



**HAL**  
open science

## **Multi-hit early life adversity affects gut microbiota, brain and behavior in a sex-dependent manner**

Marion Rincel, Philippe Aubert, Julien Chevalier, Pierre-Antoine Grohard, Lilian Basso, Camille Monchaux de Oliveira, Jean-Christophe Helbling, Grégoire Chevalier, Marion Leboyer, Gerard Eberl, et al.

### ► To cite this version:

Marion Rincel, Philippe Aubert, Julien Chevalier, Pierre-Antoine Grohard, Lilian Basso, et al.. Multi-hit early life adversity affects gut microbiota, brain and behavior in a sex-dependent manner. *Brain, Behavior, and Immunity*, 2019, 80, pp.179-192. 10.1016/j.bbi.2019.03.006 . hal-02623824

**HAL Id: hal-02623824**

**<https://hal.inrae.fr/hal-02623824>**

Submitted on 25 Oct 2021

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

1 **Multi-hit early life adversity affects gut microbiota, brain and behavior in a sex-**  
2 **dependent manner**

3 Marion Rincel<sup>1,2</sup>, Philippe Aubert<sup>3</sup>, Julien Chevalier<sup>3</sup>, Pierre-Antoine Grohard<sup>3</sup>, Lilian Basso<sup>4</sup>,  
4 Camille Monchaux de Oliveira<sup>1,2</sup>, Jean Christophe Helbling<sup>1,2</sup>, Élodie Lévy<sup>1,2</sup>, Grégoire  
5 Chevalier<sup>5</sup>, Marion Leboyer<sup>6</sup>, Gérard Eberl<sup>5</sup>, Sophie Layé<sup>1,2</sup>, Lucile Capuron<sup>1,2</sup>, Nathalie  
6 Vergnolle<sup>4</sup>, Michel Neunlist<sup>3</sup>, Hélène Boudin<sup>3</sup>, Patricia Lepage<sup>7</sup>, Muriel Darnaudéry<sup>1,2</sup>

7

8 1 Univ. Bordeaux, INRA, Nutrition and Integrative Neurobiology, UMR 1286, 33076  
9 Bordeaux, France.

10 2 INRA, Nutrition and Integrative Neurobiology, UMR 1286, 33076 Bordeaux, France.

11 3 The Enteric Nervous System in Gut and Brain Disorders, INSERM UMR1235, IMAD,  
12 Nantes, France

13 4 Institut de Recherche en Santé Digestive, INSERM UMR1220, INRA UMR1416, ENVT,  
14 UPS, Toulouse, France

15 5 Unité Microenvironnement et Immunité, Institut Pasteur, Paris, France

16 6 Université Paris-est-Créteil, Laboratoire Psychiatrie translationnelle, INSERM U955,  
17 Hôpital Chenevier-Mondor, Créteil, France

18 7 Micalis Institute, INRA, AgroParisTech, Univ. Paris-Saclay, Jouy-en-Josas, France

19

20 Corresponding author: [muriel.darnaudery@u-bordeaux.fr](mailto:muriel.darnaudery@u-bordeaux.fr).

21

22 Number of words: 7670

23

24 **Abstract** (263 words)

25 The accumulation of adverse events *in utero* and during childhood differentially increases the  
26 vulnerability to psychiatric diseases in men and women. Gut microbiota is highly sensitive to  
27 the early environment and has been recently hypothesized to affect brain development.  
28 However, the impact of early-life adversity on gut microbiota, notably with regards to sex  
29 differences, remains to be explored. We examined the effects of multifactorial early-life  
30 adversity on behavior and microbiota composition in C3H/HeN mice of both sexes exposed  
31 to a combination of maternal immune activation, maternal separation (3hr per day from  
32 postnatal day (PND)2 to PND14) and maternal unpredictable chronic mild stress. At  
33 adulthood, offspring exposed to multi-hit early adversity showed sex-specific behavioral  
34 phenotypes with males exhibiting deficits in social behavior and females showing increased  
35 anxiety and compulsive behavior in the elevated plus maze and marble burying tests. Early  
36 adversity also differentially regulated gene expression in the medial prefrontal cortex (mPFC)  
37 according to sex. Interestingly, several genes such as *Arc*, *Btg2*, *Fosb*, *Egr4* or *Klf2* were  
38 oppositely regulated by early adversity in males *versus* females. Finally, 16S-based  
39 microbiota profiling revealed sex-dependent gut dysbiosis. In males, abundance of taxa  
40 belonging to Lachnospiraceae and Porphyromonadaceae families or other unclassified  
41 Firmicutes, but also *Bacteroides*, *Lactobacillus* and *Alloprevotella* genera was regulated by  
42 early adversity. In females, the effects of early adversity were limited and mainly restricted to  
43 *Lactobacillus* and *Mucispirillum* genera. Our work reveals marked sex differences in a  
44 multifactorial model of early-life adversity, both on emotional behaviors and gut microbiota,  
45 suggesting that sex should systematically be considered in preclinical studies both in  
46 neurogastroenterology and psychiatric research.

47 **Keywords:** Gut-brain axis; early-life stress; emotional behavior; ultrasonic vocalizations;  
48 HPA axis; intestinal permeability; lipopolysaccharides; medial prefrontal cortex

## 50 1. INTRODUCTION

51 The multi-hit hypothesis (or cumulative stress hypothesis) proposes that neuropsychiatric  
52 disorders may be precipitated by a combination of two or more major adverse events in  
53 particular during development (Maynard et al., 2001; McEwen, 1998; Nederhof and Schmidt,  
54 2012). Subjects exposed to one or more childhood adversities are more likely to become  
55 depressed following exposure to stress at adulthood than subjects without early adversity  
56 (Chapman et al., 2004; Nemeroff, 2016). Maternal infection and postnatal exposure to  
57 psychological stress or trauma have been found to represent important environmental risk  
58 factors for the development of psychiatric disorders, including autism, schizophrenia, anxiety-  
59 disorders and depression (Brown, 2011; Brown et al., 2014; Nemeroff, 2016). According to  
60 the multiple-hit hypothesis, prenatal infection could render the offspring more vulnerable to  
61 the deleterious effects of a second postnatal stimulus, such as stress. Previous findings have  
62 comforted this hypothesis in animal models combining prenatal inflammation and adolescent  
63 or adult stress exposure (Deslauriers et al., 2013; Giovanoli et al., 2013; Monte et al., 2017).  
64 However, the impact of prenatal inflammation combined with an early post-natal stress  
65 remains underexplored.

66 The mechanisms underlying the long-term behavioral effects of early adversity are still  
67 unclear. A large body of evidence suggests that the medial prefrontal cortex (mPFC), a brain  
68 area involved in the regulation of emotional behavior, plays a major role in mediating the  
69 effects of early-life stress (Arnsten, 2009; Ulrich-Lai and Herman, 2009). Recent work has  
70 demonstrated that the gut microbiota affects gene expression in the mPFC (Gacias et al.,  
71 2016; Hoban et al., 2017, 2016). Gastrointestinal alterations during early-life, notably gut  
72 microbiota dysbiosis and loss of barrier function, can disturb brain development and lastingly  
73 impair gut-brain communication (Borre et al., 2014; Hsiao et al., 2013; Kim et al., 2017). In  
74 particular, maternal immune activation and maternal separation (MS) have been shown to  
75 produce intestinal defects such as visceral pain, increased intestinal permeability in

76 association with behavioral outcomes (see Labouesse et al., 2015 and O'Mahony et al., 2011  
77 for recent reviews). Animals exposed to early-life immune or psychological stress also exhibit  
78 gut dysbiosis in (Amini-Khoei et al., 2019; Bailey and Coe, 1999; Moya-Pérez et al., 2017;  
79 Murakami et al., 2017; O'Mahony et al., 2009; Pusceddu et al., 2015). Moreover, numerous  
80 studies using maternal immune activation or MS models have shown that microbiota-directed  
81 interventions such as probiotic treatments or fecal transplantation modulate brain and  
82 behavior, especially stress-related behaviors (De Palma et al., 2015; Giovanoli et al., 2016;  
83 Hsiao et al., 2013; Kim et al., 2017; Mattei et al., 2014; Moya-Pérez et al., 2017). Importantly,  
84 it has been reported that gut microbiota composition differs according to sex both in animals  
85 and humans (Fransen et al., 2017; Hollister et al., 2014; Jašarević et al., 2016; Markle et al.,  
86 2013). However, sex differences in gut microbiota in a context of early adversity remain  
87 underexplored. Indeed, most of the studies use males and the few studies involving males  
88 and females often pool data of both sexes (De Palma et al., 2015; El Aidy et al., 2017; Hsiao  
89 et al., 2013; Riba et al., 2018). This issue, which merits further investigation, is of particular  
90 importance with respect to the gender differences observed in the prevalence of psychiatric  
91 disorders. To support this notion, autism spectrum disorders are more prevalent in men  
92 (Werling and Geschwind, 2013), whereas women are more susceptible to anxiety and  
93 depression (Steel et al., 2014).

94 In the present study, we hypothesized that early adversity differentially affects the gut  
95 microbiota in males and females and that these differential effects may underlie the sex-  
96 related differences observed at the behavioral level. To test this hypothesis, we developed a  
97 mouse model of multifactorial early adversity combining prenatal inflammation  
98 (lipopolysaccharide (LPS) injection), post-natal MS and unpredictable chronic mild stress  
99 (UCMS) in dams and we investigated emotional behavior, mPFC gene expression and gut  
100 microbiota in male and female offspring (**Figure 1**).

101

## 102 **2. RESULTS**

103 **2.1. Effects of early adversity on offspring's body weight**

104 LPS injection on E17 induced significant hypothermia in dams ( $t(15)=4.98$ ,  $p<0.001$ ),  
105 indicating that bacterial immune activation was effective (**Supplementary Figure S1**). There  
106 was no significant effect of prenatal LPS on pup body weight on post-natal day (PND)2  
107 (**Supplementary Figure S2A**). However, the combination of prenatal LPS and MS (early  
108 adversity) significantly decreased the body weight of male pups at PND15 in comparison with  
109 control pups (males,  $t(15)=2.46$ ,  $p=0.003$ ; females,  $t(15)=1.62$ ,  $p=0.127$ ) (**Supplementary**  
110 **Figure S2B**). This effect was no longer present at weaning (**Supplementary Figure S2C**).

111

112 **2.2. In males, early adversity leads to impaired social communication during infancy**  
113 **and altered social interaction at adulthood**

114 Males submitted to early adversity showed lower vocalization number in response to isolation  
115 at PND8 relative to controls (early adversity effect:  $F(1,14)=4.78$ ,  $p=0.046$ , time x early  
116 adversity effect:  $F(1,42)=10.74$ ,  $p<0.001$ ) (**Figure 2A,B**). This effect was mainly due to the  
117 marked decrease in ultrasonic calls during the first minute (early adversity vs control,  
118  $p<0.001$ ). In addition, the latency to vocalize was increased in early adversity compared with  
119 controls ( $t(14)=3.10$ ,  $p=0.008$ ) (**Figure 2C**).

120 As adults, males submitted to early adversity spent significantly less time in social interaction  
121 with a conspecific ( $U=1$ ,  $p=0.004$ ) (**Figure 2D**). Surprisingly, the latency for the first social  
122 contact was significantly lower in early adversity mice relative to controls ( $U=3$ ,  $p=0.015$ )  
123 (**Figure 2E**). We further investigated social behavior in males using a resident-intruder  
124 paradigm (**Figure 2F,G**). While the aggressors displayed the same number of attacks in both  
125 groups (**Figure 2F**), early adversity males tended to spend less time in defensive behavior  
126 ( $U=13$ ,  $p=0.114$ ) (**Figure 2G**). There was no significant impact of early adversity on the  
127 percentages of time spent and distance traveled in the open arms of the elevated plus maze  
128 (EPM) (**Figure 2H** and data not shown), on the percentage of buried marbles in the marble  
129 burying test (**Figure 2I**), or on immobility time in the tail suspension test (TST) (**Figure 2J**),

130 suggesting that anxiety, compulsive-like and depressive-like behaviors were unspoiled in  
131 males exposed to early adversity. Locomotor activity was not altered by early adversity either  
132 (data not shown). Plasma corticosterone levels after restraint stress were not significantly  
133 modulated by early adversity (**Figure 2K**). There was no significant impact of early adversity  
134 on male body weight throughout behavioral assessment (data not shown); however, 3 weeks  
135 after TST and acute restraint stress, control males gained significant weight whereas early  
136 adversity group showed a blunted body weight growth (early adversity x time effect,  
137  $F(1,20)=5.00$ ,  $p=0.037$ ; control pre- vs poststress,  $p=0.001$ ; early adversity pre- vs  
138 poststress, n.s.) (**Figure 2L**). This difference resulted in significantly lower body weight gain  
139 in early adversity males relative to controls ( $t(20)=2.24$ ,  $p=0.037$ ) (**Figure 2M**).

140

### 141 **2.3. In females, early adversity leads to impaired social communication during infancy** 142 **and exacerbated anxiety and compulsive-like behaviors at adulthood**

143 In female pups, the number of ultrasonic calls in response to isolation was differentially  
144 altered by early adversity depending on time (time x early adversity effect:  $F(1,48)=2.73$ ,  
145  $p=0.054$ ) (**Figure 3A,B**). The number of ultrasonic calls was significantly reduced during the  
146 first minute (early adversity vs control,  $p=0.036$ ). This effect was not accompanied by  
147 significant change in call latency (**Figure 3C**).

148 At adulthood, females exposed to early adversity showed similar time spent in social  
149 interaction with a conspecific compared with controls (**Figure 3D**). In the EPM, early  
150 adversity females spent significantly less time in the open arms than controls ( $t(22)=2.64$ ,  
151  $p=0.015$ ) (**Figure 3E**). Moreover, the distance traveled in the open arms was also reduced in  
152 early adversity *versus* control mice ( $t(22)=2.13$ ,  $p=0.045$ ) (**Figure 3F**). Females submitted to  
153 early adversity also buried significantly more marbles compared with controls (early adversity  
154 effect,  $F(1,84)=6.78$ ,  $p=0.017$ ) (**Figure 3G**). Locomotor activity was not altered by early  
155 adversity (data not shown), suggesting that the above-mentioned behavioral differences  
156 were not due to altered locomotion. Immobility in the TST was not affected by early adversity

157 **(Figure 3H)**. Plasma corticosterone levels after restraint stress varied depending upon time  
158 and the history of early adversity ( $F(1,36)=3.88$ ,  $p=0.030$ ) **(Figure 3I)**. Planned comparisons  
159 revealed significantly higher corticosterone levels in the early adversity group 60 min after  
160 the end of restraint ( $t(90)$ ,  $p=0.006$ ). Further analysis showed that corticosterone recovery to  
161 basal levels was lower in the early adversity group ( $t(18)=3.31$ ,  $p=0.004$  **(Figure 3I, inset)**).  
162 Finally, as seen in males, the body weight evolution of females exposed to early adversity  
163 was stopped after TST and restraint stress exposures, whereas the control group exhibited a  
164 normal body weight progression ( $F(1,21)=5.11$ ,  $p=0.035$ ; control pre- vs poststress,  $p=0.003$ ;  
165 early adversity pre- vs poststress, n.s.; body weight gain: ( $t(21)=2.26$ ,  $p=0.035$ )) **(Figure**  
166 **3J,K)**.

167

#### 168 **2.4. Early adversity induces gastrointestinal dysfunctions in adult males but not** 169 **females**

170 We next assessed whether the effects of early adversity on behavior were associated with  
171 altered gut physiology. Visceral sensitivity to colorectal distension and intestinal permeability  
172 were evaluated in both sexes. In males, there was no significant effect of early adversity on  
173 visceral sensitivity to colorectal distension **(Figure 4A)** or on transcellular permeability to  
174 HRP (horseradish peroxidase) **(Figure 4B)**. However, paracellular permeability to FSA  
175 (Fluorescein–5.6 sulfonic acid) was significantly increased relative to controls ( $t(18)=2.56$ ,  
176  $p=0.020$ ) **(Figure 4C)**. We further assessed intestinal permeability in males at a later age  
177 (5.5 months) **(Supplementary Figure S3)**. The increased paracellular permeability was no  
178 longer observed in older male animals **(Supplementary Figure S3A)**, however, early  
179 adversity significantly increased males' transcellular permeability at this age ( $t(21)=2.80$ ,  
180  $p=0.011$ ) **(Supplementary Figure S3B)**. There was no effect of early adversity on intestinal  
181 motility, as indicated by similar total transit time and fecal pellet output after oral gavage  
182 **(Supplementary Figure S3C,D)**. *Ex vivo* electrical field stimulation (EFS) of distal colon  
183 longitudinal muscle segments revealed significantly higher EFS-induced contractile response



184 in mice exposed to early adversity after atropine application in the bath ( $t(14)=3.57$ ,  $p=0.003$ )  
185 **(Supplementary Figure S3E)**. Finally, Western blots of several key markers for enteric  
186 neurons and enteric glial cells showed significantly decreased expression of MAP2 in the  
187 distal colon from early adversity mice ( $U=4$ ,  $p=0.026$ ) **(Supplementary Figure S3F)**. There  
188 was no significant difference in the expression of PGP 9.5, S100 $\beta$  and GFAP between  
189 groups (see full names in legend).

190 In contrast, the effects of early adversity in females were limited. Intriguingly, early adversity  
191 females displayed visceral hyposensitivity, an effect likely driven by a hypo-response to  
192 noxious stimuli (60 mmHg) (early adversity effect:  $F(1,13)=4.90$ ,  $p=0.045$ ) **(Figure 4D)**.  
193 Intestinal permeability was similar between groups **(Figure 4E,F)**.

194

## 195 **2.5. Early adversity differentially alters gut microbiota composition in adult males and** 196 **females**

197 In order to test the hypothesis of a sex-specific impact of early-life adversity on gut  
198 microbiota composition, we used 16S-based profiling **(Figure 5 and Supplementary Figure**  
199 **S4)**. As illustrated in **Supplementary Figure S4**, in adult control animals, microbiota  
200 composition strongly differed both qualitatively and quantitatively between males and  
201 females. Moreover, there were differential effects of early adversity according to sex at  
202 several taxonomic levels. In males, principal component analysis (PCA) based on genus  
203 composition revealed a trend towards differential distribution according to the history of early  
204 adversity ( $p=0.078$ ) **(Figure 5A)**. Alpha diversity as calculated by Shannon and Simpson  
205 indices was not altered by early adversity **(Figure 5B)**. Males exposed to early adversity  
206 displayed significantly lower proportions of members of *unclassified Lachnospiraceae* ( $U=27$ ,  
207  $p=0.016$ ) and *unclassified Porphyromonadaceae* ( $U=27$ ,  $p=0.016$ ) **(Figure 5C)** and higher  
208 proportions of *Bacteroides* ( $U=34$ ,  $p=0.051$ ), *Lactobacillus* ( $U=29$ ,  $p=0.023$ ), *Porphyromonas*  
209 ( $U=31$ ,  $p=0.032$ ), *Alloprevotella* ( $U=32$ ,  $p=0.037$ ) and *unclassified Firmicutes* ( $U=34$ ,  
210  $p=0.051$ ) members **(Figure 5D)** compared with controls. Of note, the effect of early adversity

211 on *unclassified Lachnospiraceae* species (spp.), the most abundant detected taxa, was  
212 particularly striking (falling from 30% to 20%). Further analysis at the operational taxonomic  
213 unit (OTU) level, which provides a better approximation of bacterial species in presence,  
214 showed 92 significantly affected OTUs in males with a history of early adversity in  
215 comparison with controls: 59 OTUs were depleted and 33 OTUs were enriched (**Table 1 and**  
216 **Supplementary Figure S5A,B**). Notably, an important proportion of the altered OTUs  
217 belongs to *Barnesiella* and *Clostridium XIVa* spp. of the Porphyromonadaceae and  
218 Lachnospiraceae families, respectively. Although the analysis at the genus level shows  
219 overall lower proportions of *unclassified Lachnospiraceae* and *unclassified*  
220 *Porphyromonadaceae* spp., the OTU data indicate that different OTUs within the same  
221 genus or family vary in opposite directions.

222 In female offspring, PCA based on genus composition showed no significant dissociation  
223 between groups and alpha diversity indices were not altered by early adversity (**Figure**  
224 **5G,H**), suggesting that the gut microbiota of females was less sensitive to early adversity  
225 than that of males. Consistently, only two genera were significantly altered by early adversity  
226 in females (**Figure 5I**). Specifically, relative abundance of *Mucispirillum* ( $U=29$ ,  $p=0.040$ ) and  
227 *Lactobacillus* ( $U=30$ ,  $p=0.047$ ) genera was significantly decreased in early adversity female  
228 mice compared with controls. Interestingly, this depletion of *Lactobacillus* spp. in females  
229 contrasts with its enrichment in males submitted to early adversity. Further analysis at the  
230 OTU level showed 64 significantly affected OTUs in early adversity females *versus* controls,  
231 with 37 depleted and 27 enriched (**Table 1 and Supplementary Figure S5C,D**). As in  
232 males, an important proportion of the altered OTUs belongs to *Barnesiella* and *Clostridium*  
233 *XIVa* spp. of the Porphyromonadaceae and Lachnospiraceae families and different OTUs  
234 within these families vary in opposite directions. Moreover, a substantial proportion of the  
235 depleted OTUs belongs to the *Lactobacillus* genus and may account for the overall effect  
236 observed at the genus level. Detailed OTU data and taxonomic affiliation are provided in the  
237 online **Supplementary File F1**.

238

239 **2.6. Effects of early adversity on gene expression in the medial prefrontal cortex of**  
240 **adult offspring**

241 To identify molecular determinants of sex-differences in multi-hit susceptibility, we examined  
242 gene expression in the mPFC using microarrays. Overall, there were 156 genes and 108  
243 genes significantly affected ( $p < 0.05$  and foldchange  $> 20\%$ ) by multi-hit adversity in males  
244 and females, respectively. Notably, most of the genes were down-regulated in males (138  
245 down and 18 up-regulated) whereas in females, genes were predominantly up-regulated (17  
246 down and 91 up-regulated) (**Figure 6A**, see gene list and detailed statistical results in online  
247 **Supplementary File F2**). Interestingly, only 18 genes were affected by early adversity in  
248 both males and females, most often in the opposite direction. Subsequent measures by  
249 qPCR using BioMark (Fluidigm) indicated that 80% of the top genes identified by microarrays  
250 were also significantly regulated by early adversity using qPCR in females. In contrast, in  
251 males, only 30% of the top genes were validated by qPCR, suggesting a weak impact of  
252 early adversity on males' mPFC transcriptome (Figure 6B,C). qPCR results confirmed  
253 however that many genes such as *Btg2*, *Arc*, *Egr4*, *Fosb* and *Klf2* were oppositely regulated  
254 by early adversity in males *versus* females (Figure 6 B). Pearson's correlations between  
255 microarrays and qPCR data revealed good concordance between the two methods ( $r > 0.70$ )  
256 (Figure 6B, C).

257

258 **2.7. Correlation between behavior, gene expression in the mPFC and gut microbiota**

259 Males' social behavior (time spent in social interaction) was significantly correlated with the  
260 expression of *Arc* in the mPFC, but also with the abundance of several bacterial OTUs (26),  
261 in particular those belonging to the Lachnospiraceae family (including 8 OTUs among the  
262 most impacted by early adversity, **Table 2**). Females' behavior (percentage of time spent in  
263 the open arms of the EPM and percentage of buried marbles) was significantly correlated  
264 with the expression *Arc*, *Fosb*, *Junb* and *Gadd45b* in the mPFC (**Table 2**), but also with the

265 abundance of several bacterial OTUs (4 and 13, respectively) belonging to the  
266 Lachnospiraceae and Lactobacillaceae families (including 8 OTUs among the most impacted  
267 by early adversity, **Table 2**).

268

### 269 **3. DISCUSSION**

270 Several studies have reported sex differences in gut microbiota composition in both humans  
271 and animals (Hollister et al., 2014; Markle et al., 2013). There is a growing number of studies  
272 investigating the role of the gut-brain axis, especially gut microbiota, in the regulation of  
273 stress-related emotional behaviors in animal models of early-life adversity. However, it is not  
274 clear whether early stress differentially affects the gut microbiota between males and females  
275 (Jašarević et al., 2017; Moussaoui et al., 2017). Herein, using a mouse model of  
276 multifactorial early adversity combining prenatal inflammation (LPS injection), post-natal MS  
277 and UCMS in dams, we report that early adversity leads to social deficits in males and  
278 increased anxiety and compulsive-like behavior in females. Multi-hit early adversity also  
279 affects mPFC gene expression in a sex-dependent manner. More importantly, we provide  
280 clear evidence that early adversity differentially alters gut microbiota composition in males  
281 and females.

282 An increasing body of evidence suggests that early adversity does not equally affect  
283 emotional vulnerability in males and females (Farrell et al., 2016; Foley et al., 2014; Monte et  
284 al., 2017; Murlon et al., 2010; Sloten et al., 2006). Herein, extending previous findings  
285 (Fernández de Cossío et al., 2017; Hsiao et al., 2013; Kim et al., 2017; Monte et al., 2017;  
286 Rincel et al., 2016; Shin et al., 2016; Shin Yim et al., 2017; Tsuda et al., 2011), we report that  
287 multi-hit adversity induces social deficits in male offspring and increases anxiety in female  
288 offspring. These results are particularly sounded with regards to the high prevalence of  
289 autism spectrum disorders in male and anxiety disorders in female subjects. Notably,  
290 ultrasonic vocalizations (USV) data suggest early onset social communication deficits in  
291 males and show only mild alterations in females, which is consistent with their intact social

292 interaction as adults. We report here that multi-hit adversity differentially affected genes  
293 expression in the mPFC of females and males with predominantly a down regulation of gene  
294 expression in males and an up-regulation in females. Previous works demonstrate that  
295 chronic stress may produce opposite effects on brain gene expression between males and  
296 females (Hodes et al., 2015). Several genes belonging to immediate-early genes (IEGs such  
297 as *Arc*, *Junb*, *Fosb*, *Egr4*) were affected by early adversity. A large literature indicates that  
298 stress-related disorders and psychiatric disorders are associated with alterations of IEGs  
299 expression within the brain (Gallo et al., 2018 ; Russo et al., 2012). In males exposed to  
300 multi-hit early adversity, social behavior deficits were associated with a down regulation of  
301 *Arc*, *Btg2* and *H2-k1*. A decrease of *Arc* expression has been reported in the mPFC of male  
302 rodents after chronic stress and in preclinical models of depression with social withdrawal  
303 (Covington et al., 2010; Eriksson et al., 2012; Ons et al., 2010) as well as in depressed  
304 patients (Covington et al., 2010). Interestingly, increased *Btg2* expression has been reported  
305 in animal models with high levels of aggressive social behavior (Malki et al., 2016). In  
306 females, the major effect of early life adversity is the increased expression of the IEGs *Junb*,  
307 *Fosb*, *Arc* and *Egr4*. Since acute stress increases the expression of several IEGs (Kurumaji  
308 et al., 2008), this effect may be related to their exacerbated anxiety. Indeed, increased *Fosb*  
309 expression in the mPFC has been reported in animal models with hyper-anxiety (Montesinos  
310 et al., 2016). In females exposed to early adversity we also report changes in the expression  
311 of genes such as *Nr4a3* and *Egr4* involved in neural development (Bae et al., 2015; Pönniö  
312 and Conneely, 2004), suggesting that multi-hit adversity may affected mPFC  
313 neurodevelopment in females. Finally, the effect on *H2-k1* expression in males and on  
314 *Gadd45b*, *Egr4*, *Klf2* and *Nr4a3* in females suggests that multi-hit adversity may interfere  
315 with immune-related processes within the mPFC.

316 Several studies have demonstrated that correction of gut dysbiosis can reverse social deficits  
317 (Buffington et al., 2016; Hsiao et al., 2013; Kim et al., 2017) and anxiety-like behaviors  
318 (Bercik et al., 2010; Hsiao et al., 2013; Leclercq et al., 2017; Moya-Pérez et al., 2017) in

319 rodents, highlighting the role of gut microbiota in the regulation of emotional behaviors. Here,  
320 we report that the effects of early adversity on gut microbiota composition vary according to  
321 sex, with males being the most affected. *Clostridium XIVa* cluster, which represents more  
322 than 10% of the total bacteria, and more generally the *Lachnospiraceae* family, accounting  
323 for approximately 40% of total bacteria, appear highly sensitive to early adversity in both  
324 sexes, with different OTUs impacted in males *versus* females. A previous study found  
325 increased proportions of *Porphyromonadaceae*, *Prevotellaceae*, unclassified *Bacteroidales*  
326 and *Lachnospiraceae* spp. in the poly I:C-induced maternal immune activation mouse model  
327 of autism (Hsiao et al., 2013). However, males and females were pooled together in this  
328 study. Other studies using MS have reported increased *Bacteroides*, *Lachnospiraceae* and  
329 *Clostridium XIVa* spp. in male rats and mice (De Palma et al., 2015; García-Ródenas et al.,  
330 2006; Murakami et al., 2017; Zhou et al., 2016). Herein, we demonstrate that social deficits in  
331 males and anxiety and compulsive-like behaviors in females are associated with abundance  
332 of OTUs belonging to the *Lachnospiraceae* family. Interestingly, numerous OTUs of this  
333 family have been found to be either increased or decreased in stools of depressive patients  
334 (Zheng et al., 2016) suggesting that the effects of early adversity on these bacteria are  
335 relevant to human psychiatric conditions. In addition, a recent study reveals that the  
336 antidepressant effects of probiotics in male mice are associated with an increase of  
337 *Lachnospiraceae* abundance (Guida et al., 2018). Although we do not report altered  
338 depressive-like behaviors in the TST, decreases in social interactions have been reported in  
339 numerous preclinical models of depression (Nestler and Hyman, 2010). Transfer of intestinal  
340 microbiota, including members of *Clostridiales* and *Lachnospiraceae*, to microbiota-depleted  
341 C57BL/6 recipients was sufficient to induce social avoidance and change gene expression  
342 and myelination in the prefrontal cortex (Gacias et al., 2016). Overall, bacteria from the  
343 *Lachnospiraceae* family appear as good candidates for the regulation of emotional behaviors  
344 within the microbiota-gut-brain axis in males. The only genus affected in both sexes was  
345 *Lactobacillus*, yet the effects of early adversity on this genus were opposed in males and

346 females. *Lactobacillus* strains are commonly used as probiotics with beneficial effects on  
347 anxiety-like behavior in rodents (Bravo et al., 2011; Leclercq et al., 2017; Liu et al., 2016). In  
348 humans, randomized double-blind, placebo controlled trials show that women who received  
349 *Lactobacillus rhamnosus* spp. throughout pregnancy had significantly lower depression and  
350 anxiety scores in the postpartum period (Slykerman et al., 2017) and that patients with  
351 chronic fatigue syndrome displayed reduced anxiety symptoms after treatment with  
352 *Lactobacillus casei* spp (Rao et al., 2009). Whether treatment with probiotics such as the  
353 *Lactobacillus* strains could reverse the behavioral alterations induced by multi-hit early  
354 adversity remains to be tested. However, considering the sex-differences in both gut  
355 microbiota and behavior, we assume that a single strain may not be efficient in both males  
356 and females. Finally, a limitation in our study is the focus on microbiota composition at  
357 adulthood. Future studies should examine earlier timepoints to increase our understanding of  
358 the mechanisms underlying the effects of early adversity on gut microbial communities and  
359 help to identify species of which the abundance at early stages can predict the behavioral  
360 phenotype later in life. The links between specific gut microbiota alterations and gene  
361 expression in the brain remain to be elucidated. Among the genes impacted by early-life  
362 adversity, IEGs were of particular interest regarding both behavior and gut microbiota.  
363 Previous studies demonstrate that absence of gut microbiota alters transcriptional regulation  
364 in the mPFC and in the amygdala and affects immediate-early genes expression (Hoban et  
365 al., 2017, 2016). Notably, male germ-free animals exhibit changes in expression of genes  
366 such as *Arc*, *Dusp1*, *FosB* and *Klf2* also found to be regulated by multi-hit adversity in our  
367 study. In females, *Klf2* mPFC expression is substantially increased by early-life adversity and  
368 is significantly correlated with both anxiety/compulsive behavior and the abundance of  
369 *Lactobacillus* and *Clostridium XIVA* members. KLF2 is a transcription regulator highly  
370 expressed in vascular cells (Shi et al., 2013; Tang et al., 2017). Remarkably, a recent study  
371 demonstrated a link between brain KLF2 signaling and gut microbiota in an animal model of  
372 cerebral cavernous malformations (Tang et al., 2017).

373 Our work establishes the first model of multi-hit early adversity combining pre- and post-natal  
374 factors. The aim of such model was to closer mimic what happens in human, where it is  
375 assumed that the combination of early adverse events, rather than a single event occurring  
376 either pre- or post-natally, precipitate disease in adult life. Our findings show that the  
377 combination of early adversities effectively produces long-lasting alterations in mice. Here,  
378 the aim was not to tackle the specific role played by each individual factor (prenatal  
379 inflammation, maternal separation, maternal unpredictable stress), but this aspect is a  
380 limitation that will require further investigation. In particular, in light of our results on the  
381 phenotype associated with the multi-hit early adversity, one can hypothesize that the prenatal  
382 inflammation is critical in priming the organism and may modify the responses of mice to the  
383 subsequent post-natal stressors. In a similar way, we cannot rule out that the exposure to  
384 successive behavioral tests, which include mild to severe psychological stressors (EPM, tail  
385 suspension test, restraint stress) can add to the effects of the early adverse treatments.  
386 Previous findings in the literature indicate that stress during development exacerbates  
387 behavioral consequences of exposure to stress in adulthood (Avital and Richter-Levin, 2005;  
388 Tsoory et al., 2007).

389 In conclusion, our work comforts a large literature showing that early adversity affects  
390 emotional behavior. At the behavioral level, multi-hit early adversity produced social behavior  
391 deficits in males and increased anxiety in females. Importantly, we demonstrate that  
392 offspring's gut physiology is differentially impacted by multifactorial adversity in males and  
393 females. Finally, the sex-specific correlations between behavior, mPFC transcriptional  
394 changes and microbiota composition suggest that the mPFC may be a key target within the  
395 gut-brain axis. Overall, our study highlights the importance to examine gut microbiota in  
396 psychiatric research and systematically consider potential sex differences.

## 397 **4. MATERIALS AND METHODS**

### 398 **4.1. Animals**



399 Experiments were approved by the Bioethical committee of the University of Bordeaux (N°  
400 50120186-A) according to the European legislation (Directive 2010/63/EU, 22 September  
401 2010). Mice were maintained in a 12-h light/12-h dark cycle (lights on at 0800 hours) in a  
402 temperature-controlled room (22 °C) with free access to food and water, unless otherwise  
403 mentioned. Gestant female C3H/HeNRj mice (n=30) purchased (Janvier Labs, Le Genest  
404 Saint Isle, France) at gestational day 2 were individually housed throughout gestation and  
405 lactation and assigned to either early adversity or control groups.

406

#### 407 **4.2. Multifactorial early-life adversity**

408 The multiple-hit early-life adversity consisted in maternal immune activation during gestation,  
409 chronic MS and maternal exposure to UCMS during lactation. C3H/HeNRj mice were chosen  
410 for this study because this strain has been described as highly maternal and thus is more  
411 likely to be sensitive to MS, contrary to C57/BL6. On embryonic day 17 (E17), dams  
412 randomly assigned to the early adversity group (n=14) received LPS injection (120 µg/kg, i.p,  
413 *E. coli* O127B8, Sigma-Aldrich, St. Quentin Fallavier, France), while dams of the control  
414 group (n=14) received saline (Golan et al., 2005; Labrousse et al., 2018). Maternal body  
415 temperature was monitored immediately before and 3hr after the LPS injection. Five litters  
416 were delivered at gestational day (G)18 (all from LPS-injected dams), and 23 litters at G19.  
417 None of the 5 litters born the day after LPS injection survived. MS was carried out from  
418 PND2 to PND14 (180 min daily) (Rincel et al., 2016) and started randomly at 8:30, 9:00,  
419 9:30, 10:15, 10:30 or 11:00 to minimize habituation. During separation sessions, pups were  
420 individually separated and kept at 32°C ±2 while dams were placed in a new cage and  
421 submitted to UCMS (in random order: no bedding, wet bedding, 45° tilted cage, or defiled,  
422 old and dry, rat bedding). Control litters were housed in an adjacent room and remained  
423 undisturbed until weaning (PND21). Pups' body weight was monitored throughout lactation.  
424 Male and female offspring from litters with equilibrated sex-ratio (n=9 control litters and n=8  
425 early adversity litters) were weaned and kept for long-term analyses. A maximum of 2

426 siblings per dam was used to minimize the litter effect. Two batches of animals were used for  
427 the different experiments (batch 1 N=12 per group, batch 2 N=7-8 per group). The  
428 experimental design is depicted in **Figure 1**.

### 429 **4.3. Behavioral assessment in offspring**

430 From PND7 to 5 months, mice were tested on a battery of tests relevant for psychiatric  
431 disorders (USVs, social interaction, anxiety-like, compulsive-like and depressive-like  
432 behaviors). Locomotor activity was also recorded. Plasma corticosterone in response to  
433 restraint stress was evaluated at the end of behavioral testing. All experiments were  
434 performed during the light phase (8:00-14:00, except for the resident-intruder test: 16:00-  
435 17:00). For analyses involving manual quantifications, experimenters remained blind to the  
436 experimental groups. Animals were collectively housed except for visceral sensitivity and gut  
437 permeability assessment (batch 2).

#### 438 **4.3.1. Ultrasonic vocalizations (USVs) in pups.**

439 USVs were assessed in response to a 6min isolation on PND7-8 (in the afternoon, 4 hours  
440 after the end of the MS episode). Pups were gently removed from the homecage and placed  
441 in a glass crystallizer bedded with thick cotton in a different room. USVs (range 60-80 kHz,  
442 threshold -50 dB) were recorded using an ultrasound microphone coupled with the Recorder  
443 USGH software (Avisoft, Glienicke, Germany) and automatically quantified using the whistle  
444 tracking mode of the SAS LabPro software (Avisoft Bioacoustics).

#### 445 **4.3.2. Elevated plus maze (EPM) (3 months).**

446 The apparatus consisted of two opposing open arms (30 × 8 cm, light intensity: 80 lux) and  
447 two opposing closed arms (30 × 8 × 15 cm, light intensity: 20 lux) connected by a central  
448 platform (8 × 8 cm) and elevated 120 cm above the floor. Mice were placed in the center of  
449 the maze facing an open arm and allowed to explore for 10 min. Distance traveled and time  
450 spent in each arm were automatically quantified using videotracking (Smart software, Bioseb,  
451 Vitrolles, France). The percentages of distance traveled and time spent in open arms were

452 calculated ( $\text{open arms}/(\text{open arms}+\text{closed arms})\times 100$ ). A reduction of the percent of  
453 exploration of the open arms is considered as an anxiety-like index (Walf and Frye, 2007).

#### 454 **4.3.3. Locomotor activity (3.5 months).**

455 Mice were placed in individual cages (30 x 12 cm) filled with fresh bedding under dim light  
456 (light intensity: 30 lux) and videotracked for 60 min using Smart software (Bioseb). Total  
457 distance travelled was automatically quantified.

458 **4.3.4. Marble burying test (4 months).** Mice were individually housed in large cages (30 x  
459 20 cm) filled with 3L of wood chip bedding (5 cm deep). On the next day, they were  
460 transiently removed from the cages and 20 marbles were evenly placed on top of the  
461 bedding as previously described (Deacon, 2006). Mice were put back in the cage and their  
462 behavior was videorecorded during 20 min (light intensity: 80 lux). The number of buried  
463 marbles was manually quantified every 4 min. A marble was considered buried when at least  
464 2/3 of its volume was coated.

465 **4.3.5. Social interaction (4.5 months).** Time spent in social interaction was evaluated over  
466 6 min, under dim light (15 lux) in a new cage (30 x 20 cm) filled with fresh bedding. Pairs of  
467 weight-matched mice from the same experimental group (batch 1) were placed together in  
468 the cage. Since males, but not females, exhibited altered social behavior towards a  
469 conspecific, an additional test was carried out in males only to evaluate another type of social  
470 behavior that is generally male-specific, i.e. aggressive behavior. For this test, a different  
471 subset of males (batch 2) was confronted with aggressor CD1 mice (old breeders previously  
472 used for social defeat protocols, n=6). Aggressors were isolated for 2 weeks and  
473 experimental mice were introduced in their homecage for 6 min (resident-intruder paradigm).  
474 Each aggressor encountered a control mouse and a mouse from the early adversity group to  
475 avoid bias. Mice behavior was recorded using a digital camera and social interaction  
476 (sniffing, allogrooming and crawling over), aggression and submission were manually scored  
477 using an ethological software (The observer, Noldus Information Technology, Wageningen,  
478 The Netherlands).

479 **4.3.6. Tail suspension test (TST) (5 months).** Mice were hanged by the tail to a hook  
480 placed 30cm above the floor of the apparatus (Bioseb) using adhesive tape (and plastic  
481 pipes to prevent from climbing). Their behavior was recorded with a digital camera during  
482 6min. Mice demonstrated several escape attempts interspersed with immobility periods  
483 during which they hung passively and completely motionless. Time spent immobile was  
484 manually quantified using the Observer software (Noldus) (Dinel et al., 2011).

485

#### 486 **4.4. Hypothalamus-pituitary adrenal (HPA) axis reactivity to stress (5 months)**

487 Mice were restrained in perforated 50 ml falcons for 30 min. Blood samples were collected  
488 before the restraint stress (t0, facial vein) and at the end of the stress by tail nick (t30) using  
489 EDTA-coated tubes. Mice returned to their homecage and blood samples were collected 60  
490 min later (t90). Blood samples were centrifuged (4000 rpm, 4°C) for 20 min and stored at -  
491 20°C until use. Plasma corticosterone was determined with an in-house radioimmunoassay  
492 using a highly specific antibody as previously described (Richard et al., 2010). Cross  
493 reactivity with related compound such as cortisol was less than 3%. Intra- and inter-assay  
494 variations were less than 10% and less than 15%, respectively.

495

#### 496 **4.5. Intestinal phenotype of adult offspring**

497 Visceral sensitivity to colorectal distension, *in vivo* intestinal paracellular and transcellular  
498 permeability and gut microbiota composition were evaluated in mice of both sexes (2.5-5.5  
499 months). Since males, but not females, displayed altered intestinal permeability at the first  
500 timepoint (2.5 months), we investigated intestinal permeability at a later timepoint (5.5  
501 months) in males only. In addition, at 5.5 months, a deeper analysis of intestinal function was  
502 carried out also in males only, using *ex-vivo* colonic motility assessment. Enteric neuronal  
503 and glial proteins were also quantified in segments of distal colon.

##### 504 **4.5.1. Colorectal distension (3.5 months).**

505 Mice were individually housed for this experiment. Four days before colorectal distention, 2  
506 electrodes were implanted in the abdominal external oblique musculature of mice previously  
507 anesthetized with xylazine and ketamine. Electrodes were exteriorized at the back of the  
508 neck and protected by a plastic tube attached to the skin. As previously described (Boué et  
509 al., 2014), electrodes were connected to a Bio Amp, which was connected to an  
510 electromyogram acquisition system (ADInstruments, Inc, Colorado Springs, CO). A 10.5-mm-  
511 diameter balloon catheter was gently inserted into the colon at 5 mm proximal to the rectum.  
512 The balloon was inflated in a stepwise fashion. Ten-second distensions were performed at  
513 pressures of 15, 30, 45, and 60 mmHg with 5-minute rest intervals. Electromyographic  
514 activity of the abdominal muscles was recorded and visceromotor responses were calculated  
515 using Chart 5 software (ADInstruments).

#### 516 ***4.5.2. In vivo measurement of intestinal barrier permeability (2.5 or 5.5 months).***

517 Fluorescein-5.6 sulfonic acid (FSA; Invitrogen, Life Technologies, Carlsbad, CA, USA) and  
518 horseradish peroxidase (HRP; Sigma-Aldrich) were used as markers of paracellular and  
519 transcellular permeability, respectively. Mice received a solution of FSA (10 mg/mL) and  
520 HRP (10 mg/mL) diluted in 0.5% of carboxymethyl cellulose by oral gavage (vol. 100µl).  
521 Blood was collected from the tail vein 60 min after gavage. Plasma was prepared by  
522 centrifugation (1,500 g, 7 min). FSA concentration was assessed by measuring the  
523 fluorescence of plasma using a spectrofluorometer microplate reader (Varioskan,  
524 ThermoFisher Scientific, Waltham, MA, USA). HRP quantity was assessed by measuring the  
525 enzymatic activity using the chromogenic substrate 3,3',5,5' tetramethylbenzidine (BD  
526 Biosciences, Le Pont de Claix, France). Optical density was measured using a  
527 spectrofluorometer microplate reader.

#### 528 ***4.5.3. In vivo measurement of colonic motility (5.5 months).***

529 To assess fecal pellet output (FPO), mice were placed individually in a clean cage without  
530 bedding, food and water for fecal pellet collection during 1h. Fecal pellets were collected and  
531 counted every 15 min. For the measurement of total transit time, a solution of carmine red

532 (60 mg/mL) suspended in 0.5% of carboxy-methyl-cellulose was administered by gavage  
533 through a 24 gauge round-tip feeding needle. The volume of carmine red solution used for  
534 each animal was calculated based on body weight (0.25 mg/g). Fecal pellets were monitored  
535 at 5 min intervals for the presence of carmine red. Total transit time was defined as the  
536 interval between the initiation of gavage and the time of first observance of carmine red in  
537 feces.

#### 538 **4.6. Sacrifice, sample collection and ex vivo analyses**

539 After behavioral assessment (5.5 months), feces were collected and stored at -80°C (1-2 per  
540 animal) before mice were deeply anesthetized with isoflurane and killed by decapitation.  
541 Whole brains were collected and stored at -80°C until use. Segments of distal colon  
542 (approximately 1.5 cm) were collected for *ex vivo* intestinal motility experiments. Distal colon  
543 segments were finally used for assessment of protein content.

##### 544 **4.6.1. Ex vivo measurement of colonic motility (males only).**

545 Ex vivo neuromuscular transmission was evaluated as previously described (Suply et al.,  
546 2012). Segments of distal colon were placed in cold oxygenated (5% CO<sub>2</sub>-95% O<sub>2</sub>) Krebs  
547 solution containing (in mM) 117.0 NaCl, 4.7 KCl, 1.2 MgCl<sub>2</sub>, 1.2 NaH<sub>2</sub> PO<sub>4</sub>, 25.0 NaHCO<sub>3</sub>,  
548 2.5 CaCl<sub>2</sub>, and 11.0 glucose. Segments were placed in the longitudinal direction in a 7-ml  
549 organ bath containing oxygenated Krebs solution (37°C) and were stretched with a preload  
550 tension of 10 mN. Preparations were equilibrated for 60 min. Isometric contractions were  
551 recorded with force transducers (No. TRI202PAD, Panlab, Cornellã, Spain) coupled to a  
552 computer equipped with the PowerLab 8/30 System and the Labchart data analysis software  
553 (AD Instruments, Spechbach, Germany). Activation of enteric neurons was performed by  
554 electrical field stimulation (EFS) using a stimulator (STG 4008 MCS, Reutlingen, Germany)  
555 connected to two platinum ring electrodes (11 V, duration of pulse train: 10 s; pulse duration:  
556 400 µs; frequency: 20 Hz). This procedure was repeated three times with 10-min periods  
557 between stimulations. The response of colonic longitudinal muscle to EFS was also  
558 measured in the presence of the NO synthase (NOS) inhibitor, N-nitro-l-arginine methyl ester

559 (L-NAME, 50  $\mu$ M, Sigma-Aldrich), and further in presence of atropine (1  $\mu$ M, Sigma-Aldrich),  
560 an antagonist of cholinergic muscarinic receptors. Drugs were applied 15 min before EFS.  
561 Tension level, amplitude of spontaneous contractions, and area under the curve (AUC)  
562 during each EFS-induced response were measured. Data were normalized to the weight of  
563 the tissue.

#### 564 **4.6.2. Western Blot.**

565 Distal colon segments were lysed in 50 mM Tris, containing 100 mM NaCl, 1% Triton X-100,  
566 1 mM EGTA and protease inhibitors (Complete®; Roche, Boulogne-Billancourt, France), pH  
567 7.4, using the Precellys 24 tissue homogenizer (Bertin Technologies, Montigny-le-  
568 Bretonneux, France) and followed by sonication with Vibracell 75186 device (Sonics, Newton  
569 CT, USA). Total proteins were quantified using BCA protein assay kit (Thermofisher). Equal  
570 amounts of lysate (10  $\mu$ g of proteins) were separated using the Invitrogen NuPage Novex 4-  
571 12% Bis-Tris MidiGels® with lithium dodecyl sulfate running buffer and transferred to  
572 nitrocellulose membranes. Membranes were incubated overnight with either a rabbit anti-  
573 PGP9.5 antibody diluted 1/5000 (Cedarlane, TEBU-BIO France, Le Perray-en-Yvelines,  
574 France), a rabbit anti-MAP2 antibody (1/1000, Millipore, Illkirch, France), a rabbit anti-S100 $\beta$   
575 antibody (1/500, Dako) or a rabbit anti-GFAP antibody (1/5000, Dako), then with a HRP-  
576 conjugated anti-rabbit antibody (Thermoscientific; 1/5000) and visualized by  
577 chemoluminescence (Clarity Western ECL Substrate, Bio-Rad, Marnes-la-Coquette, France)  
578 using a Gel-Doc imager and the Image Lab Software (Bio-Rad). The value of the signal was  
579 normalized to the amount of  $\beta$ -actin, revealed with a mouse anti- $\beta$ -actin antibody (Sigma-  
580 Aldrich, 1/5000) and expressed as a percentage of the average of controls.

#### 581 **4.7. Fecal Microbiota assessment by high-throughput 16S-sequencing**

582 DNA extraction and sequencing were carried out at the GeT-TRiX facility (GénoToul,  
583 Génopole Toulouse Midi-Pyrénées, France). Following DNA concentration estimation by  
584 nanodrop, the V3-V4 region of the 16S rRNA gene was amplified (V3F bac339F-  
585 TACGGRAGGCAGCAG (modified from Wilson KH, et al. J Clin Microbiol. 1990) and V4R

586 bac806R-GGACTACCAGGGTATCTAAT). Illumina MiSeq sequencing was performed on 46  
587 samples. One sample provided too low sequences number (n=365) and was removed from  
588 analysis. Total reads were filtered for length (min length=300bp) and quality (min quality  
589 =25). A total of 316,668 reads was obtained (average 7,037 reads/samples). High quality  
590 reads were pooled, checked for chimeras, and grouped into operational taxonomic units  
591 (OTUs) based on a 97% similarity threshold with uclust software from QIIME. Estimates of  
592 phylotypes richness and diversity were calculated using Shannon and Simpson indices on  
593 the rarefied OTU table (n=4,000 reads). Singletons were removed and phylogenetic affiliation  
594 of each OTU (average 503 OTUs per sample) was done by using ribosomal database project  
595 taxonomy (Cole et al., 2014) and performed from phylum to species level. Wilcoxon/Mann-  
596 Whitney U tests were performed on raw abundance data to determine the effects of early  
597 adversity in each sex. Taxa with uncorrected  $p$  values < 0.05 were considered to be  
598 differentially regulated between conditions. The statistical language R was used for data  
599 visualization and to perform abundance-based principal component analysis (PCA) and inter-  
600 class PCA associated with Monte-Carlo rank testing on the bacterial genera (ade4 library).  
601 To decipher the impact of the different set-ups (sex and early adversity) on microbiota  
602 composition, principal component analyses with the different clinical factors as instrumental  
603 variables were computed based on the abundance of the different bacterial taxa for each  
604 individual (one analysis per environmental factor, data not shown). These inter-class PCA  
605 are appropriate to represent a typology displaying the diversity between individual's  
606 microbiota as it maximizes the variance between populations (here, mice fecal microbiota),  
607 instead of the total variance. Hence, inter-class PCA allows highlighting combinations of  
608 variables (bacterial phylotypes, or genera etc) that maximize variations observed between  
609 qualitative variables (e.g. environmental factors). Based on these inter-class PCA, statistical  
610  $p$ -values of the link between the different factors and microbiota profiles were assessed using  
611 a Monte-Carlo rank test (1000 replicates).

#### 612 **4.8. Gene expression analysis in the medial prefrontal cortex (mPFC)**



613 **4.8.1. Microarrays.**

614 Total mRNA was extracted from mPFC micropunches (anterioposteriority from bregma:  
615 +2.10 to +1.18 mm) using a TRIzol extraction kit (Invitrogen) according to the manufacturer's  
616 instructions. RNA concentration, purity and integrity were determined using a ND-1000  
617 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and a bioanalyzer  
618 (Agilent, Les Ulis, France) (Labrousse et al., 2012). Gene expression profiles were  
619 performed at the GeT-TRiX facility (GénoToul) using Agilent Sureprint G3 Mouse  
620 microarrays (8x60K, design 074809) following the manufacturer's instructions. For each  
621 sample, Cyanine-3 (Cy3) labeled cRNA was prepared from 25 ng of total RNA using the  
622 One-Color Quick Amp Labeling kit (Agilent) according to the manufacturer's instructions,  
623 followed by Agencourt RNAClean XP (AgencourtBioscience Corporation, Beverly,  
624 Massachusetts). Dye incorporation and cRNA yield were checked using Dropsense™ 96  
625 UV/VIS droplet reader (Trinean, Belgium). 600 ng of Cy3-labelled cRNA were hybridized on  
626 the microarray slides following the manufacturer's instructions. Immediately after washing,  
627 the slides were scanned on Agilent G2505C Microarray Scanner using Agilent Scan Control  
628 A.8.5.1 software and fluorescence signal extracted using Agilent Feature Extraction software  
629 v10.10.1.1 with default parameters. Raw data (median signal intensity) were filtered, log2  
630 transformed, corrected for batch effects (microarray washing bath and labeling serials) and  
631 normalized using quantile method (Bolstad et al., 2003). A model was fitted using the  
632 limma lmFit function (Smyth, 2004). Pair-wise comparisons between biological conditions  
633 were applied using specific contrasts. Probes with uncorrected  $p$  value  $< 0.05$  and  
634 foldchange  $> 20\%$  were considered to be differentially expressed between conditions.

635 Microarray data and experimental details are available in NCBI's Gene Expression Omnibus  
636 (Edgar et al., 2002) and are accessible through GEO Series accession number  
637 GSE116416 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE116416>). Microarray  
638 data were analyzed using R (R Development Core Team, 2008) and Bioconductor

639 packages (www.bioconductor.org, v 3.0, (Gentleman et al., 2004) as described in GEO  
640 accession GSE116416.

#### 641 **4.8.2. Validation of transcriptomic data using Microfluidics.**

642 Validation of results from microarrays by qRT-PCR was performed using the BioMark system  
643 (Fluidigm). Detailed information and sequences of primers used are provided in the  
644 **Supplemental Table S1**. The specificity of the PCR reaction was validated according to  
645 MIQE (Minimum Information for publication of Quantitative real time PCR Experiments)  
646 guidelines. Each cDNA was diluted (5 ng/ $\mu$ L) and used for target amplification. Primer pairs  
647 targeting all the genes of interest were pooled to a final concentration of 200nM for each  
648 primer pair. A multiplexed preamplification process was performed for the primers pool on  
649 every 1.25  $\mu$ L of cDNA using 14 cycles of cDNA preamplification step (at 95°C for 15 s and  
650 at 60°C 4 min) and PreAmp Master Mix (Fluidigm) in a standard PCR thermocycler.  
651 Exonuclease I treatment (NEB) was performed to degrade excess primers then preamplified  
652 cDNA was diluted 1:5 in TE low EDTA (10 mM Tris, 0.1 mM EDTA). Diluted cDNA (2  $\mu$ L) was  
653 added to DNA Binding Dye Sample Loading Reagent (Fluidigm), EvaGreen (Interchim) and  
654 TE low EDTA to constitute Sample Mix plate. In Assay Mix plate, 2  $\mu$ L of primer pairs (20  $\mu$ M)  
655 were added to the Assay Loading Reagent (Fluidigm) and TE low EDTA to a final  
656 concentration of 5  $\mu$ M. Following priming the chip in the Integrated Fluidic Circuit Controller,  
657 Sample Mix (5  $\mu$ L) were loaded into the sample inlet wells, and Assay Mix (5  $\mu$ L) were loaded  
658 into assay inlet wells. One well was loaded with water as a contamination control. The  
659 expected value of cycle quantification was around 13. The chip was placed into the IFC  
660 Controller, where 6.3nl of Sample Mix and 0.7nl of Assay Mix were mixed. Real-time PCR  
661 was performed on the Biomark System (Fluidigm) with protocol: Thermal Mix at 50 °C, 2 min;  
662 70 °C, 30min; 25°C, 10min, UNG at 50°C, 2 min, Hot Start at 95°C, 10 min, PCR Cycle of 35  
663 cycles at 95 °C, 15 s; 60 °C, 60 s and Melting curves (to 60°C until 95°C). Results were  
664 analyzed using the Fluidigm Real-Time PCR Analysis software v.4.1.3. to control specific  
665 amplification for each primer, then the raw results of the qPCR were analyzed using GenEx

666 software (MultiD analyses AB) in order to choose the best reference gene to normalize  
667 mRNA expression and to measure the relative expression of each gene between groups.

#### 668 **4.9. Statistics**

669 Data were analyzed using Statistica 6.0 (Statsoft) or R. Normality was assessed using  
670 Shapiro–Wilk tests. Student t-tests were used to test the effects of early adversity on pups'  
671 body weight, adult offspring behavior and dams' body temperature. Social interaction data  
672 were analyzed with non-parametric Mann-Whitney U tests. Two-way ANOVAs with repeated  
673 measures followed by Fisher's LSD *post-hoc* tests were used for USVs, marble burying test,  
674 locomotor activity, corticosterone response to stress and adult body weight analyses.  
675 Pearson correlations were used to examine associations between gut microbiota, behavior  
676 and mPFC gene expression. Statistical significance was set at  $p < 0.05$ . Data are expressed  
677 as means  $\pm$  SEM except for gut microbiota composition (medians).

#### 678 **5. Acknowledgements**

679 This work was supported by the University of Bordeaux, INRA, AVIESAN Immunology,  
680 Hematology and Pneumology. M.R. was supported by a stipend of the French Ministry of  
681 Research. We warmly thank Céline Monot & Karine Le Roux for technical support and  
682 logistics with the microbiological analyses. We are grateful to the INRA MIGALE  
683 bioinformatics platform (<http://migale.jouy.inra.fr>) for providing computational resources. We  
684 also thank the Genotoul Get-PlaGe sequencing platform, and Thierry Lesté-Lasserre for  
685 qPCR primer design and validation. Authors are grateful to Aline Foury and Dr. Marie-Pierre  
686 Moisan for their valuable help with the microarray analysis.

687

688 **6. References**

- 689 Amini-Khoei, H., Haghani-Samani, E., Beigi, M., Soltani, A., Mobini, G.R., Balali-Dehkordi, S., Haj-  
690 Mirzaian, A., Rafieian-Kopaei, M., Alizadeh, A., Hojjati, M.R., Validi, M., 2019. On the role of  
691 corticosterone in behavioral disorders, microbiota composition alteration and neuroimmune response  
692 in adult male mice subjected to maternal separation stress. *Int. Immunopharmacol.* 66, 242–250.  
693 <https://doi.org/10.1016/j.intimp.2018.11.037>
- 694 Arnsten, A.F.T., 2009. Stress signalling pathways that impair prefrontal cortex structure and function.  
695 *Nat. Rev. Neurosci.* 10, 410–422. <https://doi.org/10.1038/nrn2648>
- 696 Avital, A., Richter-Levin, G., 2005. Exposure to juvenile stress exacerbates the behavioural  
697 consequences of exposure to stress in the adult rat. *Int. J. Neuropsychopharmacol.* 8, 163–173.  
698 <https://doi.org/10.1017/S1461145704004808>
- 699 Bae, C.-J., Jeong, J., Saint-Jeannet, J.-P., 2015. A novel function for Egr4 in posterior hindbrain  
700 development. *Sci. Rep.* 5, 7750. <https://doi.org/10.1038/srep07750>
- 701 Bailey, M.T., Coe, C.L., 1999. Maternal separation disrupts the integrity of the intestinal microflora in  
702 infant rhesus monkeys. *Dev. Psychobiol.* 35, 146–155.
- 703 Bercik, P., Verdu, E.F., Foster, J.A., Macri, J., Potter, M., Huang, X., Malinowski, P., Jackson, W.,  
704 Blennerhassett, P., Neufeld, K.A., Lu, J., Khan, W.I., Corthesy-Theulaz, I., Cherbut, C., Bergonzelli,  
705 G.E., Collins, S.M., 2010. Chronic gastrointestinal inflammation induces anxiety-like behavior and  
706 alters central nervous system biochemistry in mice. *Gastroenterology* 139, 2102-2112.e1.  
707 <https://doi.org/10.1053/j.gastro.2010.06.063>
- 708 Bolstad, B.M., Irizarry, R.A., Astrand, M., Speed, T.P., 2003. A comparison of normalization methods  
709 for high density oligonucleotide array data based on variance and bias. *Bioinforma. Oxf. Engl.* 19,  
710 185–193.
- 711 Borre, Y.E., O'Keefe, G.W., Clarke, G., Stanton, C., Dinan, T.G., Cryan, J.F., 2014. Microbiota and  
712 neurodevelopmental windows: implications for brain disorders. *Trends Mol. Med.* 20, 509–518.  
713 <https://doi.org/10.1016/j.molmed.2014.05.002>

714 Boué, J., Basso, L., Cenac, N., Blanpied, C., Rolli-Derkinderen, M., Neunlist, M., Vergnolle, N.,  
715 Dietrich, G., 2014. Endogenous regulation of visceral pain via production of opioids by colitogenic  
716 CD4(+) T cells in mice. *Gastroenterology* 146, 166–175. <https://doi.org/10.1053/j.gastro.2013.09.020>

717 Bravo, J.A., Forsythe, P., Chew, M.V., Escaravage, E., Savignac, H.M., Dinan, T.G., Bienenstock, J.,  
718 Cryan, J.F., 2011. Ingestion of *Lactobacillus* strain regulates emotional behavior and central GABA  
719 receptor expression in a mouse via the vagus nerve. *Proc. Natl. Acad. Sci.* 108, 16050–16055.  
720 <https://doi.org/10.1073/pnas.1102999108>

721 Brown, A.S., 2011. The environment and susceptibility to schizophrenia. *Prog. Neurobiol.* 93, 23–58.  
722 <https://doi.org/10.1016/j.pneurobio.2010.09.003>

723 Brown, A.S., Sourander, A., Hinkka-Yli-Salomäki, S., McKeague, I.W., Sundvall, J., Surcel, H.-M.,  
724 2014. Elevated maternal C-reactive protein and autism in a national birth cohort. *Mol. Psychiatry* 19,  
725 259–264. <https://doi.org/10.1038/mp.2012.197>

726 Buffington, S.A., Di Prisco, G.V., Auchtung, T.A., Ajami, N.J., Petrosino, J.F., Costa-Mattioli, M., 2016.  
727 Microbial Reconstitution Reverses Maternal Diet-Induced Social and Synaptic Deficits in Offspring.  
728 *Cell* 165, 1762–1775. <https://doi.org/10.1016/j.cell.2016.06.001>

729 Chapman, D.P., Whitfield, C.L., Felitti, V.J., Dube, S.R., Edwards, V.J., Anda, R.F., 2004. Adverse  
730 childhood experiences and the risk of depressive disorders in adulthood. *J. Affect. Disord.* 82, 217–  
731 225. <https://doi.org/10.1016/j.jad.2003.12.013>

732 Cole, J.R., Wang, Q., Fish, J.A., Chai, B., McGarrell, D.M., Sun, Y., Brown, C.T., Porras-Alfaro, A.,  
733 Kuske, C.R., Tiedje, J.M., 2014. Ribosomal Database Project: data and tools for high throughput rRNA  
734 analysis. *Nucleic Acids Res.* 42, D633-642. <https://doi.org/10.1093/nar/gkt1244>

735 Covington, H.E., Lobo, M.K., Maze, I., Vialou, V., Hyman, J.M., Zaman, S., LaPlant, Q., Mouzon, E.,  
736 Ghose, S., Tamminga, C.A., Neve, R.L., Deisseroth, K., Nestler, E.J., 2010. Antidepressant Effect of  
737 Optogenetic Stimulation of the Medial Prefrontal Cortex. *J. Neurosci.* 30, 16082–16090.  
738 <https://doi.org/10.1523/JNEUROSCI.1731-10.2010>

739 De Palma, G., Blennerhassett, P., Lu, J., Deng, Y., Park, A.J., Green, W., Denou, E., Silva, M.A.,  
740 Santacruz, A., Sanz, Y., Surette, M.G., Verdu, E.F., Collins, S.M., Bercik, P., 2015. Microbiota and  
741 host determinants of behavioural phenotype in maternally separated mice. *Nat. Commun.* 6, 7735.  
742 <https://doi.org/10.1038/ncomms8735>

743 Deacon, R.M.J., 2006. Digging and marble burying in mice: simple methods for in vivo identification of  
744 biological impacts. *Nat. Protoc.* 1, 122–124. <https://doi.org/10.1038/nprot.2006.20>

745 Deslauriers, J., Larouche, A., Sarret, P., Grignon, S., 2013. Combination of prenatal immune challenge  
746 and restraint stress affects prepulse inhibition and dopaminergic/GABAergic markers. *Prog.*  
747 *Neuropsychopharmacol. Biol. Psychiatry* 45, 156–164. <https://doi.org/10.1016/j.pnpbbp.2013.05.006>

748 Dinel, A.-L., André, C., Aubert, A., Ferreira, G., Layé, S., Castanon, N., 2011. Cognitive and emotional  
749 alterations are related to hippocampal inflammation in a mouse model of metabolic syndrome. *PloS*  
750 *One* 6, e24325. <https://doi.org/10.1371/journal.pone.0024325>

751 Edgar, R., Domrachev, M., Lash, A.E., 2002. Gene Expression Omnibus: NCBI gene expression and  
752 hybridization array data repository. *Nucleic Acids Res.* 30, 207–210.

753 El Aidy, S., Ramsteijn, A.S., Dini-Andreote, F., van Eijk, R., Houwing, D.J., Salles, J.F., Olivier, J.D.A.,  
754 2017. Serotonin Transporter Genotype Modulates the Gut Microbiota Composition in Young Rats, an  
755 Effect Augmented by Early Life Stress. *Front. Cell. Neurosci.* 11, 222.  
756 <https://doi.org/10.3389/fncel.2017.00222>

757 Eriksson, T.M., Delagrange, P., Spedding, M., Popoli, M., Mathé, A.A., Ögren, S.O., Svenningsson, P.,  
758 2012. Emotional memory impairments in a genetic rat model of depression: involvement of 5-  
759 HT/MEK/Arc signaling in restoration. *Mol. Psychiatry* 17, 173–184.  
760 <https://doi.org/10.1038/mp.2010.131>

761 Farrell, M.R., Holland, F.H., Shansky, R.M., Brehm, H.C., 2016. Sex-specific effects of early life  
762 stress on social interaction and prefrontal cortex dendritic morphology in young rats. *Behav. Brain*  
763 *Res.* 310, 119–125. <https://doi.org/10.1016/j.bbr.2016.05.009>

764 Fernández de Cossío, L., Guzmán, A., van der Veldt, S., Luheshi, G.N., 2017. Prenatal infection leads  
765 to ASD-like behavior and altered synaptic pruning in the mouse offspring. *Brain. Behav. Immun.* 63,  
766 88–98. <https://doi.org/10.1016/j.bbi.2016.09.028>

767 Foley, K.A., Ossenkopp, K.-P., Kavaliers, M., Macfabe, D.F., 2014. Pre- and neonatal exposure to  
768 lipopolysaccharide or the enteric metabolite, propionic acid, alters development and behavior in  
769 adolescent rats in a sexually dimorphic manner. *PloS One* 9, e87072.  
770 <https://doi.org/10.1371/journal.pone.0087072>

771 Fransen, F., van Beek, A.A., Borghuis, T., Meijer, B., Hugenholtz, F., van der Gaast-de Jongh, C.,  
772 Savelkoul, H.F., de Jonge, M.I., Faas, M.M., Boekschoten, M.V., Smidt, H., El Aidy, S., de Vos, P.,  
773 2017. The Impact of Gut Microbiota on Gender-Specific Differences in Immunity. *Front. Immunol.* 8,  
774 754. <https://doi.org/10.3389/fimmu.2017.00754>

775 Gacias, M., Gaspari, S., Santos, P.-M.G., Tamburini, S., Andrade, M., Zhang, F., Shen, N., Tolstikov,  
776 V., Kiebish, M.A., Dupree, J.L., Zachariou, V., Clemente, J.C., Casaccia, P., 2016. Microbiota-driven  
777 transcriptional changes in prefrontal cortex override genetic differences in social behavior. *eLife* 5.  
778 <https://doi.org/10.7554/eLife.13442>

779 Gallo, F.T., Katche, C., Morici, J.F., Medina, J.H., Weisstaub, N.V., 2018. Immediate Early Genes,  
780 Memory and Psychiatric Disorders: Focus on c-Fos, Egr1 and Arc. *Front. Behav. Neurosci.* 12, 79.  
781 <https://doi.org/10.3389/fnbeh.2018.00079>

782 García-Ródenas, C.L., Bergonzelli, G.E., Nutton, S., Schumann, A., Cherbut, C., Turini, M., Ornstein,  
783 K., Rochat, F., Corthésy-Theulaz, I., 2006. Nutritional approach to restore impaired intestinal barrier  
784 function and growth after neonatal stress in rats. *J. Pediatr. Gastroenterol. Nutr.* 43, 16–24.  
785 <https://doi.org/10.1097/01.mpg.0000226376.95623.9f>

786 Gentleman, R.C., Carey, V.J., Bates, D.M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L.,  
787 Ge, Y., Gentry, J., Hornik, K., Hothorn, T., Huber, W., Iacus, S., Irizarry, R., Leisch, F., Li, C.,  
788 Maechler, M., Rossini, A.J., Sawitzki, G., Smith, C., Smyth, G., Tierney, L., Yang, J.Y.H., Zhang, J.,  
789 2004. Bioconductor: open software development for computational biology and bioinformatics.  
790 *Genome Biol.* 5, R80. <https://doi.org/10.1186/gb-2004-5-10-r80>

791 Giovanoli, S., Engler, H., Engler, A., Richetto, J., Feldon, J., Riva, M.A., Schedlowski, M., Meyer, U.,  
792 2016. Preventive effects of minocycline in a neurodevelopmental two-hit model with relevance to  
793 schizophrenia. *Transl. Psychiatry* 6, e772. <https://doi.org/10.1038/tp.2016.38>

794 Giovanoli, S., Engler, H., Engler, A., Richetto, J., Voget, M., Willi, R., Winter, C., Riva, M.A.,  
795 Mortensen, P.B., Feldon, J., Schedlowski, M., Meyer, U., 2013. Stress in puberty unmasks latent  
796 neuropathological consequences of prenatal immune activation in mice. *Science* 339, 1095–1099.  
797 <https://doi.org/10.1126/science.1228261>

798 Golan, H.M., Lev, V., Hallak, M., Sorokin, Y., Huleihel, M., 2005. Specific neurodevelopmental damage  
799 in mice offspring following maternal inflammation during pregnancy. *Neuropharmacology* 48, 903–917.  
800 <https://doi.org/10.1016/j.neuropharm.2004.12.023>

801 Guida, F., Turco, F., Iannotta, M., De Gregorio, D., Palumbo, I., Sarnelli, G., Furiano, A., Napolitano,  
802 F., Boccella, S., Luongo, L., Mazzitelli, M., Usiello, A., De Filippis, F., Iannotti, F.A., Piscitelli, F.,  
803 Ercolini, D., de Novellis, V., Di Marzo, V., Cuomo, R., Maione, S., 2018. Antibiotic-induced microbiota  
804 perturbation causes gut endocannabinoidome changes, hippocampal neuroglial reorganization and  
805 depression in mice. *Brain. Behav. Immun.* 67, 230–245. <https://doi.org/10.1016/j.bbi.2017.09.001>

806 Hoban, A.E., Stilling, R.M., M Moloney, G., Moloney, R.D., Shanahan, F., Dinan, T.G., Cryan, J.F.,  
807 Clarke, G., 2017. Microbial regulation of microRNA expression in the amygdala and prefrontal cortex.  
808 *Microbiome* 5, 102. <https://doi.org/10.1186/s40168-017-0321-3>

809 Hoban, A.E., Stilling, R.M., Ryan, F.J., Shanahan, F., Dinan, T.G., Claesson, M.J., Clarke, G., Cryan,  
810 J.F., 2016. Regulation of prefrontal cortex myelination by the microbiota. *Transl. Psychiatry* 6, e774.  
811 <https://doi.org/10.1038/tp.2016.42>

812 Hodes, G.E., Pfau, M.L., Purushothaman, I., Ahn, H.F., Golden, S.A., Christoffel, D.J., Magida, J.,  
813 Brancato, A., Takahashi, A., Flanigan, M.E., 2015. Sex differences in nucleus accumbens  
814 transcriptome profiles associated with susceptibility versus resilience to subchronic variable stress. *J.*  
815 *Neurosci.* 35, 16362–16376.



816 Hollister, E.B., Gao, C., Versalovic, J., 2014. Compositional and functional features of the  
817 gastrointestinal microbiome and their effects on human health. *Gastroenterology* 146, 1449–1458.  
818 <https://doi.org/10.1053/j.gastro.2014.01.052>

819 Hsiao, E.Y., McBride, S.W., Hsien, S., Sharon, G., Hyde, E.R., McCue, T., Codelli, J.A., Chow, J.,  
820 Reisman, S.E., Petrosino, J.F., Patterson, P.H., Mazmanian, S.K., 2013. Microbiota modulate  
821 behavioral and physiological abnormalities associated with neurodevelopmental disorders. *Cell* 155,  
822 1451–1463. <https://doi.org/10.1016/j.cell.2013.11.024>

823 Jašarević, E., Howard, C.D., Misić, A.M., Beiting, D.P., Bale, T.L., 2017. Stress during pregnancy  
824 alters temporal and spatial dynamics of the maternal and offspring microbiome in a sex-specific  
825 manner. *Sci. Rep.* 7, 44182. <https://doi.org/10.1038/srep44182>

826 Jašarević, E., Morrison, K.E., Bale, T.L., 2016. Sex differences in the gut microbiome-brain axis across  
827 the lifespan. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 371, 20150122.  
828 <https://doi.org/10.1098/rstb.2015.0122>

829 Kim, S., Kim, H., Yim, Y.S., Ha, S., Atarashi, K., Tan, T.G., Longman, R.S., Honda, K., Littman, D.R.,  
830 Choi, G.B., Huh, J.R., 2017. Maternal gut bacteria promote neurodevelopmental abnormalities in  
831 mouse offspring. *Nature* 549, 528–532. <https://doi.org/10.1038/nature23910>

832 Kurumaji, A., Ito, T., Ishii, S., Nishikawa, T., 2008. Effects of FG7142 and immobilization stress on the  
833 gene expression in the neocortex of mice. *Neurosci. Res.* 62, 155–159.  
834 <https://doi.org/10.1016/j.neures.2008.08.001>

835 Labouesse, M.A., Langhans, W., Meyer, U., 2015. Long-term pathological consequences of prenatal  
836 infection: beyond brain disorders. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 309, R1–R12.  
837 <https://doi.org/10.1152/ajpregu.00087.2015>

838 Labrousse, V.F., Leyrolle, Q., Amadiou, C., Aubert, A., Sere, A., Coutureau, E., Grégoire, S., Bretillon,  
839 L., Pallet, V., Gressens, P., Joffre, C., Nadjar, A., Layé, S., 2018. Dietary omega-3 deficiency  
840 exacerbates inflammation and reveals spatial memory deficits in mice exposed to lipopolysaccharide  
841 during gestation. *Brain. Behav. Immun.* <https://doi.org/10.1016/j.bbi.2018.06.004>

842 Labrousse, V.F., Nadjar, A., Joffre, C., Costes, L., Aubert, A., Grégoire, S., Bretillon, L., Layé, S.,  
843 2012. Short-term long chain omega3 diet protects from neuroinflammatory processes and memory  
844 impairment in aged mice. *PLoS One* 7, e36861. <https://doi.org/10.1371/journal.pone.0036861>

845 Leclercq, S., Mian, F.M., Stanisiz, A.M., Bindels, L.B., Cambier, E., Ben-Amram, H., Koren, O.,  
846 Forsythe, P., Bienenstock, J., 2017. Low-dose penicillin in early life induces long-term changes in  
847 murine gut microbiota, brain cytokines and behavior. *Nat. Commun.* 8, 15062.  
848 <https://doi.org/10.1038/ncomms15062>

849 Liu, Y.-W., Liu, W.-H., Wu, C.-C., Juan, Y.-C., Wu, Y.-C., Tsai, H.-P., Wang, S., Tsai, Y.-C., 2016.  
850 Psychotropic effects of *Lactobacillus plantarum* PS128 in early life-stressed and naïve adult mice.  
851 *Brain Res.* 1631, 1–12. <https://doi.org/10.1016/j.brainres.2015.11.018>

852 Malki, K., Du Rietz, E., Crusio, W.E., Pain, O., Paya-Cano, J., Karadaghi, R.L., Sluyter, F., de Boer,  
853 S.F., Sandnabba, K., Schalkwyk, L.C., Asherson, P., Tosto, M.G., 2016. Transcriptome analysis of  
854 genes and gene networks involved in aggressive behavior in mouse and zebrafish. *Am. J. Med.*  
855 *Genet. Part B Neuropsychiatr. Genet. Off. Publ. Int. Soc. Psychiatr. Genet.* 171, 827–838.  
856 <https://doi.org/10.1002/ajmg.b.32451>

857 Markle, J.G.M., Frank, D.N., Mortin-Toth, S., Robertson, C.E., Feazel, L.M., Rolle-Kampczyk, U., von  
858 Bergen, M., McCoy, K.D., Macpherson, A.J., Danska, J.S., 2013. Sex differences in the gut  
859 microbiome drive hormone-dependent regulation of autoimmunity. *Science* 339, 1084–1088.  
860 <https://doi.org/10.1126/science.1233521>

861 Mattei, D., Djodari-Irani, A., Hadar, R., Pelz, A., de Cossío, L.F., Goetz, T., Matyash, M., Kettenmann,  
862 H., Winter, C., Wolf, S.A., 2014. Minocycline rescues decrease in neurogenesis, increase in microglia  
863 cytokines and deficits in sensorimotor gating in an animal model of schizophrenia. *Brain. Behav.*  
864 *Immun.* 38, 175–184. <https://doi.org/10.1016/j.bbi.2014.01.019>

865 Maynard, T.M., Sikich, L., Lieberman, J.A., LaMantia, A.S., 2001. Neural development, cell-cell  
866 signaling, and the “two-hit” hypothesis of schizophrenia. *Schizophr. Bull.* 27, 457–476.

867 McEwen, B.S., 1998. Stress, adaptation, and disease. Allostasis and allostatic load. *Ann. N. Y. Acad.*  
868 *Sci.* 840, 33–44.

869 Monte, A.S., Mello, B.S.F., Borella, V.C.M., da Silva Araujo, T., da Silva, F.E.R., Sousa, F.C.F. de, de  
870 Oliveira, A.C.P., Gama, C.S., Seeman, M.V., Vasconcelos, S.M.M., Lucena, D.F.D., Macêdo, D.,  
871 2017. Two-hit model of schizophrenia induced by neonatal immune activation and peripubertal stress  
872 in rats: Study of sex differences and brain oxidative alterations. *Behav. Brain Res.* 331, 30–37.  
873 <https://doi.org/10.1016/j.bbr.2017.04.057>

874 Montesinos, J., Pascual, M., Rodríguez-Arias, M., Miñarro, J., Guerri, C., 2016. Involvement of TLR4  
875 in the long-term epigenetic changes, rewarding and anxiety effects induced by intermittent ethanol  
876 treatment in adolescence. *Brain Behav. Immun.* 53, 159–171.  
877 <https://doi.org/10.1016/j.bbi.2015.12.006>.

878 Mourlon, V., Baudin, A., Blanc, O., Lauber, A., Giros, B., Naudon, L., Daugé, V., 2010. Maternal  
879 deprivation induces depressive-like behaviours only in female rats. *Behav. Brain Res.* 213, 278–287.  
880 <https://doi.org/10.1016/j.bbr.2010.05.017>

881 Moussaoui, N., Jacobs, J.P., Larauche, M., Biraud, M., Million, M., Mayer, E., Taché, Y., 2017.  
882 Chronic Early-life Stress in Rat Pups Alters Basal Corticosterone, Intestinal Permeability, and Fecal  
883 Microbiota at Weaning: Influence of Sex. *J. Neurogastroenterol. Motil.* 23, 135–143.  
884 <https://doi.org/10.5056/jnm16105>

885 Moya-Pérez, A., Perez-Villalba, A., Benítez-Páez, A., Campillo, I., Sanz, Y., 2017. Bifidobacterium  
886 CECT 7765 modulates early stress-induced immune, neuroendocrine and behavioral alterations in  
887 mice. *Brain. Behav. Immun.* 65, 43–56. <https://doi.org/10.1016/j.bbi.2017.05.011>

888 Murakami, T., Kamada, K., Mizushima, K., Higashimura, Y., Katada, K., Uchiyama, K., Handa, O.,  
889 Takagi, T., Naito, Y., Itoh, Y., 2017a. Changes in Intestinal Motility and Gut Microbiota Composition in  
890 a Rat Stress Model. *Digestion* 95, 55–60. <https://doi.org/10.1159/000452364>

891 Nederhof, E., Schmidt, M.V., 2012. Mismatch or cumulative stress: Toward an integrated hypothesis of  
892 programming effects. *Physiol. Behav.*, Special Section: The Mismatch Hypothesis of Psychiatric  
893 Disease 106, 691–700. <https://doi.org/10.1016/j.physbeh.2011.12.008>

894 Nemeroff, C.B., 2016. Paradise Lost: The Neurobiological and Clinical Consequences of Child Abuse  
895 and Neglect. *Neuron* 89, 892–909. <https://doi.org/10.1016/j.neuron.2016.01.019>

896 Nestler, E.J., Hyman, S.E., 2010. Animal models of neuropsychiatric disorders. *Nat. Neurosci.* 13,  
897 1161–1169. <https://doi.org/10.1038/nn.2647>

898 O’Mahony, S.M., Hyland, N.P., Dinan, T.G., Cryan, J.F., 2011. Maternal separation as a model of  
899 brain-gut axis dysfunction. *Psychopharmacology (Berl.)* 214, 71–88. [https://doi.org/10.1007/s00213-](https://doi.org/10.1007/s00213-010-2010-9)  
900 010-2010-9

901 O’Mahony, S.M., Marchesi, J.R., Scully, P., Codling, C., Ceolho, A.-M., Quigley, E.M.M., Cryan, J.F.,  
902 Dinan, T.G., 2009. Early Life Stress Alters Behavior, Immunity, and Microbiota in Rats: Implications for  
903 Irritable Bowel Syndrome and Psychiatric Illnesses. *Biol. Psychiatry* 65, 263–267.  
904 <https://doi.org/10.1016/j.biopsych.2008.06.026>

905 Ons, S., Rotllant, D., Marín-Blasco, I.J., Armario, A., 2010. Immediate-early gene response to  
906 repeated immobilization: Fos protein and arc mRNA levels appear to be less sensitive than c-fos  
907 mRNA to adaptation. *Eur. J. Neurosci.* 31, 2043–2052. [https://doi.org/10.1111/j.1460-](https://doi.org/10.1111/j.1460-9568.2010.07242.x)  
908 9568.2010.07242.x

909 Pönniö, T., Conneely, O.M., 2004. nor-1 regulates hippocampal axon guidance, pyramidal cell  
910 survival, and seizure susceptibility. *Mol. Cell. Biol.* 24, 9070–9078.  
911 <https://doi.org/10.1128/MCB.24.20.9070-9078.2004>

912 Pusceddu, M.M., El Aidy, S., Crispie, F., O’Sullivan, O., Cotter, P., Stanton, C., Kelly, P., Cryan, J.F.,  
913 Dinan, T.G., 2015. N-3 Polyunsaturated Fatty Acids (PUFAs) Reverse the Impact of Early-Life Stress  
914 on the Gut Microbiota. *PloS One* 10, e0139721. <https://doi.org/10.1371/journal.pone.0139721>

915 R Development Core Team, 2008. R: A language and environment for statistical computing. R  
916 Found. Stat. Comput. Vienna Austria ISBN 3-900051-07-0.

917 Rao, A.V., Bested, A.C., Beaulne, T.M., Katzman, M.A., Iorio, C., Berardi, J.M., Logan, A.C., 2009. A  
918 randomized, double-blind, placebo-controlled pilot study of a probiotic in emotional symptoms of  
919 chronic fatigue syndrome. *Gut Pathog.* 1, 6. <https://doi.org/10.1186/1757-4749-1-6>

920 Riba, A., Olier, M., Lacroix-Lamandé, S., Lencina, C., Bacquié, V., Harkat, C., Van Langendonck, N.,  
921 Gillet, M., Cartier, C., Baron, M., Sommer, C., Mallet, V., Zill, M., Robert, H., Laurent, F., Ellero-

922 Simatos, S., Théodorou, V., Ménard, S., 2018. Early life stress in mice is a suitable model for Irritable  
923 Bowel Syndrome but does not predispose to colitis nor increase susceptibility to enteric infections.  
924 *Brain. Behav. Immun.* <https://doi.org/10.1016/j.bbi.2018.05.024>

925 Richard, E.M., Helbling, J.-C., Tridon, C., Desmedt, A., Minni, A.M., Cador, M., Pourtau, L., Konsman,  
926 J.-P., Mormède, P., Moisan, M.-P., 2010. Plasma transcortin influences endocrine and behavioral  
927 stress responses in mice. *Endocrinology* 151, 649–659. <https://doi.org/10.1210/en.2009-0862>

928 Rincel, M., Lépinay, A.L., Delage, P., Fioramonti, J., Théodorou, V.S., Layé, S., Darnaudéry, M., 2016.  
929 Maternal high-fat diet prevents developmental programming by early-life stress. *Transl. Psychiatry* 6,  
930 e966. <https://doi.org/10.1038/tp.2016.235>

931 Russo, S.J., Murrough, J.W., Han, M.-H., Charney, D.S., Nestler, E.J., 2012. Neurobiology of  
932 resilience. *Nat. Neurosci.* 15, 1475–1484. <https://doi.org/10.1038/nn.3234>

933 Shi, H., Sheng, B., Zhang, F., Wu, C., Zhang, R., Zhu, J., Xu, K., Kuang, Y., Jameson, S.C., Lin, Z.,  
934 Wang, Y., Chen, J., Jain, M.K., Atkins, G.B., 2013. Kruppel-like factor 2 protects against ischemic  
935 stroke by regulating endothelial blood brain barrier function. *Am. J. Physiol. Heart Circ. Physiol.* 304,  
936 H796-805. <https://doi.org/10.1152/ajpheart.00712.2012>

937 Shin, S.Y., Han, S.H., Woo, R.-S., Jang, S.H., Min, S.S., 2016. Adolescent mice show anxiety- and  
938 aggressive-like behavior and the reduction of long-term potentiation in mossy fiber-CA3 synapses after  
939 neonatal maternal separation. *Neuroscience* 316, 221–231.  
940 <https://doi.org/10.1016/j.neuroscience.2015.12.041>

941 Shin Yim, Y., Park, A., Berrios, J., Lafourcade, M., Pascual, L.M., Soares, N., Yeon Kim, J., Kim, S.,  
942 Kim, H., Waisman, A., Littman, D.R., Wickersham, I.R., Harnett, M.T., Huh, J.R., Choi, G.B., 2017.  
943 Reversing behavioural abnormalities in mice exposed to maternal inflammation. *Nature* 549, 482–487.  
944 <https://doi.org/10.1038/nature23909>

945 Slotten, H.A., Kalinichev, M., Hagan, J.J., Marsden, C.A., Fone, K.C.F., 2006. Long-lasting changes in  
946 behavioural and neuroendocrine indices in the rat following neonatal maternal separation: gender-  
947 dependent effects. *Brain Res.* 1097, 123–132. <https://doi.org/10.1016/j.brainres.2006.04.066>

948 Slykerman, R.F., Hood, F., Wickens, K., Thompson, J.M.D., Barthow, C., Murphy, R., Kang, J.,  
949 Rowden, J., Stone, P., Crane, J., Stanley, T., Abels, P., Purdie, G., Maude, R., Mitchell, E.A., Probiotic  
950 in Pregnancy Study Group, 2017. Effect of *Lactobacillus rhamnosus* HN001 in Pregnancy on  
951 Postpartum Symptoms of Depression and Anxiety: A Randomised Double-blind Placebo-controlled  
952 Trial. *EBioMedicine* 24, 159–165. <https://doi.org/10.1016/j.ebiom.2017.09.013>

953 Smyth, G.K., 2004. Linear Models and Empirical Bayes Methods for Assessing Differential Expression  
954 in Microarray Experiments. *Stat Appl Genet Mol Biol* 3 Artic.

955 Steel, Z., Marnane, C., Iranpour, C., Chey, T., Jackson, J.W., Patel, V., Silove, D., 2014. The global  
956 prevalence of common mental disorders: a systematic review and meta-analysis 1980-2013. *Int. J.*  
957 *Epidemiol.* 43, 476–493. <https://doi.org/10.1093/ije/dyu038>

958 Suply, E., de Vries, P., Soret, R., Cossais, F., Neunlist, M., 2012. Butyrate enemas enhance both  
959 cholinergic and nitregeric phenotype of myenteric neurons and neuromuscular transmission in newborn  
960 rat colon. *Am. J. Physiol. Gastrointest. Liver Physiol.* 302, G1373-1380.  
961 <https://doi.org/10.1152/ajpgi.00338.2011>

962 Tang, A.T., Choi, J.P., Kotzin, J.J., Yang, Y., Hong, C.C., Hobson, N., Girard, R., Zeineddine, H.A.,  
963 Lightle, R., Moore, T., Cao, Y., Shenkar, R., Chen, M., Mericko, P., Yang, J., Li, L., Tanes, C.,  
964 Kobuley, D., Vösa, U., Whitehead, K.J., Li, D.Y., Franke, L., Hart, B., Schwaninger, M., Henao-Mejia,  
965 J., Morrison, L., Kim, H., Awad, I.A., Zheng, X., Kahn, M.L., 2017. Endothelial TLR4 and the  
966 microbiome drive cerebral cavernous malformations. *Nature* 545, 305–310.  
967 <https://doi.org/10.1038/nature22075>

968 Tsoory, M., Cohen, H., Richter-Levin, G., 2007. Juvenile stress induces a predisposition to either  
969 anxiety or depressive-like symptoms following stress in adulthood. *Eur. Neuropsychopharmacol. J.*  
970 *Eur. Coll. Neuropsychopharmacol.* 17, 245–256. <https://doi.org/10.1016/j.euroneuro.2006.06.007>

971 Tsuda, M.C., Yamaguchi, N., Ogawa, S., 2011. Early life stress disrupts peripubertal development of  
972 aggression in male mice. *Neuroreport* 22, 259–263. <https://doi.org/10.1097/WNR.0b013e328344495a>

973 Ulrich-Lai, Y.M., Herman, J.P., 2009. Neural regulation of endocrine and autonomic stress responses.  
974 *Nat. Rev. Neurosci.* 10, 397–409. <https://doi.org/10.1038/nrn2647>

975 Walf, A.A., Frye, C.A., 2007. The use of the elevated plus maze as an assay of anxiety-related  
976 behavior in rodents. *Nat. Protoc.* 2, 322–328. <https://doi.org/10.1038/nprot.2007.44>

977 Werling, D.M., Geschwind, D.H., 2013. Sex differences in autism spectrum disorders. *Curr. Opin.*  
978 *Neurol.* 26, 146–153. <https://doi.org/10.1097/WCO.0b013e32835ee548>

979 Zheng, P., Zeng, B., Zhou, C., Liu, M., Fang, Z., Xu, X., Zeng, L., Chen, J., Fan, S., Du, X., Zhang, X.,  
980 Yang, D., Yang, Y., Meng, H., Li, W., Melgiri, N.D., Licinio, J., Wei, H., Xie, P., 2016. Gut microbiome  
981 remodeling induces depressive-like behaviors through a pathway mediated by the host's metabolism.  
982 *Mol. Psychiatry* 21, 786–796. <https://doi.org/10.1038/mp.2016.44>

983 Zhou, X.-Y., Li, M., Li, X., Long, X., Zuo, X.-L., Hou, X.-H., Cong, Y.-Z., Li, Y.-Q., 2016. Visceral  
984 hypersensitive rats share common dysbiosis features with irritable bowel syndrome patients. *World J.*  
985 *Gastroenterol.* 22, 5211–5227. <https://doi.org/10.3748/wjg.v22.i22.5211>

986

987

988 **7. Figure Legends**

989 **Fig 1. Experimental design.** Early adversity consisted in combined maternal immune  
990 activation and MS associated with UCMS in dams. On E17, early adversity group received  
991 LPS injection (E. Coli O127B8, 120 µg/kg, i.p.), while control group received saline. MS was  
992 carried out from PND2 to PND14 (180 min daily). Each day of separation, dams were  
993 submitted to UCMS during the 180 min. At PND21, male and female offspring were weaned  
994 and separated in two batches (batch 1 N=12 per group, batch 2 N=7-8 per group). During  
995 infancy, ultrasonic vocalizations in response to a short separation were analyzed in PND7  
996 pups. At adulthood (3-5 months), animals underwent a battery of behavioral tests for anxiety  
997 (elevated plus maze and marble burying), social behavior and depressive-like behavior (tail  
998 suspension test). *In vivo* gut permeability and visceral sensitivity to colorectal distension were  
999 evaluated in a subset of animals at 2.5 and 3.5 months, respectively. Finally, HPA axis  
1000 responsiveness to restraint stress was assessed and the animals were killed 2 weeks later  
1001 (5.5 months) for fecal microbiota composition analysis by high-throughput 16S RNA  
1002 sequencing. Gene expression in the medial prefrontal cortex was analyzed by microarrays in  
1003 the same animals. Unless stated ♂, all the experiments were conducted in both males and  
1004 females. E, Embryonic day; PND, Post-natal day; LPS, Lipopolysaccharide; USVs, Ultrasonic  
1005 vocalizations; MS, Maternal separation; UCMS, Unpredictable chronic mild stress; IP,  
1006 intestinal permeability; EPM, Elevated plus maze; Aggr, interaction with an aggressor; TST,  
1007 Tail suspension test; HPA, Hypothalamic-pituitary-adrenal axis response to stress; mPFC,  
1008 medial prefrontal cortex.

1009 **Fig 2. Early adversity leads to social behavior impairment in males at adulthood. (A-C)**  
1010 USV response to acute short separation in PND7 male pups (N=9 per group). **(A)** Each line  
1011 corresponds to one animal and each dash represents one USV call. **(B)** USV call number  
1012 across time (min) and **(C)** latency (s) to first USV. **(D-M)** Adult phenotype of male mice. **(D)**  
1013 Time (s) spent in social interaction with a conspecific over 6 min of test and **(E)** latency (s) to  
1014 first interaction (N=6 pairs per group). **(F)** Number of attacks by the aggressor and **(G)** time



1015 (s) spent in defensive behavior over 6 min in the resident-intruder paradigm (N=6-9 per  
1016 group). **(H)** Time spent (%) in the open arms of the EPM (N=10-12 per group). **(I)** Number of  
1017 buried marbles across time (min) in the marble burying test (N=11-12 per group). **(J)** Time (s)  
1018 spent immobile over the 6min of test in the TST (N=11-12 per group). **(K)** Plasma  
1019 corticosterone (ng/mL) in response to 30-min restraint stress at 0, 30 and 90 min. **(L)** Body  
1020 weight (g) before and 3 weeks after exposure to acute restraint stress (30min) and **(M)** Body  
1021 weight gain (g) 3 weeks after the TST and acute restraint stress (N=10-12 per group). Data  
1022 are mean  $\pm$  SEM. \*  $p<0.05$ , \*\*  $p<0.01$  and \*\*\*  $p<0.001$  versus control group (planned  
1023 comparisons in **B**; Student *t*-tests in **C** and **M**; Mann-Whitney *U* tests in **D**, **E** and **G**); +++  
1024  $p<0.001$  control pre- versus poststress, Fisher LSD's post-hoc tests.

1025 **Fig 3. Early adversity leads to increased anxiety in females at adulthood. (A-C)** USV  
1026 response to acute short separation in PND8 female pups (N=9 per group). **(A)** Each line  
1027 corresponds to one animal and each dash represents one USV call. **(B)** USV call number  
1028 across time (min) and **(C)** latency (s) to first USV. **(D-K)** Adult phenotype of female mice. **(D)**  
1029 Time (s) spent in social interaction with a conspecific over 6 min of test (N=6 pairs per  
1030 group). **(E)** Time spent (%) and **(F)** distance traveled (%) in the open arms of the EPM  
1031 (N=10-12 per group). **(G)** Number of buried marbles across time (min) in the marble burying  
1032 test (N=11-12 per group). **(H)** Time (s) spent immobile over the 6min of test in the TST  
1033 (N=11-12 per group). **(I)** Plasma corticosterone (ng/mL) in response to 30-min restraint stress  
1034 at 0, 30 and 90 min. Inset: recovery to basal levels (%) (N=10 per group). **(J)** Body weight (g)  
1035 before and 3 weeks after exposure to acute restraint stress (30min) and **(K)** Body weight  
1036 gain (g) 3 weeks after the TST and acute restraint stress (N=10-12 per group). Data are  
1037 mean  $\pm$  SEM. \*  $p<0.05$ , \*\*  $p<0.01$  and \*\*\*  $p<0.001$  versus control group (planned  
1038 comparisons in **B**, **G** and **I**; Student *t*-tests in **C**, **E**, **F**, inset in **I** and **K**); ++  $p<0.01$  control pre-  
1039 versus poststress, Fisher LSD's post-hoc tests.

1040

1041 **Fig 4. Early adversity leads to increased intestinal permeability in adult males but not**  
1042 **females. (A,D)** Visceral sensitivity to colorectal distension (N=7-9 per group). *In vivo*  
1043 intestinal permeability to HRP (HRP/mg plasma) **(B,E)** or FSA (AU) **(C,F)** (N=10-11 per  
1044 group). Data are mean  $\pm$  SEM. \*  $p < 0.05$  versus control group (Student *t*-test).

1045 **Fig 5. Effects of early adversity on fecal microbiota composition in adult males and**  
1046 **females. (A-F)** Males and **(G-K)** females. **(A,G)** PCA based on genus distribution in early  
1047 adversity and control mice. **(B,H)** Alpha diversity (Shannon and Simpson indices). Genera  
1048 with significantly decreased **(C,I)** or increased **(D)** relative abundance in early adversity  
1049 versus controls. Horizontal lines in **B-D** and **H-I** represent medians. Mann-Whitney U Test, \*  
1050  $p < 0.05$ . N=11-12 per group.

1051 **Fig 6. Whole genome transcript expression in the medial prefrontal cortex. (A)**  
1052 Microarrays revealed 156 genes and 108 genes significantly regulated by multi-hit adversity  
1053 ( $p < 0.05$  and foldchange  $> 20\%$ ) in males and females, respectively (N=10-12 per group). **(B-**  
1054 **C)** The heatmap shows the relative expression (Z-score for microarrays and relative quantity  
1055 for qPCR) of the 10 genes (rows) validated by qPCR in males or females (each column  
1056 represents a single animal). *Arc*, activity regulated cytoskeleton associated protein; *Btg2*, B  
1057 cell translocation gene 2; *Dusp1*, dual specificity phosphatase 1; *Egr4*, early growth response  
1058 4; *Fosb*, Fos proto-oncogene b; *Gadd45b*, growth arrest and DNA-damage-inducible 45 beta;  
1059 *H2-k1*, histocompatibility 2, K region locus 1; *Junb*, Jun proto-oncogene b; *Klf2*, Krüppel-like  
1060 factor 2; *Nr4a3*, nuclear receptor subfamily 4 group A Member 3.

1061

1062

1063

1064

1065

1066

1067

## OTUs significantly regulated by early adversity

	Total regulated			Depleted			Enriched		
	males	females	both sexes	males	females	both sexes	males	females	both sexes
<b>Total</b>	92	64	10	59	37	9	33	27	1
<b>Phylum</b>									
Firmicutes	69	45	8	51	30	8	18	15	0
Bacteroidetes	21	16	1	8	5	1	13	11	0
Proteobacteria	2	1	1	0	0	0	2	1	1
Actinobacteria	0	1	0	0	1	0	0	0	0
Deferribacteres	0	1	0	0	1	0	0	0	0
<b>Genus</b>									
<b>uncl. Lachnospiraceae</b>	<b>38</b>	<b>18</b>	<b>7</b>	<b>34</b>	<b>14</b>	<b>7</b>	<b>4</b>	<b>4</b>	<b>0</b>
<i>Clostridium XIVa</i>	10	14	0	6	8	0	4	6	0
<i>Barnesiella</i>	10	6	1	4	1	1	6	5	0
<b>Bacteroides</b>	<b>4</b>	<b>3</b>	<b>0</b>	<b>0</b>	<b>1</b>	<b>0</b>	<b>4</b>	<b>2</b>	<b>0</b>
<i>uncl. Ruminococcaceae</i>	4	2	0	1	0	0	3	2	0
<b>Lactobacillus</b>	<b>3</b>	<b>3</b>	<b>1</b>	<b>1</b>	<b>3</b>	<b>1</b>	<b>2</b>	<b>0</b>	<b>0</b>
<i>uncl. Clostridiales</i>	3	2	0	2	1	0	1	1	0
<b>uncl. Porphyromonadaceae</b>	<b>3</b>	<b>1</b>	<b>0</b>	<b>3</b>	<b>1</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>
<i>Alistipes</i>	2	3	0	0	1	0	2	2	0
<i>Clostridium IV</i>	2	2	0	2	1	0	0	1	0
<i>Roseburia</i>	2	1	0	2	0	0	0	1	0
<i>Anaerostipes</i>	2	0	0	1	0	0	1	0	0
<i>Parasutterella</i>	1	1	1	0	0	0	1	1	1
<i>Blautia</i>	1	1	0	0	1	0	1	0	0
<i>Lachnospiraceae incertae sedis</i>	1	1	0	1	1	0	0	0	0
<b>Alloprevotella</b>	<b>1</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>1</b>	<b>0</b>	<b>0</b>
<i>Oscillibacter</i>	1	0	0	0	0	0	1	0	0
<b>Porphyromonas</b>	<b>1</b>	<b>0</b>	<b>0</b>	<b>1</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>
<i>Pseudoflavonifractor</i>	1	0	0	1	0	0	0	0	0
<b>uncl. Firmicutes</b>	<b>1</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>1</b>	<b>0</b>	<b>0</b>
<i>uncl. Pasteurellaceae</i>	1	0	0	0	0	0	1	0	0
<i>Odoribacter</i>	0	2	0	0	1	0	0	1	0
<i>Anaerotruncus</i>	0	1	0	0	1	0	0	0	0
<b>Mucispirillum</b>	<b>0</b>	<b>1</b>	<b>0</b>	<b>0</b>	<b>1</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>
<i>uncl. Bacteroidale</i>	0	1	0	0	0	0	0	1	0
<i>uncl. Coriobacteriaceae</i>	0	1	0	0	1	0	0	0	0

1069 **Effects of early adversity on fecal microbiota composition at the OTU level.** Within each  
1070 phylum or genus, the number of OTUs significantly regulated by early adversity in males,  
1071 females or in both sexes is provided (at least  $p < 0.05$ ). A total of 1453 different OTUs were  
1072 detected in the fecal samples of control and early adversity mice. Genera with overall  
1073 significantly altered abundance (see Figure 4) are indicated in bold letters. N=11-12 per  
1074 group. OTU, operational taxonomic unit.

1075  
1076  
1077  
1078  
1079  
1080  
1081  
1082  
1083  
1084

1085 **Table 2**

1086

<b>Males</b>			
<b>Social interaction</b>			
<b>mPFC gene expression</b>	<i>r</i>	<i>p</i>	N
<i>Arc</i>	0.77	0.003	12
<b>OTU abundance</b>	<i>r</i>	<i>p</i>	N
OTU 2197 ( <i>Clostridium XIVa</i> )*	0.90	0.000	12
OTU 1179 ( <i>uncl. Lachnospiraceae</i> )*	0.78	0.003	12
OTU 2615 ( <i>uncl. Lachnospiraceae</i> )*	0.74	0.006	12
OTU 2752 ( <i>uncl. Lachnospiraceae</i> )	0.67	0.018	12
OTU 2730 ( <i>Lactobacillus</i> )	-0.66	0.019	12
OTU 356 ( <i>Bacteroides</i> )	-0.61	0.035	12
OTU 748 ( <i>uncl. Ruminococcaceae</i> )*	-0.60	0.039	12
OTU 1573 ( <i>Oscillibacter</i> )	-0.59	0.042	12
<b>Females</b>			
<b>EPM</b>			
<b>mPFC gene expression</b>	<i>r</i>	<i>p</i>	N
<i>Arc</i>	-0.54	0.009	22
<i>Fosb</i>	-0.46	0.030	22
<i>Gadd45b</i>	-0.50	0.016	22
<b>OTU abundance</b>	<i>r</i>	<i>p</i>	N
OTU 2656 ( <i>uncl. Lachnospiraceae</i> )*	0.52	0.014	22
OTU 2071 ( <i>Odoribacter</i> )*	0.46	0.033	22
OTU 2183 ( <i>Clostridium XIVa</i> )*	0.45	0.037	22
<b>Marble burying</b>			
<b>mPFC gene expression</b>	<i>r</i>	<i>p</i>	N
<i>Fosb</i>	0.47	0.020	21
<i>Junb</i>	0.50	0.017	21
<b>OTU abundance</b>	<i>r</i>	<i>p</i>	N
OTU 2266 ( <i>Blautia</i> )*	-0.62	0.003	21
OTU 1774 ( <i>Lactobacillus</i> )	-0.51	0.017	21
OTU 2287 ( <i>Lactobacillus</i> )*	-0.51	0.018	21
OTU 746 ( <i>Barnesiella</i> )*	0.47	0.033	21
OTU 1797 ( <i>Clostridium XIVa</i> )*	-0.44	0.046	21

1087 **Correlations between behavioral data and mPFC gene expression/bacterial OTU**  
 1088 **abundance.** Only significant correlations are shown (at least  $p < 0.05$ ). Pearson *r* coefficients,  
 1089 exact *p*-value and group size are indicated for each correlation. Only genes validated by  
 1090 PCR and only top regulated OTUs are displayed. Relative quantity (Rq in qPCR), relative  
 1091 abundance (%), time spent in social interaction (s), time spent in open arms (%) and

1092 percentage of buried marbles (%) were used for correlations between mPFC gene  
1093 expression, bacterial OTUs, social interaction, EPM and marble burying test data,  
1094 respectively. mPFC, medial prefrontal cortex; OTU, operational taxonomic unit; EPM,  
1095 elevated plus maze; *Arc*, *activity regulated cytoskeleton associated protein*; *Fosb*, *Fos proto-*  
1096 *oncogene b*; *Gadd45b*, *growth arrest and DNA-damage-inducible 45 beta*; *Junb*, *Jun proto-*  
1097 *oncogene b*.  
1098

1099

1100

1101

1102

1103

1104

1105

1106

1107

1108

1109

1110

1111

1112

1113

1114

1115

1116

1117

1118

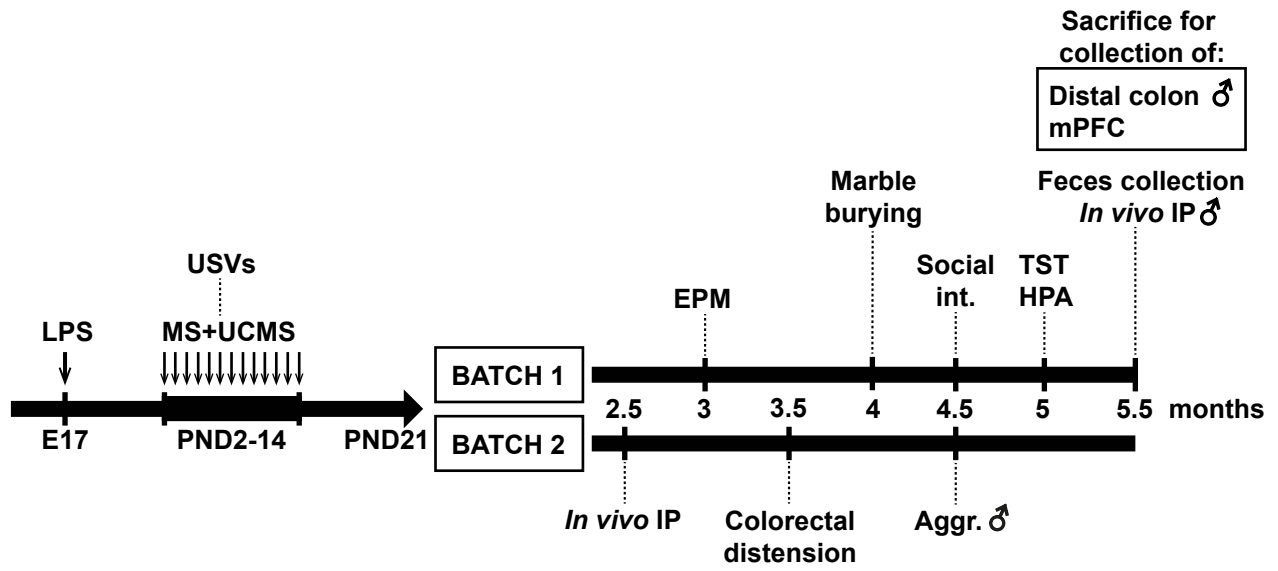
1119

1120

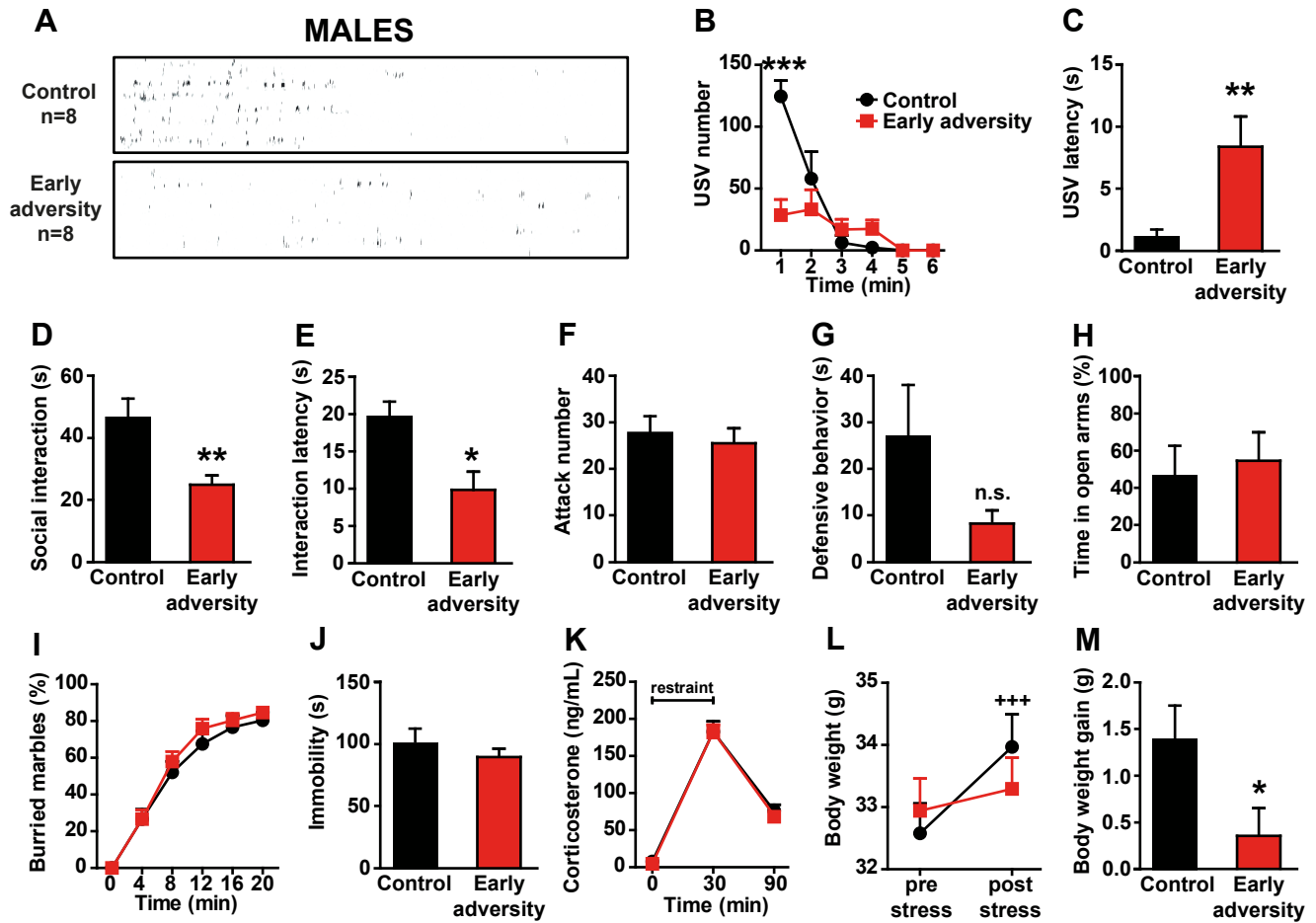
1121

1122

Figure 1



**Figure 2**



**Figure 3**

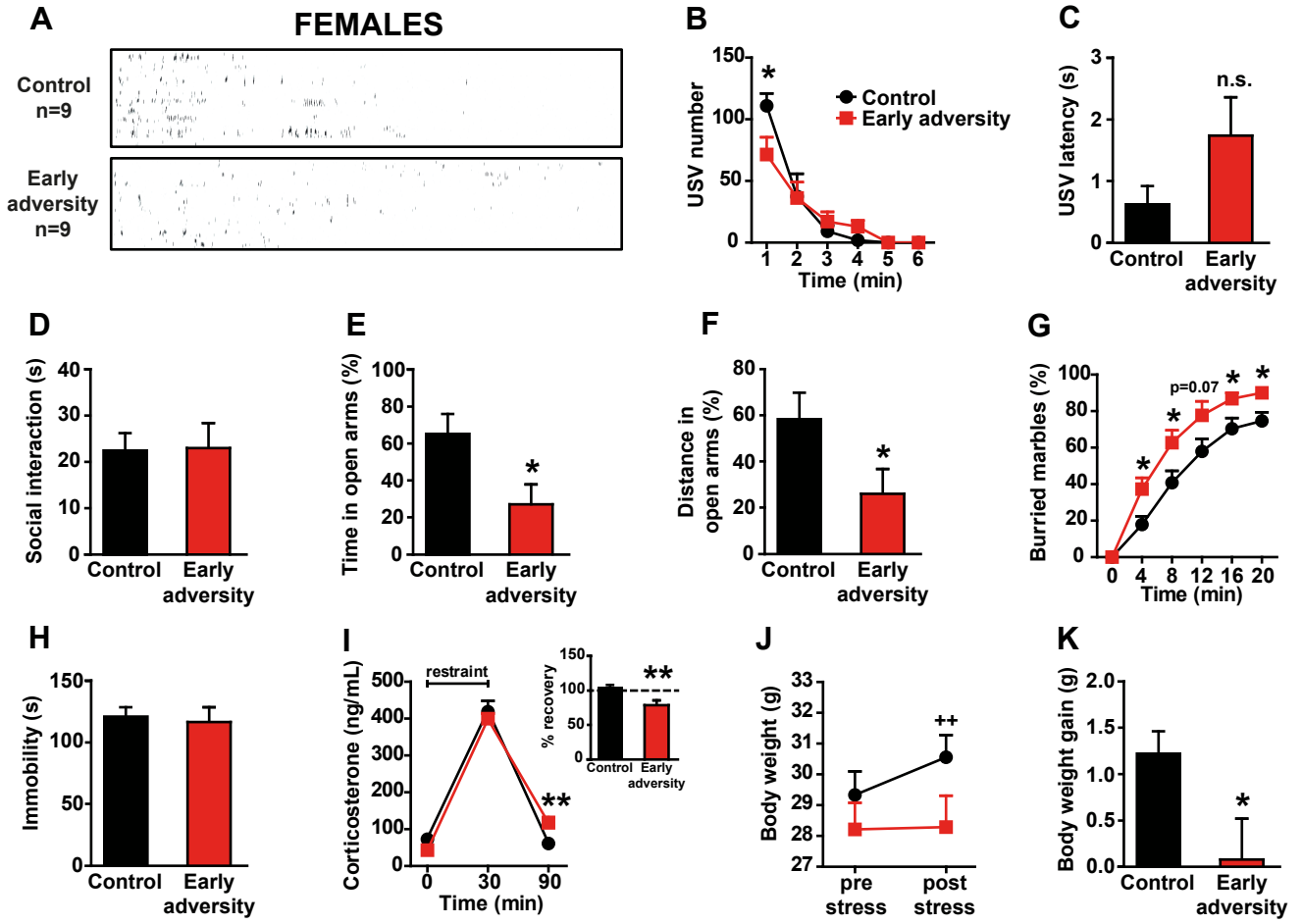
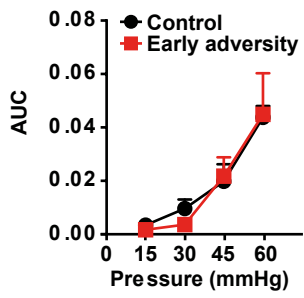




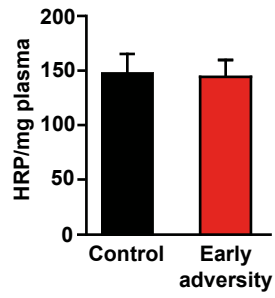
Figure 4

**MALES**

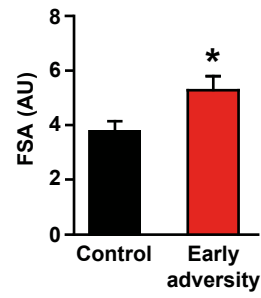
**A**



**B**

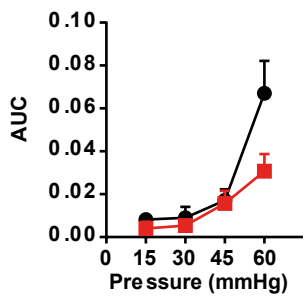


**C**

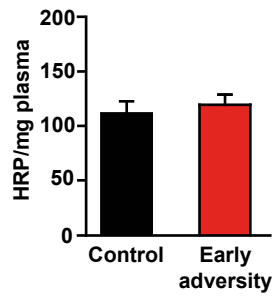


**FEMALES**

**D**



**E**



**F**

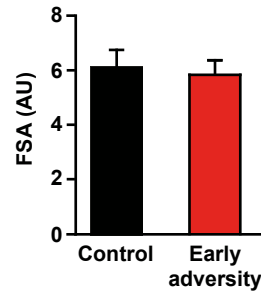
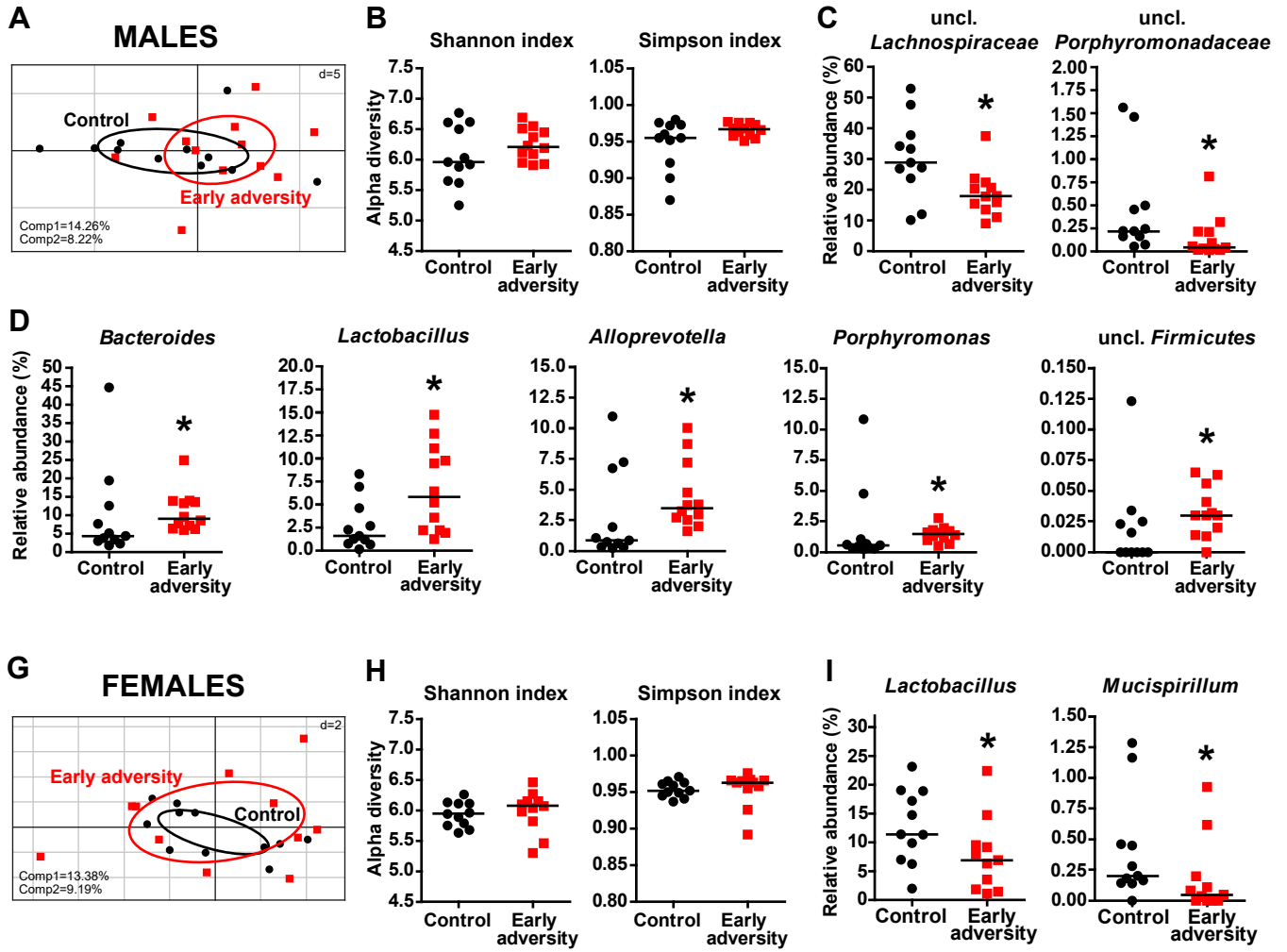


Figure 5



**Figure 6**

