

Supplementary information

Effects of a synbiotic on plasma immune activity markers and short-chain fatty acids in children and adults with ADHD —a randomized controlled trial

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Analysis of ICAM-1 expression in human aortic vascular smooth muscle cells

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Supplemental **Figure S9**. Individual scatter plots of the correlations between plasma levels of immune activity markers and short-chain fatty acids (SCFAs) shown in Fig. 6 with un-adjusted $p < 0.05$.

Supplementary **Figure S10**. IL-1 β -induced ICAM-1 mRNA expression in human aortic vascular smooth-muscle-cells after *in vitro* pre-incubation with different short-chain fatty acids (SCFAs).

Supplementary **Figure S11**. Correlation analysis between plasma levels of immune activity markers and short-chain fatty acids (SCFAs) in adult controls.

Supplementary Methods:

Analysis of plasma concentrations of short-chain fatty acids (SCFAs)

All reference compounds as well as analytical reagent-grade 3-nitrophenylhydrazine (3NPH)HCl (97%) and N-(3-dimethylaminopropyl)-N0-ethylcarbodiimide (EDC), HPLC grade pyridine, LC–MS grade acetonitrile, hypergrade LC-MS water and MeOH (Lichrosol) were purchased from Sigma–Aldrich. All reagents and solvents were used for a maximum of 5 days. ¹³C6-3NPH was custom synthesized by IsoSciences Inc. (King of Prussia, PA, USA) and used as internal standard for all SCFAs. Plasma (10 µl) was incubated with 75% methanol (60 µl) and mixed with 200 mM 3-NPH (60 µl) and 120 mM EDC-6% pyridine (10 µl) at ambient temperature for 45 min under gentle shaking. The reaction was quenched by addition of 200 mM quinic acid (10 µl) at gentle shaking at ambient temperature for 15 min. The samples were centrifuged at 15 000 g for 5 min and the supernatant was moved to a new tube. The sample was made up to 1 mL by 10% methanol in water and again centrifuged at 15 000 g for 5 min. In total 100 µl of the derivatized (¹²C) sample was mixed with 100 µl of labelled (¹³C) internal standard. Samples were analyzed by a 6500+ QTRAP triple-quadrupole mass spectrometer (AB Sciex, 11432 Stockholm, Sweden) which was equipped with an APCI source and operated in the negative-ion mode. Chromatographic separations were performed on a Phenomenex Kinetix Core-Shell C18 (2.1, 100 mm, 1.7 µm 100Å) UPLC column with SecurityGuard ULTRA Cartridges (C18 2.1mm ID) (changed at regular intervals). The column was backflushed for 60 min between each batch to ensure good chromatographic separation. LC-MS grade water (100% solvent A) and acetonitrile (100% solvent B) was the mobile phases for gradient elution. The column flow rate was 0.4 mL/min and the column temperature was 40°C, the autosampler was kept at 5°C. LC starting conditions at 0.5% B, held for 3 min, 3 min 2.5% B ramping linearly to 17% B at 6 min, then to 45% B at 10 min and 55% B at 13 min. Followed by a flush (100% B) and recondition (0.5% B), total runtime 15 min. The multiple reaction monitoring (MRM) transitions were optimized for the analytes one by one by direct infusion of the derivatives containing 50 mM of each fatty acid. The Q1/Q3 pairs were used in the MRM scan mode to optimize the collision energies for each analyte, and the two most sensitive pairs per analyte were used for the subsequent analyses. The retention time window for the scheduled MRM was 1 min for each analyte. The two MRM transitions per analyte, the Q1/Q3 pair that showed the higher sensitivity was selected as the MRM transition for quantitation. The other transition acted as a qualifier for the purpose of verification of the identity of the compound.

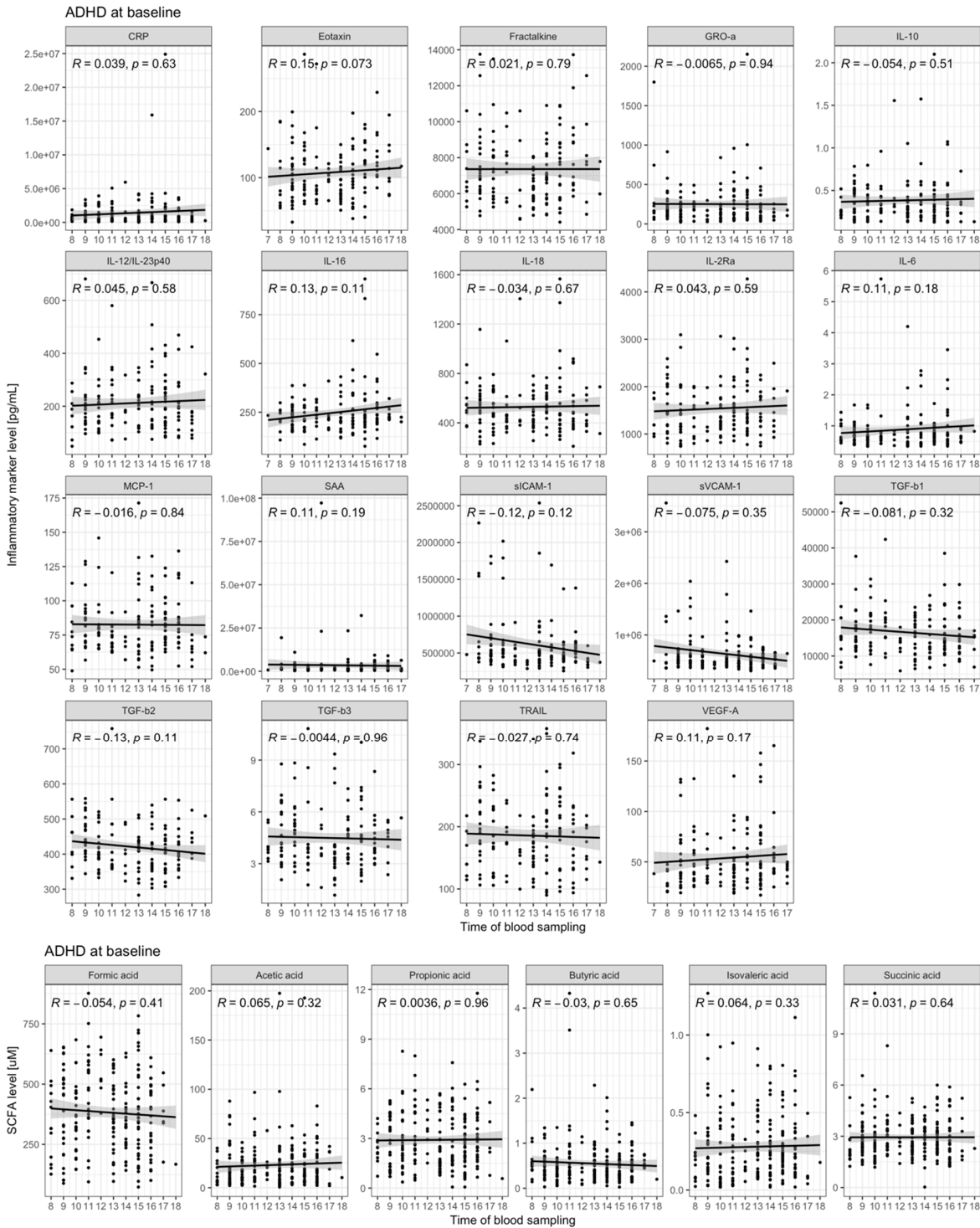
Analysis of ICAM-1 expression in human aortic vascular smooth muscle cells

Total cellular RNA was extracted using Direct-zol™ RNA MiniPrep Plus Kit (cat. #R2072, Zymo Research) according to the standard manufacturer's instructions. RNA concentration and purity (A260/A280) were measured using NanoDrop™ 2000 spectrophotometer (cat. #ND-2000). cDNA was synthesized directly after RNA extraction using SuperScript III First-Strand Synthesis System for qRT-PCR (cat. #18080051, Invitrogen) according to the standard protocol for cDNA synthesis with Veriti™ 96-Well Thermal Cycler (cat. #4375786, Applied Biosystems). qRT-PCR was carried out in a QuantStudio™ 6 Flex Real-Time PCR system (cat. #ZG21SCQSTUDIO6FLEX,

Applied Biosystems) using SYBR Green kit (cat. #4368702, Applied Biosystems) following the standard settings (5 µl SYBR green, 1 µl 10 µM primer pair, 4 µl cDNA). The specific primer pair sequences were for ICAM-1 (F: TGATGGGCAGTCAACAGCTA; R: GAAATTGGCTCCATGGTGAT) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (F: CCTGCACCACCAACTGCTTA; R: CCAGTGAGCTTCCCGTTCAG), purchased from Invitrogen. The cycle threshold values for GAPDH were 18-20, and for ICAM-1 they were 22-30. All reactions were run in triplicates and normalized to reference gene GAPDH. Levels of ICAM-1 mRNA relative to that of GAPDH were calculated applying the $2^{-\Delta\Delta C_t}$ method using the average C_t value of the triplicates and the corresponding control triplicates as the calibrator with QuantStudio Software for 6 Flex.

Supplementary Figures and Tables:

A.



B.

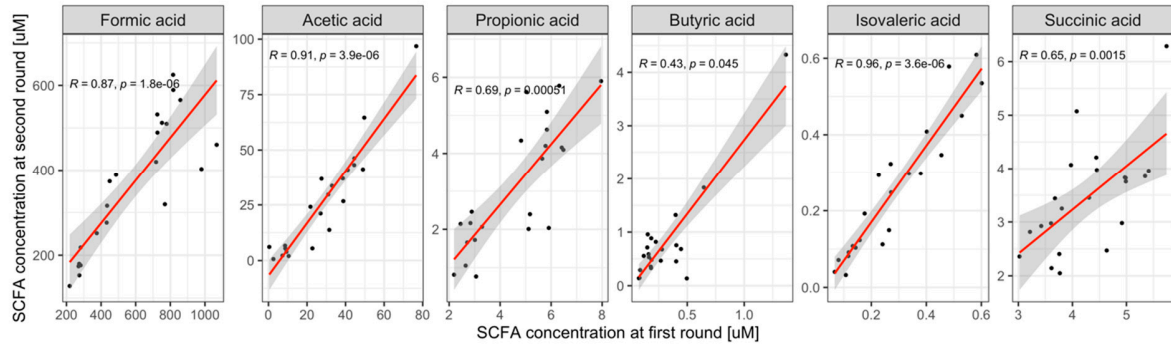


Figure S1. (A) The relationship between sampling time and the levels of analytes in ADHD patients at baseline; (B) Correlation analysis of SCFA levels between round 1 and round 2 from the samples ($n=22$) analyzed in two rounds. Spearman's rank correlation analysis. r and un-adjusted p values are shown.

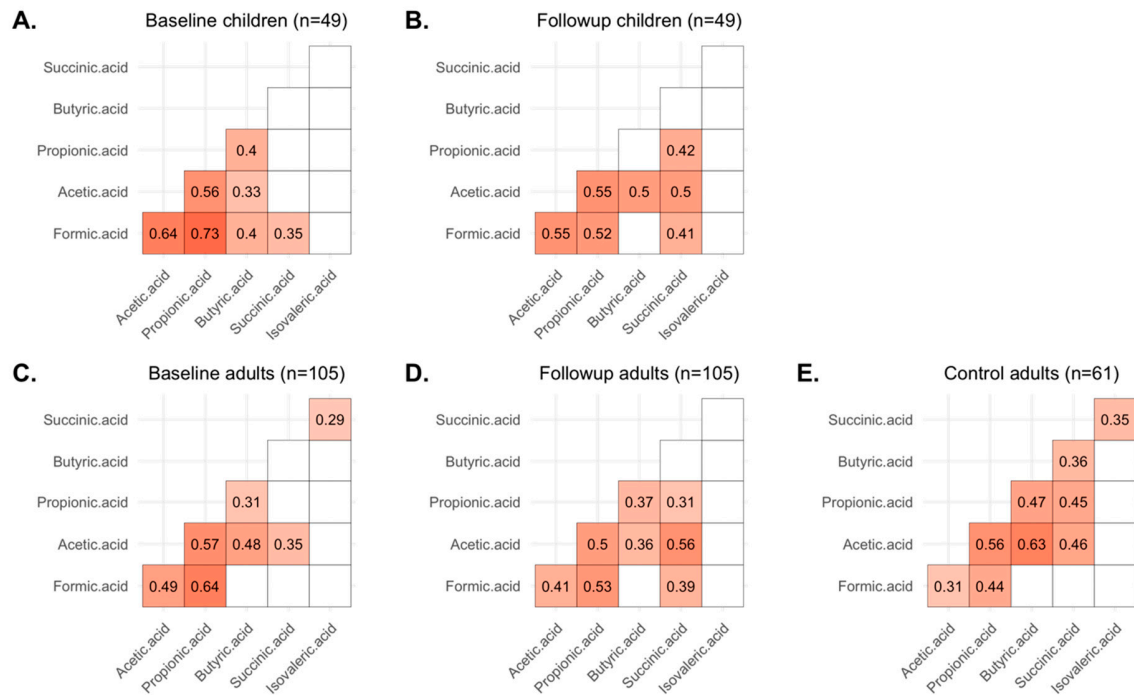


Figure S2. Correlation matrix between six SCFAs. Spearman's rank correlation coefficients for (A) child ADHD patients before treatment, (B) child ADHD patients after treatment, (C) adult ADHD patients before treatment, (D) adult ADHD patients after treatment, (E) adult healthy controls. FDR-adjusted significant correlations ($p < 0.050$) are shown with r values in the corresponding each box; Non-significant correlations are indicated by blank.

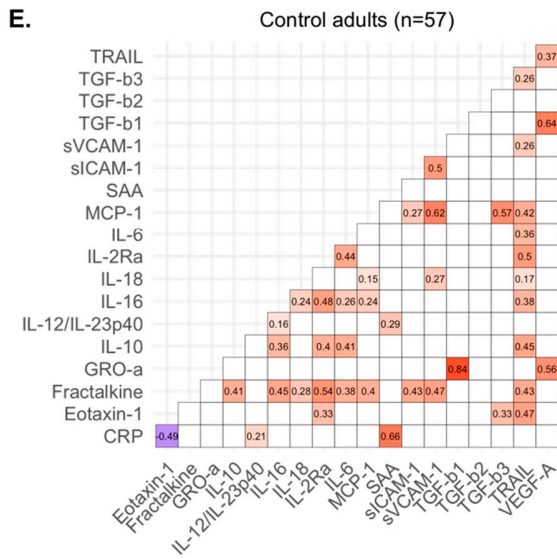
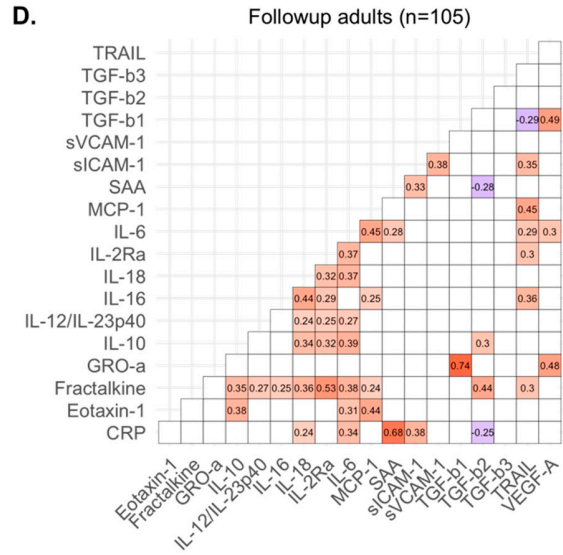
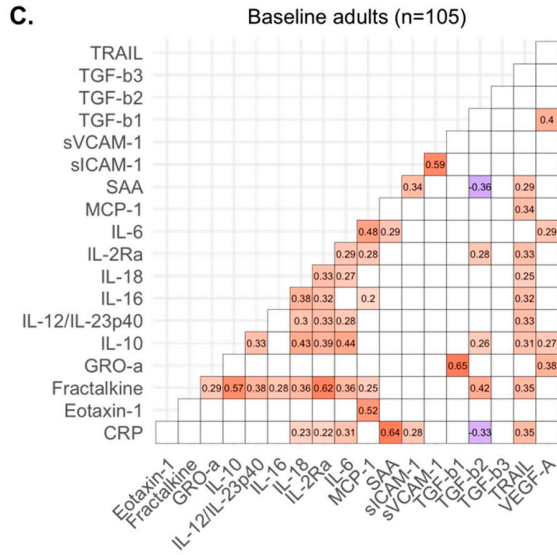
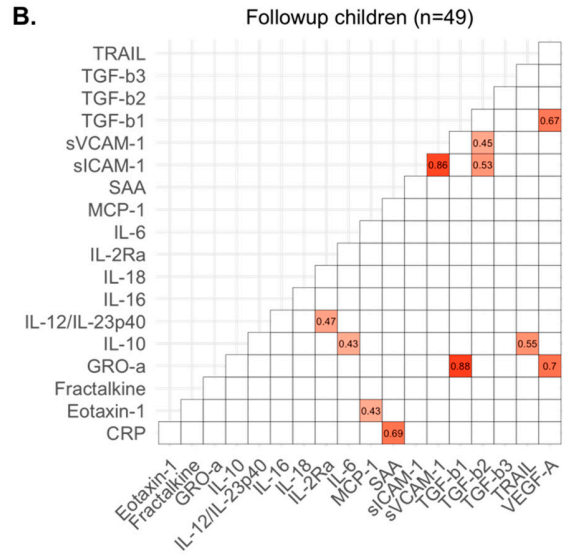
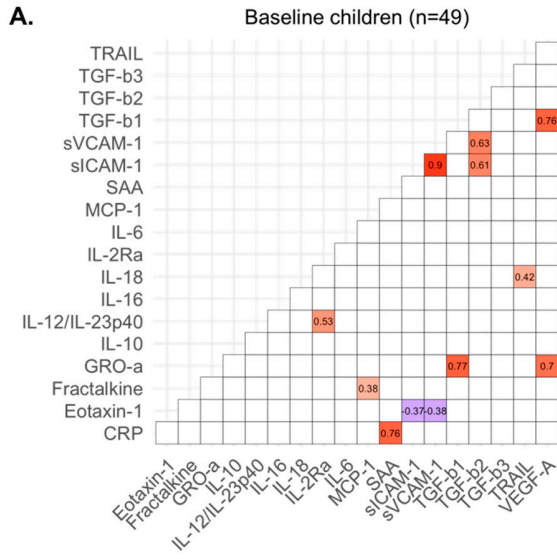
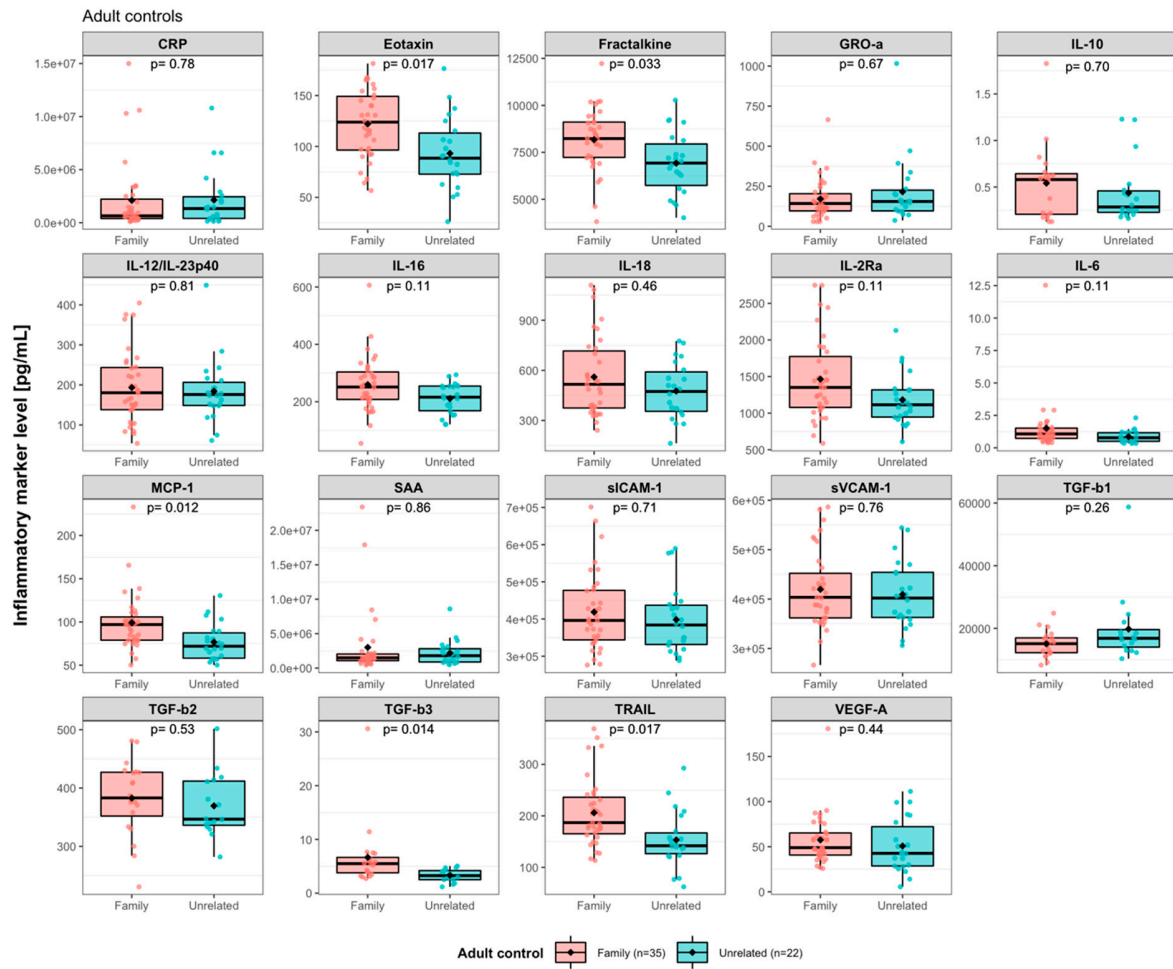
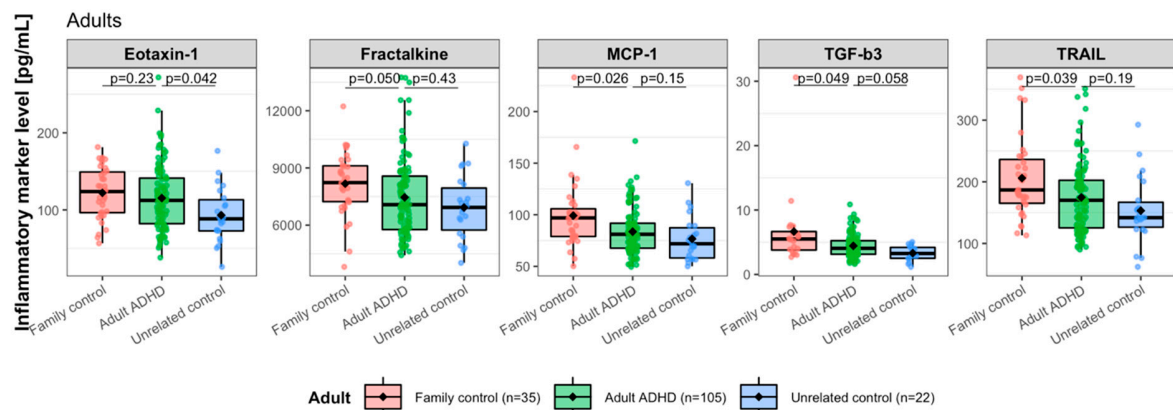


Figure S3. Correlation matrix between immune activity markers. Spearman's rank correlation coefficients for (A) child ADHD patients before treatment (B) child ADHD patients after treatment, (C) adult ADHD patients before treatment (D) adult ADHD patients after treatment, (E) adult healthy controls. FDR-adjusted significant correlations ($p < 0.050$) with r values are shown in the corresponding boxes. Red box indicates a positive correlation and blue box indicates a negative correlation. Non-significant correlations are indicated by blank. Outliers (three for CRP and two for SAA) were excluded by the defined cutoff of more than $50 \times$ interquartile range (IQR) from the median.

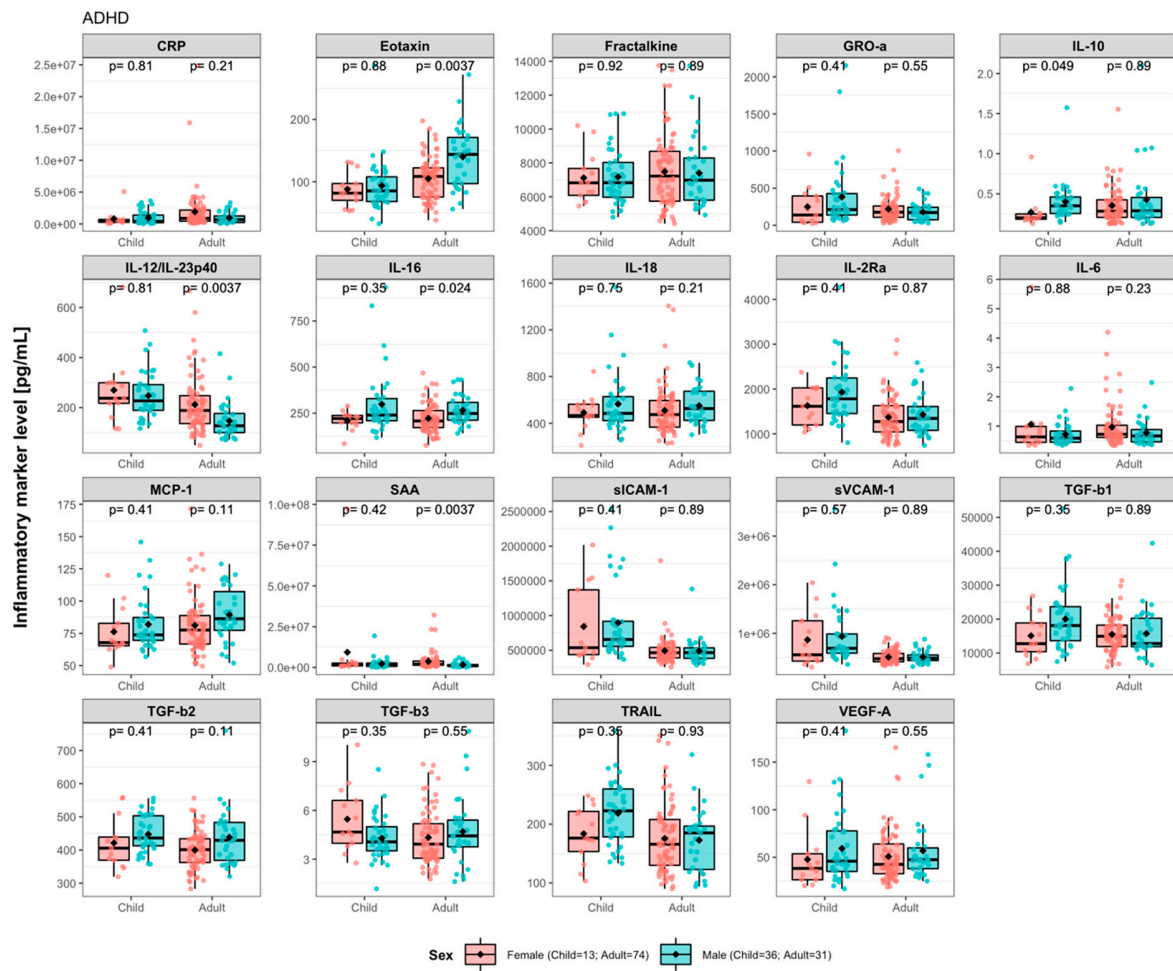
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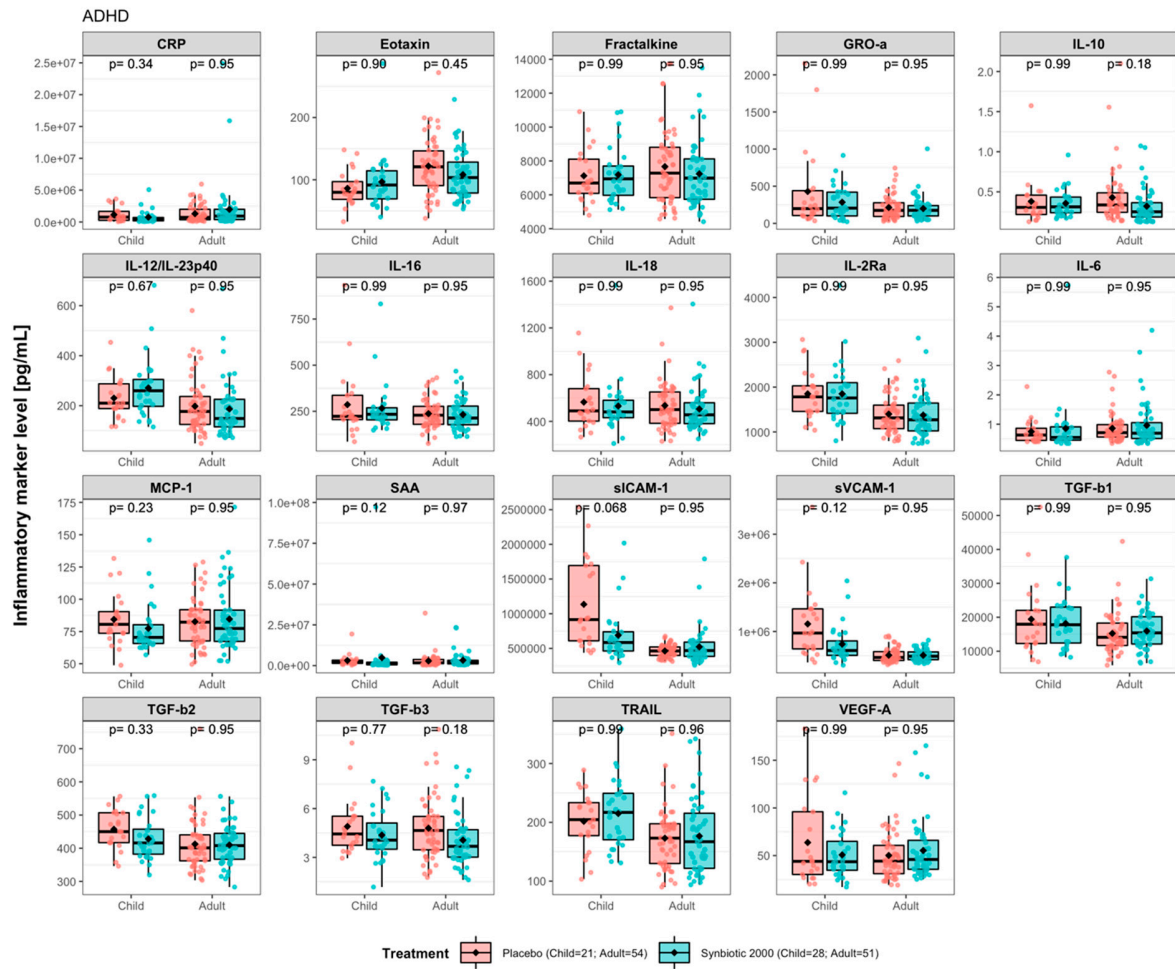
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C.



D.



E.

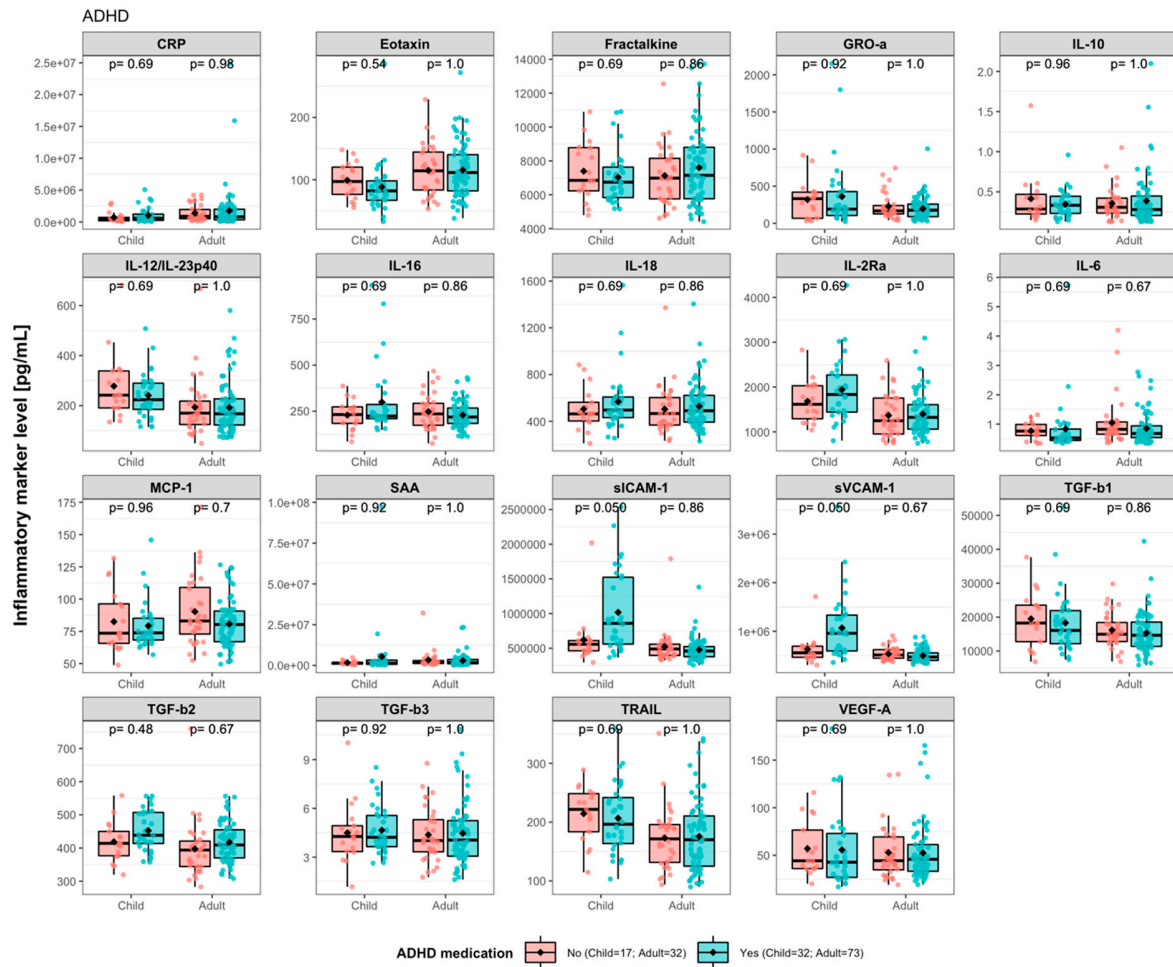
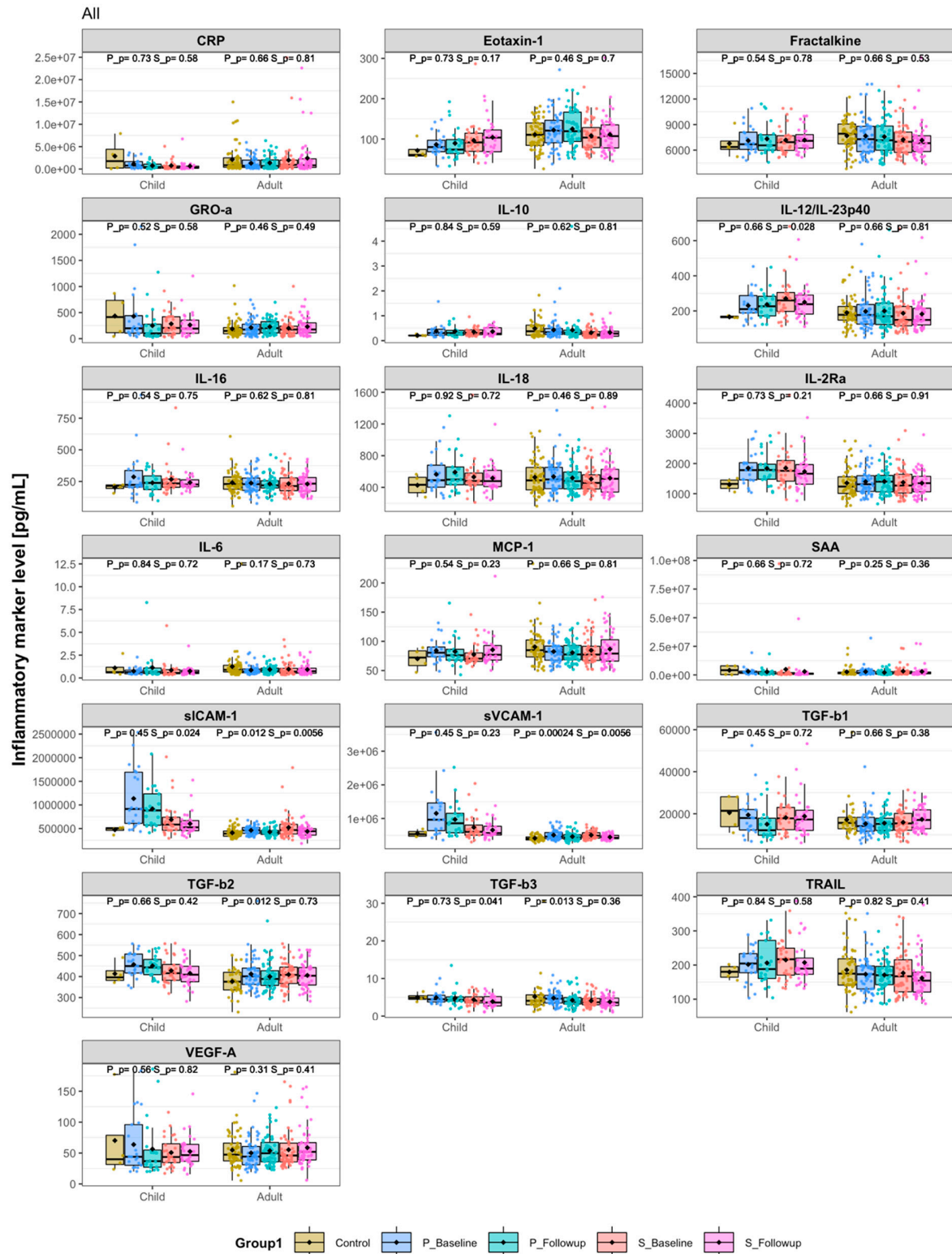


Figure S4. Immune activity marker levels at baseline with respect to control type, sex, treatment group and ADHD medication type stratified by age group. (A) Adult control types; (B) Adult control types and adult ADHD patients; (C) Sex in children and adults; (D) Treatment group (placebo and Synbiotic 2000) in children and adults; (E) ADHD medication in children and adults. Y-axes represent analyte levels. The differences between groups were analyzed using nonparametric Mann–Whitney *U* test and FDR-adjusted *p* values are shown. Each dot represents a participant. Outliers (three for CRP and two for SAA) were excluded by the defined cut-off of more than 50*interquartile range (IQR) from the median.

A.



B.

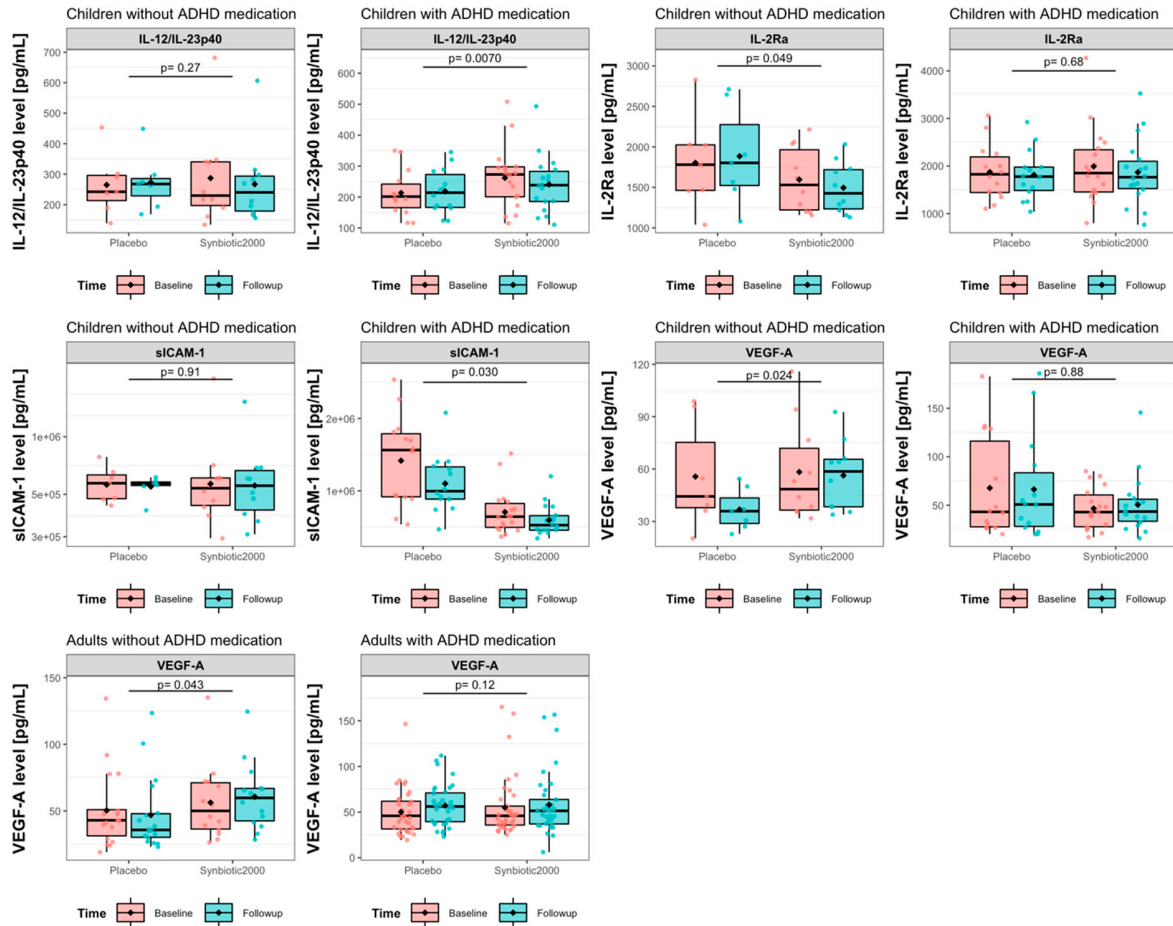


Figure S5. Plasma levels of immune activity markers: (A) Time effect (from baseline to the 9-week follow-up) for each intervention group (placebo and Synbiotic 2000); (B) Treatment effect (Synbiotic 2000 in comparison with placebo) stratified by ADHD medication. The differences from baseline to follow-up were analyzed using paired nonparametric Mann–Whitney *U* test and FDR-adjusted *p* values are shown on top. The treatment effect was determined using ANCOVA on follow-up analyte levels adjusted for sex and baseline analyte levels. “P” stands for placebo group, “S” stands for Synbiotic 2000 group. Each dot represents a participant.

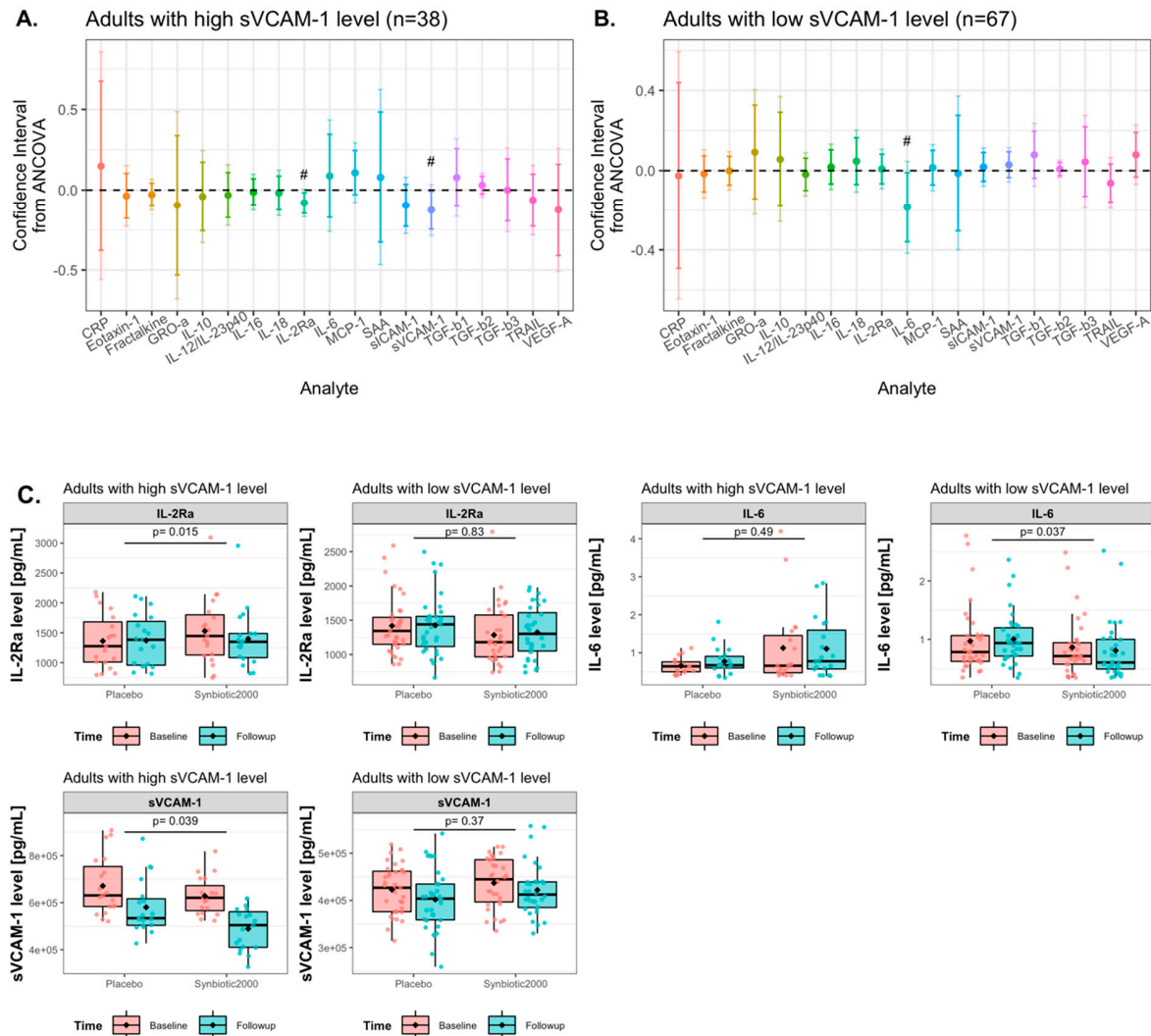
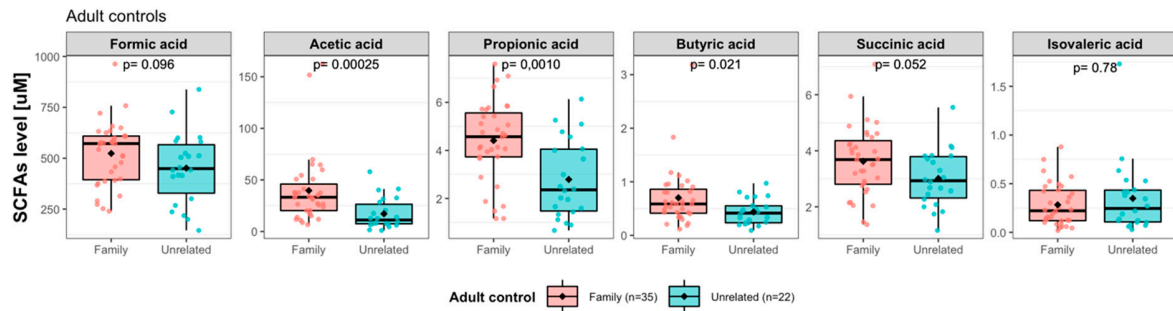
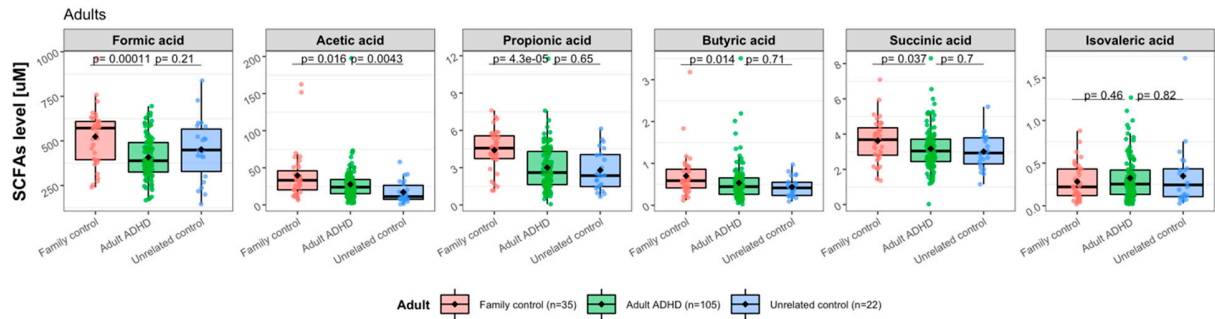


Figure S6. Plasma levels of immune activity markers: Treatment effect (Synbiotic 2000 compared to placebo) stratified by baseline plasma sVCAM-1 levels in adults with ADHD. CIs were from ANCOVA among (A) Adults with low sVCAM-1 level at baseline (< 519519.7 pg/mL); (B) Adults with high sVCAM-1 level at baseline (< 519519.7 pg/mL). Strong error bar colors indicate 95% CIs and light error bar colors indicate 99% CIs; (C) box plot for the analytes with Synbiotic 2000-specific effect in panel (A) and (B). A CI below 0 means that Synbiotic 2000, compared to placebo, reduced the analyte levels. The treatment effect was determined using ANCOVA on follow-up analyte levels adjusted for sex and baseline analyte levels. * Statistical significance ($\alpha=0.01$); # difference at $\alpha=0.05$.

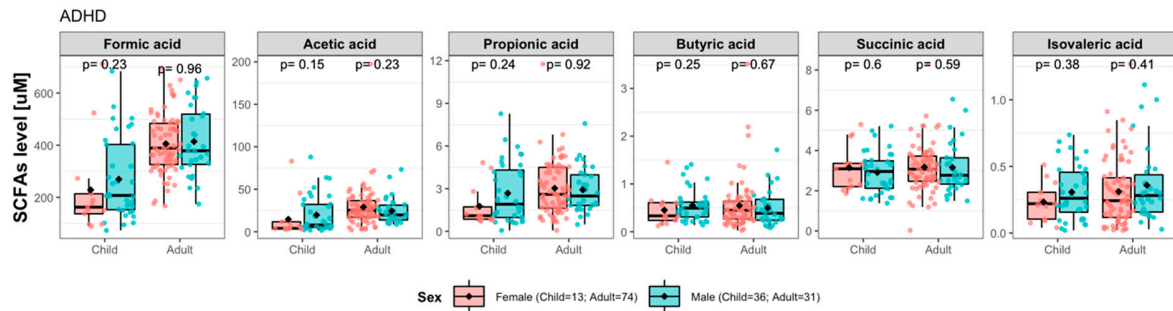
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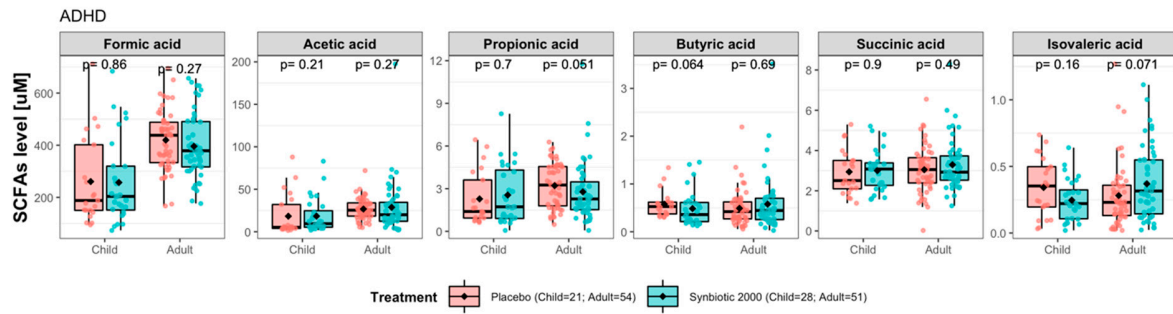
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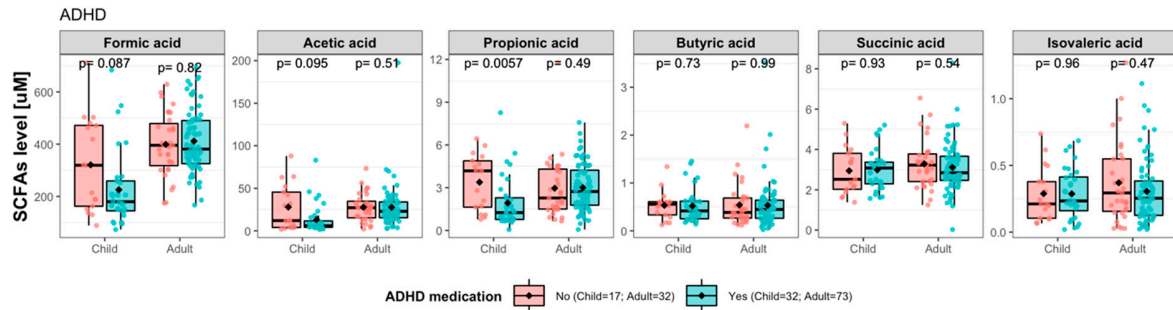
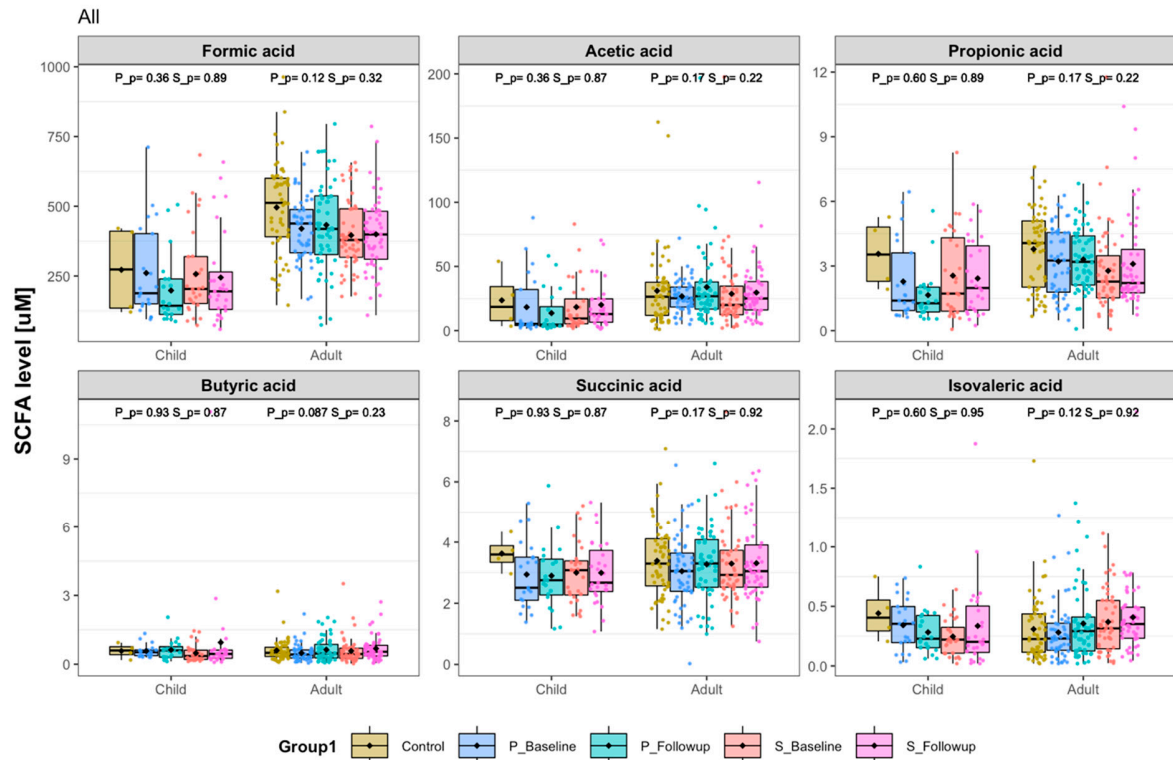


Figure S7. Short-chain fatty acid (SCFA) levels in plasma at baseline with respect to control type, sex, treatment group and ADHD medication stratified by age group. (A) Control type; (B) Diagnosis and control type; (C) Sex in children and adults (D) Treatment group in children and adults; (E) ADHD medication in children and adults. Y-axes represent analyte levels. The differences between groups were analyzed using nonparametric Mann–Whitney U test and p values are shown. Each dot represents a participant.

A.



B.

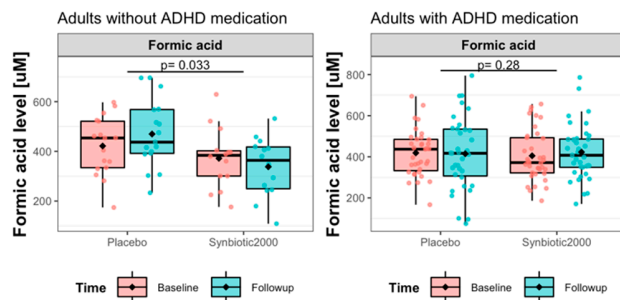
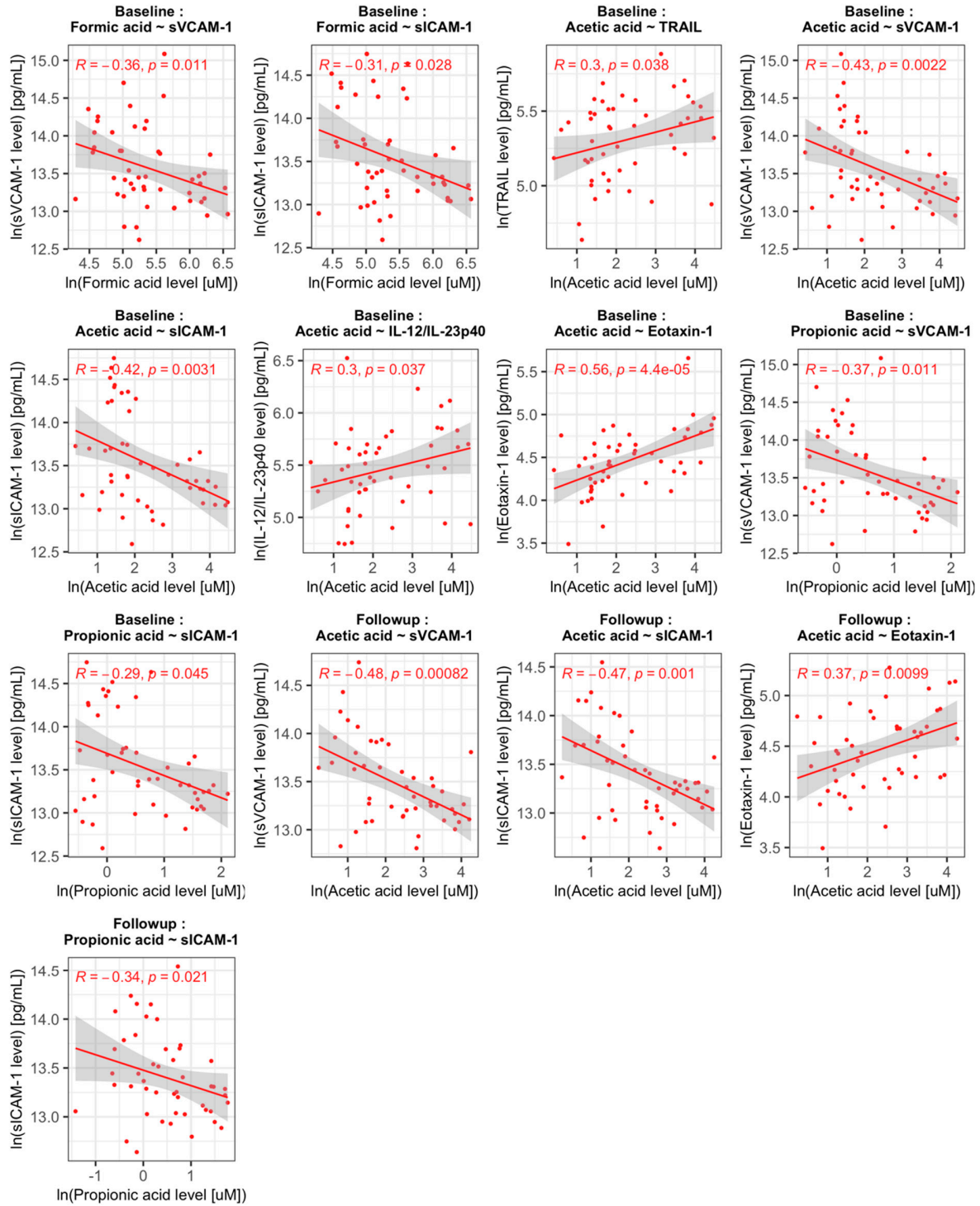


Figure S8. Plasma levels of short-chain fatty acid (SCFAs): (A) Time effect (from baseline to follow-up) on all SCFAs for each intervention group (placebo and Synbiotic 2000); (B) Treatment effect (Synbiotic 2000 in comparison with placebo) on formic acid in adults stratified by ADHD medication. The differences from baseline to follow-up were analyzed using paired nonparametric Mann–Whitney U test and p values are shown on top. The treatment effect was determined using ANCOVA on follow-up analyte levels adjusted for sex and baseline analyte levels. “P” stands for placebo group, “S” stands for Synbiotic 2000 group. Each dot represents a participant.

A. Children



B. Adults

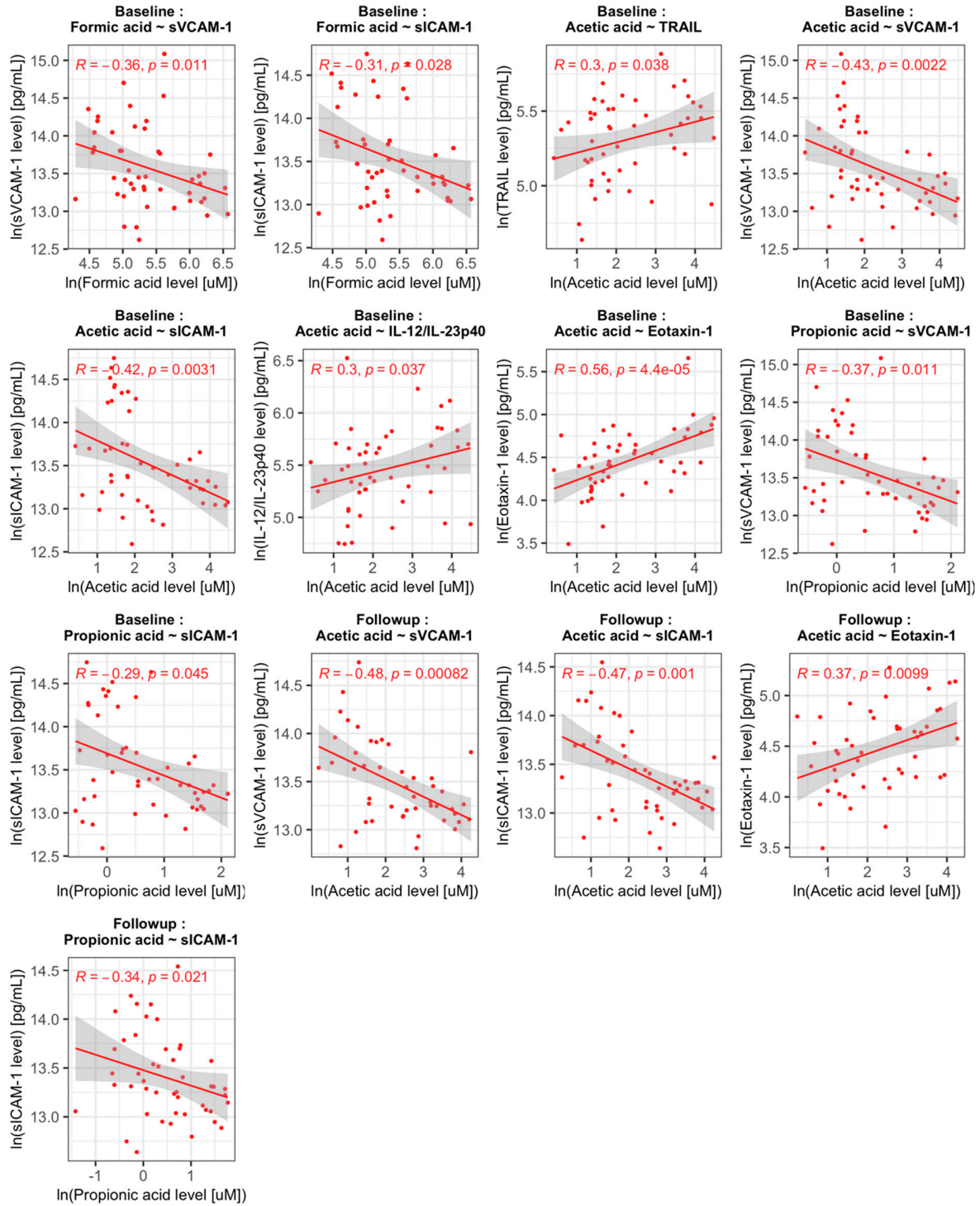


Figure S9. Individual scatter plots of the correlations between plasma levels of immune activity markers and short-chain fatty acids (SCFAs) shown in Fig. 6 with un-adjusted $p < 0.05$. (A) Children at baseline and follow-up; (B) Adults at baseline and follow-up. Spearman's correlation analysis with natural logarithm (ln)-transformed analyte levels. r and un-adjusted p values are shown. Outliers (three for CRP and two for SAA) were excluded by the defined cut-off of more than 50*interquartile range (IQR) from the median.

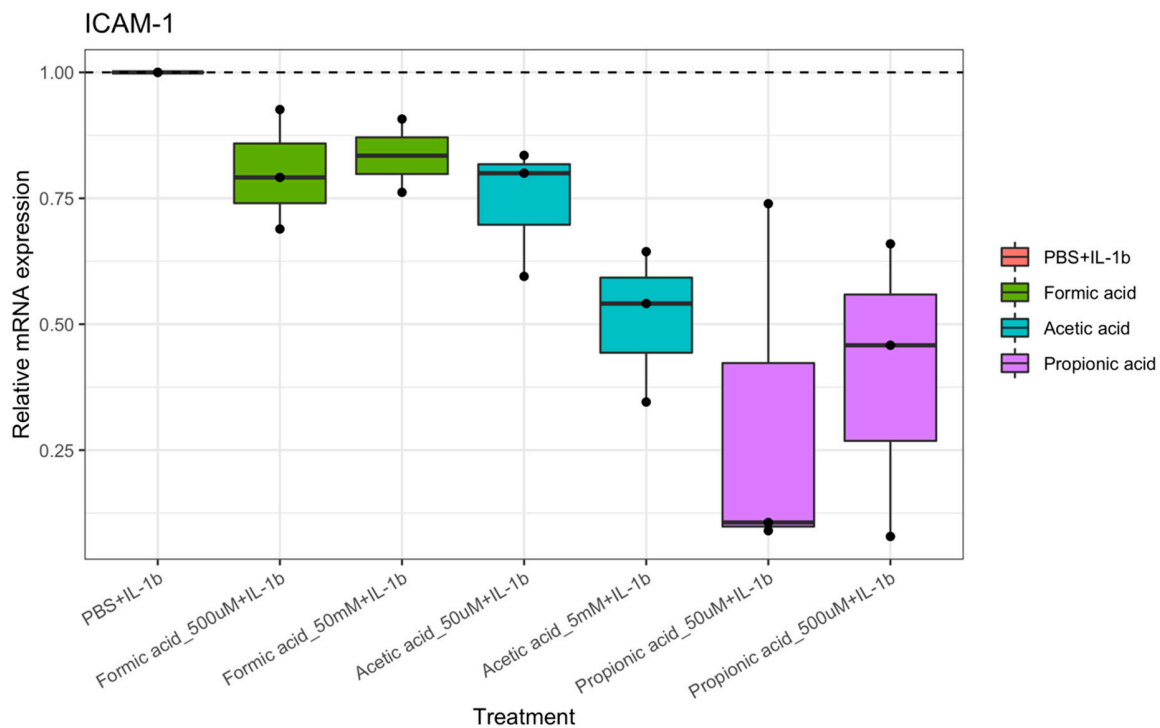


Figure S10. IL-1 β -induced ICAM-1 mRNA expression in human aortic vascular smooth-muscle-cells after *in vitro* pre-incubation with different short-chain fatty acids (SCFAs). The results from 3 independent experiments (biological replicas) are shown.

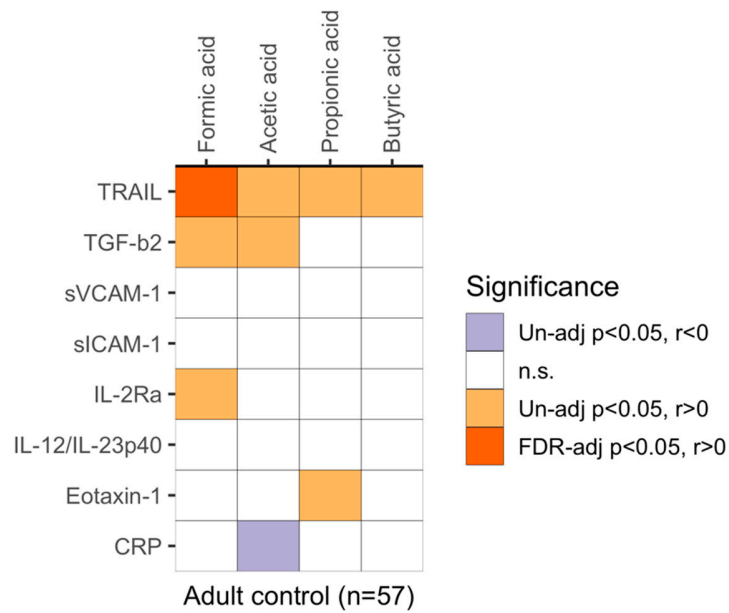


Figure S11. Correlation analysis between plasma levels of immune activity markers and short-chain fatty acids (SCFAs) in adult controls. Spearman's rank correlation coefficients were calculated. FDR-adjusted significant correlations ($p<0.05$) are indicated in strong orange colour, and unadjusted correlations ($p<0.05$) are indicated in light orange or light blue colour. Orange indicates a positive correlation, and blue a negative correlation.

Table S1. Experimental details of 24 immune activity analytes measured using Meso Scale Discovery in 61 controls and 154 ADHD patients.

Analyte	Other name/full name	Lower limit of detection (LLOD) pg/mL (n=5)	Between-plate CV (%)	Within-plate CV (%)	Number of values below LLOD: total (control/baseline/follow-up) ^a
CRP	C-reactive protein	2289-3350	4.63	2.39	0 (0/0/0)
Eotaxin-1		12.70-21.85	10.8	3.13	0 (0/0/0)
Fractalkine	CX3CL1 (C-X3-C motif chemokine ligand 1)	80.5-259.1	16.9	2.23	0 (0/0/0)
GRO- α	CXCL1 (C-X-C motif chemokine ligand 1)	0.37-0.71	11.9	1.86	0 (0/0/0)
IL-10	CSIF (cytokine synthesis inhibitory factor)/interleukin 10	0.13-0.56	7.13	3.78	56 (22/18/16)
IL-12/IL-23p40	interleukin-12 subunit p40 or β	2.25-4.14	12.3	3.48	0 (0/0/0)
IL-16	interleukin-16	6.79-16.17	10.7	1.10	0 (0/0/0)
IL-18	interleukin-18	0.57-1.52	10.2	1.52	0 (0/0/0)
IL-2R α	interleukin-2 receptor α	8.23-16.78	10.1	2.54	0 (0/0/0)
IL-6	interleukin-16	0.34-0.73	8.60	2.41	52 (11/20/21)
MCP-1	CCL2 (C-C motif chemokine ligand 2)/monocyte chemoattractant protein 1	0.72-1.53	9.93	2.00	0 (0/0/0)
SAA	serum amyloid A	7999-13025	11.0	1.48	0 (0/0/0)
sICAM-1	soluble cell adhesion molecule 1	997.4-1610.0	8.93	5.28	0 (0/0/0)
sVCAM-1	soluble vascular cell adhesion molecule 1	5429-8426	6.59	1.63	0 (0/0/0)
TGF- β 1	transforming growth factor β 1	6.70-46.36	8.67	4.90	0 (0/0/0)
TGF- β 2	transforming growth factor β 2	2.20-10.54	6.60	3.45	0 (0/0/0)
TGF- β 3	transforming growth factor β 3	1.05-1.95	7.91	4.70	8 (1/1/6)
TRAIL	TNF-related apoptosis-inducing ligand	0.83-2.66	11.0	2.60	0 (0/0/0)
VEGF-A	vascular endothelial growth factor A	3.91-6.05	6.25	3.80	1 (0/0/1)
INF- γ^b	interferon γ	4.84-15.94	25.2	6.49	166 (25/74/67)
IL-1 β^b	interleukin 1 β	0.20-0.45	8.61	2.08	308 (47/128/133)
IL-17A ^b	interleukin 17A	1.77-4.57	5.21	6.53	247 (38/15/104)
IL-2 ^b	interleukin 2	0.98-2.37	5.70	4.16	334 (54/140/140)
TNF- α^b	tumor necrosis factor α	0.59-3.70	8.71	2.71	103 (33/28/42)

CV: coefficient of variation.

a. The values below LLOD were replaced with LLOD in the data analysis;

b. These five analytes with more 25% values below LLOD were excluded from data analysis;

Table S2 Confidence intervals for treatment effects shown in **Fig 3, Fig 5, Fig S5B, Fig S6, Fig S8B.**

Analyte	Age	Group	95% CI	99% CI	<i>p</i> value
IL-12/IL-23p40	Children	All	-0.158, -0.014	-0.182, 0.010	0.020
IL-12/IL-23p40	Children	With ADHD medication	-0.157, -0.028	-0.180, -0.005	0.0070*
sICAM-1	Children	With ADHD medication	-0.547, -0.030	-0.637, 0.060	0.030
sIL-2Ra	Children	Without ADHD medication	-0.274, -0.001	-0.328, 0.053	0.049
VEGF-A	Children	Without ADHD medication	0.054, 0.644	-0.062, 0.760	0.024
VEGF-A	Adults	Without ADHD medication	0.007, 0.368	-0.056, 0.431	0.043
sIL-2Ra	Adults	With high sVCAM-1	-0.145, -0.017	-0.167, 0.005	0.015
IL-6	Adults	With low sVCAM-1	-0.359, -0.011	-0.417, 0.046	0.037
sVCAM-1	Adults	With high sVCAM-1	-0.245, -0.007	-0.286, 0.034	0.039
Propionic acid	Children	All	0.006, 0.699	-0.110, 0.816	0.046
Formic acid	Adults	Without ADHD medication	-0.522, -0.024	-0.609, 0.063	0.033

**p* < 0.01; CI: confidence interval;

Table S3. Clinical characteristics of adult participants at baseline.

		ADHD patients ^e (n=105)	Controls (n=57)
		Median (IQR)/N (%)	Median (IQR)/N (%)
Age [years]		36 (29-43)	38 (34-43)
Body mass index [kg/m ²]		23.8 (22.2-26.6)	23.6 (21.6-27.6)
Sex	Female	74 (70)	36 (63)
	Male	31 (30)	21 (37)
Antibiotic drugs ^a	≥1 time	30 (28)	11 (20)
	None	70 (67)	46 (81)
	Unknown	5 (5)	0 (0)
Melatonin medication	Yes†	26 (25)	0 (0)*
	No†	79 (75)	57 (100)
ADHD medication ^b	Yes†	73 (70)	0 (0)*
	No†	32 (30)	57 (100)
Other prescribed drug for adults ^c	Yes†	54 (51)	3 (5)*
	No†	51 (49)	54 (95)
Dietary supplements ^d	Yes	76 (72)	27 (47)*
	No	29 (28)	30 (53)

Results are given as median (25th-75th percentile [IQR]) or as number (%) of subjects; **a.** number of antibiotic drug uses in the last two years (no one was on antibiotic drug use last 6 weeks); **b.** ADHD medications for children include the stimulants Methylphenidate (n=14), Lisdexamphetamine (n=10), the nonstimulant Atomoxetine (noradrenalin re-uptake inhibitor (n=4) and Methylphenidate plus Atomoxetine (n=3), and for adults they include Methylphenidate (n=34), Lisdexamphetamine (n=34), Dexamphetamine (n=12), Atomoxetine (n=3) and Methylphenidate plus Atomoxetine (n=1); **c.** other prescribed drugs for adults reported to influence immune activity and gut microbiome include Antidepressants, Antipsychotics, Anxiolytics, Sleeping pills (mainly antihistamines), Proton-pump inhibitors and Statins; **d.** supplements (e.g. vitamins, omega-3, probiotics) taken in the last 4 weeks. The probiotics used were *L. plantarum* 299v (1 control, 11 patients), Synbiotic 15 (similar constituents as Synbiotic 2000 but 15*10⁹ CFU instead of the 4*10¹¹CFU in Synbiotic 2000, 2 patients) and other (6 patients). **e.** ADHD ICD-10 diagnosis: F90.0 (18.8%), F90.0B (57.8%), F90.0C (17.5%), F90.0X (2.6%), F90.1 (0.7%), F90.8 (0.7%), F98.8 (2.0%); † Participants on medication currently or in last 3 months as “Yes”, not on medication currently or in the last 3 months as “No”. * Difference between adult ADHD patients and adult controls at $\alpha=0.05$.

Note for ADHD medication:

Medication data was collected including medication for core ADHD symptoms (here called ADHD medication), antibiotic drugs, and other medications previously reported to influence immune activity or gut microbiome i.e., anti-inflammatory melatonin (Carrascal et al., 2018; Tarocco et al., 2019), antidepressants (Macedo et al., 2017; Więdołocha et al., 2018), antipsychotics (Bobermin et al., 2018), anxiolytics (Bretler et al., 2019; Chen et al., 2018; Lazzarini et al., 2001), sleeping pills (Tiligada et al., 2011), proton-pump inhibitors (Kanagaratham et al., 2020) and statins (Pinal-Fernandez et al., 2018).

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Table S4 Time effects over 9 weeks on inflammatory markers.

Age group	Analyte [pg/mL]	Placebo				Synbiotic 2000			
		t1 median (IQR)	t2 median (IQR)	FDR-adjusted p_{time}	Estimate of t1-t2	t1 median (IQR)	t2 median (IQR)	FDR-adjusted p_{time}	Estimate of t1-t2
Child	CRP	745053.3 (259255.4, 1346089.8)	435575 (156108.6, 1075754.4)	0.73	118080	355349.9 (179620.8, 697018.5)	279970 (189909.7, 642704.4)	0.58	82026.1
	eotaxin-1	79.9 (68.6, 97.1)	73.9 (64, 98.7)	0.73	2.2	91.6 (69.4, 114.5)	103.5 (68.4, 122.6)	0.17	-7.0
	Fractalkine	6697.4 (6084.7, 8104.7)	6560 (5979.4, 7806.4)	0.54	-304.4	6941.7 (5972, 7697.6)	7093.3 (6239.9, 7820.6)	0.78	-73.1
	GRO-a	198.4 (105.5, 440.2)	99.3 (49.9, 277.8)	0.52	84.8	205.6 (99.5, 420.6)	192.4 (103.3, 354.9)	0.54	27.8
	IL-10	0.3 (0.2, 0.5)	0.3 (0.2, 0.4)	0.84	0	0.3 (0.2, 0.4)	0.3 (0.2, 0.5)	0.59	0.02
	IL-12/IL-23p40	210.2 (188.6, 287.4)	226.3 (171.5, 283.3)	0.66	-5.1	259.8 (197.4, 305)	238.3 (182.3, 290.6)	0.028*	21.4
	IL-16	223.1 (205.2, 336.8)	238.3 (185.7, 294.4)	0.54	15.3	233.9 (203.7, 269.1)	226.7 (209, 263.1)	0.75	4.2
	IL-18	489.6 (402.1, 680)	498.7 (432.2, 657.7)	0.92	-2.4	480.2 (430.9, 580.9)	479 (409.8, 615)	0.72	7.2
	IL-2Ra	1782.6 (1457.7, 2028.4)	1786.1 (1479.4, 1979.3)	0.73	36.6	1758.1 (1415.8, 2099.3)	1662.7 (1317.5, 1963.7)	0.21	88.2
	IL-6	0.6 (0.4, 0.9)	0.6 (0.5, 1.1)	0.84	0	0.5 (0.5, 0.9)	0.6 (0.5, 0.8)	0.72	0.03
	MCP-1	80.5 (73.6, 90.4)	76.2 (67.7, 86.4)	0.54	5.9	70.4 (65.7, 80.3)	77 (67.9, 92.8)	0.23	-5.3
	SAA	1646595.2 (1252895.4, 3287193)	1161097.9 (732673.6, 2960056.1)	0.66	266012	970279.5 (585854.1, 2078620.7)	1074418.8 (582429.5, 1542974.5)	0.72	112153
	sICAM-1	914517.1 (610039.9, 1694365.8)	884480.1 (570049.7, 1234924.3)	0.45	185610	584787.8 (462437.7, 739473.6)	526133 (454646.4, 669398.4)	0.024*	46776.6
	sVCAM-1	965338.4 (638909.5, 1463139.7)	859344.7 (576357.4, 1154789.6)	0.45	104628	606946.4 (506038.6, 804800.4)	566870.8 (509538.5, 769932.6)	0.23	49783.8
	TGF-b1	17959.4 (12277.6, 22037.2)	12163.9 (9785.9, 17870.5)	0.45	4057.6	17794.4 (12419.7, 22992.3)	17284.7 (12061.5, 21661.2)	0.72	816.9
	TGF-b2	450.1 (416.9, 507)	443.1 (409.2, 481.7)	0.66	7.2	416.1 (382.2, 457.6)	408.8 (375.1, 449.1)	0.42	13.3
	TGF-b3	4.4 (3.7, 5.5)	4.3 (4, 5.1)	0.73	0.2	4.1 (3.5, 5.1)	3.6 (2.7, 5.2)	0.041*	0.6
	TRAIL	204.5 (177.4, 233.5)	188.2 (160.8, 272.2)	0.84	3.2	216.9 (170.4, 249.3)	189.6 (173, 220.5)	0.54	8.6
	VEGF-A	44 (30.2, 96)	36.9 (27.1, 54.9)	0.56	4.8	43.6 (34.6, 65)	46.7 (36.6, 64)	0.82	-1.0

Adult	CRP	721280.6 (347931.2, 1994883.4)	614275.8 (257305.5, 2054455.8)	0.66	-30546	923019.9 (365711.5, 1992913)	776434.2 (333025.4, 2016611.4)	0.81	36493.6
	eotaxin-1	120.9 (90.8, 146.4)	119.4 (93.9, 166.6)	0.45	-4.1	103.5 (78.8, 128.4)	107.3 (78.1, 135.1)	0.70	-2.5
	Fractalkine	7282.6 (5832.9, 8806.8)	7156.8 (5988.6, 8832.5)	0.66	47.6	6986 (5735.2, 8121.7)	6818 (5833.8, 7694.5)	0.53	137.1
	GRO-a	174.4 (92.6, 277)	201.4 (109.7, 326)	0.46	-13.4	175.1 (99.5, 239.5)	175.7 (112.3, 303.1)	0.49	-15.6
	IL-10	0.3 (0.2, 0.5)	0.3 (0.3, 0.4)	0.62	0	0.3 (0.2, 0.4)	0.3 (0.2, 0.4)	0.81	-0.004
	IL-12/IL-23p40	176.8 (125, 236.9)	169.1 (122.7, 245.1)	0.66	-2.5	148.6 (115.3, 225.4)	148.7 (120.3, 215)	0.81	1.4
	IL-16	228.8 (179.5, 276.9)	219.1 (198.3, 256.1)	0.62	6	213.5 (177.4, 278.3)	232.8 (172.8, 280.9)	0.81	1.7
	IL-18	500.8 (383.2, 651.6)	478.8 (380.6, 593.6)	0.46	14.6	455.7 (380.2, 559.1)	508.6 (340.8, 628.1)	0.89	1.9
	IL-2Ra	1313.5 (1073.3, 1598.1)	1400.2 (1060.2, 1605.4)	0.66	-14	1270.2 (1022.7, 1644.6)	1348.7 (1062.7, 1575.2)	0.91	4.2
	IL-6	0.7 (0.6, 1)	0.8 (0.7, 1.1)	0.17	-0.1	0.7 (0.5, 1.1)	0.7 (0.5, 1.1)	0.73	0.03
	MCP-1	82.2 (67.7, 91.9)	77.6 (68.5, 93)	0.66	0.9	77.3 (67.3, 91.6)	79.1 (66.2, 102.8)	0.81	-1.0
	SAA	1703542 (1228098.7, 3789533.2)	1245891.6 (772594.2, 2850467.6)	0.25	253102	2009562.8 (1071442.7, 3302860.7)	1426810.9 (1027341, 2502272.2)	0.36	285248
	sICAM-1	463137.4 (398350.6, 520920)	418921.3 (375803.7, 458988.9)	0.012*	30422.7	469008.3 (385456.1, 590741.4)	444406.2 (360751.2, 496985.4)	0.0056**	45113.8
	sVCAM-1	463842.8 (400746.2, 583659)	434797.5 (383524.3, 520150.9)	0.00024***	37318	488951.9 (419294.6, 577680)	428840.4 (397779.4, 498670.5)	0.0056**	50596.9
	TGF-b1	14105.1 (11680, 18298)	15167.4 (12051.4, 17848)	0.66	-352.2	15413 (12130.8, 20174.2)	17062.6 (12877, 21874)	0.38	-993.2
	TGF-b2	401.1 (362.1, 440.3)	388.7 (357.8, 428.1)	0.012*	11.4	407.9 (366.9, 444.8)	403.9 (359.9, 443.1)	0.73	2.6
	TGF-b3	4.7 (3.5, 5.5)	3.8 (3.2, 4.9)	0.013*	0.5	3.7 (3, 4.7)	3.6 (2.8, 4.8)	0.36	0.3
	TRAIL	173.2 (130, 197.6)	170.1 (142.5, 196.1)	0.82	1.1	167.1 (121.7, 215.5)	154.4 (121, 179.1)	0.41	7.6
	VEGF-A	44.2 (31, 60.7)	49.7 (35.7, 67.2)	0.31	-3.3	45.9 (35.6, 66)	51.9 (38.8, 66.8)	0.41	-2.7

a. Time effects were assessed by paired Mann–Whitney *U* test and adjusted by FDR.

t1=baseline, t2=9 weeks; IQR=interquartile range; * FDR-adjusted $p < 0.05$, **FDR-adjusted $p < 0.01$, *** FDR-adjusted $p < 0.001$.

Table S5 Time effects over 9 weeks on SCFAs.

Age group	Analyte [μM]	Placebo						Synbiotic 2000					
		t1 (IQR)	median	t2 (IQR)	median	FDR-adjusted p_{time}	Estimate of t1-t2	t1 (IQR)	median	t2 (IQR)	median	FDR-adjusted p_{time}	Estimate of t1-t2
Child	Formic acid	188 (150.1, 401.7)		143.2 (111.9, 239.4)		0.36	25.761	203.6 (151.4, 319.6)		194.9 (130.1, 264.6)		0.89	6.812
	Acetic acid	5.2 (4, 32)		4.6 (3.3, 18.7)		0.36	1.914	9.5 (5.3, 24.7)		12.9 (6.5, 24.8)		0.87	-2.561
	Propionic acid	1.4 (0.9, 3.6)		1.3 (0.9, 2)		0.60	0.115	1.7 (0.9, 4.3)		2 (1, 3.9)		0.89	-0.041
	Butyric acid												
	Succinic acid	0.5 (0.4, 0.6)		0.6 (0.3, 0.8)		0.93	0.015	0.4 (0.2, 0.6)		0.4 (0.3, 0.6)		0.87	-0.043
	Isovaleric acid	0.4 (0.2, 0.5)		0.2 (0.2, 0.4)		0.60	0.057	0.2 (0.1, 0.3)		0.2 (0.1, 0.5)		0.95	0.002
Adult	Formic acid	438.1 (333.2, 488.3)		418.6 (326.5, 537.4)		0.12	-42.469	378.9 (317.1, 490.6)		399 (310, 481.9)		0.32	-16.1
	Acetic acid	25.3 (18.4, 34)		26.8 (16.7, 37.9)		0.17	-2.573	20 (12.4, 34.6)		25 (16.1, 38.2)		0.22	-4.5
	Propionic acid	3.3 (1.8, 4.6)		3.2 (2.1, 4.4)		0.17	-0.219	2.3 (1.5, 3.5)		2.2 (1.8, 3.8)		0.22	
	Butyric acid	0.4 (0.3, 0.6)		0.5 (0.3, 0.9)		0.087	-0.097	0.4 (0.3, 0.7)		0.5 (0.4, 0.8)		0.23	-0.34
	Succinic acid	3.1 (2.4, 3.6)		3.3 (2.5, 4.1)		0.17	-0.167	2.9 (2.5, 3.7)		3 (2.5, 3.9)		0.92	-0.006
	Isovaleric acid	0.2 (0.1, 0.4)		0.3 (0.1, 0.4)		0.12	-0.053	0.3 (0.1, 0.5)		0.4 (0.2, 0.5)		0.92	-0.001

a. Time effects were assessed by paired Mann–Whitney U test and adjusted by FDR.

t1=baseline, t2=9 weeks; IQR=interquartile range; * FDR-adjusted $p<0.05$, **FDR-adjusted $p<0.01$, *** FDR-adjusted $p<0.001$.