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Abstract (263 words)

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

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

1. INTRODUCTION

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

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

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

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

2. RESULTS

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

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

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

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

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

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

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

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

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

(Figure 3H). Plasma corticosterone levels after restraint stress varied depending upon time and the history of early adversity ($F(1,36)=3.88$, $p=0.030$) (**Figure 3I)**. Planned comparisons revealed significantly higher corticosterone levels in the early adversity group 60 min after the end of restraint (t90, $p=0.006$). Further analysis showed that corticosterone recovery to basal levels was lower in the early adversity group ($t(18)=3.31$, $p=0.004$ **(Figure 3I, inset)**). Finally, as seen in males, the body weight evolution of females exposed to early adversity was stopped after TST and restraint stress exposures, whereas the control group exhibited a normal body weight progression ($F(1,21)=5.11$, $p=0.035$; control pre- *vs* poststress, $p=0.003$; early adversity pre- *vs* poststress, n.s.; body weight gain: ($t(21)=2.26$, $p=0.035$)) **(Figure 3J,K)**.

2.4. Early adversity induces gastrointestinal dysfunctions in adult males but not females

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

in mice exposed to early adversity after atropine application in the bath (t(14)=3.57, p=0.003) **(Supplementary Figure S3E)**. Finally, Western blots of several key markers for enteric neurons and enteric glial cells showed significantly decreased expression of MAP2 in the distal colon from early adversity mice (*U*=4, p=0.026) **(Supplementary Figure S3F)**. There was no significant difference in the expression of PGP 9.5, S100β and GFAP between groups (see full names in legend).

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

2.5. Early adversity differentially alters gut microbiota composition in adult males and females

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

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

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

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

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

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

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

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

3. DISCUSSION

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

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

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

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

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

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

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

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

4. MATERIALS AND METHODS

4.1. Animals

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

4.2. Multifactorial early-life adversity

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

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

4.3. Behavioral assessment in offspring

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

4.3.1. Ultrasonic vocalizations (USVs) in pups.

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

4.3.2. Elevated plus maze (EPM) (3 months).

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

calculated (open arms/(open arms+closed arms)x100). A reduction of the percent of exploration of the open arms is considered as an anxiety-like index (Walf and Frye, 2007).

4.3.3. Locomotor activity (3.5 months).

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

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

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

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

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

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

4.5. Intestinal phenotype of adult offspring

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

4.5.1. Colorectal distension (3.5 months).

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

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

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

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

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

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

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

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

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

Ex vivo neuromuscular transmission was evaluated as previously described (Suply et al., 2012). Segments of distal colon were placed in cold oxygenated (5% CO2-95% O2) Krebs solution containing (in mM) 117.0 NaCl, 4.7 KCl, 1.2 MgCl2, 1.2 NaH2 PO4, 25.0 NaHCO3, 2.5 CaCl2, and 11.0 glucose. Segments were placed in the longitudinal direction in a 7-ml organ bath containing oxygenated Krebs solution (37°C) and were stretched with a preload tension of 10 mN. Preparations were equilibrated for 60 min. Isometric contractions were recorded with force transducers (No. TRI202PAD, Panlab, Cornellã, Spain) coupled to a computer equipped with the PowerLab 8/30 System and the Labchart data analysis software (AD Instruments, Spechbach, Germany). Activation of enteric neurons was performed by electrical field stimulation (EFS) using a stimulator (STG 4008 MCS, Reutlingen, Germany) connected to two platinum ring electrodes (11 V, duration of pulse train: 10 s; pulse duration: 400 µs; frequency: 20 Hz). This procedure was repeated three times with 10-min periods between stimulations. The response of colonic longitudinal muscle to EFS was also measured in the presence of the NO synthase (NOS) inhibitor, N-nitro-l-arginine methyl ester

559 (L-NAME, 50 µM, Sigma-Aldrich), and further in presence of atropine (1 y-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 µg 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-S100β
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 β-actin, revealed with a mouse anti-β-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

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

4.8.2. Validation of transcriptomic data using Microfluidics.

Validation of results from microarrays by qRT-PCR was performed using the BioMark system (Fluidigm). Detailed information and sequences of primers used are provided in the **Supplemental Table S1**. The specificity of the PCR reaction was validated according to MIQE (Minimum Information for publication of Quantitative real time PCR Experiments) guidelines. Each cDNA was diluted (5 ng/µL) and used for target amplification. Primer pairs targeting all the genes of interest were pooled to a final concentration of 200nM for each primer pair. A multiplexed preamplification process was performed for the primers pool on every 1.25 µL of cDNA using 14 cycles of cDNA preamplification step (at 95°C for 15 s and at 60°C 4 min) and PreAmp Master Mix (Fluidigm) in a standard PCR thermocycler. Exonuclease I treatment (NEB) was performed to degrade excess primers then preamplified cDNA was diluted 1:5 in TE low EDTA (10 mMTris, 0.1 mM EDTA). Diluted cDNA (2 µL) was added to DNA Binding Dye Sample Loading Reagent (Fluidigm), EvaGreen (Interchim) and TE low EDTA to constitute Sample Mix plate. In Assay Mix plate, 2 µL of primer pairs (20 µM) were added to the Assay Loading Reagent (Fluidigm) and TE low EDTA to a final concentration of 5 µM. Following priming the chip in the Integrated Fluidic Circuit Controller, Sample Mix (5 µL) were loaded into the sample inlet wells, and Assay Mix (5 µl) were loaded into assay inlet wells. One well was loaded with water as a contamination control. The expected value of cycle quantification was around 13. The chip was placed into the IFC Controller, where 6.3nl of Sample Mix and 0.7nl of Assay Mix were mixed. Real-time PCR was performed on the Biomark System (Fluidigm) with protocol: Thermal Mix at 50 °C, 2 min; 70 °C, 30min; 25°C, 10min, UNG at 50°C, 2 min, Hot Start at 95°C, 10 min, PCR Cycle of 35 cycles at95 °C, 15 s; 60 °C, 60 s and Melting curves (to 60°C until 95°C). Results were analyzed using the Fluidigm Real-Time PCR Analysis software v.4.1.3. to control specific amplification for each primer, then the raw results of the qPCR were analyzed using GenEx

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

4.9. Statistics

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

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7. Figure Legends

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

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

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

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

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

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

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

Table 1

	OTUs significantly regulated by early adversity								
	Total regulated			Depleted			Enriched		
	males	females	both sexes	males	females	both sexes	males	females	both sexes
Total	92	64	10	59	37	9	33	27	1
Phylum									
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
Genus									
uncl. Lachnospiraceae	**38**	**18**	**7**	**34**	**14**	**7**	**4**	**4**	**0**
Clostridium XlVa	10	14	0	6	8	0	4	6	0
Barnesiella	10	6	1	4	1	1	6	5	0
Bacteroides	**4**	**3**	**0**	**0**	**1**	**0**	**4**	**2**	**0**
uncl. Ruminococcaceae	4	2	0	1	0	0	3	2	0
Lactobacillus	**3**	**3**	**1**	**1**	**3**	**1**	**2**	**0**	**0**
uncl. Clostridiales	3	2	0	2	1	0	1	1	0
uncl. Porphyromonadaceae	**3**	**1**	**0**	**3**	**1**	**0**	**0**	**0**	**0**
Alistipes	2	3	0	0	1	0	2	2	0
Clostridium IV	2	2	0	2	1	0	0	1	0
Roseburia	2	1	0	2	0	0	0	1	0
Anaerostipes	2	0	0	1	0	0	1	0	0
Parasutterella	1	1	1	0	0	0	1	1	1
Blautia	1	1	0	0	1	0	1	0	0
Lachnospiracea incertae sedis	1	1	0	1	1	0	0	0	0
Alloprevotella	**1**	**0**	**0**	**0**	**0**	**0**	**1**	**0**	**0**
Oscillibacter	1	0	0	0	0	0	1	0	0
Porphyromonas	**1**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**
Pseudoflavonifractor	1	0	0	1	0	0	0	0	0
uncl. Firmicutes	**1**	**0**	**0**	**0**	**0**	**0**	**1**	**0**	**0**
uncl. Pasteurellaceae	1	0	0	0	0	0	1	0	0
Odoribacter	0	2	0	0	1	0	0	1	0
Anaerotruncus	0	1	0	0	1	0	0	0	0
Mucispirillum	**0**	**1**	**0**	**0**	**1**	**0**	**0**	**0**	**0**
uncl. Bacteroidale	0	1	0	0	0	0	0	1	0
uncl. Coriobacteriaceae	0	1	0	0	1	0	0	0	0

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

Table 2

	Males		
	Social interaction		
mPFC gene expression	*r*	*p*	N
Arc	0.77	0.003	12
OTU abundance	*r*	*p*	N
OTU 2197 *(Clostridium XlVa)**	0.90	0.000	12
OTU 1179 *(uncl. Lachnospiraceae)**	0.78	0.003	12
OTU 2615 *(uncl. Lachnospiraceae)**	0.74	0.006	12
OTU 2752 *(uncl. Lachnospiraceae)*	0.67	0.018	12
OTU 2730 *(Lactobacillus)*	-0.66	0.019	12
OTU 356 *(Bacteroides)*	-0.61	0.035	12
OTU 748 *(uncl. Ruminococcaceae)**	-0.60	0.039	12
OTU 1573 *(Oscillibacter)*	-0.59	0.042	12

	Females		
	EPM		
mPFC gene expression	*r*	*p*	N
Arc	-0.54	0.009	22
Fosb	-0.46	0.030	22
Gadd45b	-0.50	0.016	22
OTU abundance			
OTU 2656 *(uncl. Lachnospiraceae)**	0.52	0.014	22
OTU 2071 *(Odoribacter)**	0.46	0.033	22
OTU 2183 *(Clostridium XlVa)**	0.45	0.037	22

	Marble burying		
mPFC gene expression	*r*	*p*	N
Fosb	0.47	0.020	21
Junb	0.50	0.017	21
OTU abundance			
OTU 2266 *(Blautia)**	-0.62	0.003	21
OTU 1774 *(Lactobacillus)*	-0.51	0.017	21
OTU 2287 *(Lactobacillus)**	-0.51	0.018	21
OTU 746 *(Barnesiella)**	0.47	0.033	21
OTU 1797 *(Clostridium XlVa)**	-0.44	0.046	21

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

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

Figure 1

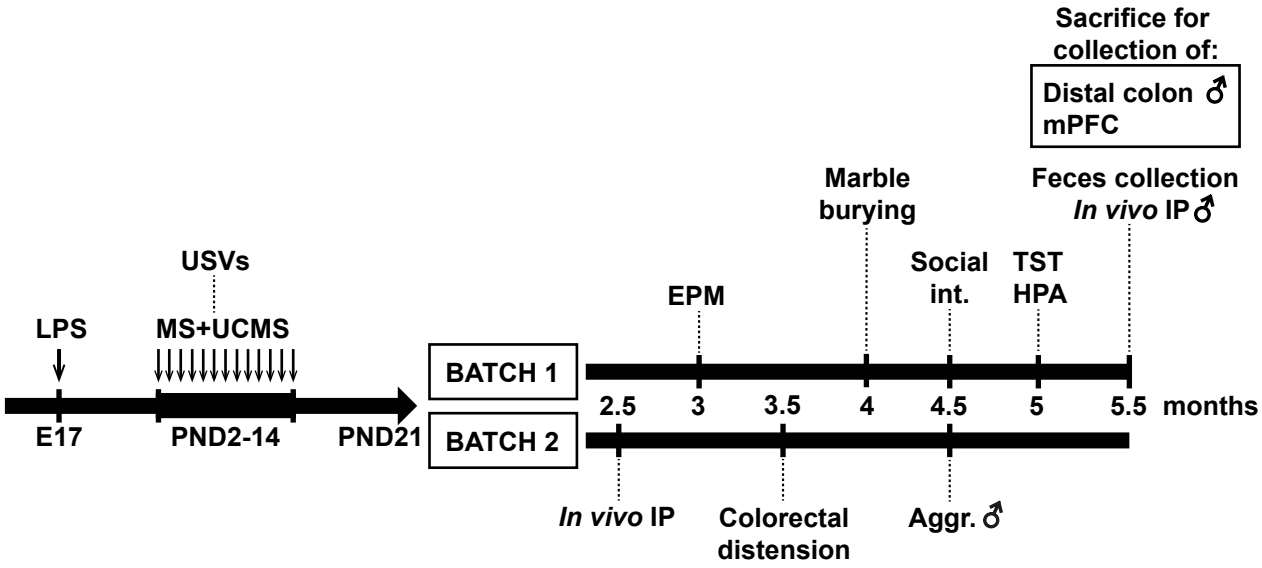

Sacrifice for collection of:
Distal colon ♂
mPFC
Marble burying
Feces collection
In vivo IP ♂
USVs
Social int.
TST
HPA
EPM
LPS
MS+UCMS
BATCH 1
BATCH 2
2.5
3
3.5
4
4.5
5
5.5
months
E17
PND2-14
PND21
In vivo IP
Colorectal distension
Aggr. ♂

Figure 2

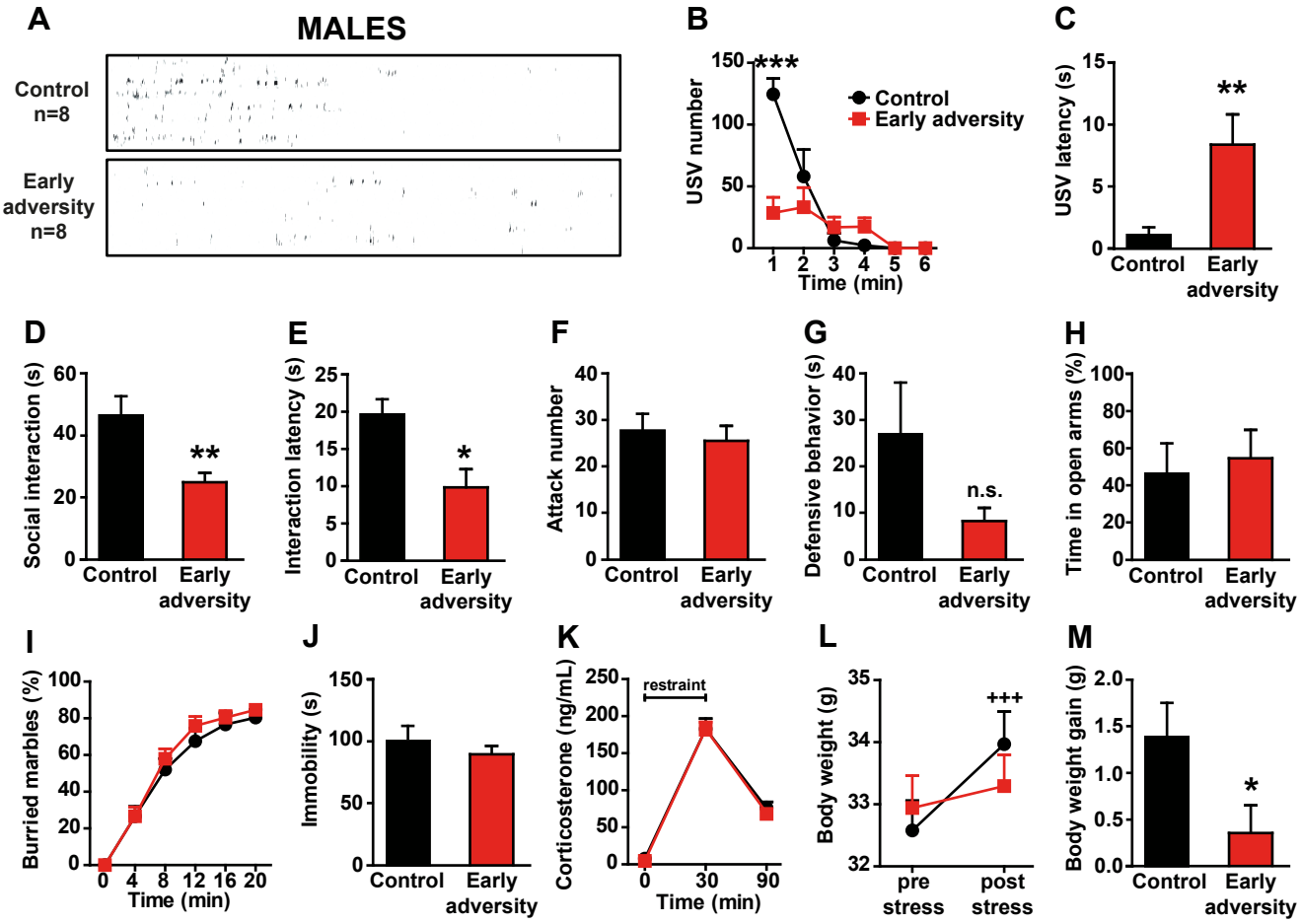

A
MALES
Control n=8
Early adversity n=8
B
USV number
150
100
50
0
***
Control
Early adversity
1 2 3 4 5 6
Time (min)
C
USV latency (s)
15
10
5
0
**
Control
Early adversity
D
Social interaction (s)
60
40
20
0
**
Control
Early adversity
E
Interaction latency (s)
25
20
15
10
5
0
*
Control
Early adversity
F
Attack number
40
30
20
10
0
Control
Early adversity
G
Defensive behavior (s)
40
30
20
10
0
n.s.
Control
Early adversity
H
Time in open arms (%)
100
80
60
40
20
0
Control
Early adversity
I
Burried marbles (%)
100
80
60
40
20
0
0 4 8 12 16 20
Time (min)
J
Immobility (s)
150
100
50
0
Control
Early adversity
K
Corticosterone (ng/mL)
250
200
150
100
50
0
restraint
0 30 90
Time (min)
L
Body weight (g)
35
34
33
32
+++
pre stress
post stress
M
Body weight gain (g)
2.0
1.5
1.0
0.5
0.0
*
Control
Early adversity

Figure 3

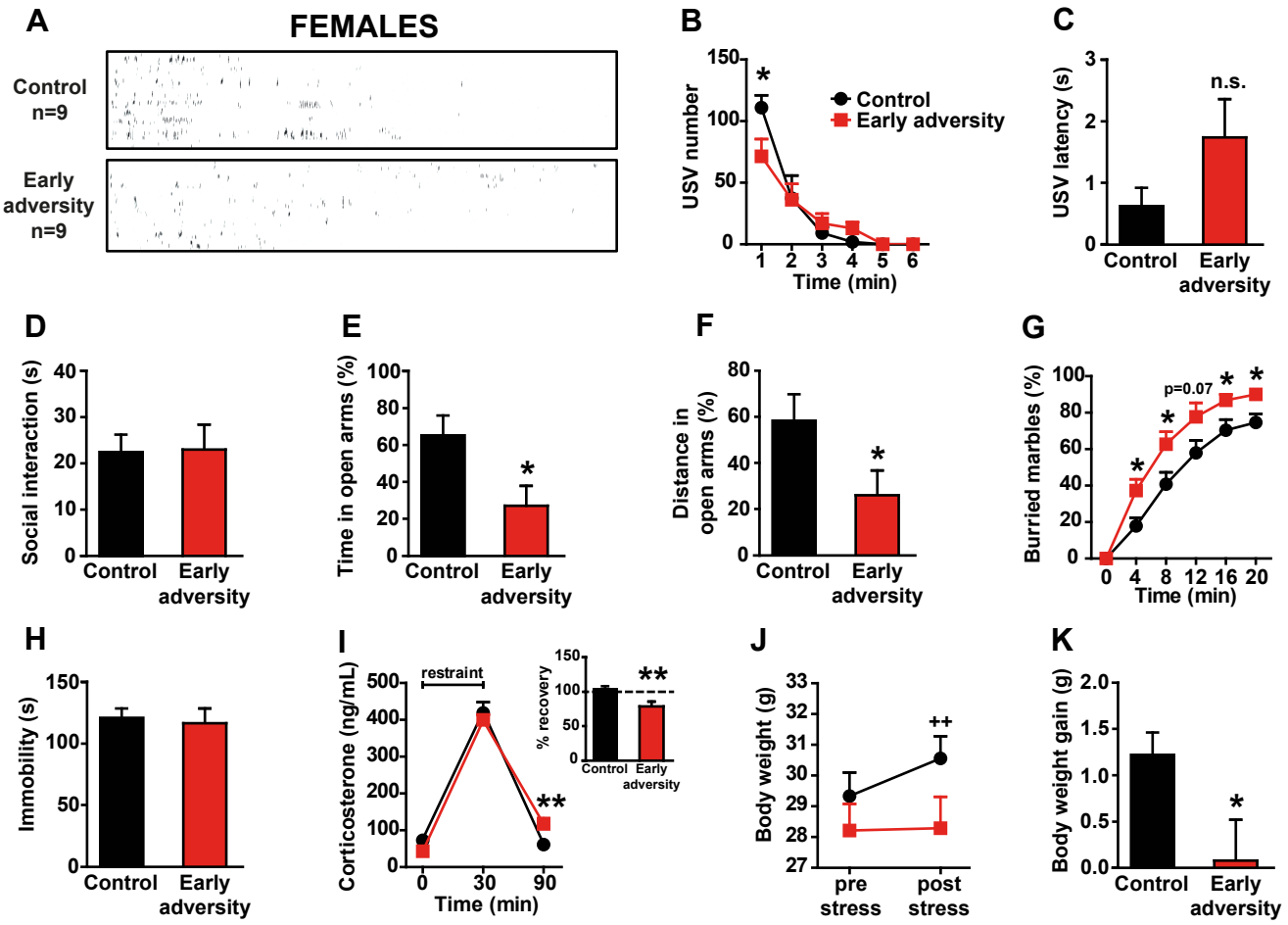

A
FEMALES
Control n=9
Early adversity n=9
B
USV number
Control
Early adversity
Time (min)
C
USV latency (s)
n.s.
Control
Early adversity
D
Social interaction (s)
Control
Early adversity
E
Time in open arms (%)
Control
Early adversity
F
Distance in open arms (%)
Control
Early adversity
G
Burried marbles (%)
p=0.07
Time (min)
H
Immobility (s)
Control
Early adversity
I
Corticosterone (ng/mL)
restraint
Time (min)
% recovery
Control
Early adversity
J
Body weight (g)
pre stress
post stress
K
Body weight gain (g)
Control
Early adversity

Figure 4

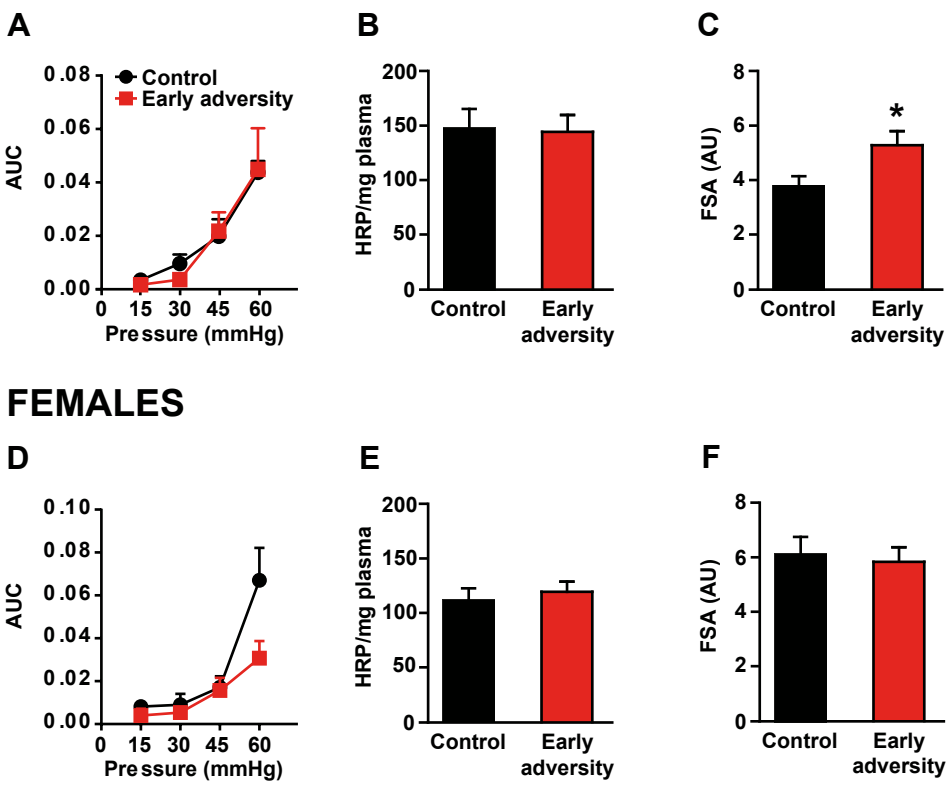

MALES
A
B
C
Control
Early adversity
AUC
0.08
0.06
0.04
0.02
0.00
0
15
30
45
60
Pressure (mmHg)
HRP/mg plasma
200
150
100
50
0
Control
Early adversity
FSA (AU)
8
6
4
2
0
*
FEMALES
D
E
F
0.10
0.08
0.06
0.04
0.02
0.00

Figure 5

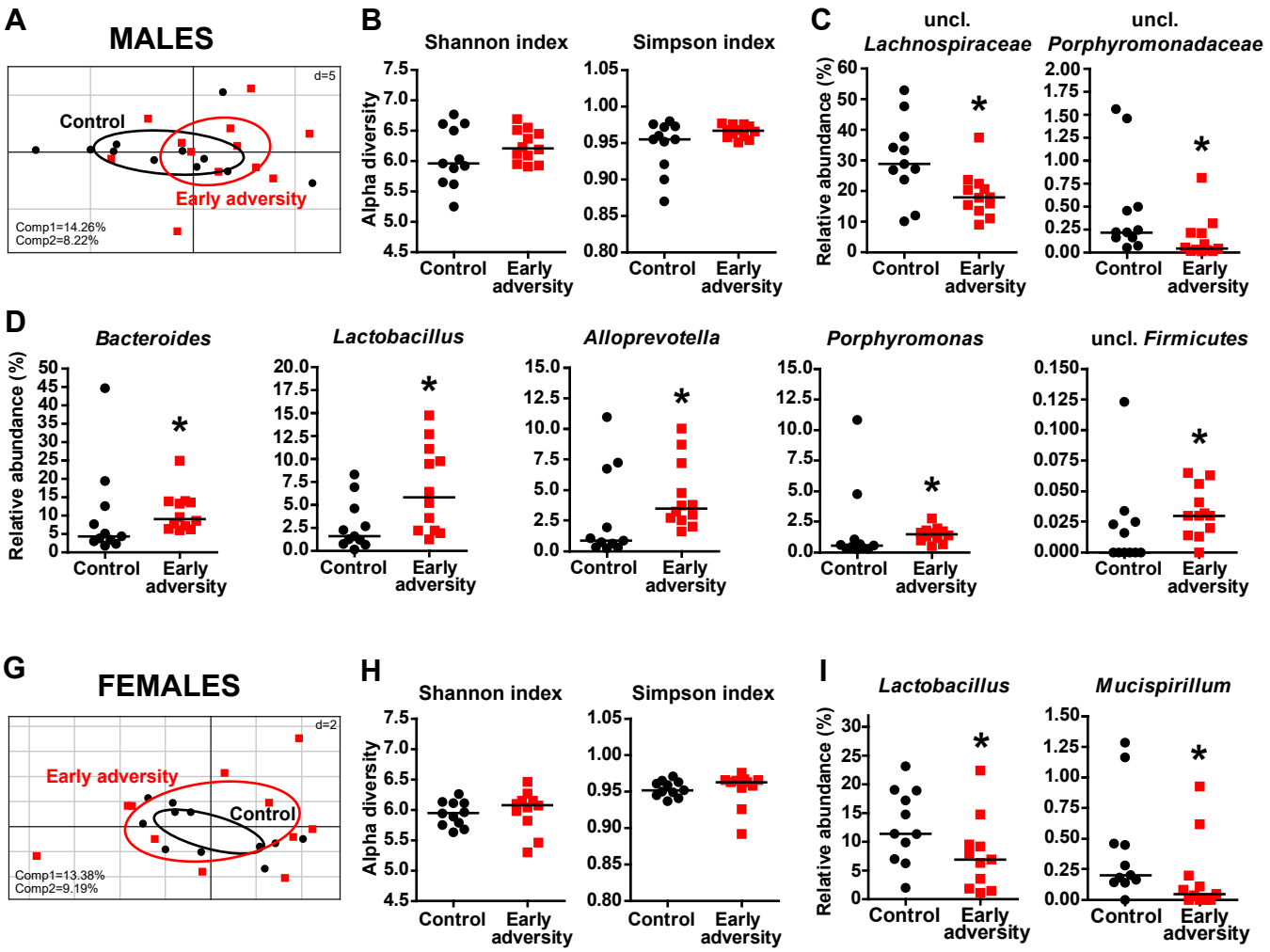
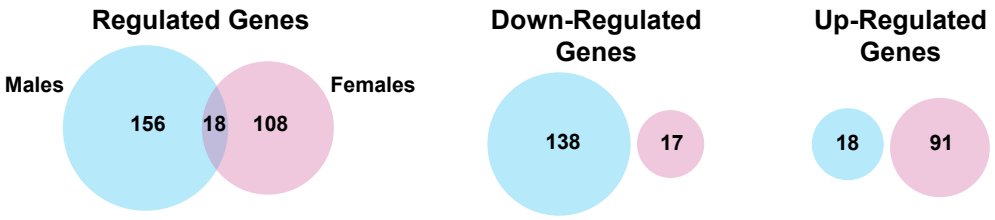
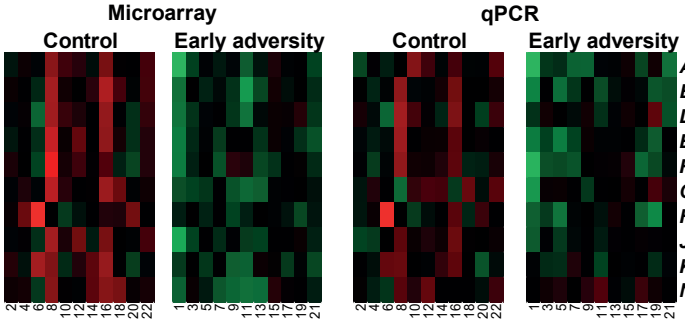

A
MALES
d=5
Control
Early adversity
Comp1=14.26%
Comp2=8.22%
B
Shannon index
Simpson index
Alpha diversity
Control
Early adversity
C
uncl. Lachnospiraceae
uncl. Porphyromonadaceae
Relative abundance (%)
D
Bacteroides
Lactobacillus
Alloprevotella
Porphyromonas
uncl. Firmicutes
G
FEMALES
d=2
Early adversity
Control
Comp1=13.38%
Comp2=9.19%
H
Shannon index
Simpson index
I
Lactobacillus
Mucispirillum

Figure 6

A

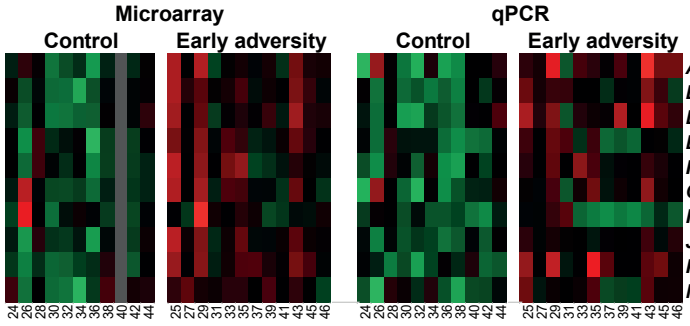


B MALES



	P-value		Pearson
	Microarray	qPCR	R value
Arc	0.010	0.011	0.77
Btg2	0.001	0.007	0.86
Dusp1	n.s.	n.s.	0.85
Egr4	0.055	0.014	0.81
Fosb	n.s.	0.025	0.73
Gadd45b	0.012	n.s.	0.08
H2-K1	0.000	0.043	0.86
Junb	0.026	0.083	0.76
Klf2	0.014	0.069	0.84
NR4A3	0.000	n.s.	0.00

C FEMALES



	P-value		Pearson
	Microarray	qPCR	R value
Arc	0.012	0.010	0.83
Btg2	0.000	0.002	0.89
Dusp1	0.001	0.001	0.95
Egr4	0.001	0.052	0.69
Fosb	0.004	0.001	0.84
Gadd45b	0.009	0.049	0.75
H2-K1	n.s.	n.s.	0.55
Junb	0.001	0.000	0.78
Klf2	0.000	0.000	0.83
NR4A3	0.007	0.035	0.10