate was 0.8 mL/min, the injection volume was 4 μL, the column temperature was 30°C, the sample temperature was 4°C, and the run time was 4 min per sample. Eluent A was 0.1% formic acid in water, eluent B was 0.1% formic acid in acetonitrile, the weak needle wash was 0.1% formic acid in water, the strong needle wash was 0.1% formic acid in acetonitrile, and the seal wash was 10% acetonitrile in water. The gradient was 70% eluent A for 2.5 minutes, gradient to 100% eluent B for 0.6 minutes, and then 70% eluent A for 0.9 minutes. The mass detection channels were: +357.35 for chenodeoxycholic acid and deoxycholic acid; +359.25 for lithocholic acid; +385.38 for obeticholic acid; +514.37 for glycoobeticholic acid; +528.39 for tauroobeticholic acid; -407.5 for cholic, alphamuricholic, betamuricholic, gamma muricholic, and omegamuricholic acids; -432.5 for glycolithocholic acid; -448.5 for glycochenodeoxycholic and glycodeoxycholic acids; -464.5 for glycocholic acid; -482.5 for taurolithocholic acid; -498.5 for taurochenodeoxycholic and taurodeoxycholic acids; and -514.4 for taurocholic acid. Samples were quantified against standard curves of at least five points run in triplicate. The range of the assay was at least 50 nM – 10,000 nM; the limit of detection was < 50 nM, and; the limit of quantitation was >10,000

221   nM. The limit of detection was 0.5 nmol/g stool. Finally, short-chain fatty acids were quantified using a

222   HSS T3 1.8 μm 2.1x150 mm column with a photodiode array detector and an autosampler (192 sample

223   capacity). The flow rate was 0.25 mL/min, the injection volume was 5 μL, the column temperature was

224   40°C, the sample temperature was 4°C, and the run time was 25 min per sample. Eluent A was 100 mM

225   sodium phosphate monobasic, pH 2.5; eluent B was methanol; the weak needle wash was 0.1% formic

226   acid in water; the strong needle wash was 0.1% formic acid in acetonitrile; the seal wash was 10%

227   acetonitrile in water. The gradient was 100% eluent A for 5 min, gradient to 70% eluent B from 5-22 min,

228   and then 100% eluent A for 3 min. The photodiode array was set to read absorbance at 215 nm with 4.8

229   nm resolution. Samples were quantified against standard curves of at least five points run in triplicate.

230   Concentrations in the samples were calculated as the measured concentration minus the internal standard.

231   The limit of detection was 1 nmol/g stool.

232   **Analysis of metabolomics data**

233   All metabolites that were detected in at least 40% of samples were included in the analysis. For these

234   metabolites, missing values were replaced by half the minimum positive value found for that metabolite,

235   assuming that missing values were the result of the concentration being below the detection limit. Random

236   Forests were applied as described above to the metabolite concentration table from the cMV-Veh samples

237   in order to reveal which individual metabolites were overrepresented during pregnancy or during lactation.

238   The resulting model was then applied to the data from the treatment groups, and heat maps were

239   generated. For predictive analysis, the open-access, online platform MetaboAnalyst 3.0 was used for

240   pathway analysis based on the metabolomics data [47]. Metabolite Set Enrichment Analysis, a

241   metabolomic version of Gene Set Enrichment Analysis, was used to investigate which sets of functionally

242   related metabolites differ between pregnancy and lactation per treatment group [48].

243   **Additional statistical methods**

244   Microbial alpha diversity was analyzed using a two-way ANOVA with period (pregnancy vs lactation)

245   and treatment group as between subject factors. Uncorrected Fisher's LSD was used for group

246   comparisons within periods (cMV-Veh vs sMV-Veh, cMV-Veh vs cMV-FLX, cMV-Veh vs sMV-FLX,

247   sMV-Veh vs sMV-FLX, cMV-FLX vs sMV-FLX within pregnancy and within lactation). Permutational

248   multivariate analysis of variance using distance matrices (PERMANOVA) was used to analyze effects of

249   pregnancy vs lactation and treatment group on weighted UniFrac distances [49]. PERMANOVA p-values

250   were based on 999 Monte Carlo simulations. Multiple comparisons of OTU relative abundances and

251   metabolite concentrations within pregnancy and within lactation between treatment groups were done by

252   nonparametric Kruskal-Wallis tests followed by uncorrected Dunn's tests (cMV-Veh vs sMV-Veh, cMV-

253   Veh vs cMV-FLX, cMV-Veh vs sMV-FLX, sMV-Veh vs sMV-FLX, cMV-FLX vs sMV-FLX within

254   pregnancy and within lactation). All of the OTUs and metabolites analyzed in this way differed between

255   pregnancy and lactation in the cMV-Veh group, following from the Random Forests analysis; further

256   statistics between periods were not conducted. A t-test was used to analyze the GD0 alpha diversity

257   difference between cMV and sMV females. For the abovementioned tests, GraphPad Prism version 7

258   (GraphPad Software, Inc., San Diego, CA) was used. The statistical significance is indicated as follows:

259   *$p<0.05$; ** $p<0.01$; and *** $p<0.001$, **** $p<0.0001$. Data are presented as mean ± SEM, except OTU

260   relative abundance which is plotted as median ± IQR.

261   **Results**

262   We examined the hypothesis that depressive-like symptoms and antidepressant treatment during

263   pregnancy and lactation affect the maternal fecal microbiome composition and function. Maternal

264   vulnerability (MV) rats were used, since early life stress produces depressive-like symptoms and shifts in

265   fecal microbiome community composition in these animals [12, 32]. In short, MV females exposed to

266   either maternal separation or control handling for the first 2 weeks of life were treated with fluoxetine or

267   vehicle daily throughout gestation and the postpartum period (Figure 1a,b). Thus, there were four groups

268   of MV females: control (cMV-Veh), maternally separated and vehicle-treated (sMS-Veh), fluoxetine-

269   treated (cMV-FLX) and maternally separated and fluoxetine-treated (sMV-FLX). It should be noted that

270   we have been confronted with a number of unexpected and thus far unexplained deaths of some MV

271   females as a result of fluoxetine treatment. About 25% of FLX-treated females died, even though there

272   was no accumulation of fluoxetine or norfluoxetine levels in the blood over the course of treatment (data

273   not shown). However, the individual females used for the current study did not show any abnormal

274   response to FLX. Fecal pellets for 16s rRNA sequencing and analysis were collected during pregnancy at

275   gestational day (GD)7, GD14, and during lactation at postnatal day (PND)7, PND14 and PND21. A subset

276   of samples was used for targeted metabolomic analysis (Figure 1b).

277   **Pregnancy and lactation have distinct fecal microbial signatures**

278   To examine whether pregnancy and lactation have distinct fecal microbial signatures, we first analyzed the

279   16S rRNA sequencing data of pregnancy versus lactation in terms of microbial alpha diversity and

280   community structure over all 4 groups. Alpha diversity was higher during pregnancy than during lactation,

281   as shown by the Shannon diversity index (Figure 2a, $p<0.0001$). The differences in phylogenetic structure

282   between samples were explored by assessing weighted UniFrac distances. A separation between samples

283   obtained during pregnancy versus lactation was revealed by Principle Coordinates Analysis (PCoA)

284   (Figure 2b, PERMANOVA $p=0.001$).

285   In order to identify the bacterial signatures that underlie the observed shift in community structure

286   between pregnancy and lactation, a machine-learning approach called Random Forests was used to

287   analyze the data of the cMV-Veh group. This generated a model containing 28 Operational Taxonomic

288   Units (OTUs) at the genus-level, discriminating between pregnancy and lactation. A large reorganization

289   of the microbiome composition from gestation to lactation was revealed, consistent with structural

290   remodeling (Figure 2c; Supplemental Figure 1a). Particularly within the *Bacteroidales* and *Clostridiales*

291   orders, OTUs changed in their relative abundance from pregnancy to lactation (Figure 2c; Supplemental

292   Figure 1a).

Fluoxetine treatment alters the maternal fecal bacterial signatures of pregnancy and lactation in a rat model for depressive-like behavior

To test whether early life stress, FLX, and their combination alter the observed fecal microbial signatures of pregnancy and lactation as observed in the cMV-Veh group, we first compared the groups with respect to alpha- and beta diversity measures. We observed pre-conception differences between the sMV- and cMV group, with the sMV group showing higher alpha diversity than the cMV group (Supplemental Figure 2a, $p<0.05$), coinciding with differences in OTU relative abundance (Supplemental Figure 2b). During pregnancy and lactation there was a similar pattern for the sMV-Veh but also the cMV-FLX and sMV-FLX groups; the mean Shannon diversity of the fecal microbial communities of the treatment groups tended to be higher than that of the cMV-Veh group. However, this was only statistically significant for the sMV-FLX group during pregnancy and the sMV-Veh group during lactation (Figure 2a, pregnancy cMV-Veh vs sMV-Veh $p=0.09$; vs cMV-FLX $p=0.25$; vs sMV-FLX $p<0.05$ | lactation cMV-Veh vs sMV-Veh $p<0.001$; vs cMV-FLX $p=0.06$; vs sMV-FLX $p=0.31$). The treatment groups dissociated in terms of community structure, based on PCoA of the weighted UniFrac distances (Supplemental Figure 1b, PERMANOVA $p=0.001$). In particular, microbial community structure in the sMV-FLX group differed from the structure in the sMV-Veh group, both during pregnancy and lactation (Figure 1d, pregnancy sMV-Veh vs sMV-FLX PERMANOVA $p<0.05$ | lactation sMV-Veh vs sMV-FLX PERMANOVA $p=0.001$). This suggested that, in addition to the dynamic restructuring of the fecal microbiome across pregnancy and lactation, fluoxetine treatment modulated community structure of the fecal microbiota in the sMV females during these periods.

Next, the relative abundance of the 28 OTUs constituting the Random Forests-generated model discriminating between pregnancy and lactation in the cMV-Veh group was examined for the treatment groups. Globally, the pattern of OTUs distinguishing between pregnancy and lactation in the treatment groups was similar to the cMV-Veh group (Figure 2c). However, specific OTUs showed variations in their relative abundance within pregnancy and/or lactation (Figure 2e; Supplemental Figure 1c). The 2 OTUs

318   with the largest contribution to the accuracy of the Random Forests model, *Bacteroides* and *Prevotella*

319   from the *Bacteroidales* order, were sensitive to FLX treatment. Specifically, both the cMV-FLX and

320   sMV-FLX had a lower relative abundance of *Bacteroides* than the cMV-Veh group during pregnancy, and

321   the sMV-FLX group had a lower relative abundance of *Bacteroides* than the sMV-Veh group during

322   lactation (Figure 2e, pregnancy cMV-Veh vs cMV-FLX $p<0.01$; vs sMV-FLX $p<0.05$ | lactation sMV-

323   Veh vs sMV-FLX $p<0.05$). On the other hand, the sMV-FLX group had a higher relative abundance of

324   *Prevotella* than the sMV-Veh group during pregnancy and relative to the cMV-Veh, sMV-Veh and cMV-

325   FLX groups during lactation, reaching almost 25% of the total sample in lactation (Figure 2e, pregnancy

326   sMV-Veh vs sMV-FLX $p<0.05$ | lactation cMV-Veh vs sMV-FLX $p<0.05$, sMV-Veh vs sMV-FLX

327   $p<0.01$, cMV-FLX vs sMV-FLX $p<0.05$). Early life stress alone did not change the abundance of many

328   OTUs relative to group that was control handled, although *Ruminococcus* was lower in the sMV-Veh

329   group than in the cMV-Veh group during lactation (Figure 2e, lactation cMV-Veh vs sMV-Veh $p<0.05$).

330   This effect was reversed by FLX, as the sMV-FLX had a higher relative abundance than cMV-Veh and

331   sMV-Veh during pregnancy, and a higher relative abundance than sMV-Veh during lactation (Figure 2e,

332   pregnancy cMV-Veh/ sMV-Veh vs sMV-FLX $p<0.001$ | lactation sMV-Veh vs sMV-FLX $p<0.01$).

333   Considering that OTUs within a community display co-occurrence and co-exclusion relationships,

334   we performed a correlation analysis to look at these relationships within our samples [50]. A correlation

335   matrix between the 28 selected OTUs revealed that *Prevotella* was negatively correlated with a cluster

336   consisting mainly of *Clostridiales* (Supplemental Figure 1d). *Ruminoccus* was the exception within the

337   *Clostridiales*, being positively correlated with *Prevotella* (Supplemental Figure 1d).

338   Together, these results confirm that pregnancy and lactation are distinct with regard to fecal

339   microbiota diversity, structure, and composition. Early life stress increased alpha diversity during

340   lactation, as was the case pre-conception. There were no profound effects of early life stress alone on

341   either microbial community structure or on the relative abundance of OTUs resulting from our Random

342   Forests model. However, FLX impacted community structure during pregnancy and lactation, as well as

343   the relative abundance of a number of OTUs selected for their ability to distinguish between pregnancy

344   and lactation. FLX in combination with early life stress had the largest effects on the maternal

345   microbiome.

Pregnancy and lactation have distinct fecal metabolic signatures

347   Bacterial communities metabolize and synthesize a number of metabolites that are essential for both dam

348   and offspring. Shifts in bacterial community composition and predictive function may suggest alterations

349   in the capacity of these communities to synthesize or metabolize these metabolites. We first performed

350   PICRUSt predictive analysis on the OTU data to explore the hypothesis that the functional potential of

351   pregnancy and lactation is different, since they have different microbial signatures. To determine which

352   distinct predictive metabolic pathways discriminate between pregnancy and lactation, Random Forests

353   analysis was performed on PICRUSt-generated data from the cMV-Veh group. Nine KEGG pathways

354   were significantly changed across pregnancy and lactation, including the pathway of lysine biosynthesis;

355   the pathway of alanine, aspartate, and glutamate metabolism; and the pathway of arginine and proline

356   metabolism (Figure 3a; Supplemental Figure 3a). Overall, pathways related to amino acid synthesis and

357   metabolism were among the metabolic pathways best able to characterize the difference in the predicted

358   function of the maternal bacterial community during pregnancy vs lactation.

359   To determine whether this altered predictive metabolic capacity of the microbiome was associated

360   with actual metabolite availability, we measured the concentration of amino acids, bile acids, and short-

361   chain fatty acids in a subset (N=4-5 per group in pregnancy and lactation) of fecal pellets. Corresponding

362   to the microbiome results, principle component analysis (PCA) revealed a clear distinction in metabolic

363   profile between samples obtained during pregnancy versus lactation (Figure 3b, upper graph).

364   To reveal the metabolic signatures of pregnancy and lactation, Random Forests analysis was

365   performed on the metabolite data from the cMV-Veh group. Fourteen metabolites were included in the

366   resulting model to distinguish between pregnancy and lactation (Supplemental Figure 3b). Most of them

367   were amino acids, and 11 out of 14 metabolites were higher in concentration during pregnancy than during

368   lactation (Figure 3c; Supplemental Figure 3c). For instance, the amino acids isoleucine and methionine

369 had higher concentrations during pregnancy than during lactation, while proline concentrations were

370 higher during lactation than during pregnancy (Figure 3c; Supplemental Figure 3c). The bile acids

371 deoxycholic acid and taurochenodeoxycholic acid were both higher in concentration during pregnancy

372 than during lactation (Figure 3c; Supplemental Figure 3c). Of the short-chain fatty acids included in the

373 Random Forests model, isocaproic acid concentrations were very low during pregnancy, whereas

374 isovaleric acid concentrations decreased during lactation (Figure 3c; Supplemental Figure 3c).

375 Next, we used Metabolite Set Enrichment Analysis (MSEA) to identify the fecal metabolite sets

376 that were enriched during either pregnancy or lactation. In the cMV-Veh group, there were several

377 pathways that significantly differed between these two periods, again containing amino acid-related

378 pathways such as glycine and serine metabolism (Figure 3e; Supplemental Figure 3e).

379 **Fluoxetine treatment alters maternal fecal metabolite availability during pregnancy**

380 **and lactation in a rat model for depressive-like behavior**

381 To investigate whether early life stress, FLX and their combination affect the abovementioned fecal

382 metabolic signatures of pregnancy and lactation as observed in the cMV-Veh group, we compared these

383 groups with respect to predicted- and measured metabolic data. The effects of early life stress and FLX on

384 the predictive pathways were mixed, with the sMV-FLX group having generally the lowest predicted

385 abundance of these metabolic pathways (Figure 3a). Visual inspection of the PCA graph based on the

386 metabolite concentration table revealed no clear effect of early life stress or FLX treatment (Figure 3b,

387 lower graph).

388 Next, the concentrations of the 14 metabolites included in the Random Forests-generated model

389 discriminating between pregnancy and lactation in the cMV-Veh group were examined for the treatment

390 groups. Similar to the microbiome results, the overall pattern of metabolite availability changes between

391 pregnancy and lactation was similar in all groups (Figure 3c). However, again, specific metabolites

392 showed variations in their concentration within pregnancy and/or lactation (Figure 3d; Supplemental

393 figure 3c). While there were no significant differences between the cMV-Veh and sMV-Veh group for any

394   metabolites, the sMV-FLX group had low fecal concentrations of some amino acids (Figure 3d).

395   Concentrations of serine were lower in the sMV-FLX group than in the sMV-Veh group during

396   pregnancy, and for proline and aspartic acid this was the case during lactation (Figure 3d, serine

397   pregnancy sMV-Veh vs sMV-FLX $p<0.01$, proline lactation cMV-Veh/ sMV-Veh vs sMV-FLX $p<0.05$,

398   aspartic acid lactation sMV-Veh vs sMV-FLX $p<0.05$). From the metabolites that were not selected by the

399   Random Forests model as distinguishing between pregnancy and lactation, the concentration of the bile

400   acids betamuricholic acid and omegamuricholic acid were decreased during pregnancy in the sMV-FLX

401   group compared to the cMV-Veh group and both the cMV-Veh- and the sMV-Veh group, respectively

402   (Supplemental Figure 3d, betamuricholic acid pregnancy cMV-Veh vs cMV-FLX/sMV-FLX $p<0.05$,

403   omegamuricholic acid pregnancy cMV-Veh/sMV-Veh vs sMV-FLX $p<0.05$). Similarly, the short-chain

404   fatty acid isobutyric acid was decreased in the sMV-FLX group relative to the Ctrl- and the sMV-Veh

405   group during lactation (Supplemental Figure 3d, cMV-Veh/sMV-Veh vs sMV-FLX $p<0.05$).

406   Then, we performed MSEA on the sMV-Veh, cMV-FLX and sMV-FLX data to determine which

407   metabolites involved in specific metabolic processes were enriched in either pregnancy or lactation. The

408   sMV-Veh group, like the cMV-Veh group, contained a number of metabolite sets that distinguished

409   between pregnancy and lactation, such as methionine metabolism, and glycine and serine metabolism

410   (Figure 3e; Supplemental Figure 3e). In contrast, in the cMV-FLX and sMV-FLX groups no metabolite

411   sets were identified that differed significantly between pregnancy and lactation (Figure 3e; Supplemental

412   Figure 3e).

413   Overall, the metabolomic results were consistent with the microbiome findings and the predictive

414   functional analysis in discriminating between pregnancy and lactation and pointing towards amino acids

415   as being central to this shift. Fluoxetine treatment lowered the fecal concentrations of several amino acids

416   during these dynamic periods in our model for depressive-like symptoms. In addition, fluoxetine treatment

417   abolished the differences in MSEA-identified pathways that distinguished the fecal metabolome during

418   pregnancy from lactation as observed in the vehicle-treated groups.

419 **The relative abundances of fecal bacterial taxa correlate with metabolite**

420 **concentrations**

421   Finally, we generated a correlation matrix to further explore the link between the variation in the

422   composition of the microbiome and variation in the availability of metabolites. A cluster consisting of

423   OTUs from the *Clostridiales* (including *Oscillospira*, *Clostridia*;other and UC *Peptococcaceae*) and

424   *Desulfovibrionales* (*Desulfovibrio* and UC *Desulfovibrionaceae*) orders was positively correlated with

425   methionine, and some of these OTUs also to other amino acids such as alanine, isoleucine glycine, lycine,

426   and threonine (Figure 3f). Showing almost the reverse pattern, the relative abundance of *Prevotella* was

427   negatively correlated with availability of isobutyric acid and a collection of amino acids; alanine,

428   isoleucine, glycine, methionine, and serine (Figure 3f). Associating to a different set of metabolites, the

429   relative abundance of *Ruminococcus* was inversely correlated with concentrations of aspartic acid and

430   some bile acids; betamuricholic acid, omegamuricholic acid, and alphamuricholic acid (Figure 3f).

431   Overall, the OTUs that were most affected in relative abundance by FLX treatment (*Prevotella* and

432   *Ruminococcus*, among others) are correlated with amino acid availability (serine, proline and aspartic

433   acid, among others).

434 **Discussion**

435   Our study shows that antidepressant treatment during pregnancy and lactation disrupts metabolite

436   availability in a rat model for maternal depressive-like symptoms, possibly through alterations in bacterial

437   community composition and function. In short, maternal vulnerability (MV) female rats were exposed to

438   early life stress (sMV) in the form of maternal separation. In adulthood, sMV and control (cMV) females

439   were treated throughout gestation and lactation with fluoxetine (FLX), a commonly prescribed selective

440   serotonin reuptake inhibitor (SSRI), or vehicle (Veh). Fecal samples were collected during pregnancy and

441   lactation, and 16s rRNA sequencing and targeted metabolomics were performed on these samples.

442   We first identified the distinct fecal microbial signatures of pregnancy and lactation in cMV-Veh

443   females. We showed that the gut microbiome alpha diversity was relatively high during pregnancy, and

444   dropped during lactation. In addition, principal coordinates analysis revealed a shift in the structure of the

445   fecal microbiome from pregnancy to lactation. Using a machine-learning procedure called Random

446   Forests we identified the set of microbial taxa that was overrepresented in either pregnancy or lactation.

447   The resulting model, including 28 OTUs, characterized the shift in the maternal gut bacterial community

448   between pregnancy and lactation. Twenty-three OTUs in the resulting model were higher in abundance

449   during pregnancy than during lactation. In the literature there are several reports describing the changes in

450   the maternal microbiome over the course of gestation in humans and rodents [23, 38, 51]. We extend these

451   studies to define distinct microbial signatures associated with pregnancy and lactation.

452   Studies have shown that the mere presence of bacteria, but also the specific community

453   composition in the gut greatly affects plasma and fecal metabolite availability [52, 53]. To begin to

454   explore the hypothesis that, since pregnancy and lactation have different microbial signatures, their

455   functional capacity is also different, we performed PICRUSt predictive analysis on the OTU data. Using

456   Random Forests to determine the set of metabolic pathways that was best able to characterize the

457   difference between pregnancy and lactation, we identified a set of 9 KEGG pathways, a large proportion

458   of which was related to amino acid synthesis and metabolism. Gut microbes utilize metabolites as fuel, but

459   also modify and produce short-chain fatty acids, bile acids and amino acids that are used by the host [54,

460   55]. We measured the concentrations of these metabolites in a subset of fecal samples. Random Forests

461   analysis identified a set of 14 metabolites that best characterized the difference in fecal metabolite

462   availability between pregnancy and lactation in cMV-Veh rats. Most of these metabolites – 11 out of 14 –

463   were higher in concentration during pregnancy than during lactation, reflecting either greater intake or

464   production of these metabolites during pregnancy, or more breakdown or transfer to the offspring during

465   lactation. Maternal (bacterial) metabolites have been shown to transfer to the developing offspring through

466   placental transfer and mother milk and play crucial roles in development [22, 56–58]. We postulate that

467   the observed shift in metabolic profile from pregnancy to lactation is an adaptive one, aiding the pregnant

468   and lactating female in providing nutrients and metabolites to the developing offspring.

469   We then hypothesized that early life stress and FLX alter the fecal microbial signatures of

470   pregnancy and lactation, and that the combination of early life stress and FLX produces the most

471   pronounced effect. An estimated 7-13% of women are diagnosed with a major depressive disorder in the

472   perinatal period [28, 29]. Numerous studies have identified differences between the gut microbiome of

473   depressed patients or depressive-like rodents, and healthy controls [13–15]. In this study, we used sMV

474   females as a rat model with a depressive-like phenotype [32]. It has been shown repeatedly that maternal

475   separation in rodents produces long-term effects not only on behavior, immune function and neurobiology,

476   but also on the gut microbiome [59–61]. Correspondingly, our lab showed previously that maternal

477   separation exacerbated the differences in the gut microbiome composition in young rats with a

478   heterozygous or complete knockout of SERT compared to wildtypes [12]. Consistent with these results,

479   we showed here that adult sMV females had a higher fecal alpha diversity than cMV females pre-

480   conception, during pregnancy and during lactation [32]. However, maternal separation did not affect the

481   structure and composition of the microbiome during pregnancy and lactation. It seems that the shift in

482   maternal microbial community structure and composition from pregnancy to lactation is robust enough to

483   withstand major perturbations by our early life stress protocol. In addition, no significant differences were

484   found between the cMV-Veh and sMV-Veh groups in any metabolite concentration.

485   However, the SSRI fluoxetine was associated with differences in the maternal fecal microbiome

486   and metabolic output in sMV rats. Around 1-10% of women use SSRI antidepressants during pregnancy

487   [30, 31]. Medication has the capacity to profoundly alter the gut microbiome composition [62, 63].

488   Indeed, there are several *in vitro* studies suggesting that SSRIs possess antimicrobial properties [16–19].

489   Moreover, it was shown recently that psychotropic drugs, including fluoxetine, are capable of altering the

490   composition of the gut microbiome in rats [20]. Our results here confirm that SSRIs have the potential to

491   modulate the fecal microbiome during pregnancy and lactation in rats. However, we only find significant

492   effects of FLX on maternal gut microbial community dynamics in sMV-, and not in cMV females.

493   Principle coordinates analysis showed an effect of FLX on the sMV bacterial community structure.

494   Similarly, when we compared the relative abundance of the Random Forests-identified taxa between the

495   treatments, most effects were seen in the sMV-FLX group relative to the sMV-Veh group. For instance,

496   fecal relative abundances of *Prevotella* and *Ruminococcus* were higher in the sMV-FLX group than in the

497   sMV-Veh group, at the cost of *Bacteroides*.

498   Thus, treatment with fluoxetine during pregnancy and lactation modulated the microbiome almost

499   exclusively in the sMV animals, and not significantly in the cMV animals. There are several possible

500   explanations for this. It might be that the sMV microbiome, belonging to a depressive-like host, is more

501   sensitive to disruptions than the cMV microbiome, especially during the dynamic perinatal period. It

502   might also be that both early life stress and FLX are "hits" that can shift the balance in the gut microbial

503   community, and that under the current experimental conditions these "hits" are not large enough to be

504   observed individually, but only when they are combined (a double-hit model). At the moment, there is a

505   paucity of studies into the effect of SSRI on the microbiome from clinical studies on depression or animal

506   studies mimicking this condition. For instance, it is unclear whether any effects SSRIs might have on the

507   microbiome are part of their therapeutic potential or should rather be seen as side effects.

508   Then, we were interested in whether the effects of FLX on the maternal fecal microbiome would

509   be reflected in altered metabolite concentrations. Indeed, FLX treatment suppressed the fecal availability

510   of some amino acids in sMV females. Specifically, we found levels of serine to be lower in sMV-FLX

511   animals compared to sMV-Veh animals during pregnancy, and proline and aspartic acid to be lower in the

512   sMV-FLX group compared to the sMV-Veh group during lactation. Similarly, a study using the

513   unpredictable mild stress paradigm showed that in stressed or depressive-like rats, serine plasma levels are

514   higher than in controls, while fluoxetine treatment lowered serine plasma levels compared to controls [64].

515   That study, however, did not include an experimental group receiving both stress and fluoxetine [64].

516   Bacteria are able to produce amino acids from nonspecific nitrogen sources [65]. Amino acids play

517   essential roles in both homeostatic physiology in adults as well as development and survival of the fetus,

518   and are involved in cell growth and differentiation, protein synthesis, hormone secretion and lactation [66,

519   67]. During early pregnancy, maternal amino acids are only second to glucose in the amount of substrate

520   that crosses the placenta to support the growth of the developing fetus [68].

521   To further examine the metabolic pathways that were characteristic of either pregnancy or

522   lactation based on fecal metabolite concentrations and the effect of early life stress and FLX on these

523   pathways, metabolite set enrichment analysis was performed. Both the cMV-Veh and the sMV-Veh group

524   showed a clear signature of pregnancy vs lactation, reflected in a number of metabolic pathways

525   (including amino acid-related pathways) that were enriched in either period. Both the cMV-FLX and the

526   sMV-FLX group showed no such significantly enriched pathways, adding to the emerging picture that

527   FLX disrupts the fecal metabolic signatures of pregnancy and lactation. Future studies should address

528   whether this results in altered metabolite transfer to the offspring and what the possible consequences are

529   for offspring development.

530   Finally, to link the 16S rRNA sequencing data with the metabolomic data, we generated

531   correlation matrices both between the 28 OTUs selected by Random Forests analysis, as well as between

532   the relative abundance of these OTUs and metabolite concentrations. We found that the relative

533   abundance of *Prevotella*, enhanced in the sMV-FLX group, was negatively correlated with a cluster of

534   *Clostridiales* and *Desulfovibrionales* genera. Interestingly, these same *Clostridiales* and

535   *Desulfovibrionales* OTUs were positively associated with availability of amino acids. Previous literature

536   describes that some of the most prevalent amino acid fermenting microbes indeed belong to the

537   *Clostridiales* and *Proteobacteria* taxa [69]. The associations we report here yield a working hypothesis

538   that FLX – at least in an animal model for depressive-like behavior – tips the balance toward higher

539   abundance *Prevotella* and lower abundance of the aforementioned cluster of amino acid-fermenting

540   OTUs, which leads to lower levels of amino acids. Further mechanistic studies are needed to test this

541   hypothesis.

542   This study has several limitations. As with any 16S rRNA marker gene sequencing approach, our

543   analysis was constrained to the genus level. In addition, we are aware that the microbiome and its

544   functional capacity varies across locations in the gastrointestinal tract whereas we only used fecal samples

545   [70]. However, our study design enabled us to sample longitudinally and to reduce the number of animals

546   needed to complete the study. Another issue is the observed mortality in about one-fourth of our SSRI-

547   treated females. This may be the result of the $SERT^{+/-}$ genotype of the MV females. Indeed, humans

548   carrying the short allele of the SERT gene polymorphism are more likely to suffer from side effects from

549   SSRIs than controls without a short allele [71]. In future studies, wildtype $SERT^{+/+}$ will have to be

550   included in order to establish whether this is indeed a genotype X drug effect. None of the females

551   included in the current study showed any signs of toxicity as a result of FLX. For future studies we also

552   suggest taking plasma samples, and possibly mother's milk, to confirm whether changes in fecal

553   metabolite concentrations correspond to systemic availability of these metabolites.

554   The benefits of performing microbiome research on laboratory rodents as opposed to humans

555   deserve mentioning here. For example, diet composition could be kept stable throughout the study,

556   whereas diet composition changes considerably from pre-conception to pregnancy in humans and even

557   short-term changes in diet are known to affect the gut microbiome [72, 73]. In addition, we are able to

558   study the independent effect of SSRI use in rodents, as well as its interactions with a depressive-like

559   phenotype. Human SSRI-users always have underlying psychopathologies that are difficult to control for

560   [74]. However, the biggest asset of the current study is our ability to link changes to the microbiome and

561   predicted functional capacity of the bacterial community to changes in metabolite availability. Especially

562   in the context of pregnancy and lactation, any changes to maternal microbial metabolite availability might

563   have far-reaching consequences and are therefore important to assess in addition to the microbiome itself.

564   Our results add to the growing body of research linking serotonin signaling to the gut microbiome.

565   Maternal depression and SSRI use during pregnancy are both associated with detrimental developmental

566   outcomes in offspring. Clinical and animal studies have identified adverse effects such as lower birth

567   weight, delayed motor development, and increased anxiety [75]. Future studies will assess whether and to

568   what extent the maternal gut microbiome and metabolome might mediate these effects. Microbial

569   molecules have been shown to transfer from mother to offspring during gestation and through lactation to

570   support the development of the brain and the immune system, emphasizing their importance for offspring

571   development [21, 22, 76, 77]. If the dampening effect of SSRI treatment on fecal amino acid

572   concentrations in a rodent model of depressive-like symptoms can be replicated in future studies, and if

573   these concentrations correlate with maternal and fetal plasma levels and to phenotypic outcomes in

574   offspring, dietary supplementation of amino acids alongside SSRI treatment during pregnancy and

575   lactation might be an important direction for future research.

576   ## Conclusions

577   We showed here that the fecal microbial and metabolic signatures of pregnancy and lactation in MV rats

578   are robust and are largely unaltered by early life stress with maternal separation. However, fluoxetine

579   treatment modulated key aspects of maternal microbial community dynamics and metabolite output in the

580   sMV females, including changes in the relative abundance of OTUs and associated decreases in amino

581   acid availability. This suggests that antidepressant use during pregnancy and lactation may lead to changes

582   in the microbiome. The current study adds to the emerging awareness that the microbiome is plastic and

583   vulnerable to environmental and pharmacological insults, especially during dynamic periods such as

584   pregnancy and lactation. We speculate that alterations to the maternal microbiome by SSRI treatment, and

585   the associated decreased availability of amino acids, reflect a compromised ability to supply nutrients to

586   the developing offspring.

587   ## List of abbreviations

588   cMV: maternal vulnerability females exposed to control handling; FLX: fluoxetine(-treated); IQR:

589   interquartile range; KEGG: Kyoto Encyclopedia of Genes and Genomes; L: lactation; MSEA: Metabolite

590   Set Enrichment Analysis; OTU: operational taxonomic unit; P: pregnancy; PCA: principal component

591   analysis; PC: principal component/coordinate; PCoA: principle coordinates analysis; PICRUSt:

592   Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; SCFA: short-chain

593   fatty acids; sMV: maternal vulnerability females exposed to early life stress; UC: unclassified; Veh:

594   vehicle-treated.

Declarations

Ethics approval and consent to participate

597   The animal study was approved by the Institutional Animal Care and Use Committee at the University of

598   Groningen (no. DEC-6936A), and was conducted in accordance with the institutional guidelines and the

599   Law on Animal Experiments.

Consent for publication

601   Not applicable.

Competing interests

603   The authors declare no competing interests.

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Authors' contributions

610   ASR, EJ, TLB and JDAO designed the study. ASR and DJH performed the animal experiment and

611   collected the samples. ASR and EJ performed the wet lab work and bioinformatics analysis. ASR drafted

612   the manuscript. All authors contributed to critical revision of the manuscript.

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815 **Figure legends**

816 **Figure 1: Overview of study design and sampling schedule.**

817 **(a) Early life stress protocol.** SERT$^{+/-}$ females were crossed with SERT$^{+/-}$ males, yielding nests with

818 offspring genotypes SERT$^{+/+}$, SERT$^{+/-}$, and SERT$^{-/-}$. The SERT$^{+/-}$ females are our model of maternal

819 vulnerability (MV). From postnatal day (PND) 2 to PND15, pups were either maternally separated for 6

820 hours per day (early life stress - sMV) or control handled (cMV) for 15 minutes per day. Pups were

821 weaned at PND21. sMV- and cMV females were group housed (same treatment) until adulthood. **(b)**

822 **Fluoxetine treatment during pregnancy and lactation, and fecal sampling schedule.** Adult sMV and

823 cMV females (N=14-18/group) were crossed with wildtype males. Through pregnancy and lactation, from

824 gestational day (GD)1 until PND21, females received a daily oral injection of either 10 mg/kg fluoxetine

825 (FLX) or methylcellulose (MC, Veh). Thus, there were 4 groups of females: cMV-Veh, sMV-Veh, cMV-

826 FLX, and sMV-FLX (N=6-11/ group). Fecal pellets for 16s rRNA gene sequencing were freshly collected

827 before conception at GD0, during pregnancy at GD7 and GD14, at PND2, and during lactation at PND7,

828 PND14 and PND21 (N=192 in total). Selected fecal samples from GD7 and PND7 were also used for

829 metabolomic analysis (N=4-5/group per time point, N=36 in total).

830 **Figure 2: Fluoxetine treatment alters the microbiome during pregnancy and lactation in rats with a**

831 **depressive-like phenotype. (a) Microbial alpha diversity.** The Shannon diversity index was used as a

832 measure of alpha diversity. A two-way ANOVA was performed with period (pregnancy vs lactation) and

833 group as factors. Uncorrected Fisher's LSD was used for group comparisons within periods. **(b) Structure**

834 **of the microbial communities during pregnancy and lactation.** Communities were clustered using

835 PCoA of the weighted UniFrac distance matrix. Each point corresponds to the microbial community of

836 one sample that was collected during pregnancy or lactation. The percentage of variation explained by the

837 PC is indicated on the axes. Colors correspond to period; all samples were grouped within pregnancy and

838 lactation. **(c) Heatmap of relative abundances of Random Forests-identified OTUs distinguishing**

839 **between pregnancy and lactation.** Random Forests was used to identify the microbial OTUs that had the

840   highest predictive value for distinguishing between pregnancy and lactation in the cMV-Veh group. The

841   heatmap depicts the relative abundance of these 28 OTUs during pregnancy and lactation for all groups,

842   ordered by unsupervised clustering. **(d) The effect of FLX on the structure of the sMV microbiome**

843   **during pregnancy and lactation.** Communities were clustered using PCoA of the weighted UniFrac

844   distance matrix. Each point corresponds to the microbial community of one sample that was collected

845   during pregnancy (upper graph) or lactation (lower graph). The percentage of variation explained by the

846   PC is indicated on the axes. Colors correspond to group; sMV-Veh in white and sMV-FLX in grey. **(e)**

847   **Relative abundance of selected Random Forests-identified OTUs.** Kruskal-Wallis tests were used, with

848   subsequent group comparisons within periods (see Methods). N=12-22 samples/group from pregnancy

849   (N=64 in total), N=18-33/group from lactation (N=96 in total).

850   **Figure 3: Fluoxetine alters fecal metabolite availability during pregnancy and lactation in rats with**

851   **a depressive-like phenotype. (a) Heatmap of Random Forests-identified PICRUSt-generated KEGG**

852   **pathways distinguishing between pregnancy and lactation.** Z-scores are depicted of the abundance of

853   the 9 identified pathways for all groups. The pathways are ordered by unsupervised clustering. **(b)**

854   **Structure of the metabolomic composition during pregnancy and lactation.** Samples were clustered

855   using PCA of the metabolomics concentration table. Each point corresponds to the metabolic capacity of

856   one sample that was collected during pregnancy or lactation. The percentage of variation explained by the

857   PC is indicated on the axes. Colors correspond to period in the upper graph, and to group in the lower

858   graph. **(c) Heatmap of Random Forests-identified metabolite concentrations distinguishing between**

859   **pregnancy and lactation.** Z-scores are depicted of the concentrations of the 14 identified metabolites for

860   all groups. **(d) Concentrations of selected amino acids.** Kruskal-Wallis tests were used, with subsequent

861   group comparisons within periods (see Methods). **(e) Metabolite Set Enrichment Analysis of pregnancy**

862   **vs lactation per group**. Only the pathways that were significantly altered between pregnancy and

863   lactation are shown here; the full list of metabolic pathways is shown in Supplemental Figure 3e. **(f)**

864   **Correlation matrix of metabolites and OTUs throughout pregnancy and lactation.** Spearman's rank

865   correlations were determined between the 14 Random Forests-identified metabolites and the 28 Random

866   Forests-identified OTUs. The circles represent the correlation between each metabolite and OTU. The size

867   of the circle increases with decreasing p-value-, and the color of the circle corresponds to the ρ-value of

868   the correlation (the strength of the correlation). Only significant ($p<0.05$) correlations are plotted, and only

869   the OTUs with at least 1 significant correlation are shown here. The matrix is ordered by hierarchical

870   clustering. For a: N=12-22 samples/group from pregnancy (N=64 in total), N=18-33/group from lactation

871   (N=96 in total). For b-f: N=4-5/group from pregnancy (N=18 in total), N=4-5/group from lactation (N=18

872   in total).

873   **Supplemental Figure 1: The effect of early life stress and fluoxetine on the microbiome during**

874   **pregnancy and lactation. (a) Random Forests model to determine which OTUs were best able to**

875   **distinguish between pregnancy and lactation in the cMV-Veh group.** The inset figure shows

876   increasing numbers of 97%-identity OTUs on the x-axis plotted against the cross-validation error of the

877   model on the y-axis. It was determined that the optimal model would include the top 28 OTUs, which are

878   shown in the bigger bar graph. From top to bottom, the OTUs are shown in descending order,

879   corresponding to their importance to discriminating between pregnancy and lactation. The length of the

880   bars represents the mean decrease in accuracy of the model if this OTU would be removed from the

881   model. **(b) Structure of the microbial communities during pregnancy and lactation.** Communities

882   were clustered using PCoA of the weighted UniFrac distance matrix. Each point corresponds to the

883   microbial community of one sample that was collected during pregnancy or lactation. The percentage of

884   variation explained by the PC is indicated on the axes. Colors correspond to group (containing samples

885   from both pregnancy and lactation). **(c) Bar graphs showing relative abundance of selected Random**

886   **Forests-identified OTUs.** A Kruskal-Wallis test was used, with subsequent group comparisons within

887   periods (see Methods). Only OTUs with a median relative abundance > 0 in at least 1 of the groups are

888   shown here. **(d) Correlation matrix of OTUs throughout pregnancy and lactation.** Spearman's rank

889   correlations were determined between the 28 Random Forests-identified OTUs. The circles represent the

890   correlation between two given OTUs, with the size of the circle increasing with decreasing p-value of the

891   correlation, and the color of the circle corresponding to the ρ-value of the correlation (the strength of the

892   correlation). Only significant ($p<0.05$) correlations are plotted. The OTUs are ordered by hierarchical

893   clustering. N=12-22 samples/group from pregnancy (N=64 in total), N=18-33/group from lactation (N=96

894   in total).

895   **Supplemental Figure 2: The effect of early life stress on the gut microbiome in adult MV female**

896   **rats. a) Pre-conception alpha diversity.** A t-test was used to test whether adult cMV and sMV females

897   differed in terms of fecal microbial Shannon Diversity. **(b) Pre-conception microbiome composition.** A

898   stack plot of the 20 most abundant OTUs was used to compare the fecal microbiome composition of cMV

899   and sMV females. N=18 for cMV; N=14 for sMV.

900   **Supplemental Figure 3: The effect of early life stress and fluoxetine on metabolite availability**

901   **during pregnancy and lactation. (a) Random Forests model to determine which predicted KEGG**

902   **metabolic pathways were best able to distinguish between pregnancy and lactation in the cMV-Veh**

903   **group.** The inset figure shows increasing numbers of KEGG metabolic pathways on the x-axis plotted

904   against the cross-validation error of the model on the y-axis. It was determined that the optimal model

905   would include the top 9 KEGG metabolic pathways. The length of the bars represents the mean decrease

906   in accuracy of the model if this KEGG pathway would be removed from the model. **(b) Random Forests**

907   **model to determine which metabolites were best able to distinguish between pregnancy and**

908   **lactation in the cMV-Veh group.** The smaller figure shows increasing numbers metabolites on the x-axis

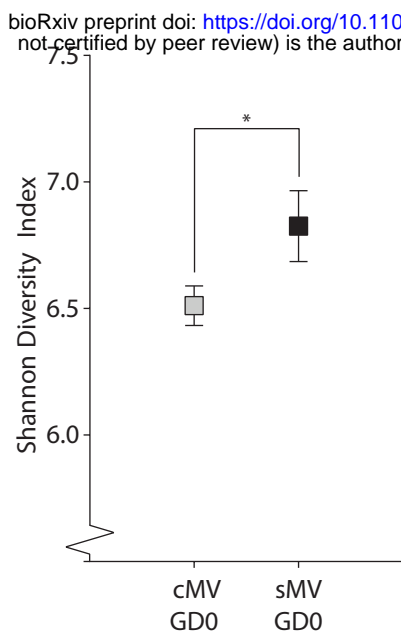909   plotted against the cross-validation error of the model on the y-axis. It was determined that the optimal

910   model would include the top 14 metabolites, which are shown in the bigger bar graph. The length of the

911   bars represents the mean decrease in accuracy of the model if this metabolite would be removed from the

912   model. **(c) Bar graphs showing concentrations of Random Forests-identified metabolites.** A Kruskal-

913   Wallis test was used, with subsequent group comparisons within periods (see Methods). **(d) Bar graphs**

914   **showing concentrations of the non-Random Forests-identified metabolites.** A Kruskal-Wallis test was

915   used, with subsequent group comparisons within periods (see Methods). **(e) Metabolite Set Enrichment**

916   **Analysis of pregnancy vs lactation per group**. MSEA was used to identify the metabolic pathways

917   distinguishing pregnancy and lactation based on the metabolomics concentration table for each group. For

918   a: N=22 cMV-Veh samples from pregnancy and N=33 from lactation. For b: N=5 cMV-Veh samples from

919   pregnancy and N=5 from lactation. For c: N=4-5/group from pregnancy and N=4-5/group from lactation

920   (N=36 in total).

921

Figure 1

a

Maternal separation 6 hrs/day

SERT$^{+/+}$
SERT$^{+/-}$(sMV)
SERT$^{-/-}$

Control handling 15 min/day

SERT$^{+/+}$
SERT$^{+/-}$(cMV)
SERT$^{-/-}$

♀ sMV

♀ cMV

Birth

Weaning

Adulthood

Maternal separation or control handling PND2-PND15

b

♀ sMV

♀ cMV

Key
🟠 Fecal sample for 16s rRNA sequencing
⊙ Fecal sample for 16s rRNA sequencing and metabolomics

Pregnancy

Lactation

GD0   GD7   GD14   PND2   PND7   PND14   PND21

Daily oral gavage of 10 mg/kg fluoxetine (FLX) or vehicle (Veh)

Figure 2

Figure 3

Supplemental Figure 1

a

b

c

Supplemental Figure 2

Supplemental Figure 3

a

b

c

Amino acids

d

Amino acids

Bile acids

Short-chain fatty acids

e