J. Microbiol. Biotechnol.
https://doi.org/10.4014/jmb.1803.03020

PICRUSt Using Filtered OTU Tables

OTU tables can be filtered out to contain only subsets of taxa. As mentioned in the main text, the use of filtered OTU tables for specific taxa to predict the metabolic profile using PICRUSt is uncommon in the literature but may shed light into the contribution of different bacterial groups to the overall functional profile (Garcia-Mazcorro *et al*., 2017). For example, in this study the separate analysis of OTUs from Lachnospiraceae (the only group that showed higher abundance in supplemented mice) revealed a total of 38 features that showed statistical significance between the groups using the same stringent criteria used for the full OTU tables (interestingly, genes related to Ribosome biogenesis, $p = 0.906$, and amino acid metabolism, $p = 0.617$, were not different between the groups when using OTUs from Lachnospiraceae). Control mice had higher abundance of genes related to toluene degradation (adjusted $p = 0.003$), inorganic ion transport and metabolism ($p = 0.004$), sporulation ($p = 0.008$), energy metabolism ($p = 0.008$), while supplemented mice had more genes related to lipid metabolism ($p = 0.008$), glycine, serine and threonine metabolism ($p = 0.009$), citrate cycle ($p = 0.009$), and D-alanine metabolism ($p = 0.013$), among others (see main text). The separate analysis of other groups such as *Bacteroides ovatus*, a group that can cause systematic antibody responses (Saitoh *et al*., 2002) and that in this study was more prevalent in the supplemented mice, did not yield any difference in the predicted profile between the groups. In our experience the separate PICRUSt analysis of all taxa is advisable but is outside the scope of this current manuscript.

References

Garcia-Mazcorro JF, Nunes Lage, N, Mertens-Talcott S, Talcott S, Chew B, Dowd SE, *et al*. 2017. Effect of dark sweet cherry powder consumption on the gut microbiota, short-chain fatty acids, and biomarkers of gut health in obese db/db mice. *PeerJ* **6:** e4195.

Saitoh S, Noda S, Aiba Y, Takagi A, Sakamoto M, Benno Y, Koga Y. 2002. *Bacteroides ovatus* as the predominant commensal intestinal microbe causing a systemic antibody response in inflammatory bowel disease. *Clin. Diagn. Lab. Immunol.* **9:** 54-59.

Proteomic Analysis of Feces

Protein extracts (500 ng) from control and raspberry supplemented groups were quantified with a Quant-iT Protein Assay Kit (ThermoFisher Scientific, USA) in compliance with the manufacturer's protocol and subjected to trypsinization before injection. Briefly, disulfide bonds in protein extracts were reduced using 100 mM dithiothreitol (DTT) at a ratio of 1:10 DTT/sample volume and incubated at 50°C for 45 min. Cysteine bonds were then alkylated with 200 mM iodoacetamide at the same volume ratio for 20 min at room temperature. Protein was digested with trypsin (G-Biosciences, St. Louis, MO, USA) at a 1:50 ratio of trypsin/protein, and incubated at 37 °C for 12 h for high resolution nano-HPLC tandem mass spectrometry analysis. Peptide samples were subjected to Thermo ScientificTM Orbitrap FusionTM TribridTM with an Easy-nLCTM 1 000 ultra-high pressure LC on a Thermo ScientificTM PepMap 100 C18 column (2 μm, 50 μm × 15 cm). The peptides were separated over 115 min gradient eluted at 400 ηl/min with 0.1% FA in water (solvent A) and 0.1% FA in acetonitrile (solvent B) (5−30% B in 85 min, followed by 30−50% B over 10 min and 50−97% B over 10 min). The run was completed by holding a 97% B for 10 min. MS1 data was acquired on an Orbitrap Fusion mass spectrometry using a full scan method according to the following parameters: scan range 400-1 500 m/z, Orbitrap resolution 120 000; AGC target 400 000; and maximum injection time of 50 ms. MS2 data were collected using the following parameters: rapid scan rate, HCD collision energy 35%, 1.6 m/z isolation window, AGC 2 000 and maximum injection time of 50 ms. MS2 precursors were selected for a 3 s cycle. The precursors with an assigned monoisotopic m/z and a charge state of 2-7 were interrogated. The precursors were filtered using a 60 s dynamic exclusion window. MS/MS spectra were searched using Thermo ScientificTM Proteome DiscovererTM software version 2.0 with SEQUEST® against Uniprot database. Fasta files were prepared based on main bacteria identified on 16S rRNA sequencing. Precursor and fragment mass tolerances were set to 10 ppm and 0.8 Da respectively and allowing up to two missed cleavages. Static modification used was carbamidomethylation (C). Protein relative quantitation was achieved by extracting peptide areas with the Proteome Discoverer (PD) 2.0 (Thermo Scientific, San Jose, CA, USA). Three unique peptides per protein were used for the protein quantitation analysis.

Metabolomics Analysis of Feces

Primary metabolite derivatization and gas chromatography time-of-flight mass spectrometry analysis was performed by the Laboratory for Cellular Metabolism and Engineering (LCME) at WSU. Defined amount of fecal matter was suspended in 300 µl methanol. After adding 1.5 µg of the surrogate standard ribitol, the material was continued extracted by shaking at room temperature for 15 min (Vortex), sonication for 20 min (Branson 450 sonication bath), and shaking for 15 min at 35°C and 1200 rpm (Eppendorf Thermomixer). The extracts were then centrifuged for 10 min at 21,000 g, and the supernatants transferred into new vial. The residue was extracted a second time in the same manner but with 500 µl of a solvent mixture containing methanol, 2-propanol, and water at a ratio of 5:2:2. The debris was again removed by centrifugation for 10 min at 21,000 g, and the supernatants combined with the products of first extraction. The combined extracts were dried in vacuo. Dry residues were suspended in 5 µl O-methoxylamine hydrochloride (40 mg ml^{-1} in pyridine, both from Sigma) and incubated for 90 min at 30°C and 1,000 rpm (Eppendorf Thermomixer). Subsequently, samples were derivatized with 45 µl of MSTFA with 1% TMCS (Thermo-Pierce cat.-no.TS-48915) for 30 min at 37°C and 1,000 rpm (Eppendorf Thermomixer). A mixture of fatty acid methyl esters was added to each sample prior to injection. Gas chromatography-mass spectroscopy analysis was performed using a Pegasus 4D time-of-flight mass spectrometer (LECO) equipped with a Gerstel MPS2 autosampler and an Agilent 7890A oven. The derivatization products were separated on a 30 m, 0.25 mm i.d., 0.25 µm df Rxi-5Sil® column (Restek) with an IntegraGuard® pre-column using ultrapure He at a constant flow of 1 ml min^{-1} as carrier gas. The linear thermal gradient started with a one-minute hold at 50°C, followed by a ramp to 330°C at 20°C min^{-1}. The final temperature was held for 5 min prior to returning to initial conditions. Mass spectra were collected at 17 spectra s^{-1}. The injection port was held at 250°C, and 2 µl of the sample were injected at an appropriate split ratio. Peak identification was conducted using Fiehn primary metabolite library (Kind *et al*. 2009). Peak alignment and spectrum comparisons were carried out using the Statistical Compare feature of the ChromaTOF® software (LECO). The surrogate standard ribitol and the initial matter weight were used for normalization. Statistical analyses were performed on normalized data using MetaboAnalyst 3.0 (Xia *et al*. 2015).

References

Xia J, Sinelnikov IV, Han B, Wishart DS. 2015. MetaboAnalyst 3.0 -- making metabolomics more meaningful. *Nucl. Acids Res.* **43 :** W251-257.

Table S1. AIM-93G Diet composition.

	Control		Raspberry	
Ingredient	Weight (g)	kcal	Weight (g)	Kcal
Casein	100	400	100	400
Maltodextrin	66	264	66	264
Sucrose	50	200	50	200
Cellulose	25	0	25	0
Mineral Mix[a]	17.5	0	17.5	0
Vitamin Mix[b]	5	0	5	0
L-Cysteine	1.5	6	1.5	6
Choline Bitartrate	1.25	0	1.25	0
t-Butylhydroquinone	0.007	0	0.007	0
Cornstarch	198.75	795	150	600
Soybean Oil	35	315	35	315
Raspberry[c]	0	0	48.75	195
Agar	20	0	20	0
Water	480	0	480	0
Total	1000.01	1980	1000.01	1980

[a]AIN-93G Vitamin Mix supplied by Dyets Inc. (Bethlehem, PA), containing (g/kg): Niacin (3), Calcium Pantothenate (1.6), Pyridoxine HCl (0.7), Thiamine HCl (0.6), Riboflavin (0.6), Folic Acid (0.2), Biotin (0.02), Vitamin E Acetate (500 IU/g) (15), Vitamin B12 (0.1%) (2.5), Vitamin A Palmitate (500000 IU/g) (0.8), Vitamin D3 (400000 IU/g) (0.25), Vitamin K1/Dextrose Mix (10 mg/g) (7.5), Sucrose (967.23).

[b]AIN-93G-MX supplied by Dyets Inc. (Bethlehem, PA), containing (g/kg): Calcium Carbonate (357), Potassium Phosphate, monobasic (196), Potassium Citrate .H20 (70.78), Sodium Chloride (74), Potassium Sulfate (46.6), Magnesium Oxide (24), Ferric Citrate, U.S.P. (6.06), Zinc Carbonate (1.65), Manganous Carbonate (0.63), Cupric Carbonate (0.3), Potassium Iodate (0.01), Sodium Selenate (0.01025), Ammonium Paramolybdate .4H20 (0.00795), Sodium Metasilicate .9H20 (1.45), Chromium Potassium Sulfate .12H20 (0.275), Lithium Chloride (0.0174), Boric Acid (0.0815), Sodium Fluoride (0.0635), Nickel Carbonate (0.0318), Ammonium Vanadate (0.0066), Sucrose, finely powdered (221.026).

[c]Final raspberry supplementation was 5.3% due to water evaporation during agar-based diet preparation (~ 8%). Raspberry contributed with polyphenolics (963 mg extractable GAE/kg agar-based diet and 111 mg non-extractable PA/kg agar-based diet) and 18.6 g dietary fibre/kg agar-based diet. The election of an agar-based diet allowed fulfilling the nutrients and part of the water requirement of mice.

Table S2. PCR primers used in this study for qPCR analyses.

Bacterial group	Primer sequences (5′-3′)	Reference
Faecalibacterium	GAAGGCGGCCTACTGGGCAC GTGCAGGCGAGTTGCAGCCT	Garcia-Mazcorro *et al*. (2012)
Enterobacteriaceae	CATTGACGTTACCCGCAGAAGAAGC CTCTACGAGACTCAAGCTTGC	Bartosch *et al.* (2004)
B. fragilis	CTGAACCAGCCAAGTAGCG CCGCAAACTTTCACAACTGACTTA	Liu *et al.* (2003)
Akkermansia	CAGCACGTGAAGGTGGGGAC CCTTGCGGTTGGCTTCAGAT	Collado *et al.* (2007)
Prevotella	CACCAAGGCGACGATCA GGATAACGCCYGGACCT	Larsen *et al.* (2010)
Bacteroidetes	GGARCATGTGGTTTAATTCGATGAT AGCTGACGACAACCATGCAG	Guo *et al*. (2008)
Firmicutes	TGAAACTYAAAGGAATTGACG ACCATGCACCACCTGTC	Bacchetti De Gregoris *et al.* (2011)
E. coli	CATGCCGCGTGTATGAAGAA CGGGTAACGTCAATGAGCAAA	Huijsdens *et al.* (2002)
Ruminococcaceae	ACTGAGAGGTTGAACGGCCA CCTTTACACCCAGTAAWTCCGGA	Garcia-Mazcorro *et al.* (2012)
B. bifidum	CCACATGATCGCATGTGATTG CCGAAGGCTTGCTCCCAAA	Matsuki *et al.* (1998)
Turicibacter	CAGACGGGGACAACGATTGGA TACGCATCGTCGCCTTGGTA	Suchodolski *et al.* (2012)

References for Table S2

Bacchetti De Gregoris T, Aldred N, Clare AS, Burgess JG. 2011. Improvement of phylum- and class-specific primers for real-time PCR quantification of bacterial taxa. *J. Microbiol. Methods* **86:** 351-356.

Bartosch S, Fite A, Macfarlane GT, McMurdo ME. 2004. Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using real-time PCR and effects of antibiotic treatment on the fecal microbiota. *Appl. Environ. Microbiol.* **70:** 3575-3581.

Collado MC, Derrien M, Isolauri E, de Vos WM, Salminen S. 2007. Intestinal integrity and *Akkermansia muciniphila*, a mucin-degrading member of the intestinal microbiota present in infants, adults, and the elderly. *Appl. Environ. Microbiol.* **73:** 7767-7770.

Garcia-Mazcorro JF, Suchodolski JS, Jones KR, Clark-Price SC, Dowd SE, Minamoto Y, Markel M, Steiner JM, Dossin O. 2012. Effect of the proton pump inhibitor omeprazole on the gastrointestinal microbiota of healthy dogs. *FEMS Microbiol. Ecol.* **80:** 624-636.

Guo X, Xia X, Tang R, Zhou J, Zhao H, Wang K. 2008. Development of a real-time PCR method for Firmicutes and Bacteroidetes in faeces and its application to quantify intestinal population of obese and lean pigs. *Lett. Appl. Microbiol.* **47:** 367-373.

Huijsdens XW, Linskens RK, Mak M, Meuwissen SG, Vandenbroucke-Grauls CM, Savelkoul PH. 2002. Quantification of bacteria adherent to gastrointestinal mucosa by real-time PCR. *J. Clin. Microbiol.* **40:** 4423-4427.

Larsen N, Vogensen FK, van den Berg FW, Nielsen DS, Andreasen AS, Pedersen BK, Al-Soud WA, Sørensen SJ, Hansen LH, Jakobsen M. 2010. Gut microbiota in human adults with type 2 diabetes differs from non-diabetic adults. PLoS ONE **5:** e9085

Liu C, Song Y, McTeague M, Vu AW, Wexler H, Finegold SM. 2003. Rapid identification of the species of the *Bacteroides fragilis* group by multiplex PCR assays using group- and species-specific primers. *FEMS Microbiol. Lett.* **222:** 9-16.

Matsuki T, Watanabe K, Tanaka R, Oyaizu H. 1998. Rapid identification of human intestinal bifidobacteria by 16S rRNA-targeted species- and group-specific primers. *FEMS Microbiol. Lett.* **167:** 113-121

Suchodolski JS, Markel ME, Garcia-Mazcorro JF, Unterer S, Heilmann RM, Dowd SE, Kachroo P, Ivanov I, Minamoto Y, Dillman EM, Steiner JM, Cook AK, Toresson L. 2012. The fecal microbiome in dogs with acute diarrhea and idiopathic inflammatory bowel disease. *PLoS One* **7:** e51907.

Table S3. Primers used for mRNA analysis.

Target	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
IL-1β	TCGCTCAGGGTCACAAGAAA	CATCAGAGGCAAGGAGGAAAAC
TNF-α	AAATGGGCTCCCTCTCATCAGTTC	TCTGCTTGGTGGTTTGCTACGAC
IFN-α	ACTCATTCTGCAATGACCTC	CTCCAGACTTCTGCTCTGAC
PPARγ	CTGTGGGGATAAAGCATCAG	TGATGGCATTGTGAGACATC
*ATF4**	GAGCTTCCTGAACAGCGAAGTG	TGGCCACCTCCAGATAGTCATC
CHOP	CCTAGCTTGGCTGACAGAGG	CTGCTCCTTCTCCTTCATGC
Occ	ATGTCCGGCCGATGCTCTC	TTTGGCTGCTCTTGGGTCTGTAT
F4/80	TGACAACCAGACGGCTTGTG	CAGGCGAGGAAAAGATAGTGT
RPL19	GAAGGTCAAAGGGAATGTGTTCA	CCTTGTCTGCCTTCAGCTTGT

IL-1 β; interleukin-1β, TNF-α; tumor necrosis factor alpha; IFN-α, interferon alpha; PPARγ, Peroxisome proliferator-activated receptor gamma; ATF4, activating transcription factor 4; CHOP, CCAAT/enhancer binding protein homologous protein; Occ, occludin; F4/80, macrophage F4/80 receptor; RPL19, ribosomal protein L19.

Table S4. mRNA levels of biomarkers involved in inflammation, cellular stress, tight junction and barrier function in colonic mucosal cells.

Genes	mRNA levels/β-actin mRNA	Obese	Raspberry	*p* value
Inflammation/ Cellular stress	IL-1β	5.24 (0.1-17)	0.984 (0.04-2.4)	<0.001
	TNF-α	0.364 (0.1-0.9)	0.399 (0.2-3.6)	0.331
	IFNα	14.4 (2.7-29.1)	19.3 (2.8-60.6)	0.446
	PPARγ	0.7 (0.02-6.6)	1.6 (0.2-4.4)	0.575
	ATF4 (mean, SEM)	11.4 (1.2-37.5)	17.69 (3.2-37.9)	0.117
	CHOP (mean, SEM)	1.0 (0.1-4.9)	4.88 (0.1-10.8)	0.001
	IL-8 (mean, SEM)	0.2 (0.02-0.7)	0.09 (0.02-0.7)	0.312
Tight junction and barrier function	OCC (mean, SEM)	5.5 (1.2-18.7)	16.9 (0.8-46.3)	0.035
Monocyte infiltration	F4/80 (mean, SEM)	0.1 (0.05-0.2)	0.1 (0.03-0.3)	0.778

Data are medians (min-max). Data analyzed with Kruskal-Wallis test, and outlier detection were performed with GraphPad Prism 6.0.

Table S5. Fecal metabolites involved in the discrimination of samples analyzed by PLS-DA.

Metabolite	Obese controls	Obese supplemented	*p* value
Serine	5(1.99-13.73)	13.3 (6.2-23.3)	<0.001
Alpha tocopherol	20 (11-32)	32 (24-45)	0.001
Gamma aminobutyric acid	1 (0.7-2)	2 (1-3)	0.003
Hydroxicinnamic acid	0.2 (0-1)	1 (0-3)	0.002
Sitosterol	67 (44-90)	94 (53-131)	0.002
Alanine	43 (11-120)	110 (50-212)	0.001
Methionine sulfoxide	0.8 (0-2)	2 (0.7-4)	0.004
N-acetyltryptophan	1 (0-5)	5 (0-14)	0.006
D-glycerol	0.1 (0-0.3)	0.3 (0.1-0.9)	0.012
Stigmasterol	9 (0-44)	39 (0-89)	0.009
Tyrosine	7 (0-19)	21 (0-58)	0.013
Mannose	0 (0-0)	0 (0-170)	0.018
Glucose1phosphate	1 (0.3-2)	2 (1-6)	0.015
Norleucine	18 (2-49)	42 (10-72)	0.012
Xylose	34 (19-76)	51 (32-171)	0.019
Threonine	3 (0-8)	8 (4-40)	0.034
Glycine	19 (7-47)	30 (23-82)	0.022
Cholestan-3-betaol	4 (2-6)	6 (2-15)	0.038
Isoleucine	18 (4-34)	28 (13-89)	0.044
Leucine	24 (6-53)	36 (17-118)	0.045
Valine	31 (5-63)	41 (18-220)	0.056
Glycine	19 (7-47)	30 (23-82)	0.022
Oxoproline	7 (0-31)	12 (0-104)	0.061
8-Aminocaprylic acid	0.4 (0-1)	0.6 (0.3-2)	0.048
Zymosterol 1	0 (0-4)	0 (0-8)	0.809

Values are medians (min-max). Data was analyzed with unpaired t test with Welch's correction with GraphPad Prism 6.0.

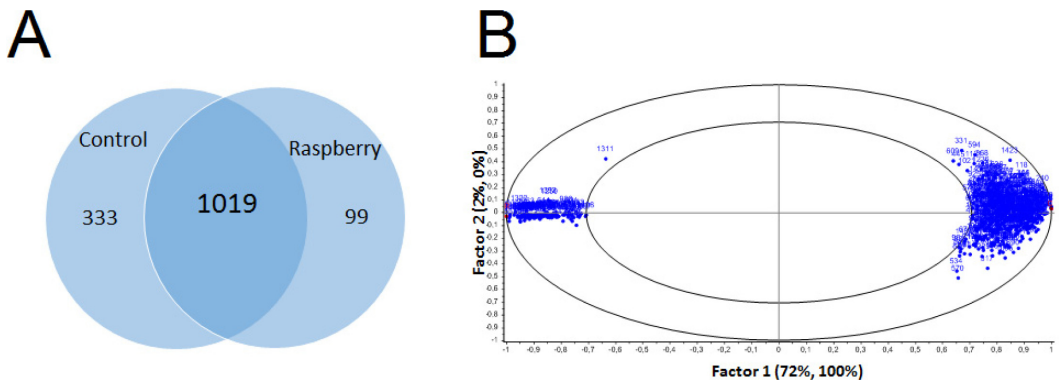

Fig. S1. Proteins differentially expressed in Control and Raspberry groups.
(**A**) Venn diagram. (**B**) Correlation loadings (X and Y) from partial least-squares-discriminant analysis (PLS-DA). This model shows a 72% (X-variables) and 100% discrimination (Y-variables) of raspberry (left) and obese control (right).

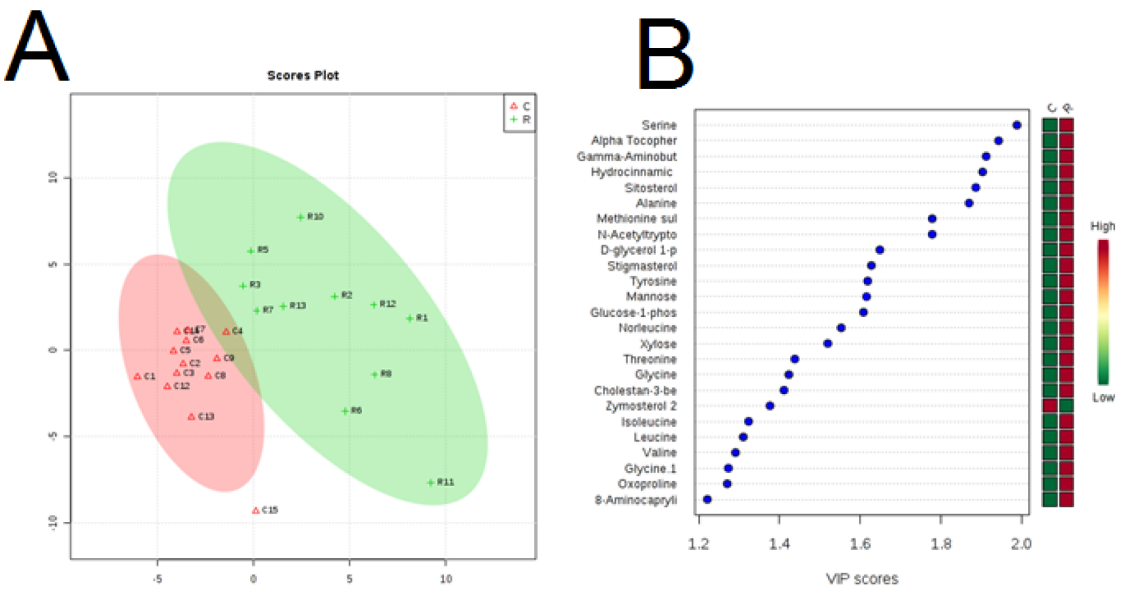

Fig. S2. Comparison of fecal metabolites in raspberry supplemented [R] versus obese control [C].
(**A**) Partial least-squares-discriminant analysis (PLS-DA) in two components discriminated [C] from [R] group and explained 36.7% of the total variance. (**B**) Random forest classification based on 25 fecal metabolites in order of importance from right to left (VIP 2.0 to 1.2). Concentrations for each experimental group are visualized on the right with higher relative concentrations (red) for raspberry and lower (green) for obese control, except for zymosterol 2.

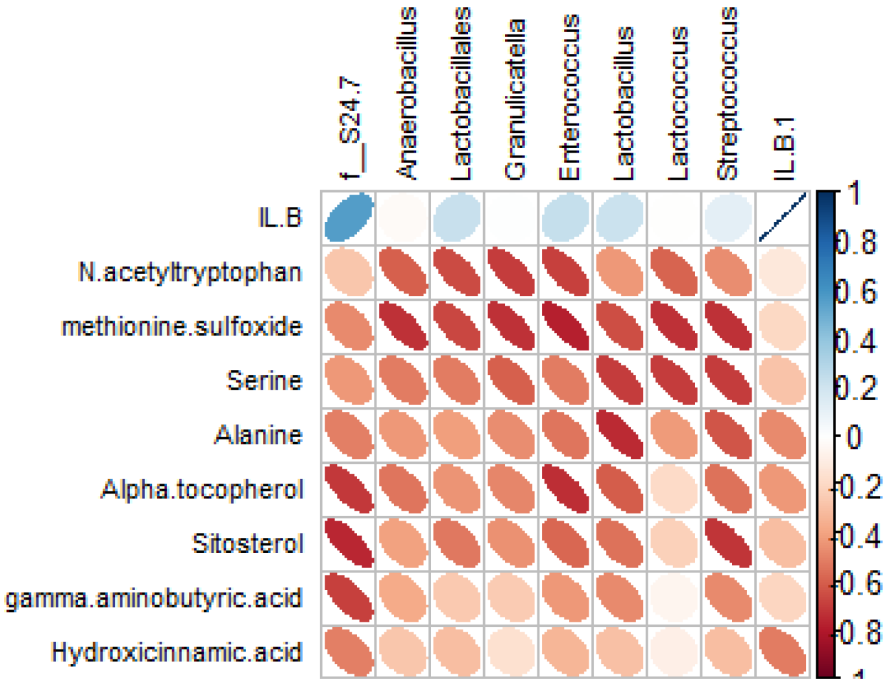

Fig. S3. Spearman's correlation matrix of fecal bacteria versus fecal metabolites and mRNA levels of IL-1β.

Data variables were significant different between experimental groups ($p < 0.05$). Bacteria are listed to the Genus/Specie level. The direction of ellipses represents positive or negative correlations and the width of ellipses represents the strength of correlation (narrow ellipse = stronger correlation).