

Supporting Information

Regulatory effect of volatile compounds in fermented alcoholic beverages on gut microbiota and serum metabolism in mouse model

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1. Supplementary Methods

1.1 Volatile Compounds Analysis.

Each liquor sample was diluted with deionized water to a final concentration of 10% (v/v) ethanol. A total of 5 mL diluted solution saturated with sodium chloride was placed into a 20 mL screw-capped vial. And adding 10 µl menthol as the internal standard (100.00 ppm). Then, the vial was tightly capped with a silicon septum.

For the SPME, an automatic headspace sampling system (Multi-Purpose Sample MPS 2 with a SPME adapter, Gerstel Inc., Baltimore, MD, USA) with a 50/30 µm DVB/CAR/PDMS fiber (2 cm, Supelco Inc., Bellefonte, PA, USA) was used for the analyses. For the GC-MS analysis, an Agilent 6890 N GC and 5975 mass selective detector (Agilent Technologies Inc., Santa Clara, CA, USA) were used.

The extraction used DVB/CAR/PBDS for 45 min extraction at 45 °C. For the GC, the inlet temperature was 250 °C, the column carrier gas was helium (purity 99.9995%) at a constant flow rate of 2 mL/min, using the splitless mode. A CP-Wax column was used (60 m × 0.25 mm i.d. × 0.25 µm, Varian Inc., Palo Alto, CA, USA). The temperature program for detection was: constant temperature of 50 °C for 2 min; then raised to 230 °C at 6 °C/min over 30 min¹. For MS, the conditions were: the electron impact energy was 70eV, and the ion source temperature was set at 230 °C. Full-scan acquisition was used across a range of masses (30-350 amu). After detection, the peaks with matching similarity greater than 70.00% were screened and normalized.

1.2 Serum Metabolite Assessment.

Sample Preparation. Pooled quality control (QC) samples were prepared by mixing

20 μL of each the serum sample. An aliquot of a 50 μL serum sample was spiked with two internal standards (10 μL of L-2-chlorophenylalanine in water, 0.3 mg/mL; 10 μL of heptadecanoic acid in methanol, 1 mg/mL) and vortexed for 10s. The mixed solution was extracted with 175 μL of methanol/chloroform (3:1) and vortexed for 30s. The samples were centrifuged at 8000 rpm for 10 min after stored for 10 min at $-20\text{ }^{\circ}\text{C}$. An aliquot of the 200 μL supernatant was transferred to a glass sampling vial to vacuum-dry at room temperature. Derivatization procedure was described in reported method ². When the reaction was finished, the samples were placed at room temperature for 1 h waiting for GC-TOFMS analysis.

Instrumental Analysis. The samples were analyzed by a GC-TOFMS in a randomized order to minimize the systematic analytical error. One QC sample and one blank vial were run after each 10 plasma samples. The injection volume was 1 μL using a splitless mode. The separation of metabolites were achieved on a DB-5MS capillary column (30 m \times 250 μm i.d., 0.25- μm film thickness; 5% diphenyl cross-linked 95% dimethylpolysiloxane). The carrier gas was helium with a constant flow rate of 1 mL/min. The GC and MS condition were described in previously published papers with minor modifications ³. The acquisition rate was 20 spectra/second in the TOFMS setting.

Data Preprocessing. The acquired data files from GC-TOFMS were processed by Chroma TOF software (v.4.51.6.0; Leco, Tustin, CA). After the pretreatment for baseline correction, denoising, smoothing, alignment, deconvolution, raw data containing retention time, intensity, and the m/z of each peak were obtained. Both

mass-spectrum and retention times were used to achieve precise compound annotations. The metabolites were annotated by comparing mass fragments and retention time with NIST 05 standard mass spectral databases and our in-house standard libraries (covering more than 800 metabolites and still expanding).

2. Supplementary Figures

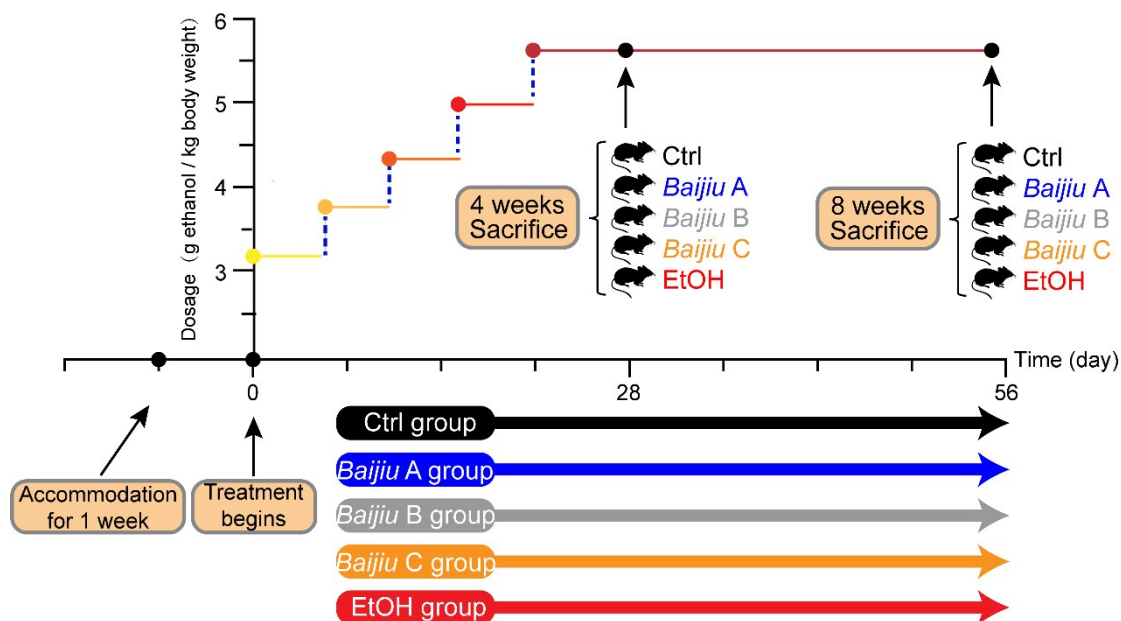


Figure S1. Animal feeding schedule. Mice of intervention groups were given Ctrl ($n = 20$; 10 replicates for each time point), type A of *Baijiu* ($n = 20$; 10 replicates for each time point), type B of *Baijiu* ($n = 20$; 10 replicates for each time point), type C of *Baijiu* ($n = 20$; 10 replicates for each time point) and EtOH ($n = 20$; 10 replicates for each time point) by gavage. The final dosage (5.6 g EtOH/kg body weight) is equivalent to ~ 3.12 standard human drinks.

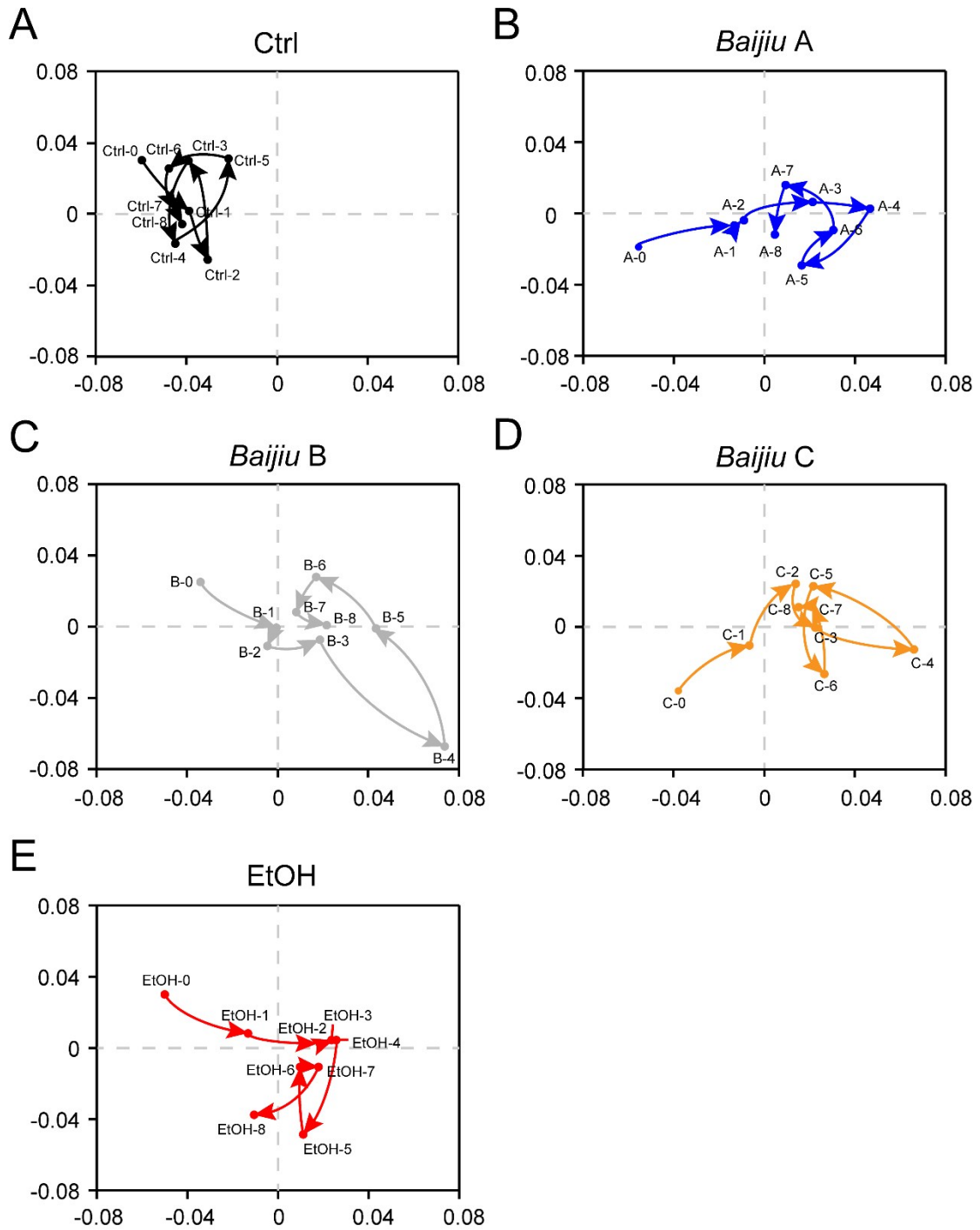


Figure S2. Alteration of gut microbiome depicted by trajectories. PCoA scores plots of the time-dependent microbial ‘footprints’ in feces samples of five groups across the 8 weeks intervention period. Letters represent different interventions. The numbers after the letters represent the intervention periods.

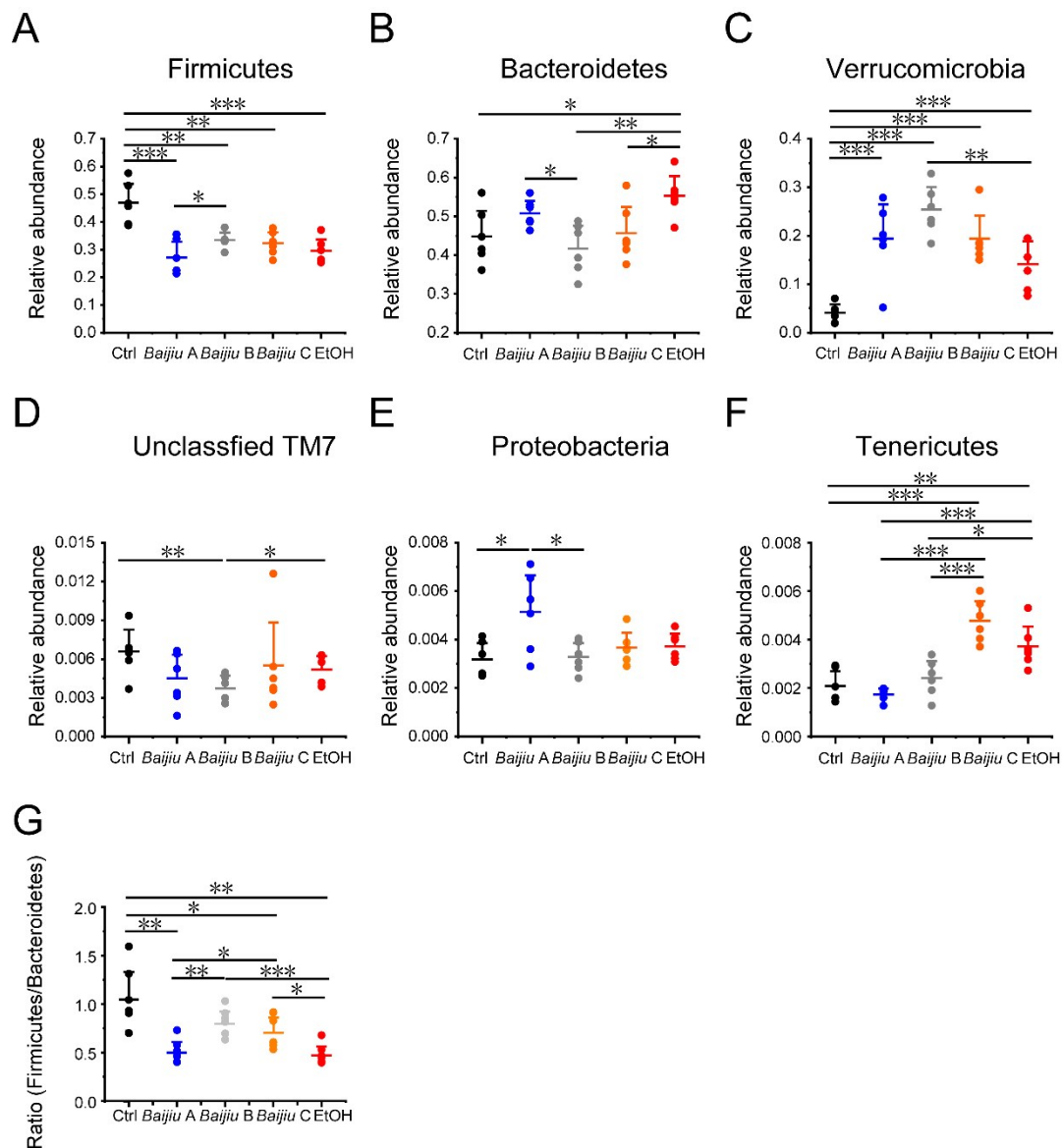


Figure S3. The 6 most abundant phyla and the ratio of Firmicutes to Bacteroidetes in the Ctrl, type A, B, and C of *Baijiu* and EtOH groups. Group differences were assessed by using the 2-tailed, unpaired Student's *t*-test ($n = 6$ mice/group). $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

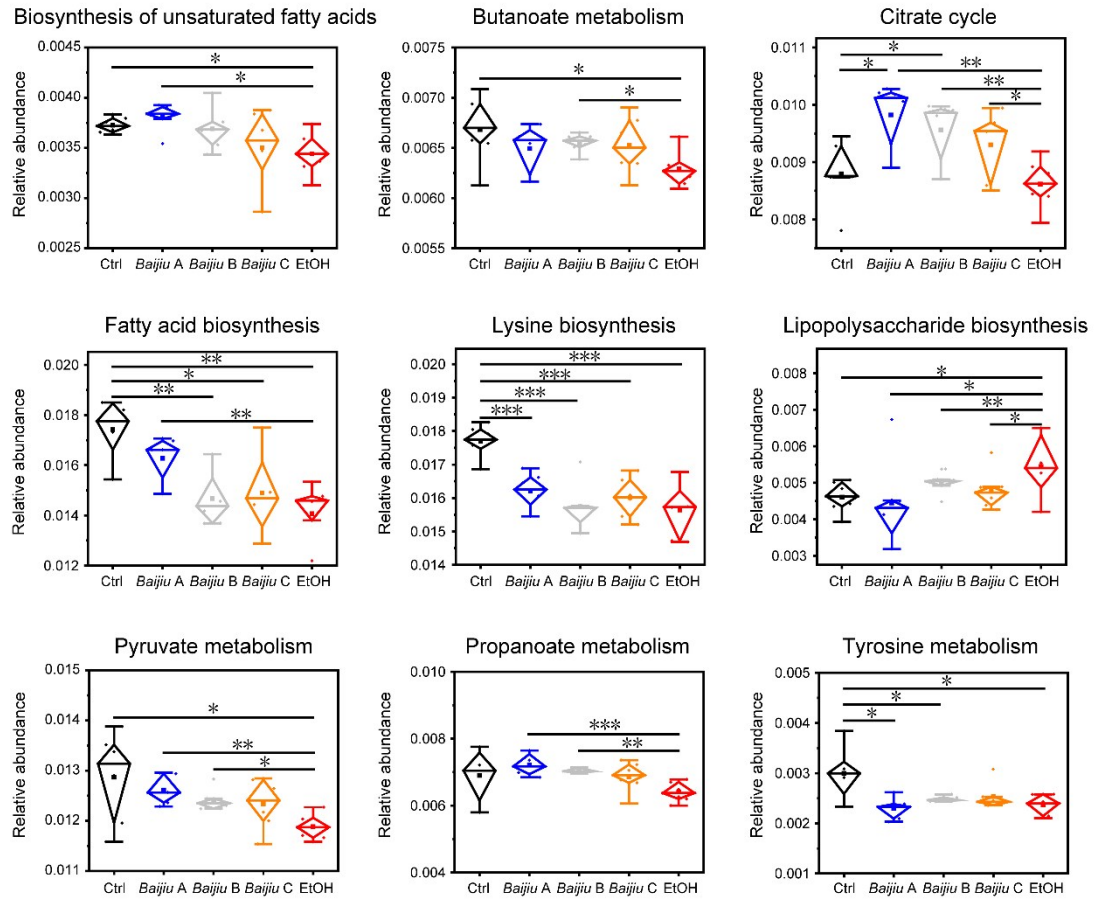


Figure S4. Comparison of predicted microbial function among groups based on KEGG level-3. Group differences were assessed by using the 2-tailed, unpaired Student's *t*-test (n = 6 mice/group). **P* < 0.05, ***P* < 0.01 and ****P* < 0.001.

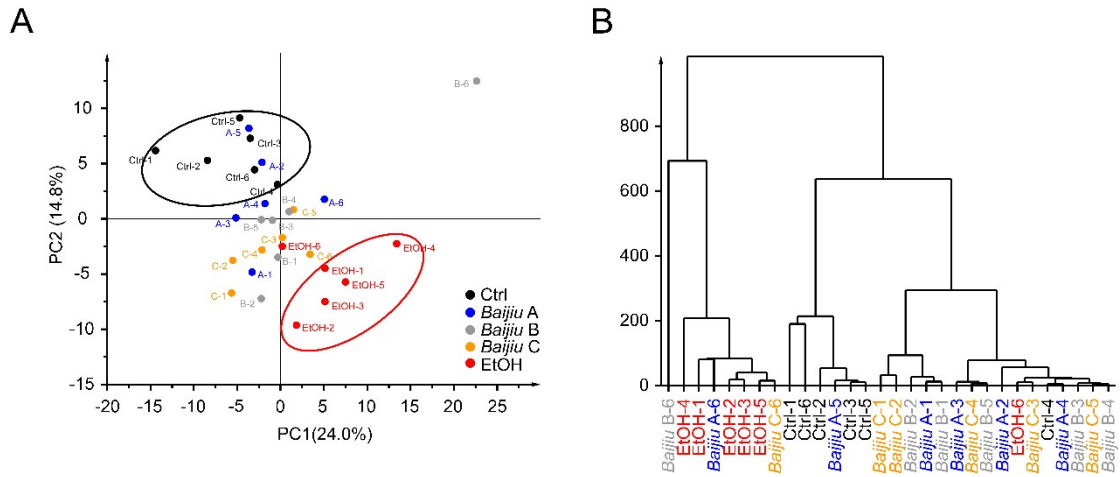


Figure S5. Effects of different *Baijiu* interventions on the serum metabolism. (A) The PCA score plot of metabolites generated by using the metabolite concentrations from the Ctrl, type A, B, and C of *Baijiu* and EtOH groups. (B) Hierarchical cluster analysis plot was based on PCA modelling.

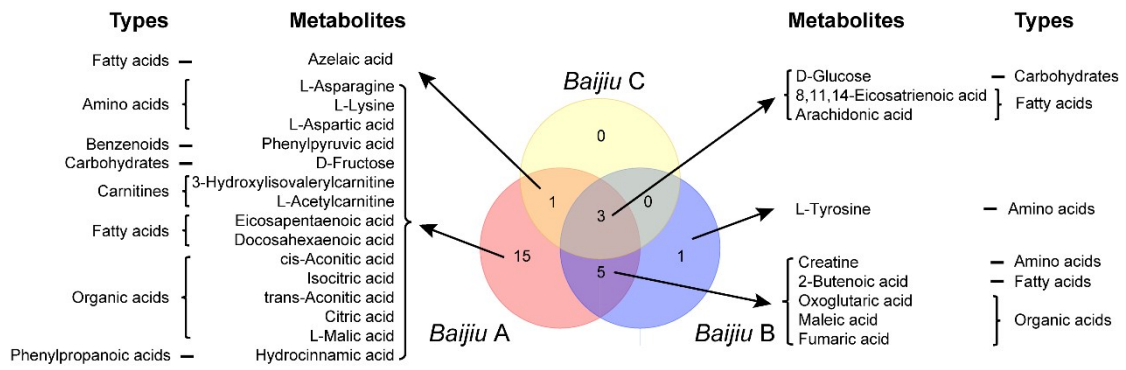


Figure S6. Differential serum metabolites caused by three types of *Baijiu*

interventions. The Venn plot showed the serum metabolites of mice in type A, B and C of *Baijiu* groups significantly different from EtOH groups.

3. Supplementary Tables

Table S1. Relative abundance of 64 key OTUs analyzed by RDA

| OUT Number | Family | Genus | Relative abundance (%) ^a | | | | |
|--|---------------------|----------------------|-------------------------------------|-----------------|-----------------|-----------------|-----------|
| | | | Ctrl | <i>Baijiu</i> A | <i>Baijiu</i> B | <i>Baijiu</i> C | EtOH |
| 14 OTUs responded to <i>Baijiu</i> interventions | | | | | | | |
| Otu2 | Lactobacillaceae | <i>Lactobacillus</i> | 0.31±0.25 | 1.97±0.78 | 11.16±1.52 | 6.22±1.22 | 1.80±0.66 |
| Otu362 | Muribaculaceae | <i>Duncaniella</i> | 0.10±0.02 | 0.24±0.06 | 0.08±0.03 | 0.09±0.03 | 0.09±0.02 |
| Otu367 | Muribaculaceae | <i>Duncaniella</i> | 0.06±0.02 | 0.19±0.04 | 0.14±0.02 | 0.13±0.02 | 0.12±0.02 |
| Otu7 | Muribaculaceae | <i>Muribaculum</i> | 5.56±0.64 | 12.73±1.84 | 9.35±0.54 | 7.15±1.49 | 7.34±0.84 |
| Otu427 | Muribaculaceae | <i>Muribaculum</i> | 0.15±0.02 | 0.39±0.12 | 0.29±0.05 | 0.28±0.03 | 0.20±0.04 |
| Otu26 | Oscillospiraceae | <i>Ruminococcus</i> | 0.02±0.02 | 0.03±0.01 | 0.01±0.00 | 0.00±0.00 | 0.00±0.00 |
| Otu353 | Lachnospiraceae | PAC002367 | 0.08±0.02 | 0.12±0.04 | 0.16±0.03 | 0.22±0.05 | 0.12±0.01 |
| Otu235 | Muribaculaceae | PAC001112 | 0.32±0.04 | 0.29±0.08 | 0.32±0.04 | 0.22±0.08 | 0.25±0.08 |
| Otu46 | Muribaculaceae | PAC001692 | 0.46±0.05 | 0.41±0.19 | 0.50±0.06 | 0.67±0.23 | 0.44±0.08 |
| Otu727 | Muribaculaceae | PAC001692 | 0.04±0.01 | 0.08±0.03 | 0.09±0.02 | 0.09±0.03 | 0.06±0.01 |
| Otu758 | Muribaculaceae | PAC001692 | 0.05±0.01 | 0.12±0.03 | 0.09±0.02 | 0.09±0.03 | 0.04±0.01 |
| Otu366 | Oscillospiraceae | PAC000661 | 0.04±0.01 | 0.10±0.04 | 0.13±0.05 | 0.12±0.03 | 0.06±0.01 |
| Otu621 | Oscillospiraceae | PAC000661 | 0.04±0.01 | 0.14±0.06 | 0.07±0.02 | 0.09±0.02 | 0.04±0.01 |
| Otu59 | Oscillospiraceae | PAC000661 | 0.20±0.06 | 0.59±0.16 | 0.56±0.08 | 0.62±0.15 | 0.45±0.07 |
| 50 OTUs responded to EtOH intervention | | | | | | | |
| Otu35 | Bacteroidaceae | <i>Bacteroides</i> | 0.35±0.08 | 0.10±0.05 | 0.10±0.03 | 0.14±0.02 | 0.15±0.04 |
| Otu25 | Deferribacteraceae | <i>Mucispirillum</i> | 0.30±0.08 | 0.01±0.00 | 0.00±0.00 | 0.01±0.00 | 0.01±0.01 |
| Otu113 | Desulfovibrionaceae | <i>Bilophila</i> | 0.08±0.02 | 0.02±0.00 | 0.02±0.01 | 0.04±0.02 | 0.03±0.01 |
| Otu144 | Desulfovibrionaceae | <i>Mailhella</i> | 0.11±0.04 | 0.04±0.02 | 0.03±0.01 | 0.09±0.02 | 0.07±0.01 |

| | | | | | | | |
|--------|--------------|---------------------|-------|--------|--------|-------|-------|
| | onaceae | | 0.03 | .01 | .01 | 0.03 | 0.02 |
| Otu97 | Lachnospira | <i>Anaerotignu</i> | 0.23± | 0.02±0 | 0.02±0 | 0.06± | 0.07± |
| | ceae | <i>m</i> | 0.09 | .01 | .00 | 0.02 | 0.02 |
| Otu586 | Lachnospira | <i>Clostridium</i> | 0.24± | 0.04±0 | 0.03±0 | 0.06± | 0.10± |
| | ceae | | 0.05 | .01 | .01 | 0.02 | 0.03 |
| Otu194 | Lachnospira | <i>Enterocloste</i> | 0.27± | 0.02±0 | 0.01±0 | 0.06± | 0.03± |
| | ceae | <i>r</i> | 0.11 | .01 | .00 | 0.04 | 0.01 |
| Otu516 | Lachnospira | <i>Enterocloste</i> | 0.42± | 0.02±0 | 0.02±0 | 0.03± | 0.06± |
| | ceae | <i>r</i> | 0.14 | .01 | .01 | 0.02 | 0.01 |
| Otu40 | Lachnospira | <i>Eubacterium</i> | 0.58± | 0.10±0 | 0.09±0 | 0.22± | 0.18± |
| | ceae | | 0.23 | .03 | .03 | 0.06 | 0.03 |
| Otu784 | Lachnospira | <i>Eubacterium</i> | 0.11± | 0.02±0 | 0.01±0 | 0.03± | 0.03± |
| | ceae | | 0.05 | .01 | .00 | 0.01 | 0.01 |
| Otu724 | Lachnospira | <i>Kineothrix</i> | 0.40± | 0.01±0 | 0.04±0 | 0.05± | 0.06± |
| | ceae | | 0.10 | .00 | .03 | 0.02 | 0.01 |
| Otu49 | Lachnospira | <i>Roseburia</i> | 0.95± | 0.06±0 | 0.02±0 | 0.09± | 0.18± |
| | ceae | | 0.37 | .04 | .01 | 0.05 | 0.06 |
| Otu74 | Muribaculac | <i>Duncaniella</i> | 0.37± | 0.32±0 | 0.17±0 | 0.35± | 0.33± |
| | eae | | 0.10 | .05 | .03 | 0.04 | 0.05 |
| Otu421 | Muribaculac | <i>Muribaculu</i> | 0.10± | 0.24±0 | 0.19±0 | 0.12± | 0.30± |
| | eae | <i>m</i> | 0.03 | .06 | .05 | 0.05 | 0.06 |
| Otu537 | Muribaculac | <i>Muribaculu</i> | 0.06± | 0.15±0 | 0.08±0 | 0.09± | 0.10± |
| | eae | <i>m</i> | 0.01 | .03 | .02 | 0.02 | 0.02 |
| Otu179 | Oscillospira | <i>Pseudoflavo</i> | 0.34± | 0.09±0 | 0.09±0 | 0.10± | 0.15± |
| | ceae | <i>nifractor</i> | 0.08 | .02 | .01 | 0.02 | 0.03 |
| Otu67 | Oscillospira | <i>Pseudoflavo</i> | 0.34± | 0.10±0 | 0.09±0 | 0.14± | 0.18± |
| | ceae | <i>nifractor</i> | 0.09 | .04 | .02 | 0.06 | 0.06 |
| Otu137 | Lachnospira | LLKB | 0.17± | 0.03±0 | 0.02±0 | 0.06± | 0.04± |
| | ceae | | 0.08 | .01 | .01 | 0.03 | 0.02 |
| Otu487 | Lachnospira | AM932595 | 0.12± | 0.02±0 | 0.02±0 | 0.06± | 0.03± |
| | ceae | | 0.05 | .01 | .01 | 0.02 | 0.01 |
| Otu93 | Lachnospira | KE159538 | 0.09± | 0.04±0 | 0.04±0 | 0.15± | 0.05± |
| | ceae | | 0.03 | .02 | .01 | 0.10 | 0.01 |
| Otu30 | Lachnospira | KE159810 | 1.40± | 0.32±0 | 0.06±0 | 0.06± | 0.11± |
| | ceae | | 0.73 | .19 | .02 | 0.02 | 0.03 |
| Otu65 | Lachnospira | RAYQ | 0.26± | 0.03±0 | 0.02±0 | 0.02± | 0.04± |
| | ceae | | 0.17 | .01 | .01 | 0.01 | 0.01 |
| Otu604 | Lachnospira | HM124219 | 0.09± | 0.02±0 | 0.02±0 | 0.02± | 0.03± |
| | ceae | | 0.02 | .01 | .01 | 0.01 | 0.01 |
| Otu86 | Lachnospira | KE159538 | 0.48± | 0.09±0 | 0.07±0 | 0.35± | 0.18± |
| | ceae | | 0.11 | .05 | .02 | 0.22 | 0.03 |
| Otu593 | Lachnospira | PAC000671 | 0.26± | 0.06±0 | 0.02±0 | 0.07± | 0.07± |
| | ceae | | 0.07 | .02 | .01 | 0.03 | 0.02 |
| Otu56 | Lachnospira | PAC001043 | 0.68± | 0.02±0 | 0.02±0 | 0.04± | 0.05± |

| | | | | | | | |
|--------|-------------|-----------|-------|--------|--------|-------|-------|
| | ceae | | 0.24 | .01 | .01 | 0.03 | 0.01 |
| Otu28 | Lachnospira | PAC001090 | 1.49± | 0.44±0 | 0.40±0 | 0.45± | 0.69± |
| | ceae | | 0.32 | .17 | .11 | 0.18 | 0.16 |
| Otu88 | Lachnospira | PAC001092 | 0.32± | 0.03±0 | 0.03±0 | 0.08± | 0.05± |
| | ceae | | 0.05 | .01 | .01 | 0.02 | 0.01 |
| Otu136 | Lachnospira | PAC001092 | 0.65± | 0.05±0 | 0.04±0 | 0.07± | 0.08± |
| | ceae | | 0.20 | .02 | .01 | 0.02 | 0.01 |
| Otu261 | Lachnospira | PAC001116 | 0.57± | 0.19±0 | 0.03±0 | 0.11± | 0.19± |
| | ceae | | 0.19 | .07 | .01 | 0.04 | 0.04 |
| Otu379 | Lachnospira | PAC001124 | 0.32± | 0.17±0 | 0.20±0 | 0.50± | 1.28± |
| | ceae | | 0.12 | .08 | .15 | 0.34 | 0.56 |
| Otu248 | Lachnospira | PAC001165 | 0.30± | 0.09±0 | 0.07±0 | 0.26± | 0.16± |
| | ceae | | 0.10 | .03 | .01 | 0.12 | 0.04 |
| Otu37 | Lachnospira | PAC001165 | 0.60± | 0.03±0 | 0.01±0 | 0.05± | 0.03± |
| | ceae | | 0.32 | .02 | .00 | 0.01 | 0.01 |
| Otu700 | Lachnospira | PAC001390 | 0.06± | 0.05±0 | 0.03±0 | 0.03± | 0.05± |
| | ceae | | 0.03 | .02 | .01 | 0.01 | 0.02 |
| Otu34 | Lachnospira | PAC001588 | 2.47± | 0.26±0 | 0.35±0 | 0.35± | 0.27± |
| | ceae | | 0.69 | .11 | .10 | 0.10 | 0.06 |
| Otu20 | Lachnospira | PAC002367 | 1.64± | 0.16±0 | 0.12±0 | 0.51± | 0.75± |
| | ceae | | 1.10 | .06 | .06 | 0.24 | 0.16 |
| Otu742 | Lachnospira | PAC002367 | 0.58± | 0.05±0 | 0.07±0 | 0.14± | 0.25± |
| | ceae | | 0.36 | .02 | .04 | 0.09 | 0.05 |
| Otu51 | Lachnospira | RAYR | 0.49± | 0.15±0 | 0.06±0 | 0.13± | 0.06± |
| | ceae | | 0.19 | .07 | .03 | 0.05 | 0.01 |
| Otu14 | Lachnospira | RAYR | 1.86± | 0.28±0 | 0.15±0 | 0.56± | 0.86± |
| | ceae | | 1.26 | .20 | .06 | 0.28 | 0.25 |
| Otu793 | Lachnospira | RAYR | 0.69± | 0.07±0 | 0.08±0 | 0.19± | 0.17± |
| | ceae | | 0.36 | .05 | .03 | 0.10 | 0.04 |
| Otu13 | Lachnospira | RAYR | 2.09± | 0.27±0 | 0.29±0 | 0.35± | 0.51± |
| | ceae | | 0.49 | .13 | .12 | 0.16 | 0.04 |
| Otu523 | Lachnospira | RAZD | 0.31± | 0.02±0 | 0.04±0 | 0.06± | 0.07± |
| | ceae | | 0.05 | .01 | .02 | 0.03 | 0.01 |
| Otu292 | Muribaculac | PAC000198 | 0.13± | 0.25±0 | 0.29±0 | 0.42± | 0.51± |
| | eae | | 0.05 | .04 | .07 | 0.07 | 0.07 |
| Otu324 | Muribaculac | PAC001112 | 0.11± | 0.06±0 | 0.06±0 | 0.07± | 0.13± |
| | eae | | 0.02 | .02 | .02 | 0.02 | 0.03 |
| Otu394 | Muribaculac | PAC001112 | 0.06± | 0.04±0 | 0.04±0 | 0.06± | 0.12± |
| | eae | | 0.01 | .02 | .01 | 0.02 | 0.03 |
| Otu564 | Muribaculac | PAC001112 | 0.29± | 0.28±0 | 0.25±0 | 0.36± | 0.57± |
| | eae | | 0.04 | .06 | .05 | 0.07 | 0.09 |
| Otu671 | Muribaculac | PAC001112 | 0.07± | 0.10±0 | 0.11±0 | 0.16± | 0.26± |
| | eae | | 0.02 | .03 | .03 | 0.04 | 0.07 |
| Otu84 | Muribaculac | PAC001127 | 0.00± | 0.27±0 | 0.12±0 | 0.02± | 0.45± |

| | | | | | | | |
|--------|--------------|-----------|-------|--------|--------|-------|-------|
| | eae | | 0.00 | .16 | .07 | 0.01 | 0.09 |
| Otu182 | Oscillospira | PAC000661 | 0.05± | 0.04±0 | 0.09±0 | 0.07± | 0.10± |
| | ceae | | 0.01 | .02 | .01 | 0.01 | 0.02 |
| Otu83 | Oscillospira | PAC000661 | 0.09± | 0.04±0 | 0.02±0 | 0.04± | 0.25± |
| | ceae | | 0.04 | .02 | .01 | 0.02 | 0.12 |

a: All data are presented as means ± standard error of the mean (n = 6 mice/group).

Table S2. Post hoc multiple comparison tests (MCTs) performed using Tukey–Kramer based on X-axis of PCA modelling.

| ANOVA five-group comparison ($F = 3.222$, $P = 0.029^*$) | | | | | |
|---|-----------------------------|------------|--------|----------------------------|----------------|
| Tukey–Kramer analysis | | | | | |
| Group comparisons (X-axis) | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
| | | | | Lower Bound | Upper Bound |
| Ctrl vs <i>Baijiu</i> A | -3.89338 | 3.52575 | 0.803 | -14.2480 | 6.4613 |
| Ctrl vs <i>Baijiu</i> B | -9.10774 | 3.52575 | 0.104 | -19.4624 | 1.2469 |
| Ctrl vs <i>Baijiu</i> C | -4.36417 | 3.52575 | 0.730 | -14.7188 | 5.9905 |
| Ctrl vs EtOH | -11.26308* | 3.52575 | 0.028* | -21.6177 | -0.9084 |
| <i>Baijiu</i> A vs <i>Baijiu</i> B | -5.21436 | 3.52575 | 0.585 | -15.5690 | 5.1403 |
| <i>Baijiu</i> A vs <i>Baijiu</i> C | -0.47079 | 3.52575 | 1.000 | -10.8255 | 9.8839 |
| <i>Baijiu</i> A vs EtOH | -7.36970 | 3.52575 | 0.255 | -17.7244 | 2.9850 |
| <i>Baijiu</i> B vs <i>Baijiu</i> C | 4.74357 | 3.52575 | 0.666 | -5.6111 | 15.0982 |
| <i>Baijiu</i> B vs EtOH | -2.15534 | 3.52575 | 0.972 | -12.5100 | 8.1993 |
| <i>Baijiu</i> C vs EtOH | -6.89891 | 3.52575 | 0.315 | -17.2536 | 3.4558 |

*. $P < 0.05$ indicated a significant difference between groups.

Table S3. Post hoc multiple comparison tests (MCTs) performed using Tukey–Kramer based on Y-axis of PCA modelling.

| ANOVA five-group comparison (F = 6.801, P = 0.001*) | | | | | |
|---|-----------------------------|------------|--------|----------------------------|----------------|
| Tukey–Kramer analysis | | | | | |
| Group comparisons (Y-axis) | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
| | | | | Lower Bound | Upper Bound |
| Ctrl vs <i>Baijiu</i> A | 3.95030 | 2.35784 | 0.466 | -2.9744 | 10.8750 |
| Ctrl vs <i>Baijiu</i> B | 5.50010 | 2.35784 | 0.168 | -1.4246 | 12.4248 |
| Ctrl vs <i>Baijiu</i> C | 8.79756 | 2.35784 | 0.008* | 1.8729 | 15.7222 |
| Ctrl vs EtOH | 11.23411 | 2.35784 | 0.001* | 4.3094 | 18.1588 |
| <i>Baijiu</i> A vs <i>Baijiu</i> B | 1.54980 | 2.35784 | 0.964 | -5.3749 | 8.4745 |
| <i>Baijiu</i> A vs <i>Baijiu</i> C | 4.84726 | 2.35784 | 0.270 | -2.0774 | 11.7719 |
| <i>Baijiu</i> A vs EtOH | 7.28382 | 2.35784 | 0.036* | 0.3591 | 14.2085 |
| <i>Baijiu</i> B vs <i>Baijiu</i> C | 3.29746 | 2.35784 | 0.634 | -3.6272 | 10.2221 |
| <i>Baijiu</i> B vs EtOH | 5.73401 | 2.35784 | 0.140 | -1.1907 | 12.6587 |
| <i>Baijiu</i> C vs EtOH | 2.43656 | 2.35784 | 0.838 | -4.4881 | 9.3612 |

*. $P < 0.05$ indicated a significant difference between groups.

References

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