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Supplemental Information

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

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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)
A
Investigation Time (s)
150
100
50
0
*
**
**
Object N F N F N F N F
VB
VB_CH
CS
CS_CH
C
Investigation Time (s)
120
100
80
60
40
20
0
***
***
Object N F N F N F N F
Control
Control
B.breve
Prebiotic
VB
CS
B
Investigation Index
0.80
0.60
0.40
0.20
0.00
-0.20
-0.40
-0.60
Co-housing − + − +
VB
CS
D
Investigation Index
0.80
0.60
0.40
0.20
0.00
-0.20
-0.40
*
#
Control
Control
B.breve
Prebiotic
VB
CS

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.

A
Before
After
Marbles Buried (n)
Co-housing
VB
CS
B
Open Arm Entries (n)
*
C
Closed Arm Entries (n)
D
Time in the Centre Zone (s)
*
#
E
Locomotion (distance, m)
F
Marbles Buried (n)
Control
Control
B.breve
Prebiotic
G
Open Arm Entries (n)
H
Closed Arm Entries (n)

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.E.M (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 Entries	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.