Enduring Behavioral Effects Induced by Birth by Caesarean Section in the Mouse

Graphical Abstract

Highlights

- C-section leads to changes in *Bifidobacterium spp.* abundance in early life

- Mice born by C-section have behavioral deficits throughout their lifespan

- Co-housing C-section-born mice with vaginally born mice corrects social deficits

- *B. breve* or a dietary prebiotic mixture improves behavior in C-section mice
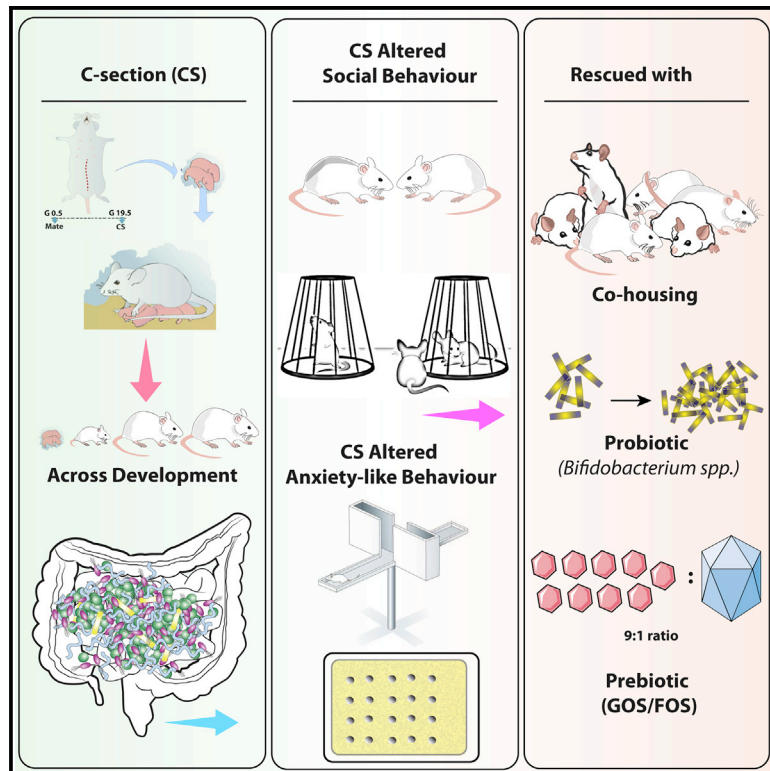
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In Brief

Recent evidence points to an important role for the microbiome in regulating brain function and behavior. Here, Morais *et al*. show that birth by C-section results in a different pattern of microbiota colonization with long-term behavioral consequences in the mouse. Targeting the gut microbiota reverses social behavioral effects of C-section.

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Article

Enduring Behavioral Effects Induced by Birth by Caesarean Section in the Mouse

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SUMMARY

Birth by Caesarean (C)-section impacts early gut microbiota colonization and is associated with an increased risk of developing immune and metabolic disorders. Moreover, alterations of the microbiome have been shown to affect neurodevelopmental trajectories. However, the long-term effects of C-section on neurobehavioral processes remain unknown. Here, we demonstrated that birth by C-section results in marked but transient changes in microbiome composition in the mouse, in particular, the abundance of *Bifidobacterium spp.* was depleted in early life. Mice born by C-section had enduring social, cognitive, and anxiety deficits in early life and adulthood. Interestingly, we found that these specific behavioral alterations induced by the mode of birth were also partially corrected by co-housing with vaginally born mice. Finally, we showed that supplementation from birth with a *Bifidobacterium breve* strain, or with a dietary prebiotic mixture that stimulates the growth of bifidobacteria, reverses selective behavioral alterations in C-section mice. Taken together, our data link the gut microbiota to behavioral alterations in C-section-born mice and suggest the possibility of developing adjunctive microbiota-targeted therapies that may help to avert long-term negative consequences on behavior associated with C-section birth mode.

INTRODUCTION

The gut microbiota—the collection of *bacteria*, archaea, and eukarya residing in the gastrointestinal tract—have co-evolved with their hosts over thousands of years, resulting in an intricate mutual relationship wielding significant benefit to the host health [1]. Interactions between the gut microbiota and the host involve signaling via chemical neurotransmitters and metabolites, neuronal pathways, and the immune system [2]. There is a

growing appreciation that microbiota, especially in early life, influences the development and function of multiple hosts' physiological systems, including the central nervous system [3, 4]. Thus, it has been posited to be a key pillar in understanding the developmental origins of mental health and disease [3, 5]. Preclinical studies using mice born and raised without exposure to micro-organisms, germ-free mice, have highlighted the long-lasting effects of the disruption of the normal acquisition and maturation of the gut microbiota on cognition [6], social behavior

[7], and brain development [8]. However, germ-free animals are specialized model systems, and it is unclear whether more medically relevant alterations in microbiome composition in early life can have enduring psychological and neurobehavioral effects.

In mammals, the composition of the gut microbiota starts to develop mainly upon birth and continues to mature and change throughout life, influenced by several factors, including breast-feeding patterns [9], diet [10], antibiotic exposure [11], and birth mode [12]. In humans, birth by Caesarean (C)-section results in a different pattern of microbiota colonization, and it is associated with increased likelihood of developing immune and metabolic disorders in childhood [13–16]. Moreover, babies born by C-section exhibit lower relative abundance of maternally transmitted commensal bacteria and higher relative abundance of opportunistic micro-organisms that are commonly found in the hospital environment [15]. Despite this, the number of infants delivered by C-section birth mode worldwide has rapidly increased over recent years, in many jurisdictions far exceeding the World Health Organization guidelines of between 10% and 15% [17]. Until recently, there have been limited epidemiological data examining behavioral and psychiatric outcomes in individuals born by C-section. Associations have been made with autism, psychosis, depression, attention deficit disorder, and school performance [18–21], though some of these associations fail to stand up when familial confounding is considered [18, 20]. Although the importance of maternal vaginal microbiome transmission for programming of the offspring brain has been recently demonstrated [22], C-section-induced changes in the microbiome have been largely neglected in the context of brain health.

Within the gut microbiota, bifidobacteria are among the earliest and most-abundant bacterial colonizers of the gut and are essential for appropriate immune, metabolic, and gastrointestinal development in infancy [23, 24]. The establishment of *Bifidobacterium spp*. seeding in the neonatal gut is largely influenced by vertical transmission from mother to infant during vaginal delivery [25, 26]. Birth by C-section circumvents early bifidobacterial colonization and, compared to vaginally born babies, C-section babies have decreased *Bifidobacterium spp*. relative abundance in their gut microbiota [12, 15, 17, 27]. Although this difference tends to normalize somewhere between 6 months and 4 years, it may [17, 28] lead to maladaptive programming of brain and behavior. Intervention strategies that promote a healthy balance of the gut microbiota in babies born by C-section have included the use of prebiotics and probiotics to promote growth of *Bifidobacterium spp*. and other beneficial bacteria [29].

Given the importance that initial colonization of the gut microbiota has on brain development, we used a mouse model to assess the long-term consequences of birth by C-section on neurobehavioral outcomes and the potential role of gut-microbiota-based interventions in remediating such effects. To interrogate these interactions, we used three different approaches. First, we compared the gut microbiota composition and neurobehavior of pups delivered by C-section and given to foster dams (C-section [CS]) with pups delivered spontaneously and nursed by their own mother (vaginally born [VB]) or by a foster dam (cross-fostered [CF]; Figure 1A). To prove the importance of the microbiome in mediating such an effect, we transferred microbiota from VB to CS-born mice at weaning through co-housing. Co-housing may be the simplest and most convenient

technique for microbial transfer, as it offers opportunities of microbiota mixing between co-housed partners due to coprophagic nature of mice [30]. Finally, we treated pups from birth with a *Bifidobacterium breve* strain or with a dietary prebiotic mixture that stimulates the growth of bifidobacteria to investigate whether it could avert the long-term negative consequences on behavior associated with delivery by C-section.

RESULTS

Gut Microbiota Alterations Induced by CS Mode of Birth across Lifespan

To address our hypothesis that birth by CS can affect the programming of the microbiota-gut-brain-behavior axis, we used 16S rRNA gene sequencing to profile the gut microbiota composition in CS, VB, and CF offspring in early life (postnatal day [P] 9), pre-weaning adolescence (P21), and adulthood (week 20).

Regardless of the delivery mode, the composition of the gut microbiota was the most diverse, with regard to alpha diversity, and exhibited the highest inter-animal variability in early life (P9), with the overall dominance of the *Lactobacillus* genus from the Firmicutes phylum (Data S1A; Figure S1A). Principal component analysis (PCA) and canonical correspondence (CCA) analyses showed that the structure of the intestinal microbial community was significantly altered in both CS and CF offspring across the lifespan (Figures 1B–1D; see also Data S1A). Indeed, CS clustered separately from the VB and CF groups at P9, and the separation persisted throughout adolescence and adulthood (Data S1B and S1C). From weaning onward, the microbiota successfully re-shaped toward an approximately equal dominance of Bacteroidetes and Firmicutes phyla (see also Data S1A–S1C), which is typical for the adult murine microbiota [31].

Analysis of individual bacterial taxa abundance at the phylum, family, and genus levels revealed that, although both the CS model of delivery and the CF procedure itself had a long-lasting impact on the gut microbiota in the affected offspring, the profile of observed changes was unique for each intervention. The latter can be illustrated by the CCA plots, with CS and CF groups diverging from the VB mice (Figures 1B–1D). For instance, at P9, CF offspring displayed a dramatic increase in the relative abundance of *Gammaproteobacteria* species, although CS offspring was characterized by an increase in the proportion of a few Bacteroidetes genera (*Odoribacter* and *Parabacteroides*) and a marked reduction in the *Lactobacillus* bacteria (see also Data S1A). Similarly, at P21 and week 20, various genera from the Actinobacteria and Tenericutes phyla, as well as *Rikenellaceae*, *Lachnospiraceae*, and *Ruminococcaceae* families of the Firmicutes phylum, were differentially affected by CS and CF (see also Data S1C). Differences in the composition of the microbiota among treatment groups were associated with alterations in the short-chain fatty acid (SCFA) profile, whereby cecal levels of acetate were different among groups in adolescence, but *post hoc* testing did not yield significant results. Butyrate levels were higher in adulthood in CS compared with CF, but not with VB, mice (see also Table S1).

CS Delivery Mode Leads to Neurobehavioral Changes in Early Life

We then compared the consequences of mode of delivery on offspring behavior in early life, particularly focusing on social
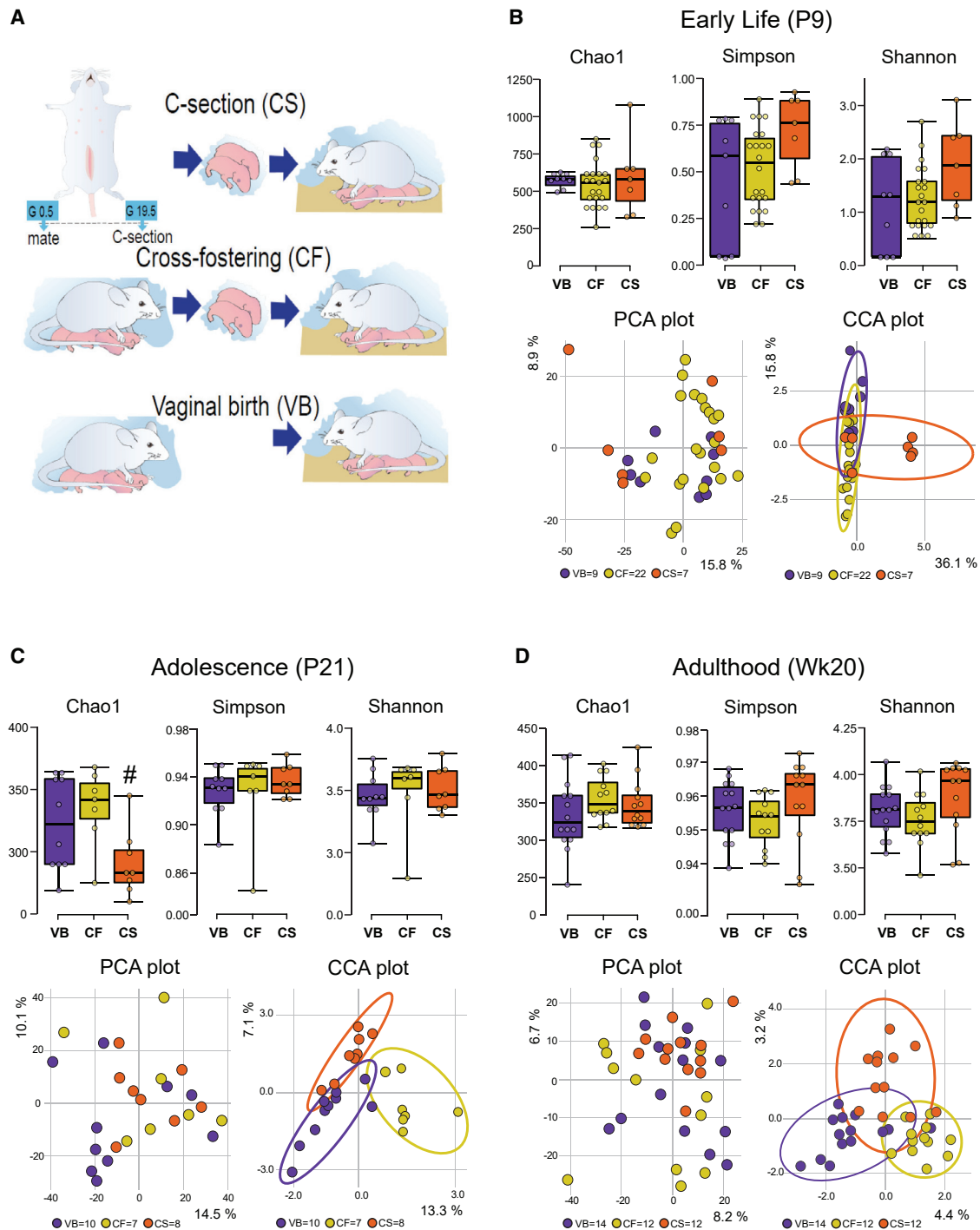
Figure 1. Mode of Delivery Affects Microbial Beta-Diversity throughout the Lifespan

(A) CS animal model and experimental design.

(B–D) Principal component analysis (PCA) and canonical correspondence analysis (CCA) showed that beta-diversity of intestinal (cecal) microbial community was significantly altered in the CS offspring in early-life (P9), adolescence (P21), and adulthood (week 20). CS did not impact alpha-diversity indices (Chao1, Simpson, and Shannon) at any time point. Alpha-diversity indices are presented as median and interquartile range with whiskers representing minimum and maximum values. The x and y axes explain the variability between samples.

(B) Early life (P9; VB n = 9, 4 litters; CF n = 22, 4 litters; CS n = 7, 4 litters).

(C) Adolescence (P21; VB n = 10, 4 litters; CF n = 7, 4 litters; CS n = 8, 4 litters), #p < 0.05 CS versus CF.

(D) Adulthood (VB n = 14, 4 litters; CF n = 12, 4 litters; CS n = 12, 4 litters).
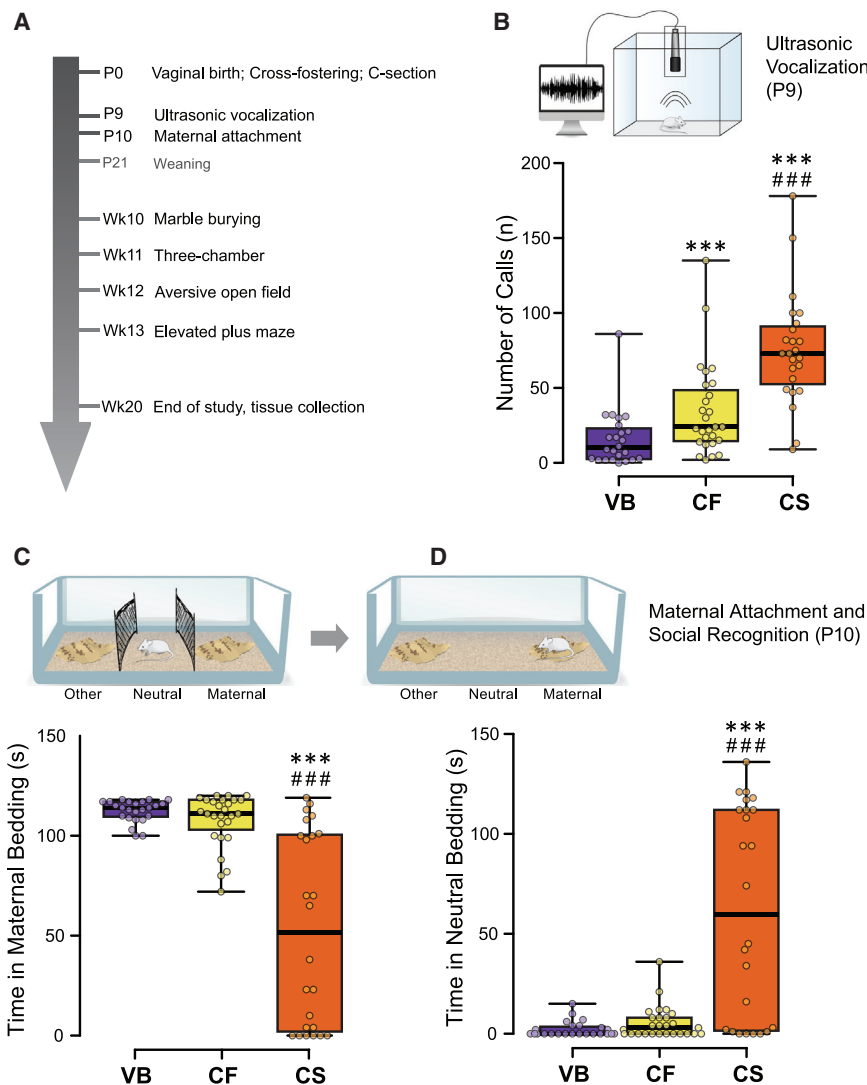
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life communication, perception of relevant signals, and association with particular environmental contexts.

Enduring Neurobehavioral Effects Induced by CS Mode of Birth

Alterations in sociability are a common feature among a variety of neuropsychiatric conditions, and microbiota-deficient mice develop social deficits [7]. Here, we investigated whether mice born by CS exhibit deficits in social behavior in adulthood. Although CS mice displayed normal sociability in the three-chamber test (i.e., preference for mouse over object; Figure 3A), a specific deficit in social novelty recognition (i.e., preference for novel over familiar social partner) was revealed in CS mice compared with VB and CF offspring (Figure 3B). Interestingly, during the subsequent intervention studies where we probed adult CS mice against non-social cognitive cues in the novel object recognition test, CS failed to discriminate between a novel and a familiar object in active investigation time (the effect was not significant in investigation index; see also Figure S2).

Given the role of the microbiome in early life in shaping anxiety [34], we next assessed the impact of CS on relevant behaviors. Indeed, CS mice exhibited exaggerated anxiety-like

behavior, cognitive, and anxiety-like aspects of their behavioral phenotype (Figure 2A). Quantification of ultrasonic vocalization (USV) is widely used to measure early communicative behavior and aversive affective reactions to stress separation [32]. Here, we found that, in early life (P9), CS offspring exhibited a higher number of USV calls when isolated from their littermates and mother than CF or VB animals (Figure 2B). It has been previously demonstrated that, by P10, pups are normally able to respond to relevant social stimuli and to efficiently discriminate their mother's nest when physically separated [33]. Unlike VB or CF animals, CS pups had less preference for the maternal versus neutral bedding (Figures 2C and 2D), thus expressing early social recognition and maternal attachment deficits. Together, these results suggest that birth by C-section is interfering with early-
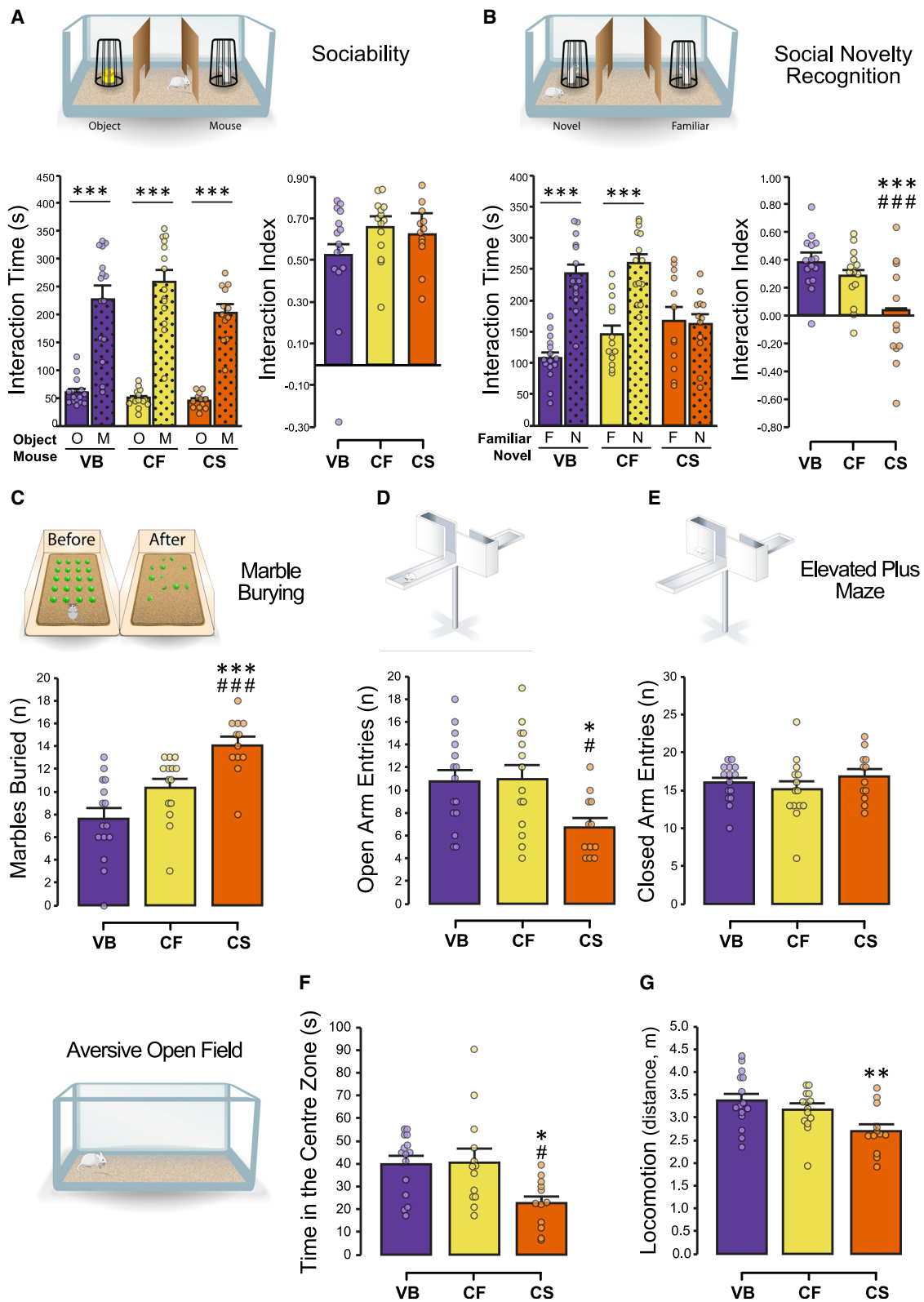
Figure 3. Enduring Neurobehavioral Effects Induced by CS

(A and B) CS delivery mode had an impact on social behavior in adulthood (three-chamber test).

(A) CS did not impair sociability. ***p < 0.001 mouse versus object for the interaction time data.

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behavior as observed by increased number of buried marbles in the marble-burying test (Figure 3C), reduced number of entries into the open arms in the elevated plus maze (EPM) test (Figures 3D and 3E), and reduced locomotion and time spent in the central zone of the open-field (OF) test (Figures 3F and 3G). Most of the CS-associated effects on anxiety remained significant after adjustment for the litter effect (see also Table S2) but failed to be robustly evident in subsequent cohorts (see also Figure S3). This suggests a subtle nature of the pro-anxious behavioral phenotype in the CS offspring and/or the importance of postnatal environment for the development of these outcomes. In contrast, the CS-induced deficit in social novelty recognition not only withstood the adjustment for the litter effect (see also Table S2) but was consistently observed across all experimental cohorts (Figures 5C and 6H), thus indicating the robustness of the observed effects. Controlling for the early environment exposure was an important goal of the initial experiments, and that was why, for this first set of experiments, we included all three groups (CS, CF, and VB), which would give us a fully balanced stratified experimental design. Although the CF procedure itself resulted in a unique effect on the gut microbiota (Figures 1B–1D), these changes did not manifest in many behavioral alterations throughout (Figures 2 and 3).

The hippocampus is an important brain area for learning and memory as well as for the regulation of the stress response [35]. Accumulating data also show that it represents a key node in the microbiome-gut-brain axis, with alterations in the gut microbiome being associated with changes in hippocampal gene expression, neurogenesis, and neurotransmission [36–39]. In addition, the hippocampus is required for proper social recognition [40] and social memory formation [41]. Thus, it was important to investigate whether the hippocampal transcriptome was sensitive to CS-induced changes in the gut microbiota. In agreement with the behavioral data, the transcriptome analysis of the hippocampal brain region in adult mice revealed substantial transcriptional differences in the CS offspring (Figures 4A and 4B). CS mice clustered separately from either VB or CF counterparts, although no differences between CF and VB groups were observed. Interestingly, of the 38 genes upregulated in CS mice, nine belonged to extracellular matrix (ECM)-associated group (*Col8a1*, *Col8a2*, *Col4a3*, *Ctsc*, *Frdc9*, *Itih5*, etc.). The ECM regulates brain mechanical properties [42], homeostatic plasticity [43],

and the immune response [44], and alterations of the ECM are linked to the development of neurological disorders [45]. For statistical, logistical, and ethical reasons (in order to meet 3R requirements and minimize animal usage), we chose to only use the VB group as the control in the intervention studies.

Microbiota Transfer by Co-housing Reverses Specific Neurobehavioral Changes Induced by CS

To investigate a potential causal role for the gut microbiota in mediating the observed behavioral changes, we examined whether transferring microbiota from VB to CS-born mice at weaning could prevent CS-mediated behavioral deficits. We exploited the coprophagic nature of mice and performed fecal transfer by co-housing one CS mouse with three VB mice in adolescence (based on the strategy utilized by Buffington and colleagues) [46]. Littermates originating from multiple litters were randomly assigned to the different housing systems to minimize the litter effect. Behavior was assessed in adulthood (Figure 5A). Although CS mice displayed normal sociability in the three-chamber test (i.e., mouse versus object; Figure 5B), co-housing CS with VB mice selectively reversed CS-induced cognitive deficits, restoring social novelty recognition (Figure 5C). Despite not affecting the marble-burying or EPM readouts (see also Figures S3A, S3B, and S3C), co-housing had anxiolytic effects in CS mice, increasing the time spent in the central zone of the OF (see also Figure S3D).

Next, we investigated the gut microbiota composition in VB, CS, and CS co-housed offspring at week 4 (i.e. 1 week following the commencement of the co-housing regimen). Co-housing did not affect alpha-diversity indices (see also Figure S4A and Data S2). Moreover, the PCA analysis did not show significant differences in the microbial community's structure across groups (Figure 5D), though all three groups clustered separately on the CCA plot (Figure S4B; p < 0.05; PERMANOVA; Data S2; beta-diversity analysis). When we looked at the individual bacterial taxa that showed the strongest response to mode of delivery or co-housing regimen, we observed that co-housing reversed CS-associated reduction in the *Bacteroidetes* genus (Figure 5E). Furthermore, co-housing had a unique effect on the relative abundance of *Blautia* and *Rikenella* bacteria, although not affecting *Bacteroidales* S24-7 group and *Anaeroplasma* species in CS mice (Figure 5E). These data support the concept of plasticity within the microbiota-gut-brain axis and show that the

(B) CS-born mice had deficits in social novelty recognition. ***p < 0.001 novel versus familiar mouse for the interaction time data. ***p < 0.001 CS versus VB and ###p < 0.001 CS versus CF for the interaction index data.
(C–G) In adulthood, mice delivered by CS displayed enhanced anxiety-like behavior across various tests.
(C) Increased number of buried marbles in the CS group. ***p < 0.001 CS versus VB and ###p < 0.001 CS versus CF.
(D) Decreased number of entrances into the open arms in the CS group. *p < 0.05 CS versus VB and #p < 0.05 CS versus CF.
(E) Number of entrances in the closed arms were unchanged.
(F) Reduced time spent in the center zone of an aversive open-field arena in the CS group *p < 0.05 CS versus VB; #p < 0.05 CS versus CF. VB n = 15, 4 litters; CF n = 13, 4 litters; CS n = 12, 4 litters.
(G) Reduced total distance traveled in the aversive open field in the CS group. **p < 0.05 CS versus VB.
Data are presented as mean + standard error of the mean (SEM). (A–E and G) VB n = 15, 4 litters; CF n = 14, 4 litters; CS n = 12, 4 litters. Statistical details: (A) interaction time: VB t (14) = 6.341, p < 0.0001; CF t (13) = 9.776, p < 0.0001; CS t (11) = 9.811, p < 0.0001, paired Student's t test. Interaction index: F (2,38) = 1.555; p = 0.224; one-way ANOVA followed by Tukey post hoc tests. (B) Interaction time: VB t (14) = 7.8; p < 0.001: CF t (13) = 5.1; p < 0.0002: CS t (11) = −0.167; p = 0.8707; paired Student's t test. Interaction index: F (2,38) = 14.73; p < 0.0001; one-way ANOVA followed by Tukey post hoc tests. (C) Marbles: F (2,38) = 14.73; p < 0.0001. (D) Entrances to open arms: F (2,38) = 4.74; p = 0.015. (E) Entrances to closed arms: F (2,38) = 0.614; p = 0.4047. (F) Time in the center zone: F (2,37) = 1.077; p = 0.0076. (G) Distance: F (2,38) = 5.22; p = 0.01. (C–G) One-way ANOVA, followed by Tukey post hoc tests. See also Data S3 and S4 and Table S2.
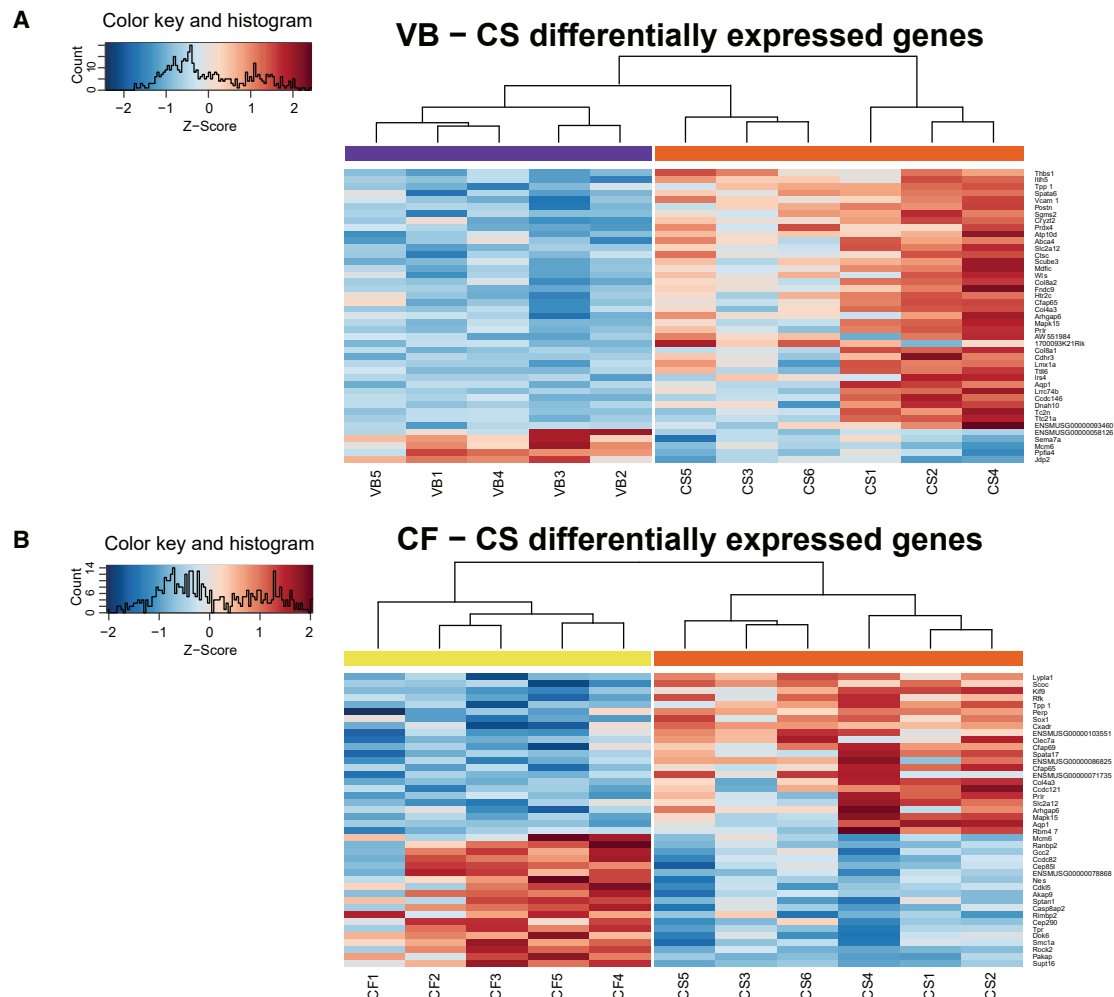
Figure 4. CS Mode of Birth Induces Enduring Changes in the Hippocampal Transcriptome

Heatmap showing differentially expressed genes in the adult hippocampus of CS versus VB offspring (A) and CS versus CF offspring (B). Differential gene expression was determined using the DESeq2 R-package (v1.6.2) with default parameters on pairwise comparisons of all possible group combinations. An adjusted p ≤ 0.1 (Benjamini-Hochberg method) was considered statistically significant. Red color indicates increased expression, and blue color indicates decreased expression levels of the affected genes. VB n = 5, 4 litters; CF n = 5, 4 litters; CS n = 6, 4 litters. See also Data S3 and S4.

enduring effects of CS can be at least partially restored via microbial transfer.

Bifidobacterium spp. Contribute to CS-Induced Neurobehavioral Changes

Because 16S rRNA gene sequencing provides a general overview of microbial community structure, we next employed a quantitative reverse transcription polymerase chain reaction (qRT-PCR) approach to look at the absolute abundance of specific bacterial taxa. We focused on the *Bifidobacterium* genus, because mode of delivery was shown to be an important factor in shaping bifidobacteria colonization in infants [12, 28, 47]. We quantified *Bifidobacterium* species in the feces of VB and CS mice at weaning, adolescence, and in adulthood. Herein, we demonstrate a transient significant decrease in *Bifidobacterium spp.* abundance in CS offspring at weaning (week 3), which was no longer observable 1 week or 4 weeks later (Figure 6A). Given the fact that bifidobacteria are among the earliest bacterial

colonizers of the neonatal gut and are essential for appropriate immune, metabolic, and gastrointestinal development in infancy, disturbances in their appropriate establishment at the beginning of life could have long-term neurobehavioral effects. To this end, we used two different methods of dietary intervention to augment *Bifidobacterium* levels in our mouse model (Figure 6B). We supplemented CS-nursing dams through their diet with either human commensal *Bifidobacterium breve* M16-V (*B. breve*) or a prebiotic mixture of short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides (scGOS/lcFOS) in a 9:1 ratio, known to promote *Bifidobacterium* growth [48]. At week 3, CS pups were weaned onto the corresponding maternal diet. Both scGOS/lcFOS and *B. breve* supplementation successfully restored early-life deficit in the *Bifidobacterium spp.* abundance associated with CS (Figure 6C). Notably, even as early as at P9, treatment with the prebiotic mixture prevented communication deficits by reducing the number of USV calls emitted by the CS pups when they were isolated from their
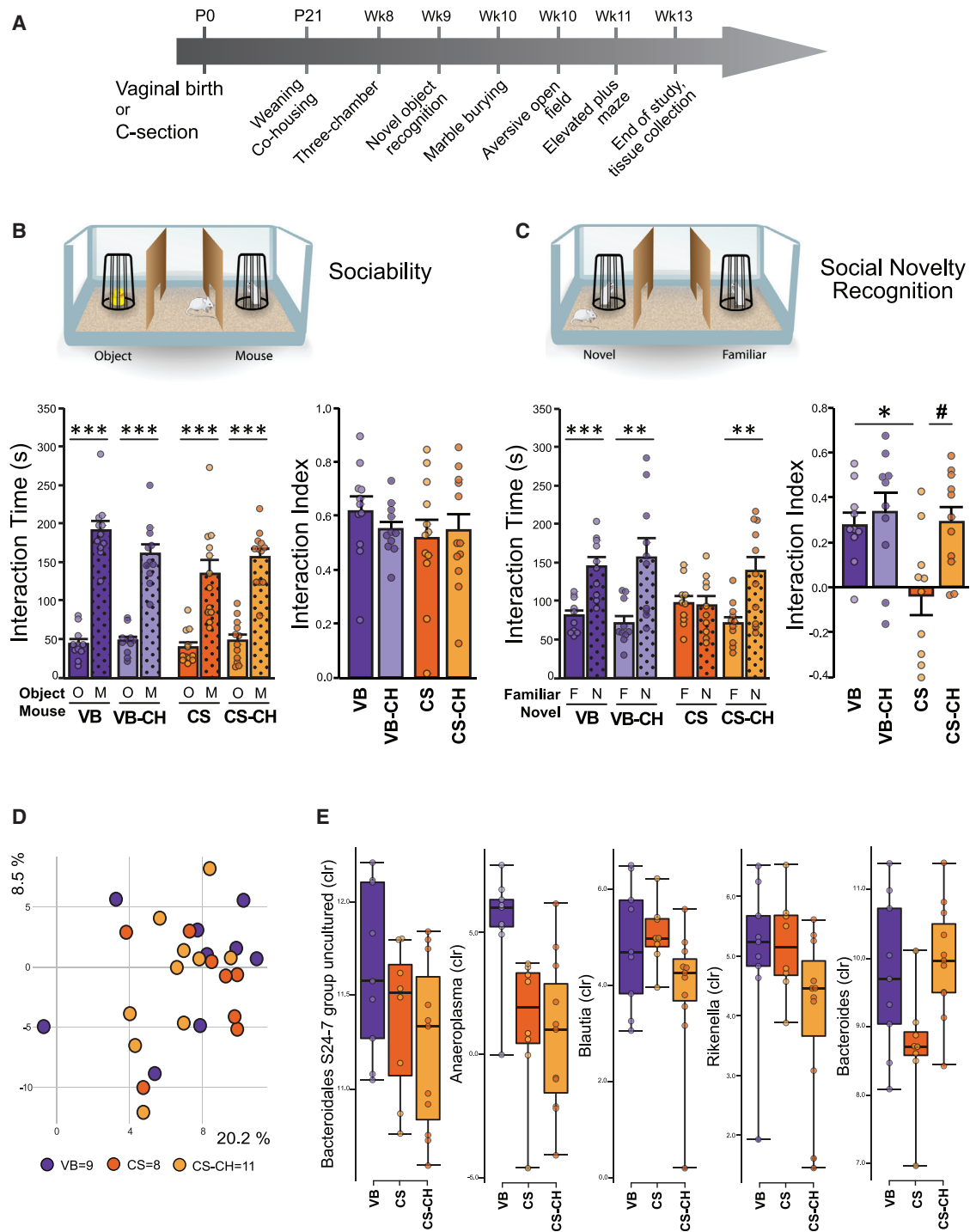
Figure 5. Microbiota Transfer by Co-housing Partially Restores CS Behavioral Phenotype

(A) Experimental timeline of the co-housing study.

(B) Co-housing did not affect sociability; ***p < 0.001 for mouse versus object for the interaction time data. VB n = 11, 9 litters; CS n = 12, 6 litters; VB-CH, n = 11, 9 litters; and CS-CH, n = 12, 7 litters.

(C) Co-housing reversed social novelty recognition deficits in CS-born mice; **p < 0.01 and ***p < 0.001 for novel versus familiar mouse for the interaction time data. *p < 0.05 CS versus VB and #p < 0.05 CS versus CS-CH for the interaction index data. Social novelty: VB n = 10, 9 litters; CS n = 10, 6 litters; VB-CH, n = 10, 9 litters; and CS-CH, n = 11, 7 litters.

(D) PCA did not show significant differences in the beta diversity among all groups in the intestinal (fecal) microbiome community (see also Data S2). The x and y axes explain the variability between samples.

nest (Figure 6D). Moreover, at P10, both interventions successfully restored neonatal recognition abilities and maternal attachment deficits in the CS pups (Figures 6E and 6F). As in Figure 3, social and non-social recognition, as well as anxiety-like behavior, were assessed in adulthood (Figures 6G and 6H; see also Figure S2 and Figure S3). In adulthood, CS-induced social recognition impairment persisted in mice treated with *B. breve*, although treatment with scGOS/lcFOS completely reversed this deficit (Figure 6H). Moreover, scGOS/lcFOS treatment restored novel object recognition deficits (see also Figure S2C and S2D) in the CS group, with all positive cognitive effects remaining significant after controlling for litter effect.

DISCUSSION

Thousands of years of interkingdom symbiosis between gut micro-organisms and their animal hosts have influenced host physiological systems development, including the central nervous system [49]. Birth is one of the key factors shaping the gut microbiota structure in mammals, and maternal transmission of the gut microbiota has likely contributed to the establishment of this evolutionary symbiotic relationship in many different species [50]. In mammals, thus, mode of delivery at birth is one of the defining regulators of early-life gut microbiota composition [14, 47]. Here, we establish a mouse model of CS mode of delivery, which recapitulates structural changes in the intestinal microbial community in early life that endured through adolescence. Previous human studies have demonstrated that CS significantly reduces *Bifidobacterium spp.* abundance in infant intestine, with the observed deficit normalizing later in life [12, 28]. In agreement, our model shows a significant and transient depletion of *Bifidobacterium spp.* in the CS offspring in early life. Altered microbiome composition at critical stages of early life, during which rapid development and maturation of central nervous system occurs, has been implicated in a variety of behavioral alterations in animals [51] and humans [52–54]. However, until recently, there have been limited epidemiological data examining behavioral and psychiatric outcomes in individuals born by CS, and the scarce data that exist from animal models were inconclusive [21, 55–59]. Here, we demonstrate that structural alterations in the intestinal microbial community induced by CS are associated with robust and persistent behavioral changes in the affected offspring. CS mice display social communication and maternal attachment deficits in early life and specific impairment of social novelty recognition in adulthood. CS-induced deficits in recognition also extend to discrimination of non-social cognitive cues.

In order to establish whether disturbances in the appropriate colonization of bifidobacteria at the beginning of life is implicated

in the observed behavioral deficits, we used two alternative approaches to counteract the reduction in *Bifidobacterium spp.* abundance induced by CS (dietary supplementation of either *B. breve* strain or a prebiotic mixture of scGOS/lcFOS). Treatment with both strategies successfully reversed social and non-social recognition deficits in the CS offspring. Thus, we provide here a causal link between deficits in early-life bifidobacteria colonization of the gut and the behavioral phenotype associated with CS. Strikingly, in a recent human study, maternal supplementation with a *B. breve* strain completely reversed the impact of birth by CS and antibiotic treatment on the microbiota composition in infants [60].

In the co-housing experiment, we demonstrated that non-specific fecal microbiota transfer from the VB to the CS offspring at weaning was similarly effective in reversing CS-induced behavioral deficits and was associated with partial restoration of gut microbiota composition in the CS offspring. This further supports the concept of gut bacteria mediating specific behavioral changes associated with CS. Here, we showed that co-housing had a specific effect on the relative abundance of *Blautia* and *Rikenella* bacteria, although not affecting *Bacteroidales* S24-7 group and *Anaeroplasma* species in CS mice. Thus, the exact bacteria involved in restoring behavioral effects are unclear and remain to be explored in future intervention studies [61]. It should be acknowledged that transmission of the microbiota via coprophagy may have limited efficacy on microbiota standardization, as it selects for bacteria that are more tolerant of certain environments and able to conquer resident microbiota response to colonization in the recipient mouse [30]. Further, co-housing mice that express different behaviors may in itself have an effect [62]. Thus, the effects of co-housing CS with VB may not be entirely due to fecal microbiome transfer.

A growing body of work implicates the gut microbiota in social behavior and cognitive performance, and alterations of microbiota have been recently associated with neurodevelopmental disorders [7, 46, 63]. The precise mechanism by which CS affects the developing brain and behavior remains to be determined. However, pathways of communication that may be involved include alterations in vagus nerve signaling, immune system response, metabolite production(including bile acids), tryptophan metabolism, enteroendocrine signaling; and changes in blood-brain and gastrointestinal barriers permeability [2]. Future studies should integrate behavioral outcomes with more functional analysis of the gut microbiota, including metabolomic and metagenomic profiling, which will allow for a more mechanistic view of microbiota gut-brain axis alterations in CS.

From a brain perspective, we observed differential expression of genes belonging to the extracellular-matrix-associated group

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(E) Co-housing restored CS-associated reduction in the *Bacteroidetes* genus. Relative abundance of the bacterial taxa (clr) with the strongest response to mode of delivery and/or housing regimen.

Data are presented as mean + SEM on (B) and (C) and as median and interquartile range with whiskers representing minimum and maximum values (E). (D and E) VB n = 9, 9 litters; CS n = 8, 6 litters; and CS-CH, n = 11, 7 litters. CS-CH, CS co-housed. Statistical details: (B) interaction time: VB t (10) = 8.863, p = 0.001; VB co-housed t (10) = 11.94, p = 0.0001; CS t (11) = 4.920, p = 0.0005; CS co-housed t (11) = 8.835, p < 0.0001, paired Student's t test. Interaction index: group effect F(1,42) = 0.146, p = 0.705; mode of delivery effect F(1, 42) = 0.557, p = 0.646; group × mode of delivery effect F(1,42) = 0.692, p = 0.410, two-way ANOVA followed by Tukey post hoc. (C) Interaction time: VB t (9) = 4.566, p = 0.001; VB co-housed t (8) = 2.902, p = 0.0198; CS t (9) = 0.7423, p = 0.7873; CS co-housed t (10) = 4133, p = 0.002, paired Student's t test. Interaction index: group effect F(1,37) = 6.49, p = 0.0151; mode of delivery effect F(1,37) = 5.565, p = 0.0237; group × mode of delivery effect F(1,37) = 3.203, p = 0.0817, two-way ANOVA, followed by Tukey post hoc. (D) Beta-diversity, PCA plots, pairwise PERMANOVA, p < 0.001, Data S2 and Figures S2–S4. See also Data S3 and S4 and Table S2.
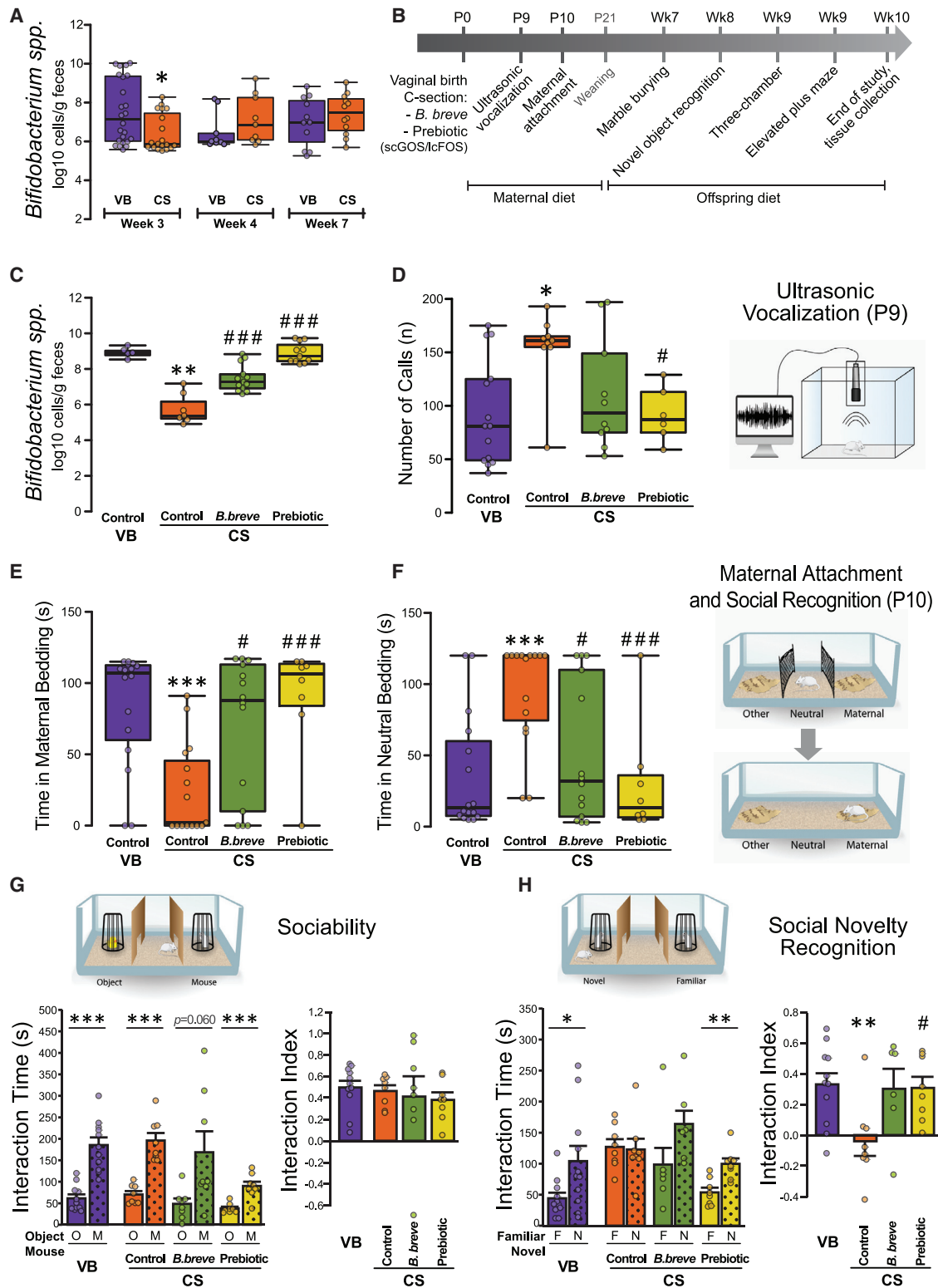
Figure 6. Targeting *Bifidobacterium* Genus from Birth Restores Behavioral Deficits in CS Mice

(A) Transient significant decrease in *Bifidobacterium spp.* abundance (log10 cell/g feces) was seen in the CS offspring at weaning (week 3). Week 3 VB n = 24, 9 litters; CS n = 19, 6 litters; week 4 VB n = 9, 9 litters; CS n = 11, 6 litters; and week 7 VB n = 7, 4 litters; CS n = 6, 7 litters. *p < 0.05 CS versus VB.

(B) *B. breve* and scGOS/lcFOS administration and experimental timeline.

in the hippocampus of the CS offspring. Changes to this gene cluster have been previously associated with formation of memory [64], cognitive flexibility [65], synaptic plasticity, and autistic-like behaviors in animal models [32]. In line with our behavioral findings, CS has been previously suggested to alter the dopaminergic system [57, 59], to increase neuronal cell death in the mouse brain, and specifically affect vasopressin neurons in the hypothalamus [66], the latter being important for social behavior and recognition. The role of microbiota in the remodeling of these pathways has yet to be elucidated.

Together, our findings raise significant concerns regarding the overuse of elective CS deliveries in modern medicine because of likely consequential changes in the microbiome and neurobehavioral effects. However, it is worth noting that, along with the microbiota, CS can affect other physiological changes, such as stress and immune priming during the birthing process, all of which may also contribute to the phenotype [67]. It is clear that certain keystone species (including *Bifidobacterium spp.*) are vitally important in critical windows of development; they contribute to essential immune priming and represent a viable target for dietary intervention in mothers and infants. Restoration of bifidobacteria imbalance in CS-delivered infants represents a challenge that can be addressed in many ways. Recently, partial restoration of the gut microbiota of infants born by CS was demonstrated via vaginal microbial transfer [68]. Vaginal seeding, performed by swabbing babies with vaginal fluid over their entire bodies, successfully colonized the newborn gut with maternal vaginal microbes for up to 30 days [68]. It should be noted though, in cases of CS, vaginal seeding is currently considered unsafe due to the potential transfer of pathogenic bacteria to the newborn infant [69, 70]. Dietary intervention may represent a more acceptable approach: both interventions (dietary supplementation of either *B. breve* strain or a prebiotic mixture of scGOS/lcFOS) did not interfere with the further colonization of native bifidobacteria and represent a safer alternative to vaginal seeding [29].

Our study is not without limitations; we use only male mice, mainly to allow us to compare our findings with previously published data from both our group and others on the role of the microbiome in behavior and neurodevelopment [71]. Future studies should focus on interrogating the impact of CS-induced microbiota changes on behavior in female mice [67]. Moreover, these studies now call for the investigation of the long-term impact of CS on brain and behavior in other mouse strains and other species, including humans. Finally, because CS deliveries, when medically indicated, are unavoidable lifesaving interventions, our data point to the possibility of developing adjunctive microbiota-targeted therapies [17, 29] in this vulnerable population. Such interventions may help to avert any long-term negative consequences for microbiota-gut-brain axis and behavior.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead Contact
  - Materials Availability
  - Data and Code Availability

(C) Treatment with scGOS/lcFOS and *B. breve* restored early-life deficit in the *Bifidobacterium* spp. abundance (log10 cell/g feces) associated with CS. **p < 0.01 CS versus VB; ###p < 0.001 CS versus CS+*B. breve* and CS versus CS+prebiotic. VB n = 5, 4 litters; CS n = 7, 3 litters; CS+*B. breve*, n = 13, 3 litters; and CS+prebiotic, n = 11, 3 litters.

(D) Prebiotic mixture attenuated communication deficits in CS-born mice at P9; *B. breve* supplementation had no effect on early-life communication and anxiety. *p < 0.05 CS versus VB; #p < 0.05 CS versus CS+prebiotic. VB n = 14, 4 litters; CS n = 9, 3 litters; CS+*B. breve*, n = 10, 3 litters; and CS+prebiotic, n = 6, 3 litters.

(E and F) scGOS/lcFOS and *B. breve* treatments restored maternal attachment deficits in the CS pups at P10. VB n = 16, 4 litters; CS n = 15, 3 litters; CS+*B. breve*, n = 14, 3 litters; and CS+prebiotic, n = 8, 3 litters.

(E) Time spent on the maternal bedding. ***p < 0.001 CS versus VB; #p < 0.05 CS versus CS+*B. breve*; ###p < 0.05 CS versus CS+prebiotic.

(F) Time spent on the neutral bedding ***p < 0.001 CS versus VB; #p < 0.05 CS versus CS+*B. breve*; ###p < 0.05 CS versus CS+prebiotic.

(A–F) Data are presented as median and interquartile range with whiskers representing minimum and maximum values.

(G) Treatment with prebiotic did not affect sociability. ***p < 0.001 for mouse versus object for the interaction time data. VB n = 11, 4 litters; CS n = 8, 3 litters; CS+*B. breve*, n = 8, 3 litters; and CS+prebiotic, n = 8, 3 litters.

(H) Treatment with prebiotic reversed social novelty recognition deficits in CS-born mice. B. breve supplementation had no effect on social novelty recognition. *p < 0.05 and **p < 0.01 for novel versus familiar mouse for the interaction time data. **p < 0.01 CS versus VB and p < 0.05 CS versus CS+prebiotic for the interaction index data. VB n = 11, 4 litters; CS n = 8, 3 litters; CS+*B. breve*, n = 6, 3 litters; and CS+prebiotic, n = 8, 3 litters.

(G and H) Treatment with prebiotic did not affect sociability but reversed social novelty recognition deficits in CS-born mice. *B. breve* supplementation had no effect on social novelty recognition. *p < 0.05, **p < 0.01, and ***p < 0.001 for mouse versus object and novel versus familiar mouse for the interaction time data. **p < 0.01 CS versus VB and p < 0.05 CS versus CS+prebiotic for the interaction index data.

Data are presented as mean + SEM. Statistical details: (A) week 3, U = 129.00, p = 0.015; week 4, U = 56.5, p = 0.161; and week 7, U = 18.00, p = 0.070, Mann-Whitney U test. (C) *Bifidobacterium* spp. abundance: CS versus VB, x^2 = 0.000, p = 0.004, Mann-Whitney U test; CS versus CS+treatment, x^2 = 20.472, p < 0.0001, Kruskal-Wallis test followed by multiple comparisons. (D) Number of calls: CS versus VB, U = 27.500, p = 0.025, Mann-Whitney U test; CS versus CS+treatment; x^2 = 6.203, p = 0.045, Kruskal-Wallis test followed by multiple comparisons. (E) Time spent on the home/maternal bedding; CS versus VB, x^2 = 35.000, p = 0.001, Mann-Whitney U test; CS versus CS+treatment; x^2 = 10.484, p = 0.005, Kruskal-Wallis test followed by multiple comparisons. (F) Time spent on the neutral bedding: CS versus VB, x^2 = 35.000, p = 0.001, Mann-Whitney U test; CS versus CS+treatment; x^2 = 10.484, p = 0.005, Kruskal-Wallis test followed by multiple comparisons test. (G) Sociability. Interaction time: VB t (10) = 6.150, p = 0.0001; CS t (7) = 6.813, p = 0.001; CS+*B. breve* t (7) = −2.236, p = 0.060; CS+prebiotic t (7) = 4.662, p = 0.0023, paired Student's t test. Interaction index: CS versus VB t (17) = 0.349, p = 0.731, unpaired Student's t test; CS versus CS+treatment groups F (2,21) = 0.1374, p = 0.8724, one-way ANOVA followed by Tukey post hoc tests. (H) Social novelty recognition. Interaction time: VB t (10) = 2.974, p = 0.014; CS t (7) = 0.1795, p = 0.8626; CS+*B. breve* t (5) = 1.588, p = 0.1232, CS+prebiotic t (7) = 3.776, p = 0.0069, paired Student's t test. Interaction index: CS versus VB, t (17) = 3.053, p = 0.007, unpaired Student's t test; CS versus CS+treatment groups, F (2,21) = 4.379, p = 0.027, one-way ANOVA followed by Tukey post hoc. See also Data S3 and S4, Table S2, and Figures S2 and S3.

AUTHOR CONTRIBUTIONS

L.H.M. designed and performed the research, discussed and analyzed the data, and wrote the paper. G.M.M., A.V.G., A.M.P., and Y.B. designed and performed the research and analyzed the data. O.O.S., A.P.V.-S., K.S., E.P., R.S., A.E.H., T.F.S.B., P.C., S.E.A., S.B., S.A., and A.M.S. performed the research. K.R. assisted on the data analyses. R.P.R., J.K., S.W., I.B.R., P.W.O., P.D.C., and C.S. designed and discussed the data and assisted in writing the manuscript. J.F.C. and T.G.D. conceived the study, supervised, discussed and analyzed the data, and wrote the paper.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and Virus Strains		
Bifidobacterium longum NCIMB8809	National Collection of Industrial and Marine Bacteria Ltd., Aberdeen, Scotland, United Kingdom	strain NCIMB 8809
Bifidobacterium breve M16V (B. breve)	Danone Nutricia Research, Utrecht, the Netherlands	strain M16V
Chemicals, Peptides, and Recombinant Proteins		
Inhibitex Buffer for stool dissociation	QIAGEN GmbH, Hilden, Germany	Catalog number 19593
2-ethylbutyric acid	Sigma-Aldrich, Ireland	Catalog number 109959-1L
Acetic acid	Sigma-Aldrich, Ireland	Catalog number A6283
Propionic Acid	Sigma-Aldrich, Ireland	Catalog number 94425
Isobutyric Acid	Sigma-Aldrich, Ireland	Catalog number I1754
Butyric Acid	Sigma-Aldrich, Ireland	Catalog number 19215
2X Kapa HiFi Hotstart ReadyMix	Kapa Biosystems Ltd, Sigma, Dublin, Ireland	Catalog number KK4609
Critical Commercial Assays		
QIAamp DNA stool kit	(QIAGEN GmbH, Hilden, Germany)	Catalog number 51604
mirVana miRNA Isolation Kit, with phenol	Ambion, Thermo Fisher, Altrincham, United Kingdom	Catalog number AM1560
Nextera XT Index Kit	Great Abington Cambridge, Cambridgeshire CB21 6DF, United Kingdom	Catalog number FC-131-1002
Qubit® dsDNA HS Assay Kit	Life Technologies, Dublin, Ireland	Catalog number Q32851
AMPure XP	London Road, High Wycombe HP11 1JU, United Kingdom	Catalog number A63882
Deposited Data		
RNA-seq	This paper	GEO: PRJNA635779.
Microbiome Data: 16 S	This paper	SRA: PRJNA635779
Experimental Models: Organisms/Strains		
Mus musculus (NIH Swiss mice)	Breeders from Harlan laboratories, Oxford, UK	N/A
Oligonucleotides		
Genus-specific primers Bifidobacteria F (5′-CTCCTGGAAACGGGTGG-3′) R (5′-GGTGTTCTTCCCGATATCTACA-3′)	Eurofins Genomics, Ebersburg, Germany	N/A
16S rRNA gene F (5′- TCGTCGGCAGCGTCAGATGTGTATA AGAGACAGCCTACGGGNGGCWGCAG-3′) and	Primer bank	N/A
16S rRNA gene R (5′-GTCTCGTGGGCTCGGAGATGTGTATAAG AGACAGGACTACHVGGGTATCTAATCC-3′)	Primer bank	N/A
Software and Algorithms		
Ethovision video tracking system, version 3.1	Noldus Information Technology, Nottingham, UK	N/A
Statistical analysis SPSS	IBM Corp. Released 2016. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp.	Version 24

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
R software environment	RStudio Team (2015). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA URL http://www.rstudio.com/.	Version 3.5.1
Trimmomatic	[72]	Version 0.32
Star Aligner	https://github.com/alexdobin/STAR	Version 2.4.0f1
DESeq2	[73]	Version 1.6.2
Varian Star Chromatography Workstation	Varian Star	Version 6.0
Paired End Sequence Assembly FLASH	LASH: Fast length adjustment of short reads to improve genome assemblies.	https://ccb.jhu.edu/software/FLASH/
QIIME	[74]	Version 1.8.0
USEARCH	https://www.drive5.com/usearch/	Version 7 0.0-64bit
Taxonomic ranks were assigned with a BLAST search against the SILVA SSU database.	https://www.arb-silva.de/	Version 123
Other		
Ultrasound sensitive microphone, bat detector for measurement of ultrasonic vocalisations	Summit, Birmingham, USA	US Mini-2 bat detector
Glass marbles (15 mm diameter)	N/A	N/A
Three chambers apparatus, (36x20x20 cm) divided into 3 chambers (left and right chambers 13.5 × 20 × 20 cm; center chamber 9 × 20 × 20 cm) -constructed in house	[7]	N/A
Open-field apparatus (Perspex box with white base: 30 × 30 × 20 cm)-constructed in house	[36]	N/A
Elevated plus maze Perspex gray plastic cross-shaped maze 1 m elevated from the floor, comprising two open and two closed arms (50 × 5 × 15 cm walls or 1 cm no wall) -constructed in house	[36]	N/A
ZB-FFAP column (30 m X 0.32 mm X 0.25 μm;	Phenomenex, Macclesfield, Cheshire, UK	Catalog number 7HM-G009-11
Rodent diet, prebiotic mixture of short-chain galacto-oligosaccharides (scGOS) and long-chain fructo-oligossaccharides (lcFOS)	ssniff Spezialdiäten GmbH, D-59494 Soest, Germany	Catalog number S9262-E364
Rodent diet, control	ssniff Spezialdiäten GmbH, D-59494 Soest, Germany	Catalog number S9262-E360
Objects for Novel object test. 1. Purple plastic drink bottles. 2. Tissue Culture Flask filled with blue dye-constructed in house.	N/A	N/A

RESOURCE AVAILABILITY

Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, John F. Cryan (j.cryan@ucc.ie).

Materials Availability
This study did not generate new unique reagents.

Data and Code Availability
The accession numbers for mouse microbiome (16S rRNA gene sequencing) raw data reported in this paper are: PRJNA635779. Raw brain RNA sequencing data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/assembly/GCF_000001635.23/) and are accessible through GEO accession number PRJNA635779. Behavioral

source data and relevant study codes are available upon reasonable request and should be directed to and will be fulfilled by the Lead Contact, John F. Cryan (j.cryan@ucc.ie).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals

The experiments were performed in male NIH Swiss mice of different ages. Maternal care was a key consideration in our choice of strain, and the NIH Swiss outbred female mice are attentive mothers with a lower pup retrieval latency compared to B6 and 129Sv mice [75]. 8-week-old female and male breeders were obtained from Harlan laboratories, Oxford, UK. Breeding began after 1-2 weeks of acclimatization to the animal holding room. The animals were kept under a strict 12:12-h dark-light cycle, controlled temperature and humidity (20 ± 1°C, 55.5%), with food and water given *ad libitum* unless specified. Male offspring were weaned on P21 and group-housed with 3-4 mice per cage. Experimental groups consisted of offspring from 3-10 litters (litter numbers for each experiment are specified in the figure legends). 10-week old Swiss mice, purchased from Harlan laboratories, UK, were used as conspecifics in the three-chamber sociability test. All procedures used in the present study were conducted in accordance with the Directive 2010/63/EU for the protection of animals used for scientific purposes and were approved by the Animal Experimentation Ethics Committee of University College Cork # 2012/036.

METHOD DETAILS

C-section surgery

Mice were time-mated, and the presence of a vaginal plug was marked as gestational day 0.5 (G0.5). Males were removed from the cage, and pregnant females were not disturbed unless for cage cleaning. At full term (G19.5) female mice were euthanized by cervical dislocation. To reduce bacterial contamination of the abdominal cavity, the abdominal skin was prepped by application of 70% ethanol. The abdomen was incised, the uterus was removed and placed on a sterile gauze. After this step, an incision was made in the uterus. To prevent hypothermia of the fetuses in the uterus, a heating pad was placed underneath to provide thermal support. The pups were then removed by gentle pressure with a sterile swab, and the umbilical cord was cut. Sterile cotton swabs were used to tear the amniotic membrane and massage each pup until spontaneous breathing was noted. The pups were given to a foster dam that gave birth on the same day. The pups were dried by smearing them with the bedding material from the cage of foster dam (CS). In addition, pregnant females were allowed to deliver spontaneously, and these litters were used as full-term vaginal delivery controls (VB). To control for the effects of fostering, a cross-fostered vaginally born group was included in the experimental design (CF, see next section) (Figure 1A).

Cross-fostering

Cross-fostering was performed on litters born within 12 h of each other. On the day of the birth, the litters were removed and put with their foster mothers. The pups were nursed by their respective foster mothers until weaning. Given that CF and VB animals showed a very similar behavioral phenotype across the lifespan, we focused on the VB control group for later experiments to meet the 3R requirements and minimize animal usage.

Co-housing procedure

At three weeks of age, male offspring born by VB or CS were weaned, and mice were split across three different housing conditions: 1) VB group, where each cage consisted of three to four VB mice housed together (from 9 litters); 2) CS group, where each cage consisted of four CS mice housed together (from 6 different litters); 3) co-housing groups, where in each cage one CS born mouse was housed together with three VB mice (CS co-housed, from 7 litters and VB co-housed, from 10 litters). For this experiment, mice from different litters were randomly distributed across different cages and housing regimens. The co-housing system was adopted from Buffington et al. (2016) [46]. In the VB co-housed group, one animal per cage was randomly selected to pass through behavioral tests.

Probiotic and prebiotic administration

CS offspring were exposed to either probiotic *Bifidobacterium breve M16V* (*B. breve*), a commercially available probiotic and supplied by Danone Nutricia Research (Utrecht, the Netherlands) or a prebiotic mixture of short-chain galacto-oligosaccharides (scGOS) and long-chain fructo-oligossaccharides (lcFOS) (ssniff Spezialdiäten GmbH, D-59494 Soest, Germany) starting from birth and throughout the experiment (see also Data S3). *B. breve* was given in drinking water at a concentration of 10^9 c.f.u./mL (freeze-dried bacterial stocks were re-suspended in MediDrop clear H_2O (75-02-1001), and drinking bottles were changed daily in the evening). Prebiotic mixture was given in the custom-made AIN93G rodent diet in a 9 (scGOS): 1 (lcFOS) ratio at the final concentration of 1%. Both interventions were given to the nursing dams starting from birth and throughout the lactation period till weaning. Male offspring were weaned on P21 onto the corresponding treatment. Control VB dams and offspring were given MediDrop clear H_2O as drinking water and AIN93G diet *ad libitum*.

16S rRNA gene sequence-based microbiota analysis

Total DNA extraction from caecal and faecal matter was performed using the QIAmp Fast DNA Stool Mini Kit (QIAGEN, Manchester, UK) coupled with an initial bead-beating step. Extracted DNA was kept frozen at −20°C until further analysis. The V3-V4

hypervariable region of the 16S rRNA gene was amplified and prepared for sequencing as outlined in the Illumina 16S Metagenomic Sequencing Library Protocol http://www.illumina.com/content/dam/illuminasupport/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf). PCR was performed using forward primer (5′- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′) and reverse primer (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′). Each 25 μL PCR reaction contained 5 ng/μL microbial genomic DNA, 1 μM of each primer and 12.5 μL 2X Kapa HiFi Hotstart ReadyMix (Kapa Biosystems Ltd, Sigma, Dublin, Ireland.). The PCR conditions were as follows: initial denaturation at 95°C for 3 min; 25 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s; and 72°C for 5 min for final extension. PCR products were purified with Agencourt AMPure XP system (Beckman Coulter Genomics, Indianapolis, IN, USA). In the next step, dual indices and Illumina sequencing adapters were attached to PCR products using the Nextera XT Index Kit (Illumina, San Diego, USA). Each 50 μL PCR reaction contained 5 μL purified DNA, 5 μL index primer 1 (N7xx), 5 μL index primer 2 (S5xx), 25 μL 2x Kapa HiFi Hot Start Ready mix and 10 μL PCR grade water. PCR amplification was completed using the previous program but with only 8 amplification cycles. Following this, a second clean-up step with the Agencourt AMPure XP system was done. PCR products were quantified, normalized and pooled in an equimolar fashion using the Qubit® dsDNA HS Assay Kit (Life Technologies, Dublin, Ireland). 2 × 300 (bp) paired-end sequencing was performed on the Illumina MiSeq platform, using standard Illumina sequencing protocols.

Microbiota bioinformatic sequence analysis
Paired-end sequences were assembled using FLASH (min overlap of 30 bp and min length of 460 bp) and analyzed using QIIME v1.8.0. Sequences were quality-checked and clustered into OTUs using USEARCH (v7.0-64bit). Taxonomic ranks were assigned with a BLAST search against the SILVA SSU database v123. Alpha diversity indices were generated in QIIME [74].

Behavioral testing
The short and long-term effects of C-section on behavior were evaluated in male offspring in early-life (P9/P10) and in adulthood (weeks 8-16). Mice were habituated to the behavioral room for 30 minutes prior to each test. The experimental procedures are described below; the experimental timelines are illustrated in Figures 2A, 5A, and 6B. The order of behavioral tests and between-test recovery intervals were chosen to minimize the potential confounding carryover effects from the previous behavioral test. Behavioral tests were analyzed by three independent experimenters blinded to experimental groups. All tests were performed during the lights on phase and between the hours of 9am and 2pm.

Isolation-induced ultrasonic vocalizations test
Isolation-induced ultrasonic vocalizations (USV) are produced by mouse pups during the first two weeks of life when separated from their mother and littermates [76]. USV was performed as described by Robertson et al. [77]. Pups were isolated and placed into a clean plastic container enclosed in a sound-attenuating chamber. Emission of USV calls were monitored by an ultrasound sensitive microphone – a bat detector (US Mini-2 bat detector, Summit, Birmingham, USA) tuned in the range of 60-80 kHz – suspended above the isolated pup for 3 min. The number of calls was recorded.

Maternal attachment test (homing test)
Maternal attachment test evaluates the ability of pups to differentiate their mother's and littermates' nest [33]. Maternal attachment was evaluated accordingly to Morais et al. [78]. At P10, the floor of a clean mouse cage was subdivided into three areas by wire-mesh dividers. One area was uniformly covered with home cage bedding, thus containing familiar odour stimuli. The opposite area was covered with bedding from the cage of another litter (born at approximately the same time). The middle section was covered with clean bedding material. Pups were placed individually in the middle section for 1 min; the dividers were then removed, and the pups were allowed to freely explore the arena for 2 min. Total time spent in each area was recorded.

Three-chamber test
Sociability and social novelty recognition were evaluated as previously described by Desbonnet et al. [7]. Animals were placed in a rectangular apparatus divided into three chambers (left and right, and a smaller center chamber) with transparent partitions; small circular openings allowed easy access to all compartments. The test was composed of three sequential 10 min trials: (1) habituation (the test animal was allowed to explore three empty chambers); (2) sociability (an unfamiliar con-specific animal was placed in an inner mesh wire cage in either left or right chamber, the alternative chamber had an empty inner cage); (3) social novelty recognition (a novel con-specific animal was placed into the previously empty inner cage). All animals were age- and sex-matched; each chamber was cleaned and lined with fresh bedding between trials. For each of the three stages, behaviors were recorded by a video camera mounted above the apparatus; time spent in active exploration of inner cages (t) was measured and sociability index was calculated by using the formula (t novel mouse- t familiar mouse)/ (t novel mouse+ t familiar mouse).

Marble burying test
Marble burying test was used to measure anxiety-like behavior, indicating higher levels of anxiety at higher number of marbles buried as described by Burokas et al. [36]. Clean cages were filled with a 4-cm layer of chipped wood bedding. Twenty glass marbles (15 mm diameter) were gently laid on top of the bedding, equidistant from each other in a 4 × 5 matrix arrangement. Each mouse was placed

in the cage and allowed to explore it for 30 min. The number of buried marbles (> 2/3 marble covered by bedding material) was recorded.

Aversive open-field (OF) test

The aversive open-field (OF) test is used to assess the locomotor activity and the response to a novel stressful environment. Light was set at 1000 lux. A test mouse was placed in the center of an aversive open-field arena (Perspex box with white base: 30 × 30 × 20 cm) and was allowed to explore the arena for 10 min. The distance moved and time in zone were recorded using Ethovision videotracking system (Noldus Information Technology, Nottingham, UK). Using this technology, a center zone was demarcated as (20 × 15 cm, L x W). The time spent in each zone and the frequency of entries into each zone were also measured. The box was cleaned with 10% ethanol and allowed to dry between animals.

Novel object recognition test

Novel object recognition test was performed as Burokas et al. [36]. On day one, a test mouse was habituated to a square open-field box (Perspex sides and base: 34.5cm x 42.7 cm for mice) for 10 min in a dimly lit room (60 lux). On day two, two identical objects were positioned in adjacent corners of the arena approximately 5 cm from the walls, and the test mouse was placed in the arena for a 10 min exploration period. Following a 24 h inter-trial interval, one familiar object was replaced with a novel object, and the test mouse was introduced for a 10 min exploration period. Object exploration was defined as when the animal's nose comes within a 2 cm distance to the object. In-between trials, objects and testing arena were cleaned with 10% ethanol. Novel object recognition index was calculated by using the formula (t novel object- t familiar object)/ (t novel object+ t familiar object).

Hippocampal RNA sequencing

Ventral hippocampus was dissected from adult VB, CF and CS mice (Figure 2A) using micro punch technique. Briefly, whole brains were snap-frozen on dry ice and stored at −80°C until ready for use. Using a mouse brain slicer, 1-mm thick sections were obtained from the entire brain, and micro punches of ventral hippocampal tissue were taken using a mouse brain atlas reference [72]. Total RNA was isolated from the ventral hippocampus using the mirVana miRNA Isolation Kit as per manufacturer's instructions (Thermo Fisher Scientific, Dublin, Ireland). RNA concentration was quantified using the ND-1000 spectrophotometer (NanoDrop®). RNA from hippocampal micro-punches from each group VB (N = 5), CF (N = 5) and CS (N = 6) were subsequently sequenced. Library preparation and sequencing, as well Fastq-file generation was done by Beckman Coulter Genomics service (Danvers, MA, USA). Paired-end reads of 2 × 100 bp were produced on an Illumina HiSeq2500 sequencer.

Bioinformatic analysis pipeline

Fastq-format reads were quality filtered and trimmed using Trimmomatic (v0.32) [73] with the following non-default parameters: *AVGQUAL*: 20; *SLIDINGWINDOW*: 4:20; *LEADING*: 10; *TRAILING*: 10; *MINLEN*: 60. Alignment to the mouse reference genome (GRCm38.p3) was achieved using the STAR aligner (v2.4.0f1) with default options and an index compiled with gene models retrieved from the Ensembl database (release 78). These gene models were also used for read counting for each gene using HTSeq-Count (v0.6.0) with the following non-default parameters: -s: no; -r: pos; -q –f bam –m intersection-nonempty. Differential gene expression was determined using the DESeq2 R-package (v1.6.2) with default parameters on pairwise comparisons of all possible group combinations [79]. An adjusted p value $\leq$ 0.1 (Benjamini-Hochberg method) was considered significantly differentially regulated. Raw and processed original data is deposited in NCBI's Gene Expression Omnibus and made accessible through a unique GEO accession number (PRJNA635779) upon publication.

Short chain fatty acid analysis

Caecal content (30-40mg) from adolescent and adult mice was vortex-mixed with 1ml Milli-Q water and incubated at room temperature for 10 min and subsequently centrifuged at 10,000 g for 5mins to pellet bacteria and other solids. The supernatant was filtered, transferred to a clear gas chromatography (GC) vial and 2-ethylbutyric acid (Sigma-Aldrich, Ireland) was added as an internal standard. Standard solutions of 10.0 m mol/L, 8.0 m mol/L, 6.0 m mol/L, 4.0 m mol/L, 1.0 m mol/L and 0.5 m mol/L of acetic acid, propionic acid, isobutyric acid and butyric acid (Sigma-Aldrich), respectively were used for calibration. The concentrations of SCFA were measured using a Varian 3800 GC-flame-ionization system fitted with a ZB-FFAP column (30 m X 0.32 mm X 0.25 μm; Phenomenex, Macclesfield, Cheshire, UK). Initial oven temperature was set at 100°C for 30 s and raised to 180°C at 8°C per min and subsequently held for 1 min, then increased to 200°C at 20°C per min and finally held at 200°C for 5 min. Helium was used as the carrier gas at a flow rate of 1.3ml/min. The temperature of injector and the detector were set at 240°C and 250°C respectively. A standard curve was constructed with different concentrations of a standard mix containing acetic acid, propionic acid, isobutyric acid and N-butyric acid (Sigma-Aldrich). Peaks were integrated using the Varian Star Chromatography Workstation v6.0 software. Technical outliers were excluded when problems in samples processing occurred (see also Data S4).

Quantitative determination of Bifidobacterium breve in faecal pellets

Absolute quantification of *Bifidobacterium breve* species was determined by quantitative PCR using genus-specific primers *Bifidobacterium spp.* F (5′-CTCCTGGAAACGGGTGG-3′) and R (5′-GGTGTTCTTCCCGATATCTACA-3′) [80]. Standard curves were created using bacterial DNA extracted from a pure culture of *Bifidobacterium longum* NCBIM8809 as previously reported [81].

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis for microbiota data

Statistical analysis was done in SPSS (IBM, SPSS Statistics 24) and R software environment. The OTUs detected only in $\leq$ two animals in each group were excluded from the analysis, as were the OTUs that did not give any BLAST hits or were unidentified or unknown on the genus level. Relative abundance of bacterial taxa on the phylum, family and genus level was expressed as % of identified sequences. Among-group differences in alpha-diversity indices and in the relative abundance of bacterial taxa were analyzed with independent Mann-Whitney U test. p value < 0.05 was deemed significant; Benjamini-Hochberg (BH) adjustment with Q = 0.2 was used to correct p values for multiple testing. p values are presented in Data S1 and S2. For beta diversity, the Aitchison distance was calculated using the *ALDEx2* library in R to account for zeroes. Recommended settings were used, with 1000 permutations per sample. Variance-based Principal Component Analysis was done using the prcomp() function in R (version 3.5.1) using Rstudio (version 1.1.456). The *vegan* implementation of PERMANOVA followed by PERMANOVA as a post hoc was used to test for differences on a beta-diversity level. Canonical correspondence analysis was performed using the vegan package in R (version 3.5). CCA plots on the OTU level were generated with the *vegan* library, ellipses represent 95% confidence interval visualized and calculated by the *ggplot*2 library [82]. In order to investigate the impact of co-housing on the microbiome in CS mice, a linear model was constructed based on the effect sizes for all identified bacteria in these groups in base R. Bacteria with the highest Cook's distance from the model were selected for further analysis. Samples samples with < 40000 reads were excluded from the analysis (technical outliers) (See also Data S4).

Statistical analysis for animal behavioral data

Statistical analysis was done in SPSS (IBM, SPSS Statistics 24) and R software environment (version 3.5.1). The normality of data distribution was checked with Shapiro-Wilk test, and the homogeneity of variances across groups was compared using Levene's test. For parametric data, two-tailed un-paired Student's t test, two-tailed paired Student's t test, one-way ANOVA followed by Tukey post hoc tests or two-way ANOVA with Tukey post hoc tests were used to compare means between groups where appropriate. Extreme outliers were excluded when values exceeded *2 x Standard Deviation* from the *mean.* Technical outliers were excluded when animals were unable to perform the test (see also Data S4). All parametric data were expressed as mean ± SEM. For nonparametric data, a Kruskal-Wallis test and Mann-Whitney U test were used to compare differences across groups. Non-parametric data were expressed as median and interquartile range. p value < 0.05 was deemed significant; F and p values are presented in the figure legends or Supplemental tables. Mixed-effects regression model was used to re-analyze adult behavioral data using litter or cage as a fixed effect in order to examine the covariance structure that is inherent in the experimental design using R (version 3.4.1). p value < 0.05 was deemed significant; F and p values are presented in the Table S2.

Supplemental Information

Enduring Behavioral Effects Induced

by Birth by Caesarean Section in the Mouse

Livia H. Morais, Anna V. Golubeva, Gerard M. Moloney, Angela Moya-Pérez, Ana Paula Ventura-Silva, Silvia Arboleya, Thomaz F.S. Bastiaanssen, Orla O'Sullivan, Kieran Rea, Yuliya Borre, Karen A. Scott, Elaine Patterson, Paul Cherry, Roman Stilling, Alan E. Hoban, Sahar El Aidy, Ana M. Sequeira, Sasja Beers, Rachel D. Moloney, Ingrid B. Renes, Shugui Wang, Jan Knol, R. Paul Ross, Paul W. O'Toole, Paul D. Cotter, Catherine Stanton, Timothy G. Dinan, and John F. Cryan
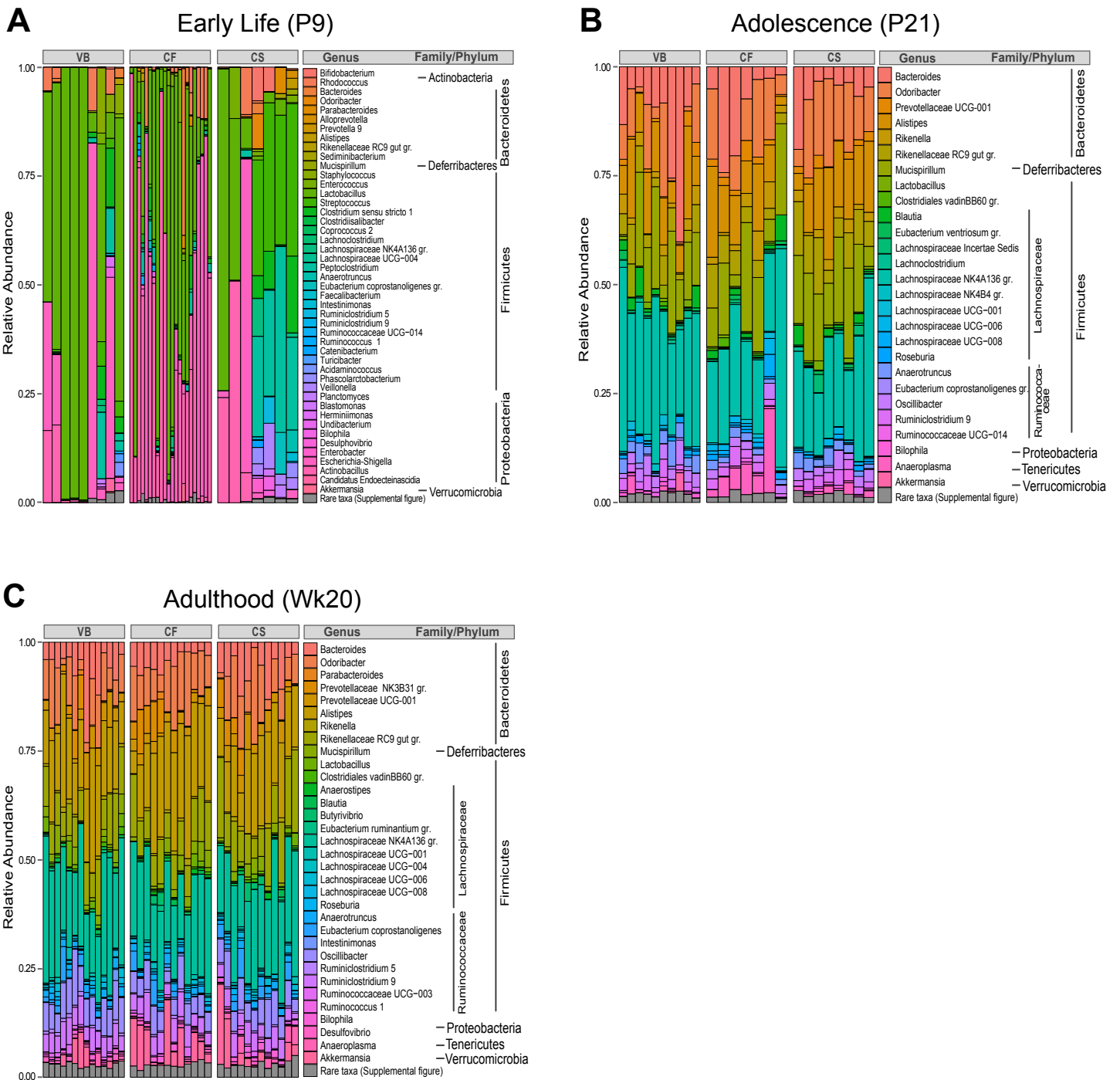
Figure S1. Gut microbiota composition in the vaginal born (VB), cross-fostered (CF) and C-section (CS) offspring throughout the lifespan. Related to Figure 1. Stacked barplots show the relative abundance of dominant bacterial genera in individual animals in (A) Early Life (P9), (B) Adolescence (P21) and (C) Adulthood (Wk20) (using a cut-off of a relative abundance of 0.001 in at least one animal). Bacterial genera are arranged in taxonomic order with phylogenetic tree presented on the right. VB, vaginal birth; CF, cross-fostering; CS, C-section. (A) VB, n=9, 4 litters; CF, n=22, 4 litters; CS, n=7, 4 litters. (B) VB, n=10, 4 litters; CF, n=7, 4 litters; CS, n=8, 4 litters. (C) VB, n=14, 4 litters; CF, n=12, 4 litters; CS, n=12, 4 litters.
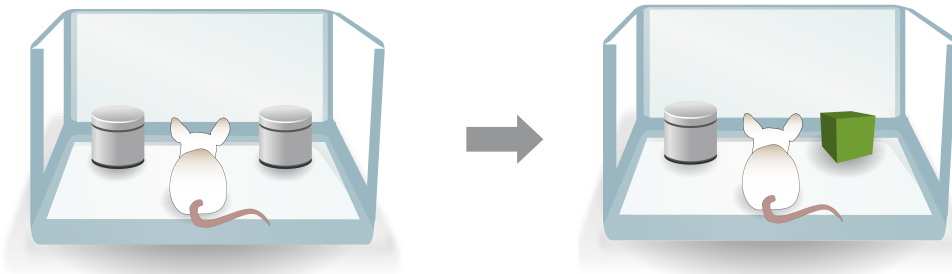
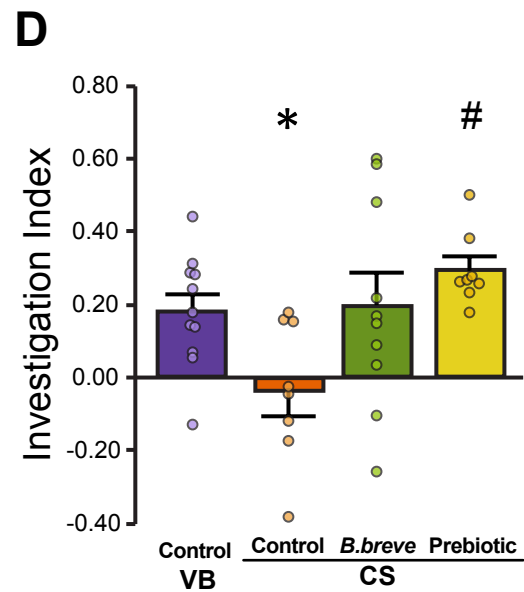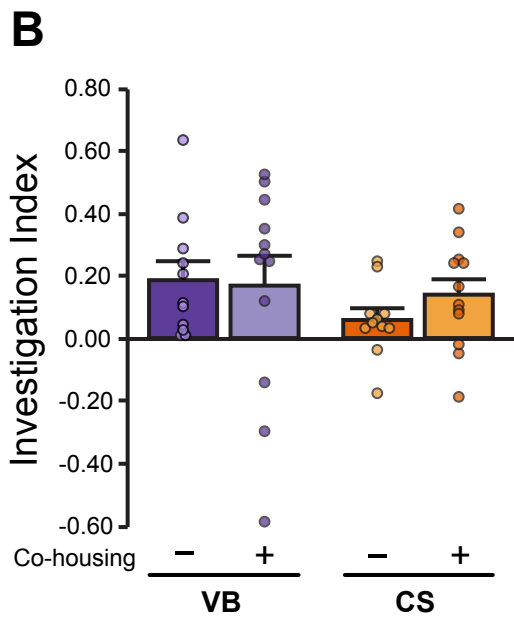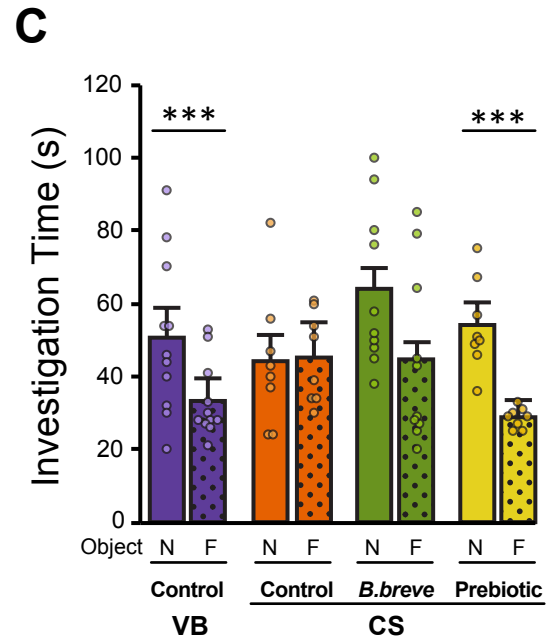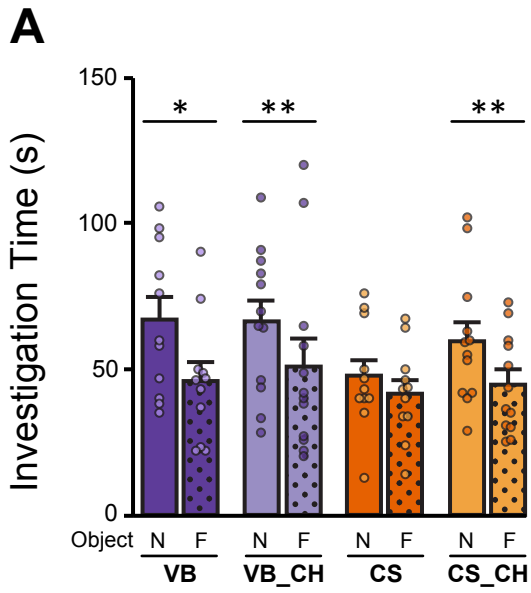Novel Object Recognition (Novel vs Familiar)

Figure S2. C-section impairs recognition of non-social clues in novel object recognition test; effects of co-housing and dietary interventions. Related to Figure 5 and Figure 6. (A-B) Novel object recognition test in the Co-Housing study. (A) CS mice failed to show preference for Novel vs Familiar object in investigation time. Co-housing improved the recognition of novel object in CS-born offspring. *p<0.05 and **p<0.01 for Novel vs. Familiar object. (B) No effect was seen in investigation index. VB, vaginal birth, n=11, 9 litters; VB-CH, vaginal birth co-housed, n=12, 9 litters; CS, C-section, n=11, 6 litters; and CS-CH, C-section co-housed, n=12, 7 litters. (C-D) Novel object recognition test in the *B. breve* and Prebiotic study. CS control mice did not show preference for Novel vs Familiar object in investigation time (C) and investigation index (D). Treatment with prebiotic reversed recognition deficits in CS–born mice. *B.breve* supplementation had no effect on novel object recognition. ***p<0.001 for Novel vs. Familiar object for the investigation time data. *p<0.05 for CS vs. VB and #p<0.05 for CS vs. CS+prebiotic for the investigation index data. VB, n=11, 4 litters; CS, n=8, 3 litters; CS+*B.breve*, n=10, 3 litters; and CS+prebiotic, n=8, 3 litters. Data are presented as Mean + S.E.M. Statistical details: (A-B) Co-housing study. Investigation time: VB t (10) =2.817, p=0.0183; CS t (10) =1.909, p=0.085; VB_CH t (9) =4.146, p=0.002; CS_CH t(12) =2.445, p=0.035, paired Student's t-test. Investigation index: group effect F(1,42)=0.207, p=0.651; mode of delivery effect F(1,42)=0.1368, p=0.249; group x mode of delivery effect F(1,42)=0.312, p=0.438, two-way ANOVA, followed by Tukey post hoc test. (C-D) *B.breve* and prebiotic study. Investigation time: VB t (10)=3.935, p=0.003; CS t (7)=-0.211, p=0.839; CS+*B.breve* t (9)=1.876, p=0.093; CS+prebiotic t(7)=5.772, p=0.001, paired Student's t-test. Investigation index: CS vs. VB t (17)=2.67, p=0.016, unpaired Student's t- test; CS vs. CS+treatment groups F (2,23)=4.838, p=0.018, one-way ANOVA, followed by Tukey post hoc test.
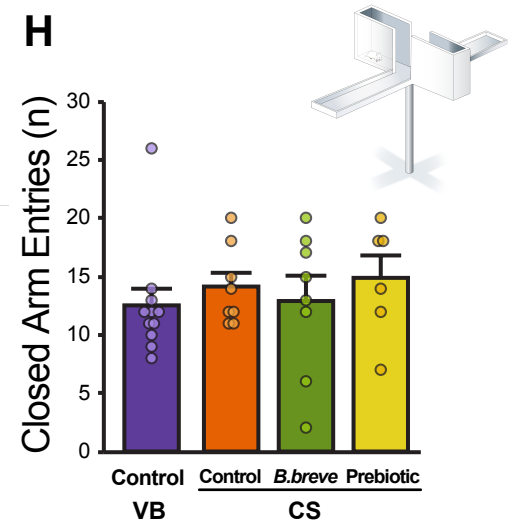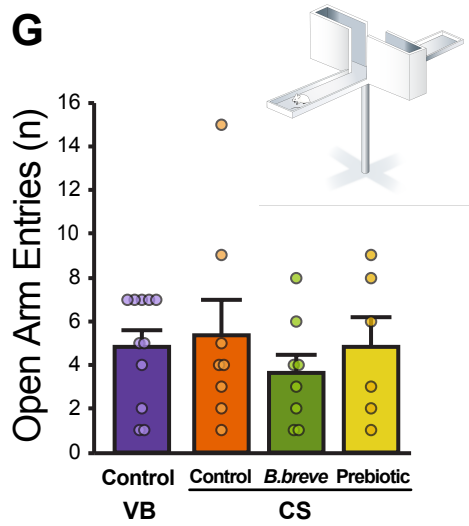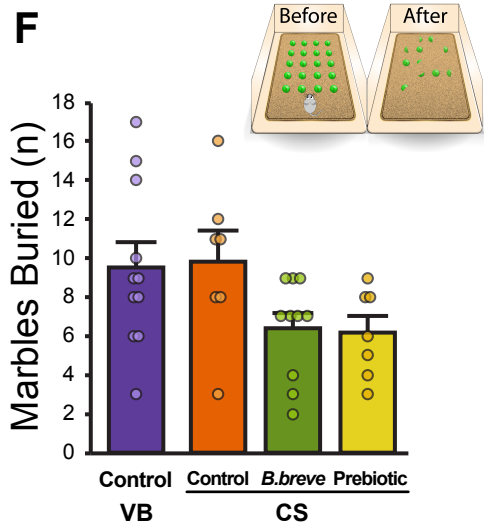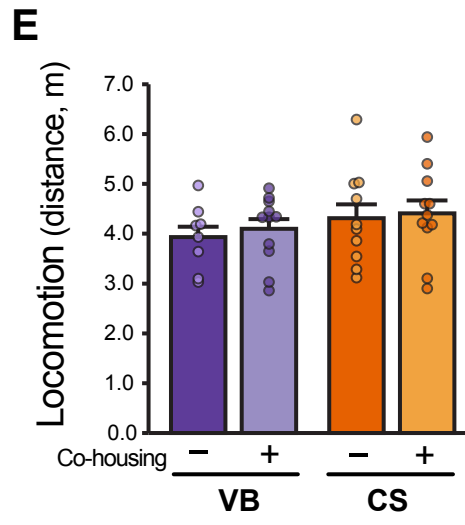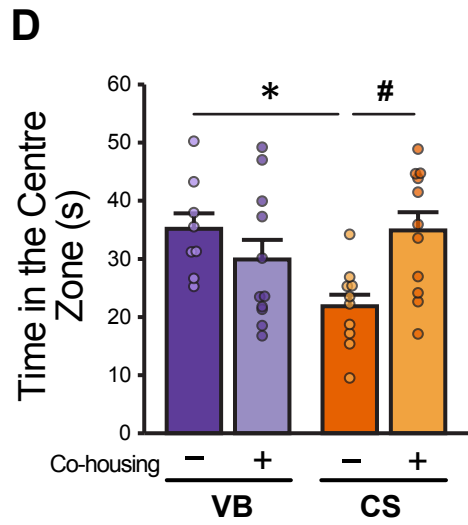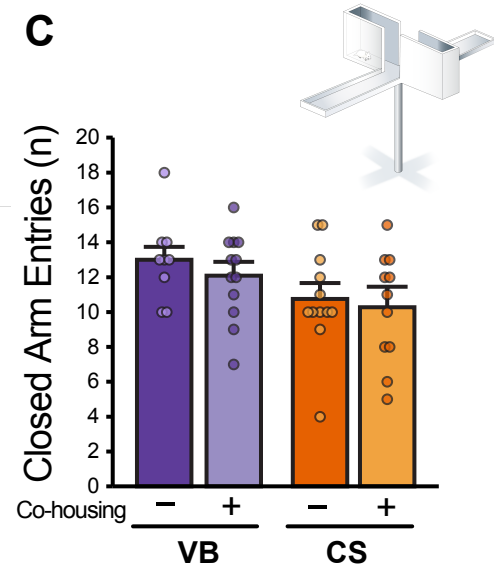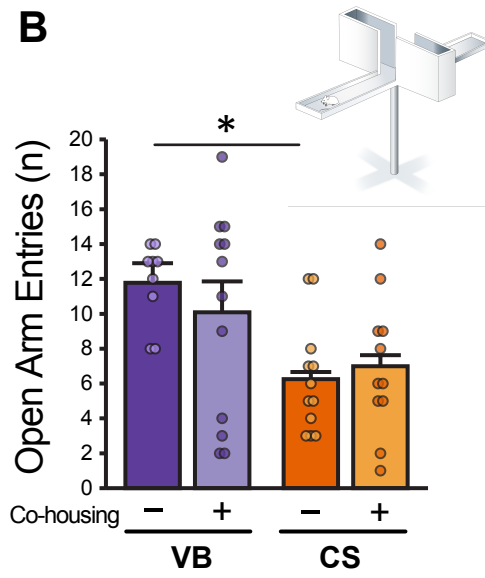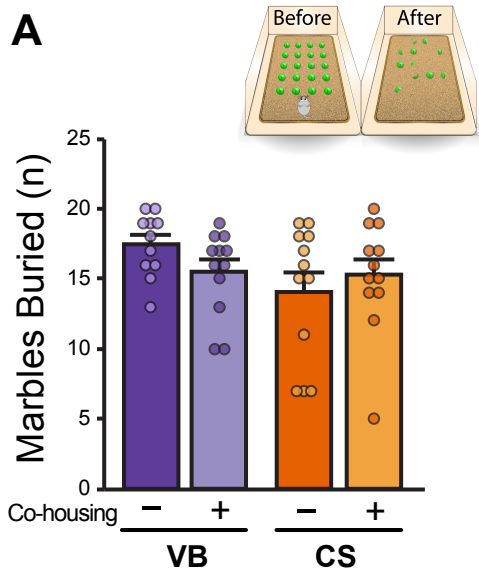
Figure S3. Effects of co-housing, *B. breve* and prebiotic interventions on anxiety-like behaviour in C-section offspring. Related to Figure 5 and Figure 6. (A-E) Co-Housing study. Co-housing selectively improved anxiety-like phenotype in CS-born mice in the aversive open field test. (A) Marble burying test. No differences were observed among groups. VB, vaginal birth, n=11, 9 litters; VB co-housed, n=9, 9 litters; CS, C-section, n=12, 6 litters; and CS co-housed, n=12, 7 litters. (B-C) Elevated plus maze. Co-housing did not affect open arm and closed arm entries. *p<0.05 for CS vs. VB. VB, n=9, 9 litters; VB-CH, n=12, 10 litters; CS, n=12, 6 litters; and CS-CH, n=11, 7 litters (D-E) Aversive open field test. Co-housing increased time spent in the centre zone of the arena in CS mice. Locomotion was similar among groups. *p<0.05 for CS vs. VB and #p<0.05 for CS vs. CS-CH. VB, n=8, 9 litters; VB-CH, n=10, 10 litters; CS, n=11, 6 litters; and CS-CH, n=10, 7 litters. (F-H) *B. breve* and Prebiotic study. No significant changes in anxiety-like behaviour were observed. (F) Marble burying test. VB, n=11, 4 litters; CS, n=7, 3 litters; CS+*B.breve*, n=10, 3 litters; and CS+prebiotic, n=7, 3 litters. (G-H) Elevated plus maze, open arm and closed arm entries. VB, n=11, 4 litters; CS, n=8, 3 litters; CS+*B.breve*, n=9, 3 litters; and CS+prebiotic, n=6, 3 litters. Data are presented as Mean + S.E.M. Statistical details: (A-E) Co-Housing study. Marbles buried: group effect $F(1, 45)=0.181$, $p=0.672$; mode of delivery effect $F(1, 45)=3.207$, $p=0.081$; group x mode of delivery effect $F(1, 45)=2.611$, $p=0.114$. Open arm entries: group effect $F(1, 43)=0.139$, $p=0.711$; mode of delivery effect $F(1, 43)=11.580$, $p=0.002$; group x mode of delivery effect $F(1, 43)=0.933$, $p=0.340$. Closed arm entries: group effect $F(1, 43)=0.681$, $p=0.414$; mode of delivery effect $F(1, 43)=0.068$, $p=0.796$; group x mode of delivery effect $F(1, 43)=5.783$, $p=0.21$. Time spent in the centre: group effect $F(1, 39)=1.566$, $p=0.219$; mode of delivery effect $F(1, 39)=1.790$, $p=0.189$; group x mode of delivery effect $F(1, 39)=8.648$, $p=0.006$. Locomotion: group effect $F(1, 39)=0.254$, $p=0.618$; mode of delivery effect $F(1, 39)=1.758$, $p=0.193$; group x mode of delivery effect $F(1, 39)=0.016$, $p=0.901$. Two-way ANOVA followed by Tukey post hoc tests. (F-H) B.breve and Prebiotic study. Marbles buried: CS vs. VB, $t(16)=-0.155$, $p=0.879$; CS vs. CS+treatment groups, $F(2,21)=3.529$, $p=0.048$. Open arm entries: CS vs. VB, $t(17)=-0.343$, $p=0.736$; CS vs. CS+treatment groups $F(2,21)=0.499$, $p=0.615$. Closed arm entries: CS vs. VB $t(17)=-0.7.99$, $p=0.435$; CS vs. CS+treatment groups $F(2,21)=0.289$, $p=0.752$. Unpaired Students's t-test for CS vs. VB comparison; one-way ANOVA followed by Tukey post hoc for CS vs. CS+treatment comparisons.
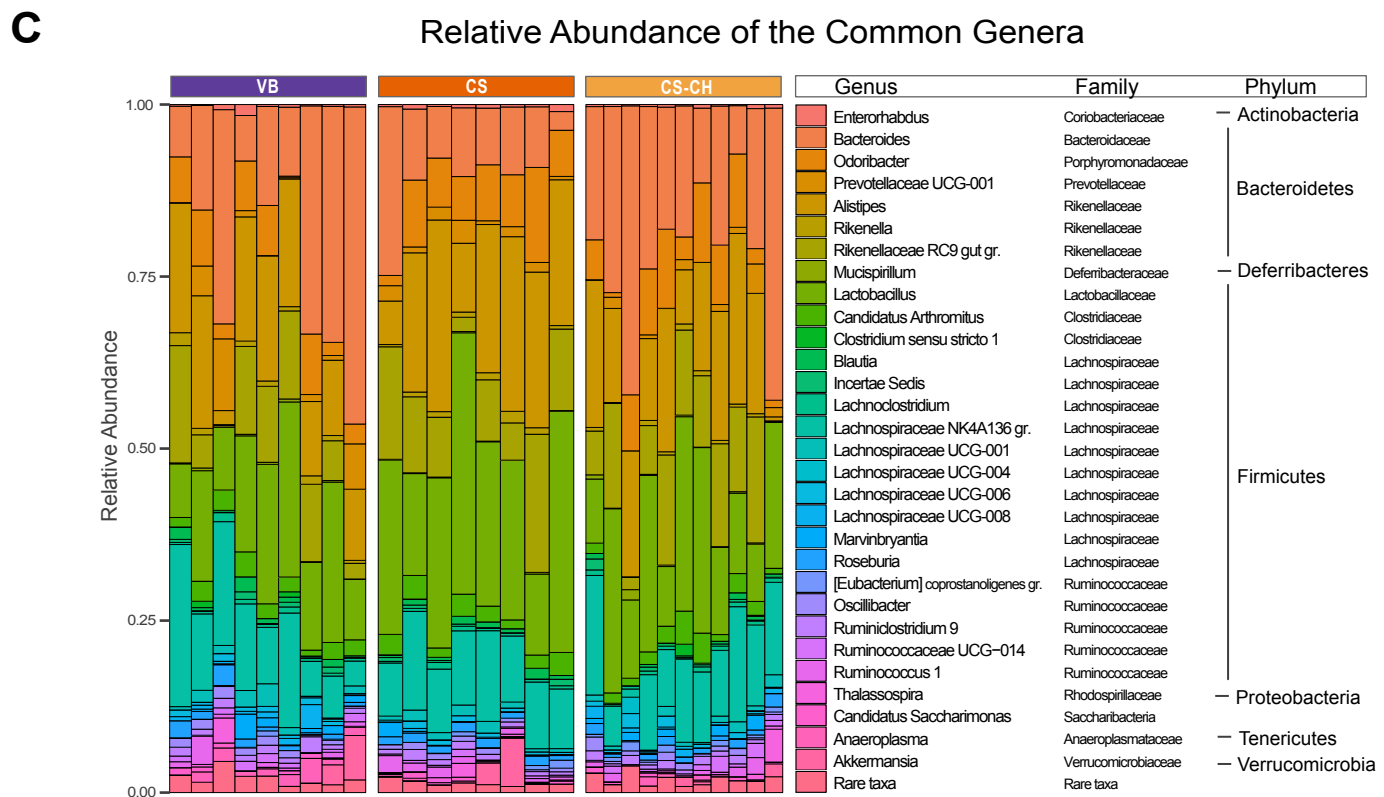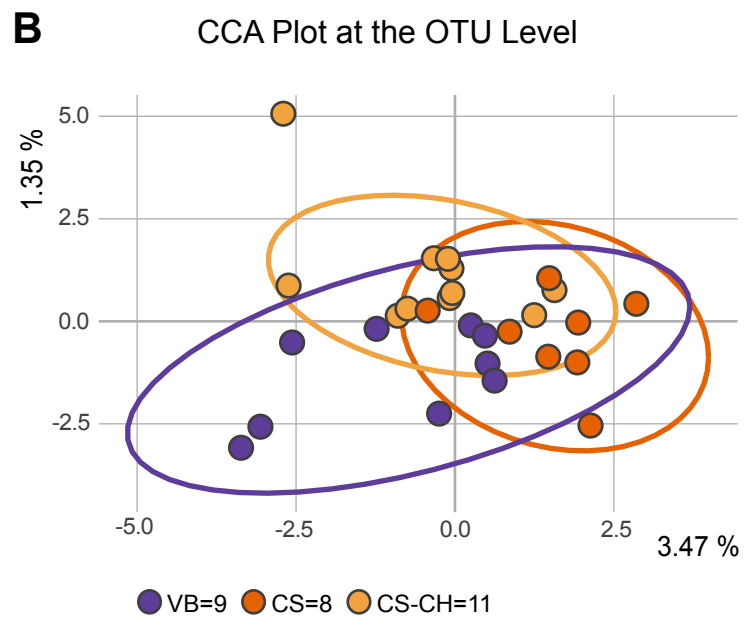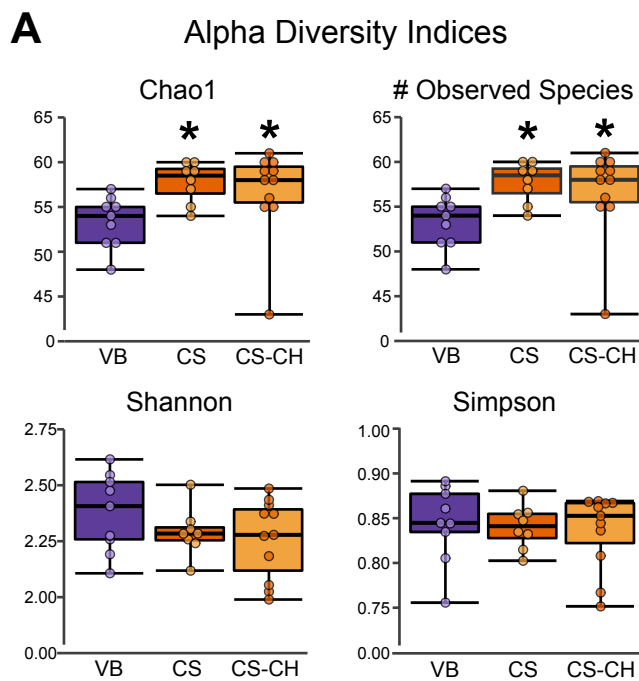
Figure S4. Gut microbiota composition in the vaginal born (VB), CS-section (CS) and CS Co-Housed (CS-CH) offspring one week following the commencement of co-housing. Related to Figure 5. (A) Alpha-diversity indices (Chao1, Observed species, Shannon and Simpson). Co-housing had no effect on alpha-diversity in CS animals. Data are presented as median and interquartile range with whiskers representing minimum and maximum values. (B) CCA analysis showed significant separation of all groups, p<0.01, pairwise PERMANOVA. (C) Stacked barplots show the relative abundance of dominant bacterial genera in individual animals (using a cut-off of a relative abundance of 0.001 in at least one animal). Bacterial genera are arranged in taxonomic order with phylogenetic tree presented on the right. VB, vaginal birth, n=9, 9 litters; CS, C-section, n=8, 6 litters; CS-CH, C-section co-housed, n=11, 7 litters. Statistical details: (A) Between-group differences in alpha-diversity indices were analysed with Mann-Whitney U test. Benjamini-Hochberg adjustment with Q=0.2 was used to correct p values for multiple testing (see Data S2). (B) CCA plot at the OTU level was generated with the *vegan* library, ellipses represent 95% confidence interval calculated by the *ggplot2* library. The *vegan* implementation of PERMANOVA followed by PERMANOVA as a post hoc was used to test for differences at beta-diversity level (see Data S2).

SCFA		Dependent measure	Mean ±S.EM (n)			One-way ANOVA			Tukey post-hoc
			VB	CF	CS	P-value	F-value	DF	
Adolescent	Acetate	Conc. (µmol/g)	33.67±1.62(8)	30.29 ±1.37(8)	36.96±1.87(7)	**0.030**	4.178	2, 20	*ns*
	Proprionate		5.56±0.88(8)	5.87±.67(8)	6.17±0.93(7)	0.877	0.132	2, 20	*ns*
	Iso-butyrate		3.04±0.57(8)	2.65±0.55(8)	3.05±0.68(7)	0.861	0.150	2, 20	*ns*
	Butyrate		16.58±0.94(8)	17.47±1.16(8)	19.84±1.32(7)	0.148	2.107	2, 20	*ns*
	Total SCFA		58.84±3.05(8)	56.28±2.69(8)	66.01±3.90(7)	0.117	2.391	2, 20	*ns*
Adult	Acetate		25.6±1.58(14)	24.70±1.57(13)	21.97±1.19 (12)	0.219	1.584	2, 37	*ns*
	Proprionate		2.87±0.2(14)	2.81±0.18(13)	2.58±0.16(12)	0.503	0.700	2, 37	*ns*
	Iso-butyrate		0.17±0.01(14)	0.16±0.01(13)	0.16±0.02(12)	0.860	0.151	2, 37	*ns*
	Butyrate		10.54±1.21(13)	10.8±1.0(13)	8.21±0.48(12)	**0.036**	3.674	2, 38	CF *vs* CS*
	Total SCFA		40.27±2.56(14)	38.47±2.53(13)	32.93±1.53(12)	0.083	2.664	2, 36	*ns*

Table S1. Short chain fatty acids (SCFAs) in the caecum content in adolescent and adult VB, CF and CS offspring. Related to Figure 1. The table compares the concentrations (conc., µmol/g caecum content) of acetate, propionate, iso-butyrate, butyrate and total SCFA among vaginal born (VB), cross-fostered (CF) and C-section (CS) groups. Data were analysed with one-way ANOVA followed by Tukey´s post hoc test. The post hoc analysis revealed significant differences in the acetate between CF and CS mice in the adolescence, and in butyrate between CF and CS offspring in the adulthood. Data are presented as Mean ± S.E.M. Group size is specified in parenthesis. The table also reports the corresponding degrees of freedom (DF) and F-values. Significant effects (p<0.05) are given in bold font.

C-section study	Mixed-effects ANOVA	Multi-comparisons test
Figure 3 A. Sociability (interaction interaction time- mouse)	F=1.5753, p=0.2442	N/A
Figure 3 A. Sociability (interaction interaction time- object)	F=2.63, p=0.08	N/A
Figure 3 A. Sociability (interaction index)	F=1.555, p=0.2535	N/A
Figure 3 B. Social Novelty Recognition (interaction time-novel mouse)	F=12.245, p<0.001	p<0.001 VB *vs* CS; p<0.001 CF *vs* CS
Figure 3 B. Social Novelty Recognition (interaction time-familiar mouse)	F=2.9073, p=0.09	N/A
Figure 3 B. Social Novelty Recognition (interaction index)	F=5.7464, p=0.017	p<0.01 VB *vs* CS; p<0.05 CF *vs* CS
Figure 3 C. Marbles Buried	F=11.455, p=0.001	p<0.001 VB *vs* CS; p<0.01 CF *vs* CS
Figure 3 D. Open Arm Entires	F=2.9458, p= 0.090	N/A
Figure 3 E. Closed Arm Entries	F=0.971, p=0.4	N/A
Figure 3 F. Time in the Centre zone	F=6.0287, p= 0.005	p<0.001 VB vs CS; p<0.001 CF *vs* CS
Figure 3 G. Locomotion	F=5.6331, p=0.0068	p<0.001 VB vs CS
Co-Housing study		
Figure 5 B. Sociability (interaction time-mouse)	F=2.8723, p=0.059	N/A
Figure 5 B. Sociability (interaction time-object)	F=0.4658, p=0.7076	N/A
Figure 5 B. Sociability (interaction index)	F=0.61, p=0.612	N/A
Figure 5 C. Social Novelty Recognition (interaction time-novel mouse)	F=2.6337, p=0.06261	N/A
Figure 5 C. Social Novelty Recognition (interaction time-familiar mouse)	F=2.342, p=0.08724	N/A
Figure 5 C. Social Novelty Recognition (interaction index)	F=5.5441, p=0.0027	p<0.001 VB vs CS; p<0.001 CS vs CS-CH
Figure S2 B. Novel Object Recognition (investigation time-novel object)	F=1.8537, p=0.1507	N/A
Figure S2 B. Novel Object Recognition (investigation time-familiar object)	F=0.3757, p=0.7709	N/A
Figure S2 B. Novel Object Recognition (investigation index)	F=0.3953, p=0.6864	N/A
Figure S3 A. Marbles Buried	F=0.0044, p=0.949	N/A
Figure S3 B. Open Arm Entires	F=4.4695, p=0.007	p<0.05 VB vs CS
Figure S3 C. Closed Arm Entries	F=2.17, p=0.1322	N/A
Figure S3 D. Time in the Centre Zone	F=4.42, p=0.008	p<0.05 VB vs CS; p<0.001 CS vs CS-CH
Figure S3 E. Locomotion	F=07181, p=0.547	N/A
***B. breve* and Prebiotics study**		
Figure 6 G. Sociability (interaction index)	VB *vs*. CS, F=0.1364, p=0.7159, CS vs CS+ treatment groups, F=0.1219, p=0.8868	N/A
Figure 6 G. Sociability (interaction time-object)	VB vs. CS, F=0.501, p=0.50, CS vs CS+ treatment groups, F=4.255, p=0.0261	p<0.05 CS *vs* CS+prebiotic
Figure 6 G. Sociability (interaction time-mouse)	VB vs. CS, F=-0.057, p=0.81, CS vs CS+ treatment groups, F=1.6038, p=0.2533	N/A
Figure 6 H. Social Novelty Recognition (interaction index)	VB *vs*. CS, F=10.414, p=0.04436, CS vs CS+ treatment groups, F=5.0701, p=0.01546	p<0.001 VB *vs* CS; p<0.01 CS *vs* CS+prebiotic; p<0.05 CS vs CS+probiotic
Figure 6 H. Social Novelty Recognition (interaction time-novel mouse)	VB *vs*. CS, F=0.36, p=0.55, CS vs CS+ treatment groups, F=5.05, p=0.015	N/A
Figure 6 H. Social Novelty Recognition (interaction time-familiar mouse)	VB *vs*. CS, F=33.817, p<0.0001, CS vs CS+ treatment groups, F=5.4, p=0.01207	p<0.001 VB *vs* CS; p<0.01 CS *vs* CS+prebiotic
Figure S2 D. Novel Object Recognition (interaction time-novel object)	VB *vs*. CS, F=-0.5530, p=0.4662, CS vs CS+ treatment groups, F=-2.9782, p=0.06846	p<0.05 CS vs CS+probiotic
Figure S2 D. Novel Object Recognition (interaction time-familiar object)	VB *vs*. CS, F=3.9627, p=0.08, CS vs CS+ treatment groups, F=1.4, p=0.2873	p<0.05 VB vs CS
Figure S2 D. Novel Object Recognition (investigation index)	VB *vs*. CS, F=-7.0396, p=0.03, CS vs CS+ treatment groups, F=3.55, p=0.06	p<0.01 VB *vs* CS; p<0.05 CS *vs* CS+prebiotic;p=0.09 CS vs CS+probiotic
Figure S3 F. Marble Burying	VB *vs*. CS, F=0.0044, p=0.949, CS vs CS+ treatment groups, F=4.023, p=0.03	p<0.05 CS vs CS+B.breve and p<0.05 CS *vs* CS+prebiotic
Figure S3 G. Open Arm Entires	VB *vs*. CS, F=-0.1188, p=0.7405, CS vs CS+ treatment groups, F=0.3953, p=0.6864	N/A
Figure S3 G. Closed Arm Entires	VB *vs*. CS, F=0.7153, p=0.4295 CS vs CS+ treatment groups, F=0.1054, p=0.90	N/A
VB, vaginal birth; CF, cross-fostering; CS, C-section; CH, cohousing		

Table S2. Litter and cage effects for behavioural data in adult mice. **Related to Figure 3, Figure 5, Figure 6, Figure S2 and Figure S3.** The table compares litter and cage effects for behavioural data in adult mice. Data were analysed with mixed-effects ANOVA followed by multi-comparisons between groups. VB, vaginal birth; CF, cross-fostering; CS, C-section; CH, cohousing.