Electronic Supplementary Material (ESI) for Food & Function.

Supplementary Information

Potential protective mechanism of Tibetan kefir underlying gut-derived liver injury induced by ochratoxin A

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Supplementary Methods

Analysis of residual OTA in organs and feces

For OTA extraction in organs, 0.5 g of frozen tissue was homogenized with 2 mL of sodium phosphate buffer (0.05 mol/L, pH 6.5) in a plastic tube for 1 min. Then, the samples were stored at -80°C for 1 d. After storage at room temperature for 30 min, 250 μL of tissue homogenates was added with 400 μL of ice-cold ethanol and 50 μL of 20% (v/v) trichloroacetic acid to enhance protein precipitation and OTA release. Next, the mixture was vortexed and shaken for 15 min before centrifugation (6200 ×*g*, 15 min). Finally, the supernatant was evaporated under nitrogen gas, re-dissolved in the mobile phase, filtered through 0.22-μm filters, and stored for further detection. For OTA extraction in feces, 1 g of fecal sample was ground with a mortar and pestle, and then 10 mL of water was added and sonicated for 30 min. After mixing with 100 μL of 25% (w/w) hydrochloric acid, the mixture was treated with 10 mL of chloroform and centrifuged (3500 ×*g*, 10 min). The chloroform phase was collected and the extraction step was repeated 2×. Finally, the sample was evaporated, re-dissolved, filtered, and stored as per the same method described for organ samples. HPLC analyses were conducted with a Waters 2695 HPLC system (Waters, Milford, MA). OTA was analyzed on an XDB-C18 reverse-phase column (5 mm, 4.6 mm × 250 mm; Agilent Technologies, Santa Clara, CA, USA). The mobile phase consisted of acetonitrile, water and acetic acid (49.5:49.5:1 v/v/v). The recovery percentages were higher than 85% in all matrices studied.

16S rRNA microbiome sequencing

Total DNA was extracted from the TK, liver, and cecal contents with a FastDNA Spin Kit for Soil (MP Biomedicals, USA). The V3–V4 hypervariable regions were amplified by using the following primers: 338F (5′- ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The ITS1 region was amplified using the primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2R (5′- GCTGCGTTCTTCATCGATGC-3′). Then, samples were sequenced with an Illumina MiSeq platform (Illumina, San Diego, USA) for paired end sequencing. Sequencing reads were imported into Quantitative Insights into the Microbial Ecology 2 (QIIME 2) framework and then de-noised with Divisive Amplicon Denoising Algorithm 2 (DADA2) to obtain the ASVs. The Silva 138 and Unite 8.0 databases were used as references for annotation. PCoA was performed with R (v3.3.1) at the ASV based on Bray-Curtis distances and then a permutational multivariate ANOVA test was used for differences between groups. Taxa with differential abundance among groups was evaluated by the LEfSe with LDA scores higher than 3. Subsequently, ASV sequences and abundance were adopted to analyze the enrichment of functional genes with Tax4Fun. Venn diagrams and heatmap analysis were performed with R software (v3.3.1).

Metabolite Profiling

Cecum contents (50 mg) were weighed into a 2-mL centrifuge tube and mixed with 400 μL of methanol/water (4:1, v/v). Then, the tube was ground with a Wonbio-96c freezing grinder (Shanghai Wanbai Biotechnology, Shanghai, China) for 6 min, and then ultrasonicated for 30 min. After stand for 30 min at -20℃, the samples were

centrifuged (13000 ×*g*, 15 min) at 4℃. Finally, the supernatant was collected and pipetted into the injection vial for further use. In addition, 20 μL of supernatant was taken from each sample and mixed as quality control (QC) samples. The analysis was performed with an UHPLC -Q Exactive HF-X system (Thermo Fisher Scientific, Shanghai, China) using an ACQUITY UPLC HSS T3 column (100 mm × 2.1 mm i.d., 1.8 μm; Waters, Milford, USA). A quality control sample was inserted periodically throughout the analysis to examine the stability of the entire assay process. The mass spectrometry signals were acquired in positive and negative ion scanning mode, respectively. The original data were preprocessed with Progenesis QI (WatersCorporation, Milford, USA) for baseline filtering, peak identification, integration, retention time correction, and peak alignment. The MS and MS/MS data were matched against online databases (e.g., Human Metabolome Database, METLIN) with a 10-ppm mass accuracy threshold to identify the metabolites. Multivariate statistical analysis was performed with SIMCA-P (version 14.1, Umetrics, Umea, Sweden). The database Kyoto Encyclopedia of Genes and Genomes (KEGG) library was employed to find metabolic pathways. Correlation analysis was executed based on Pearson's correlation coefficients and mapped with Cytoscape (3.8.2) software.

SCFA content in feces

Approximately 0.2 g of feces samples were mixed with 1 mL of distilled water and vortexed for 10 min. Then, 0.15 mL of 50% (w/w) H_2SO_4 and 1.6 mL of ether were added and incubated at 4°C for 30 min. Afterward, the mixture was centrifuged at 7000 ×*g* for 10 min. Finally, 1 ml of supernatant was collected and passed through 0.22-μm

filters. The detection was conducted with a DB-FFAP capillary column (30 m × 0.25 μm × 0.25 mm) (Agilent Technologies, Wilmington, DE, USA) by GC-2014C gas chromatography (Shimadzu Corporation, Kyoto, Japan) with a flame ionization detector.

Supplementary Tables and Figs

Table S1. Primers sequences used for RT-qPCR

Gene name	Forward Primer	Reverse Primer
Muc1	AGTGCCAAGTCAATACCCTGT	CTGGGGTGAACTGTTACTGGA
Muc2	AGGGCTCGGAACTCCAGAAA	CCAGGGAATCGGTAGACATCG
Muc3	GCCGTGAATTGTATGAACGGA	CGCAGTTGACCACGTTGACTA
TFF3	TTGCTGGGTCCTCTGGGATAG	TACACTGCTCCGATGTGACAG
Claudin-1	TGCCCCAGTGGAAGATTTACT	CTTTGCGAAACGCAGGACAT
Claudin-4	TGGAGGACGAGACCGTCAA	CACGGGCACCATAATCAGCA
Occludin	TGAAAGTCCACCTCCTTACAGA	CCGGATAAAAAGAGTACGCTGG
ZO-1	GAGCGGGCTACCTTACTGAAC	GTCATCTCTTTCCGAGGCATTAG
E-cadherin	CAGGTCTCCTCATGGCTTTGC	CTTCCGAAAAGAAGGCTGTCC
IL-1β	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
IL-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
TNF-α	CCTGTAGCCCACGTCGTAG	GGGAGTAGACAAGGTACAACCC
TLR4	GCCTTTCAGGGAATTAAGCTCC	GATCAACCGATGGACGTGTAAA
MYD88	ATCGCTGTTCTTGAACCCTCG	CTCACGGTCTAACAAGGCCAG
NF-kB	AGGCTTCTGGGCCTTATGTG	TGCTTCTCTCGCCAGGAATAC
GAPDH	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA

Muc1, mucin 1; Muc2, mucin 2; Muc3, mucin 3; TFF3, trefoil factor 3; ZO-1, zonula occludens-1; IL-1β, interleukin-1β; IL-6, interleukin-6; TNF-α, tumor necrosis factor α; TLR4, toll-like receptor 4; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor kappa B; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table S2. Identification of different metabolites from each group of Con, OTA and OTA+TK

Metabolites	VIP	P value	Trend	m/z
Con vs OTA				
7-Ketolithocholic Acid	2.51	0.000027	↓	408.31
Xanthosine	2.39	0.036	↓	283.07
Deoxycholic acid	2.29	0.0017	↓	391.28
Xanthosine 5'-Monophosphate	2.24	0.048	↓	329.03
N-Acetyl-L-glutamic acid	2.20	0.000016	↑	190.07
Cholecalciferol	1.99	0.0046	↑	385.35
3-hydroxydodecanoyl carnitine	1.99	0.017	↑	404.26
Uridine	1.87	0.015	↓	243.06
N'-Formylkynurenine	1.63	0.0013	↓	271.05
Cholic acid	1.58	0.027	↓	407.28
Phosphocholine	1.38	0.020	↑	184.07
LysoPC[16:1(9Z)]	1.33	0.013	↑	494.32
Adipic acid	1.33	0.027	↑	145.05
N-Acetyl-D-glucosamine	1.21	0.027	↓	222.10
L-Serine	1.15	0.028	↓	106.05
9,10-DiHOME	1.15	0.025	↑	313.24
Ochratoxin A	1.07	0.022	↑	404.09
Acetylcholine	1.04	0.043	↓	146.12
9-HOTrE	1.04	0.026	↑	293.21
Ochratoxin A	1.07	0.022	↑	404.09
OTA vs OTA+TK				
Reduced glutathione	2.49	0.015	↓	346.05
Indoxylsulfuric acid	2.39	0.045	↓	212.00
Hydroquinone	2.23	0.021	↓	109.03
3-hydroxydodecanoyl carnitine	2.04	0.0040	↓	404.26
Deoxyinosine	2.02	0.0072	↓	251.08
N-formylanthranilic acid	2.00	0.0073	↓	164.03
Isocitrate	1.95	0.023	↓	191.02
N-Acetylindoxyl	1.89	0.045	↓	176.07
Citric acid	1.81	0.041	↓	191.02
L-Citrulline	1.71	0.00078	↑	140.08
Malic acid	1.66	0.016	↓	133.01
Linoleoyl ethanolamide	1.65	0.017	↑	306.28
Glycocholate	1.60	0.0087	↓	446.29
Thymidine	1.55	0.00052	↑	260.12
Acetylcholine	1.55	0.0019	↑	146.12
Quinoline-2-carboxylic acid	1.49	0.015	↓	218.05
Deoxyadenosine	1.47	0.0058	↑	315.12
DG [15:0/22:6(4*Z*,7*Z*,10*Z*,13*Z*,16*Z*,19*Z*)/0:0]	1.47	0.023	↓	661.47
Oxindole	1.42	0.0045	↑	134.06

Indole-3-carboxaldehyde	1.42	0.00017	↑	146.06
Ectoine	1.41	0.000015	↑	425.22
Phenylacetaldehyde	1.40	0.047	↓	121.06
Ochratoxin A	1.39	0.0092	↑	404.09
N-Acetyl-L-glutamic acid	1.34	0.021	↓	190.07
5-Hydroxyindoleacetate	1.34	0.0098	↓	236.06
2-Deoxycastasterone	1.32	0.000096	↑	447.35
16R-HETE	1.31	0.00071	↑	303.23
Equol	1.29	0.00016	↑	287.09
9,10-DiHOME	1.23	0.0033	↓	313.24
Taurocholic acid	1.20	0.016	↑	516.30
Phenol	1.14	0.043	↓	95.05
L-Histidine	1.13	0.0023	↑	156.08
N′-Formylkynurenine	1.13	0.018	↑	271.05
L-Threonine	1.10	0.030	↓	120.07
Galactinol	1.09	0.033	↑	363.09
Taurine	1.052	0.00024	↑	124.01
Myo-inositol	1.04	0.023	↓	179.06
Palmitoleoyl ethanolamide	1.03	0.0070	↑	342.26

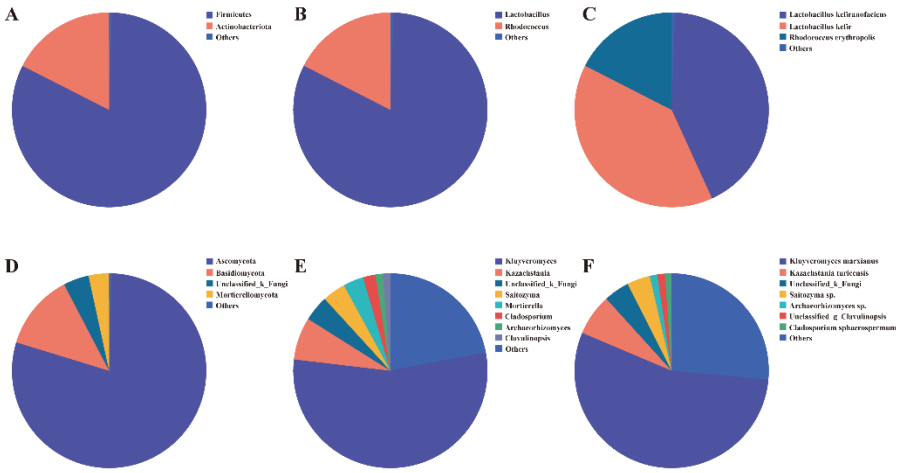

Fig S1. Microbial composition of Tibetan kefir (TK). Relative abundance of bacterial composition at the (A) phylum, (B) genus, and (C) species levels; Relative abundance of fungal composition at the (D) phylum, (E) genus, and (F) species levels.

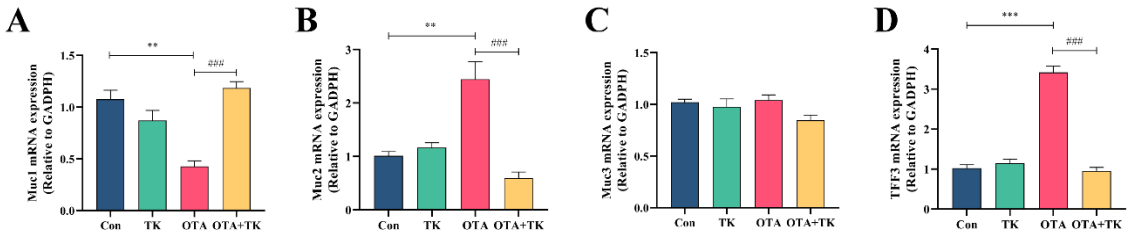

Fig S2. Effect of ochratoxin A (OTA) and Tibetan kefir (TK) on mucus proteins expression. The mRNA expression of (A) Muc1, (B) Muc2, (C) Muc3 and (D) TFF3. Data show mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 versus Con group; #p < 0.05, ##p < 0.01, ###p < 0.001 versus OTA group.

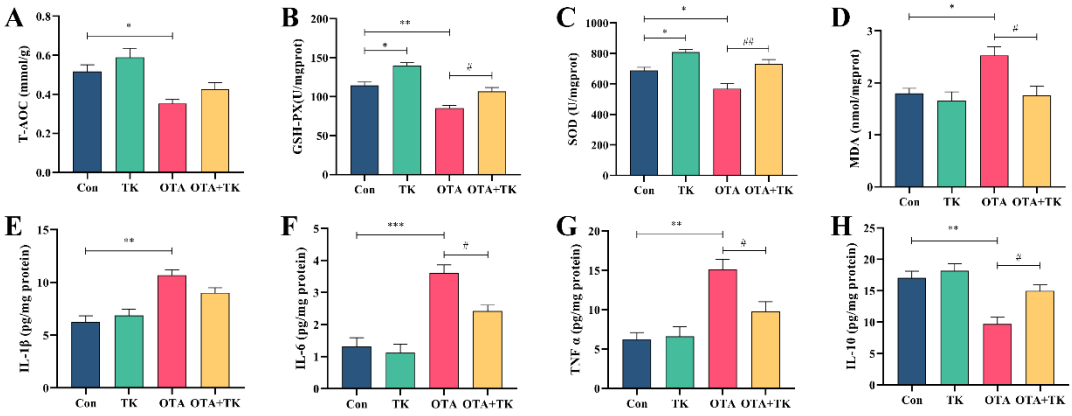

Fig S3. Effect of ochratoxin A (OTA) and Tibetan kefir (TK) on oxidative stress and inflammatory

responses in jejunal tissue. (A) T-AOC level, (B) GSH-PX activity, (C) SOD activity and (D) MDA content in jejunal tissue. (E) IL-1β level, (F) IL-6 level, (G) TNF α level and (H) IL-10 level in jejunal tissue. Data show mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 versus Con group; #p < 0.05, ##p < 0.01, ###p < 0.001 versus OTA group.

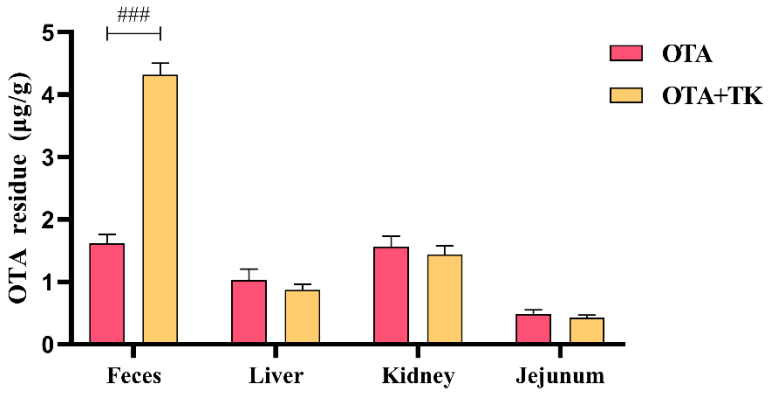

Fig S4. Effect of ochratoxin A (OTA) and Tibetan kefir (TK) on the OTA residues in organs and feces. Data show mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 versus Con group; #p < 0.05, ##p < 0.01, ###p < 0.001 versus OTA group.

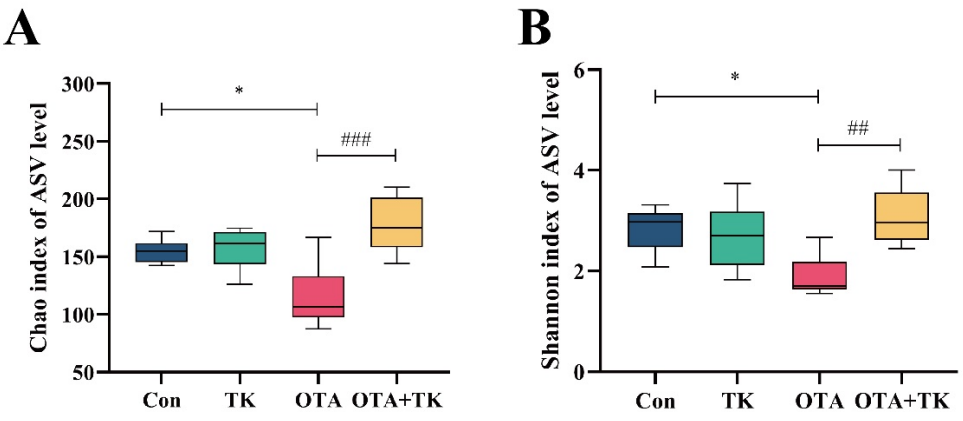

Fig S5. Effect of ochratoxin A (OTA) and Tibetan kefir (TK) on alpha diversity of gut microbiota. (A) Chao, and (B) Shannon indices in α-diversity analysis. *p < 0.05, **p < 0.01, ***p < 0.001 versus Con group; #p < 0.05, ##p < 0.01, ###p < 0.001 versus OTA group.

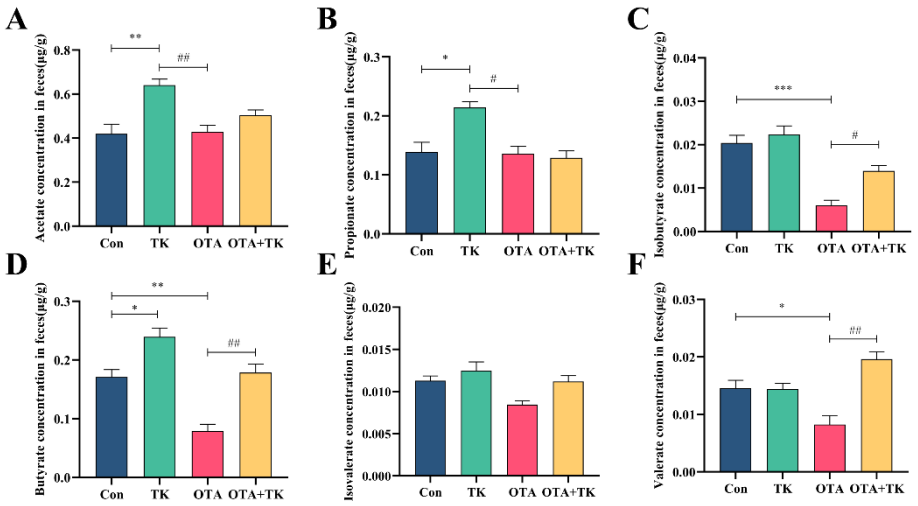

Fig S6. Effect of ochratoxin A (OTA) and Tibetan kefir (TK) on the short chain fatty acids content in feces. Concentration of (A) acetate, (B) propionate, (C) isobutyrate, (D) butyrate, (E) isovalerate and (F) valerate. Data show mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 versus Con group; #p < 0.05, ##p < 0.01, ###p < 0.001 versus OTA group.

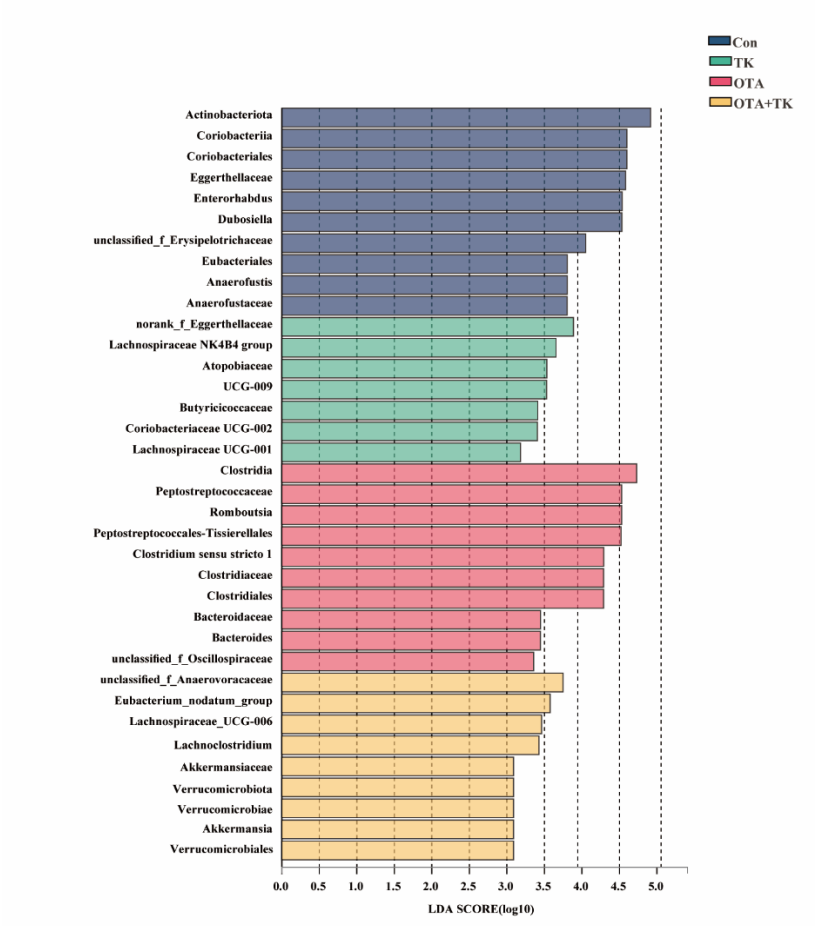

Fig S7. Linear discriminant analysis (LDA) of gut microbiota in each group with LDA scores (log 10) above 4.

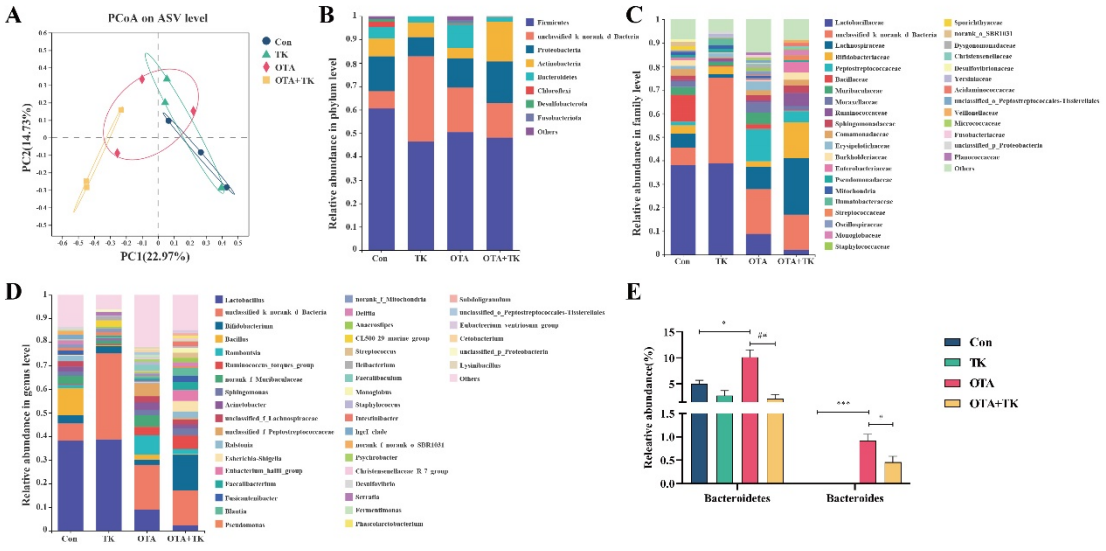

Fig S8. Effect of ochratoxin A (OTA) and Tibetan kefir (TK) on liver microbiota. (A) Principal coordinates analysis of each group; Relative abundance of gut microbiota in (B) phylum (C) family and (D) genus levels; (E) Relative abundance of Bacteroidetes (at the phylum level) and Bacteroides (at the genus level) in each group. Data show mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 versus Con group; #p < 0.05, ##p < 0.01, ###p < 0.001 versus OTA group.

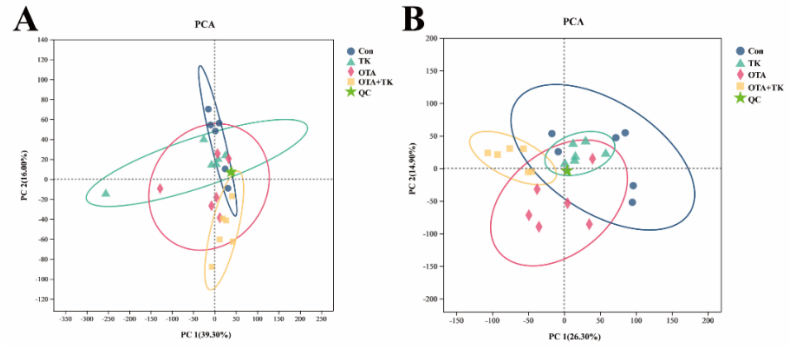

Fig S9. Differential metabolites analysis by principal component analysis (PCA). PCA score plot from (A) positive ion mode and (B) negative ion mode.

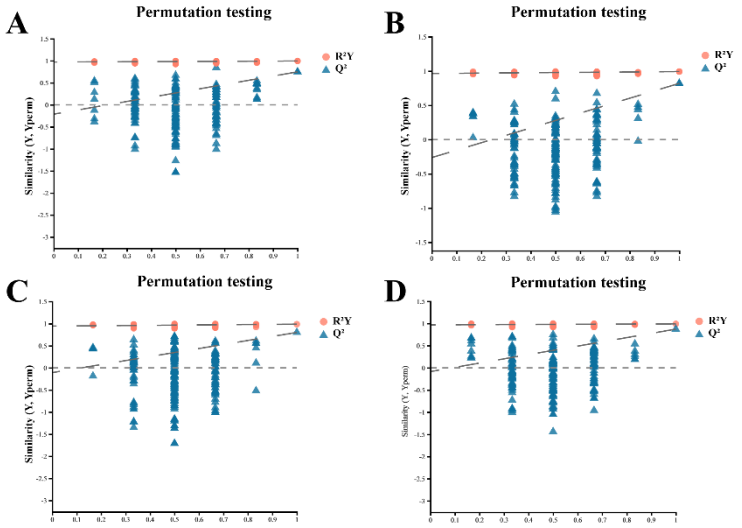

Fig S10. Permutation test analysis (200 permutations) for orthogonal partial least-squares discriminant analysis (OPLS-DA) model. Permutation test plot for the comparison group Con vs OTA; (A) Positive ion mode and (B) negative ion mode; Permutation test plot for the comparison group OTA vs OTA+TK; (C) Positive ion mode and (D) negative ion mode.

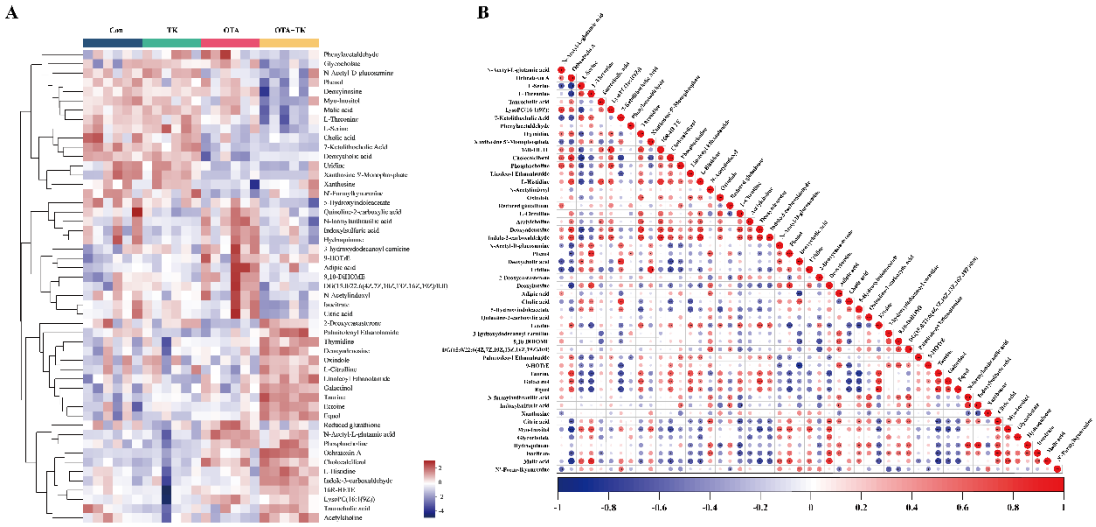

Fig S11. Heat map of hierarchical cluster analysis and correlation analysis of metabolites. (A) Heat map of cecum content metabolites within different groups (red: upregulation; blue: downregulation). (B) Correlation analysis of differential metabolites. The color and size of circles used to represent the correlation index level (red: positive correlation; blue: negative correlation). *p < 0.05, **p < 0.01, ***p < 0.001