Supplementary Information

Protein hydrolysates and xanthine oxidase inhibitory peptides from sunflower capitulum exert antihyperuricemic effects: A comprehensive study based on serum metabolomics and gut microbiota analysis

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Materials

Hexane (AR), anhydrous ethanol (AR), sodium hydroxide (AR), hydrochloric acid (AR), and pepsin (≥ 1.2×10^5 U/g) was purchased from China National Pharmaceutical Group Corporation. Trypsin (≥ 2.5×10^6 U/g) and alkaline protease (≥ 2×10^5 U/g) were purchased from Shanghai Yuanye Bio-Technology Co., Ltd. Alcalase 2.4 L (protease from *Bacillus licheniformis*, > 2.4 AU/g solution) were obtained from Novozymes (Bagsvaerd, Denmark). Papain (≥ 2×10^6 U/g), O-phthalaldehyde, and phosphate buffered saline (PBS, 1×, pH 7.2-7.4) were bought from Biorigin (Beijing) Inc. Serine (> 98%) were purchased from Beijing Solarbio Science & Technology Co., Ltd. Xanthine was purchased from Shanghai Yuanye Technology Co., Ltd. (Shanghai, China). XOD (53.1 U/mg) was purchased from Nanjing Jiancheng Bioengineering Research Institute (Jiangsu, China). All HPLC grade reagents were purchased from Merck Millipore (Billerica, MA, USA). All other reagents were of the highest grade commercially available.

Process 1. Determination of degree of hydrolysis.

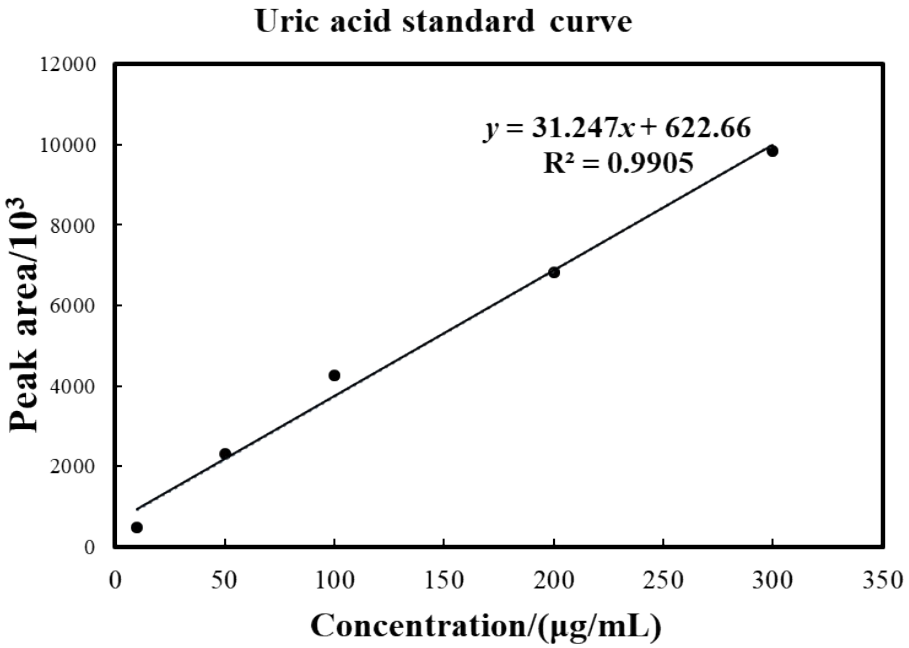
Measurement of degree of hydrolysis (DH). The DH was calculated based on the determination of free amino groups by the O-phthalaldehyde (OPA) method as previously described. Serine (0.9516 mol/L) was used as the standard. A total of 400 μL of hydrolysate sample or serine standard was mixed with 3 mL OPA reagent for 5 s. The mixture was incubated for 2 min at ambient temperature and the absorbance was measured at 340 nm.

Process 2. Determination of xanthine oxidase inhibitory (XOI) rate.

Measurement of xanthine oxidase inhibitory (XOI) rate. XOI rate assay was conducted according to the method with slight modifications. First, 50 μL of XO (0.1 U/mL) and 50 μL of the samples (protein hydrolysates at a concentration of 20 mg/mL) in PBS buffer (pH 7.2-7.4) were preincubated at 37 °C for 5 min. Then, 150 μL of xanthine solution (0.6 M) was added to start the reaction, which were incubated at 37°C for 30 min. The reaction was terminated by adding 80 μl of 1 M HCl. SHIMADZU LC-20A equipped with an Agilent dC18 column (250 × 4.6 mm, 5 μm) was employed for UA content detection. The mobile phase consisting of 85% $NH_4H_2PO_4$ aqueous solution (10 mM) and 15% methanol was at a flow rate of 0.7 mL/min. The amount of UA in test samples was determined from the absorbance at 290 nm. XO inhibitory percentage (%) = [(UA_{blank} - UA_{sample}) / UA_{blank}] × 100%.

The calibration curve for UA demonstrated excellent linearity (y = 31.247 x + 622.66, R^2 = 0.9905), indicating a reliable response across the concentration range tested (10-300 μg/mL).

Figure S1. Standard curve of uric acid in HPLC method.



Process 3. Optimization of enzymatic hydrolysis for Alcalase 2.4 L.

Optimization of the primary hydrolysis. Sunflower capitulum protein was suspended in distilled water and preheated at 100℃ for 15 min to improve the capacity of hydrolysis. Then, Alcalase 2.4 L, pepsin, alkaline protease, papain, and trypsin were added to the mixture at a certain enzyme to substrate ratio under their most suitable reaction conditions, respectively. Meanwhile, pH for the hydrolysis system was maintained with 1 M sodium hydroxide solution. The hydrolysates were then heated at 100°C for 10 min to terminate the reaction and centrifuged. Finally, the supernatants were collected to obtain primary hydrolysates for further detection.

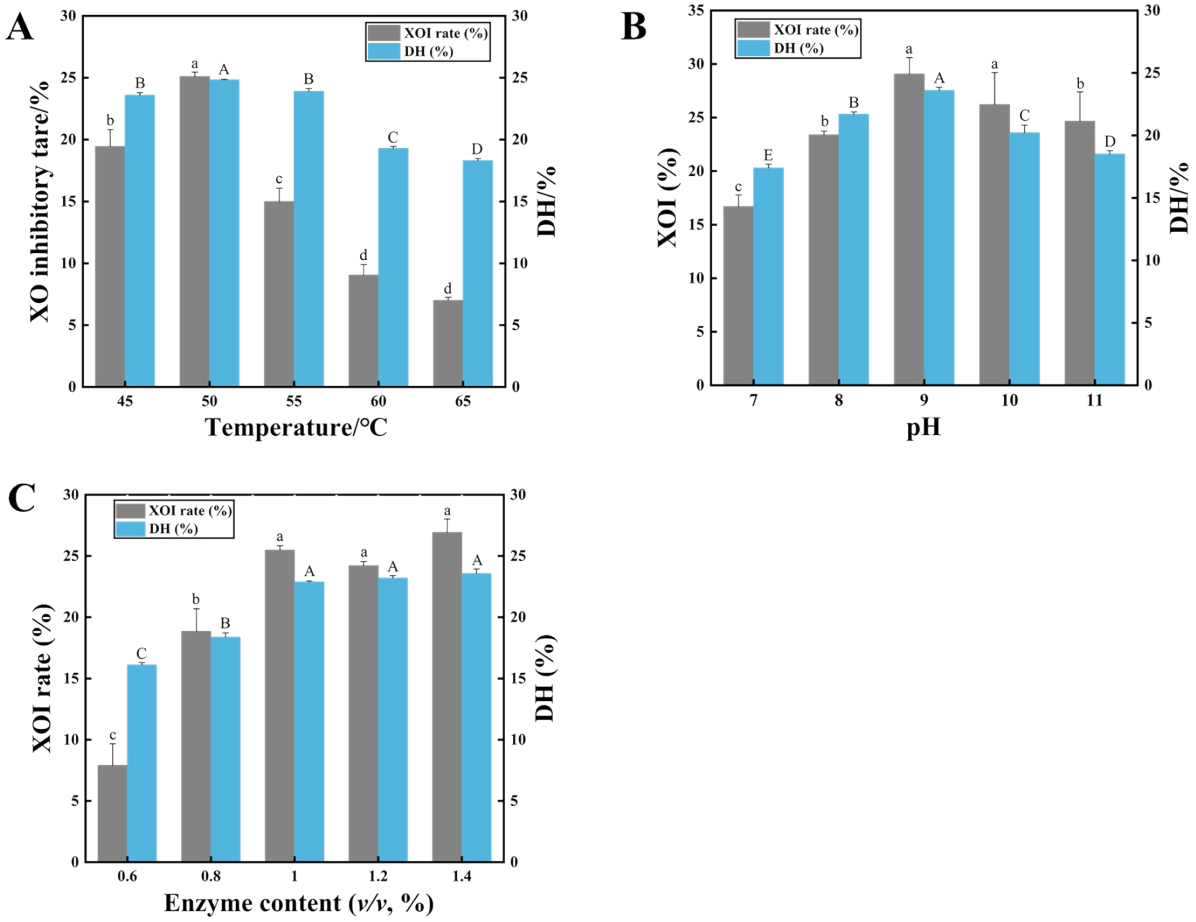
Optimization of hydrolysis conditions. Enzyme content, temperature, and pH were considered to optimize the hydrolysis process of sunflower capitulum protein based on the XOI activity and degree of hydrolysis.

Optimization of the secondary hydrolysis. The primary hydrolysate was obtained according to the optimized enzymatic hydrolysis process, pH of which was adjusted by 1 M sodium hydroxide solution or hydrochloric acid solution. Then, pepsin, alkaline protease, papain, and trypsin was added for the second hydrolysis, conditions of which was presented in Table S1. The hydrolysates were then heated at 100°C for 10 min to terminate the reaction and centrifuged for 20 min. Finally, the supernatants were collected to obtain SCPH for further detection.

Table S1. Key parameters applied in the two-step enzymatic hydrolysis.

Protease type	pH	Temperature/℃	E/S	Reaction time
Primary hydrolysis				
Pepsin	3.0	37	90000 U / 0.1 g protein	3.0 h
Trypsin	8.0	37		
Papain	7.0	40		
Alkaline Protease	10.	40		
Alcalase 2.4 L	8.0	50		
Secondary hydrolysis				
Pepsin	3.0	37	90000 U / 0.1g protein	3.0 h
Trypsin	8.0	37		
Papain	7.0	40		
Alkaline Protease	10.0	40		

Figure S2. Optimization of enzymatic conditions for Alcalase 2.4 L.



Process 4. Molecular weight determination of SCPH.

The molecular weight distribution of SCPH was monitored by using a TSK-Gel G2000 SWXL gel filtration column (7.8 mm × 300 mm, Tosoh Corporation, Tokyo, Japan) attached to a Shimadzu LC-20A HPLC system (Shimadzu, Japan). The samples were dissolved in the mobile phase consisting of acetonitrile/water (45/55, *v*/*v*) in the presence of 0.1% (v/v) trifluoroacetic acid and the flow rate was set at 0.5 mL/min. The calibration curve was obtained using the following standards from J&K Chemical Ltd.: cytochrome C (12,384 Da), aprotinin (6,510 Da), zinc bacitracin (1,422 Da), Gly-Gly-Tyr-Arg (451 Da) and Gly-Gly-Gly (189 Da). Absorbance at 214 nm was measured.

Process 5. Peptide identification through LC-MS/MS.

The peptides were re-dissolved in solvent A (0.1% formic acid in water) and analyzed by Orbitrap Exploris 480 coupled to an EASY-nanoLC 1200 system (Thermo Fisher Scientific, MA, USA). Peptide sample (1 μL) was loaded onto an Acclaim PepMap C_{18} column (75 μm × 25 cm, 1.9 μm) and separated with 60 min-gradient starting at 2.2% solvent B (80% ACN with 0.1% FA) followed by a stepwise increase to 50% in 51 min, 90% in 3.5 min and stayed there for 5.5 min. The column flow rate was maintained at 350 nL/min with the column temperature of 40°C. The electrospray voltage was set to 2 kV.

The mass spectrometer was run under data dependent acquisition (DDA) mode, and automatically switched between MS and MS/MS mode. The survey of full scan MS spectra (*m/z*: 200-1500) was acquired in the Orbitrap with 60,000 resolutions. The normalized automatic gain control (AGC) target is 300% and the maximum injection time is 25 ms. Then the precursor ions were selected into collision cell for fragmentation by higher-energy collision dissociation (HCD), and the normalized collection energy was 30%. The MS/MS resolution was set at 15,000, the normalized AGC target of 50%, the maximum injection time of 22 ms, and dynamic exclusion was 30 s.

Tandem mass spectra were processed by PEAKS Studio version 10.6 (Bioinformatics Solutions Inc., Waterloo, Canada). The database, *Helianthus annuus* (version 2023, 80941 entries), was downloaded from uniprot. None was set as the digestion enzyme. PEAKS databases were searched with a fragment ion mass tolerance of 0.02 Da and a parent ion tolerance of 10ppm. Oxidation on methionine, deamidation on asparagine and glutamine were specified as variable modifications. The peptides with $-10\lg P \geq 20$ and the proteins containing at least one unique peptide were filtered.

Figure S3. IC_{50} diagram of SCPH (A) and allopurinol (B).

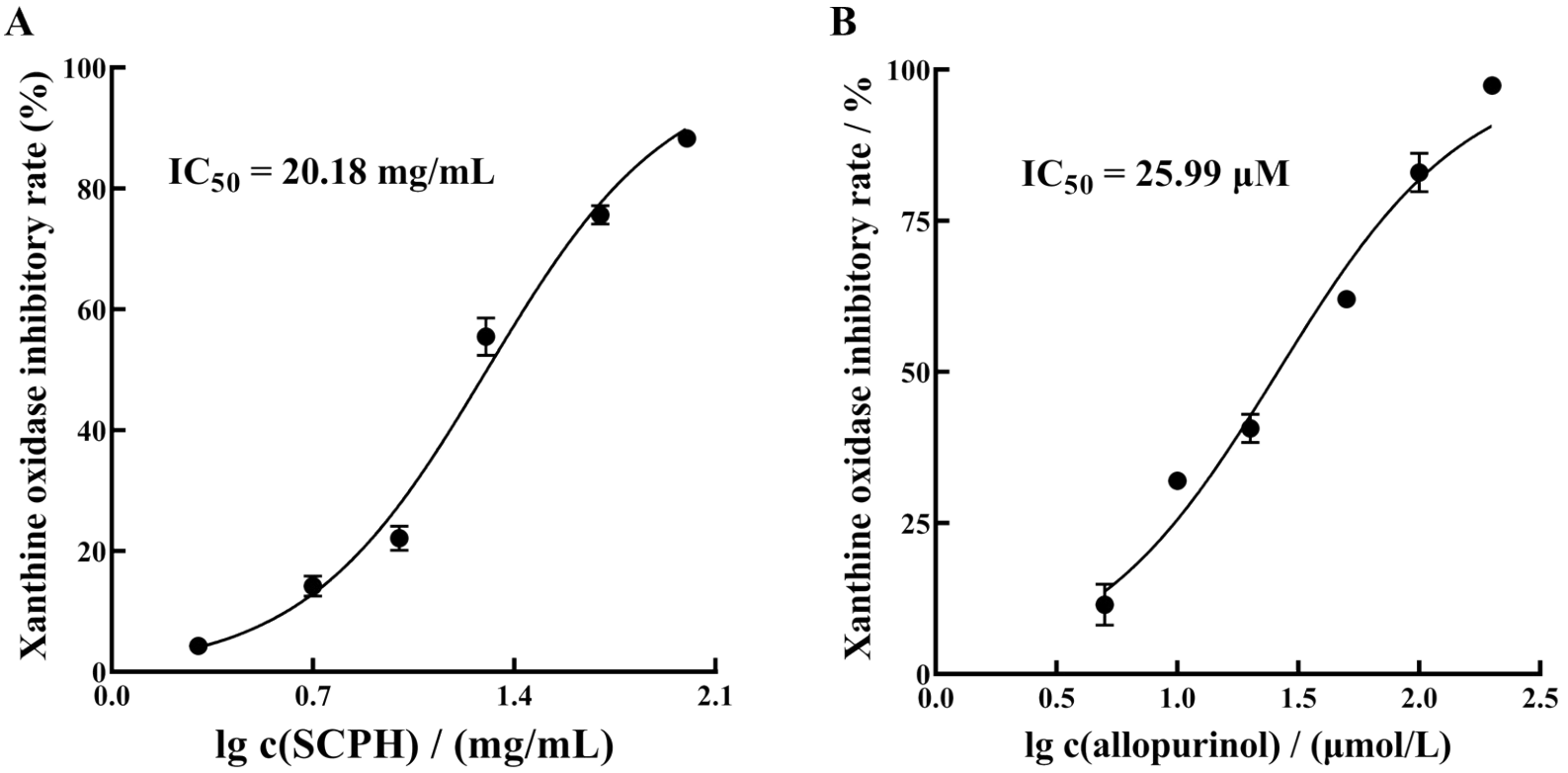
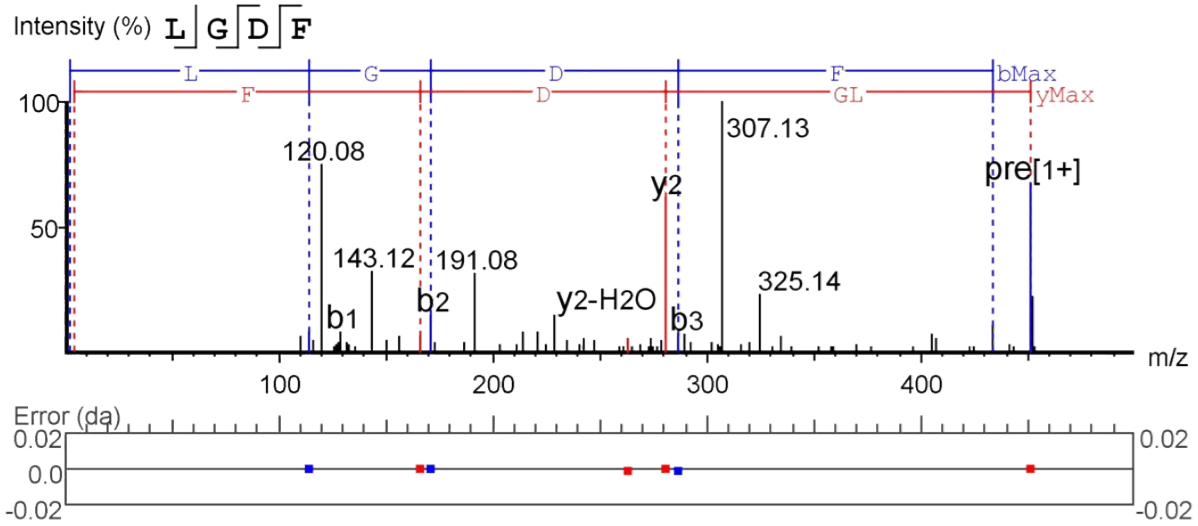


Figure S4. Secondary mass spectrum of LGDF and DML.



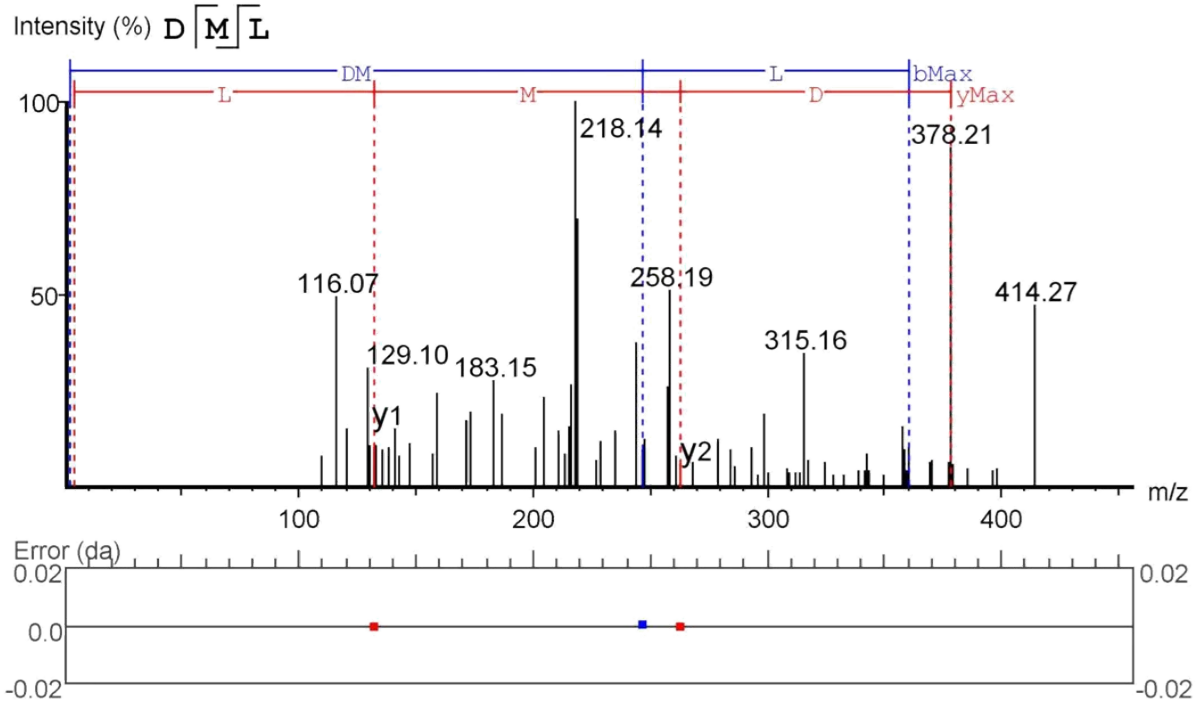


Figure S5. Total ion chromatogram (TIC) of LC-MS/MS identification.

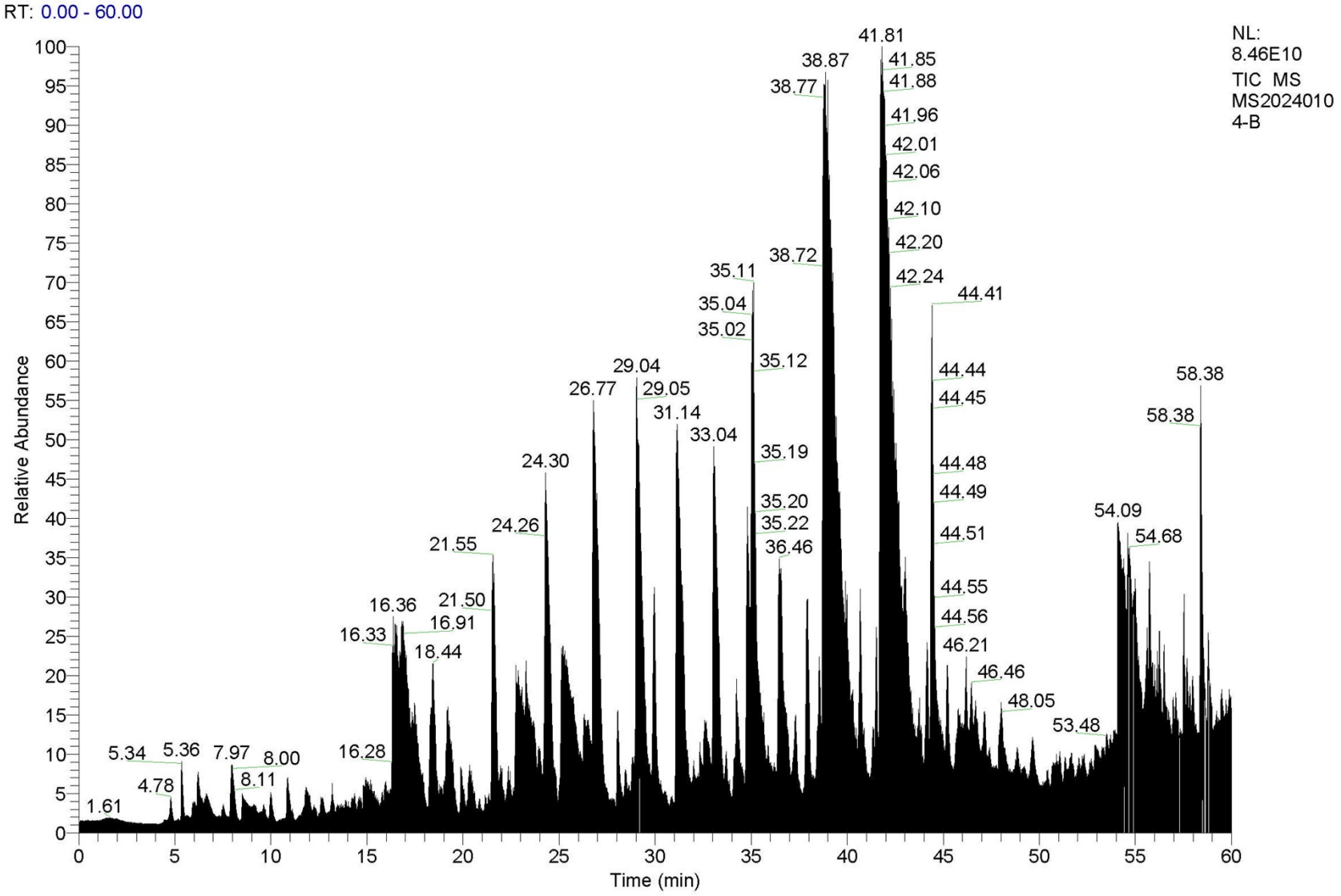


Figure S6. Quality control evaluation for serum metabolomics analysis. (A) OPLS-DA score plot including QC samples. (B) Correlation analysis among QC samples.

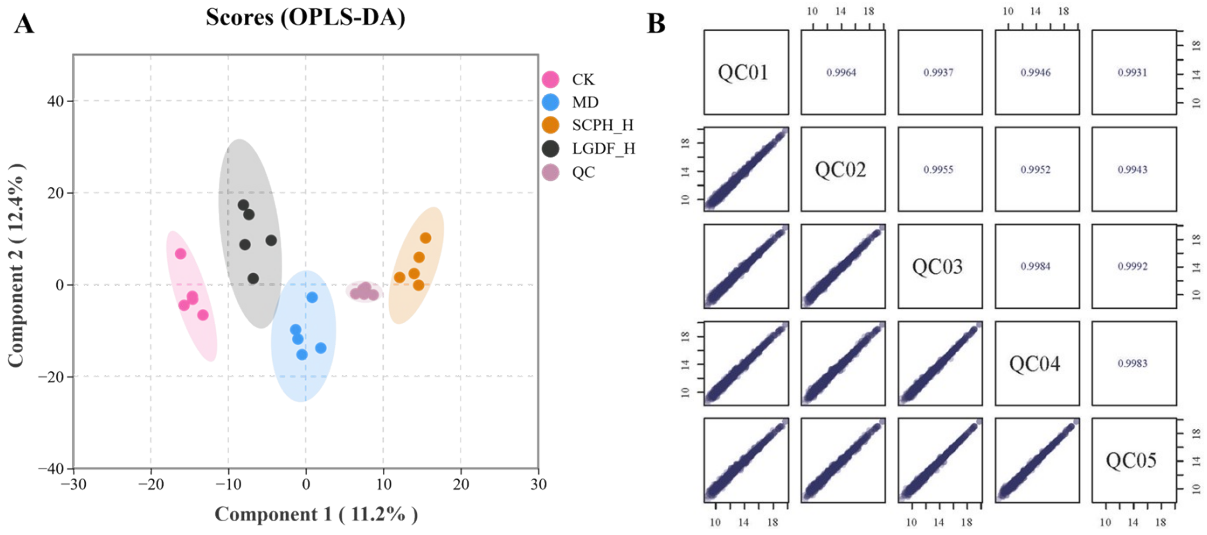
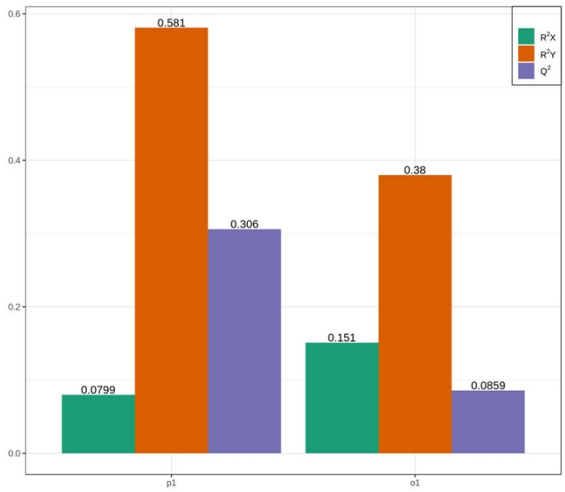
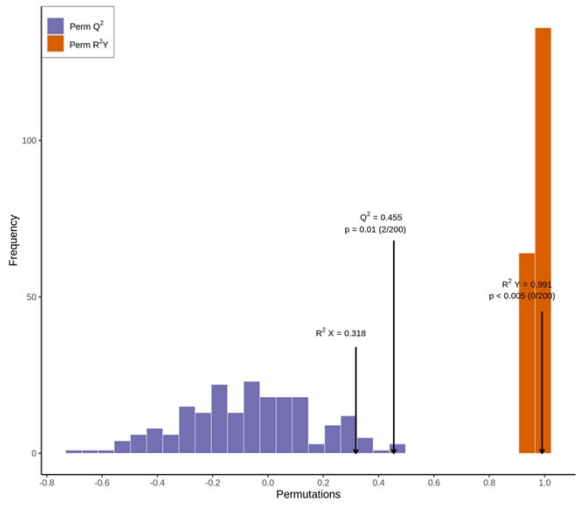
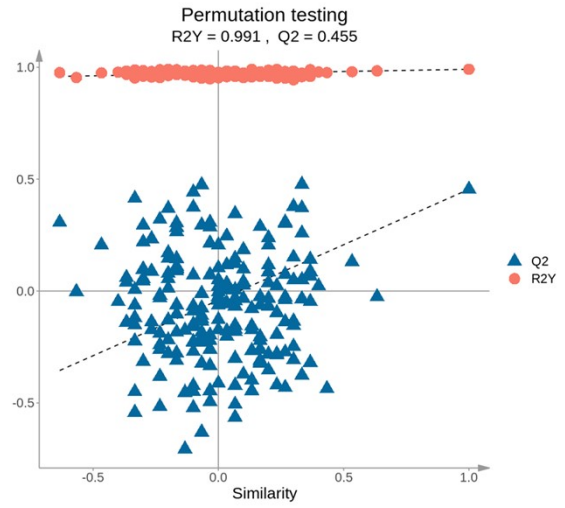


Figure S7. Permutation testing results of OPLS-DA analysis for serum metabolomics.







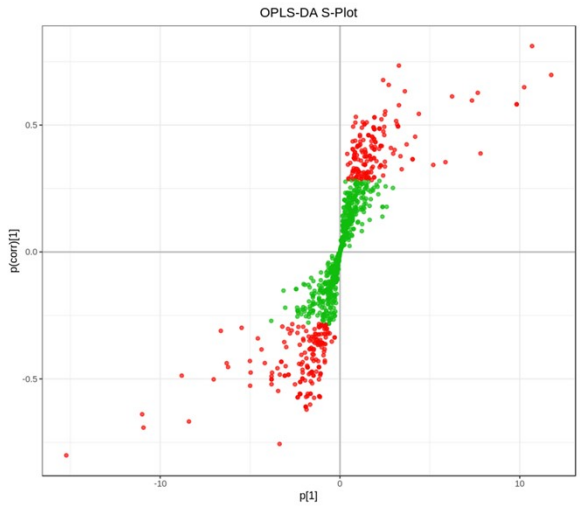


Figure S8. Permutation testing results of OPLS-DA analysis for gut microbiota.

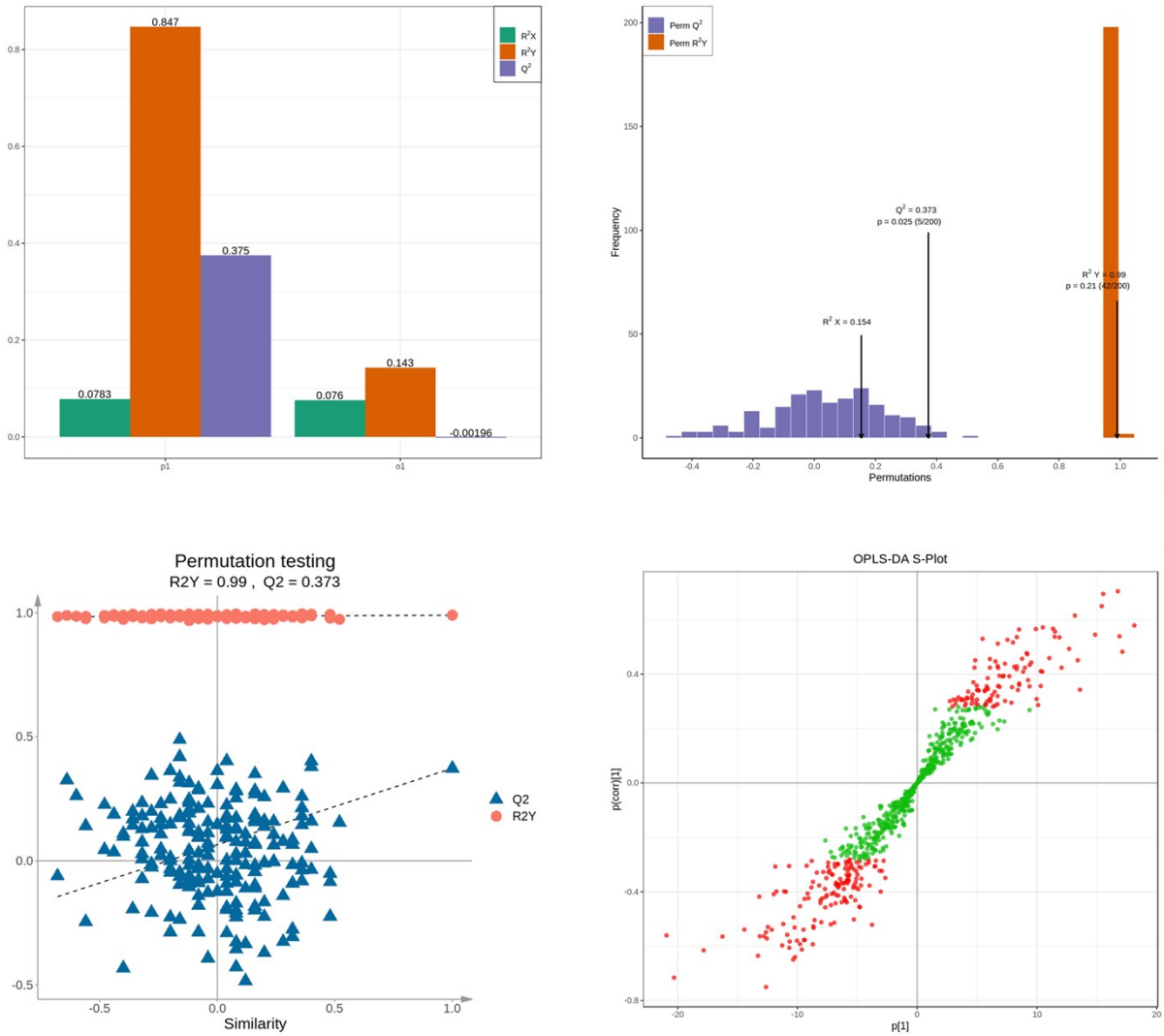


Table S2. Sources, sequence, and IC_{50} of food-derived XOI peptides

Sources	Peptide sequence	IC_{50}	Reference
Walnut	WPPKN (Trp-Pro-Pro-Lys-Asn)	17.75 ± 0.12 mg/mL	1
	ADIYTE (Ala-Asp-Ile-Tyr-Thr-Glu)	19.01 ± 0.23 mg/mL	
Macadamia nut	PGPR (Pro-Gly-Pro-Arg)	24.84 ± 0.02 mM	2
	HGGR (His-Gly-Gly-Arg)	24.89 ± 0.19 mM	
	GPY (Gly-Pro-Tyr)	30.44 ± 0.33 mM	
Small yellow croaker	WDDMEKIW (Trp-Asp-Asp-Met-Glu-Lys-Ile-Trp)	3.16 ± 0.03 mM	3
	APPERKYSVW (Ala-Pro-Pro-Glu-Arg-LysTyr-Ser-Val-Trp)	5.86 ± 0.02 mM	
Auxis thazard	PDL (Pro-Asp-Leu)	4.37 ± 0.11 mg/mL	4
	SVGGAL (Ser-Val-Gly-Gly-Ala-Leu)	5.59 ± 0.09 mg/mL	5
Tuna	FH (Phe-His)	25.7 mM	6
Katsuwonus pelamis (Skipjack tuna)	ACECD (Ala-Cys-Glu-Cys-Asp)	13.4 mM	7
Pacific cod	FF (Phe-Phe)	0.52 mM	8
	YF (Tyr-Phe)	0.80 mM	
	WPW (Trp-Pro-Trp)	1.68 mM	
	WPDARG (Trp-Pro-Asp-Ala-Arg-Gly)	0.40 mM	
	YNVTGW (Tyr-Asn-Val-Thr-Gly-Trp)	0.23 mM	
Trachinotus ovatus	FPAW (Phe-Pro-Ala-Trp)	3.81 ± 0.18 mM	9
	LLPW (Leu-Leu-Pro-Trp)	4.17 ± 0.12 mM	
Ostrea rivularis Gould	ALSGSW (Ala-Leu-Ser-Gly-Ser-Trp)	2.17 ± 0.09 mM	10
	GGYGIF (Gly-Gly-Tyr-Gly-Ile-Phe)	4.28 ± 0.43 mM	
	MAIGLW (Met-Ala-Ile-Gly-Leu-Trp)	3.48 ± 0.09 mM	

Continue Table S2

Sources	Peptide sequence	IC_{50}	Reference
Pacific White Shrimp and Swimming Crab	AEAQMWR (Ala-Glu-Ala-Gln-Met-Trp-Arg)	8.85 ± 0.05 mM	[11]
Pacific white shrimp	AGDY (Ala-Gly-Asp-Tyr)	21.82 ± 0.15 mM	[12]
	GDEY (Gly-Asp-Glu-Tyr)	20.67 ± 0.30 mM	
	YNITGW (Tyr-Asn-Ile-Thr-Gly-Trp)	9.78 ± 0.13 mM	
	PDARG (Pro-Asp-Ala-Arg-Gly)	35.01 ± 1.18 mM	
Whey protein	ALPM (Ala-Leu-Pro-Met)	7.23 ± 0.22 mM	[13]
	LWM (Leu-Trp-Met)	5.01 ± 0.31 mM	
	PEW (Pro-Glu-Trp)	3.46 ± 0.22 mM	[14]
	LLW (Leu-Leu-Trp)	3.02 ± 0.17 mM	
α-lactalbumin and colostrum casein	VYPFPGPI (Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile)	5.75 ± 0.12 mM	[15]
	GPVRGPFPIIV (Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val)	4.67 ± 0.24 mM	
	AVFPSIVGR (Ala-Val-Phe-Pro-Ser-Ile-Val-Gly-Arg)	5.04 ± 0.24 mM	
	YPWTQ (Tyr-Pro-Trp-Thr-Gln)	0.97 ± 0.03 mM	
	LITGLW (Leu-Ile-Thr-Gly-Leu-Trp)	1.09 ± 0.03 mM	
Egg	EEK (Glu-Glu-Lys)	0.14 mM	[16]
Pleurotus ostreatus	FCH (Phe-Cys-His)	0.90 mg/mL	[17]
Feather Keratin	GNQQVHLQSQDM	12.15 mg/mL	[18]
Hemoglobin	IVYPW (Ile-Val-Tyr-Pro-Trp)	0.63 ± 0.03 mM	[19]
	YPWTQ (Tyr-Pro-Trp-Thr-Gln)	0.97 ± 0.03 mM	
	LITGLW (Leu-Ile-Thr-Gly-Leu-Trp)	1.09 ± 0.03 mM	

References:

1 Q. Li, X. Kang, C. Shi, Y. Li, K. Majumder, Z. Ning, J. Ren, Moderation of hyperuricemia in rats via consuming walnut protein hydrolysate diet and identification of new antihyperuricemic peptides, *Food Funct*, 2018, **9**, 107-116.

2 L. Zhao, X. Ai, F. Pan, N. Zhou, L. Zhao, S. Cai, X. Tang, Novel peptides with xanthine oxidase inhibitory activity identified from macadamia nuts: integrated in silico and in vitro analysis, *Eur Food Res Technol*, 2022, **248**, 2031-2042.

3 X. Chen, W. Guan, Y. Li, J. Zhang, L. Cai, Xanthine oxidase inhibitory peptides from *Larimichthys polyactis*: Characterization and *in vitro*/*in silico* evidence, *Foods*, 2023, **12**, 982.

4 Y. Li, X. Kang, Q. Li, et al., Anti-hyperuricemic peptides derived from bonito hydrolysates based on in vivo hyperuricemic model and in vitro xanthine oxidase inhibitory activity, *Peptides*, 2018, **107**, 45-53.

5 L. Wei, H. Ji, W. Song, et al., Identification and molecular docking of two novel peptides with xanthine oxidase inhibitory activity from Auxis thazard, *Food Sci Tech*, 2022, **42**, e106921.

6 W. He, G. Su, D. Sun-Waterhouse, G.I.N. Waterhouse, M. Zhao, Y. Liu, *In vivo* anti-hyperuricemic and xanthine oxidase inhibitory properties of tuna protein hydrolysates and its isolated fractions, *Food Chem*, 2019, **272**, 453-461.

7 H. Zhong, Abdullah, Y. Zhang, et al., Exploring the potential of novel xanthine oxidase inhibitory peptide (ACECD) derived from Skipjack tuna hydrolysates using affinity-ultrafiltration coupled with HPLC–MALDI-TOF/TOF-MS, *Food Chem*, 2021, **347**, 129068.

8 Q. Zhao, Y. Meng, J. Liu, Z. Hu, Y. Du, J. Sun, X. Mao, Separation, identification and docking analysis of xanthine oxidase inhibitory peptides from pacific cod bone-flesh mixture, *LWT*, 2022, **167**, 113862.

9 M.F. Hou, H. Xiang, X. Hu, S.J. Chen, Y.Y. Wu, J.C. Xu, X.Q. Yang, Novel potential XOD inhibitory peptides derived from *Trachinotus ovatus*: Isolation, identification and structure-

function analysis, *Food Biosci*, 2022, **47**.

10 Q. Zhao, X. Jiang, Z. Mao, J. Zhang, J. Sun, X. Mao, Exploration, sequence optimization and mechanism analysis of novel xanthine oxidase inhibitory peptide from *Ostrea rivularis* Gould, *Food Chem*, 2023, **404**, 134537.

11 Z. Mao, H. Jiang, X. Mao, Identification and anti-hyperuricemic activity of xanthine oxidase inhibitory peptides from pacific white shrimp and swimming crab based on molecular docking screening, *J Agr Food Chem*, 2023, **71**, 1620-1627.

12 Z. Mao, H. Jiang, J. Sun, X. Mao, Virtual screening and structure optimization of xanthine oxidase inhibitory peptides from whole protein sequences of Pacific white shrimp via molecular docking, *Food Chem*, 2023, **429**, 136837.

13 X. Qi, H. Chen, K. Guan, Y. Sun, R. Wang, Q. Li, Y. Ma, Novel xanthine oxidase inhibitory peptides derived from whey protein: identification, in vitro inhibition mechanism and in vivo activity validation, *Bioorg Chem*, 2022, **128**, 106097.

14 X. Qi, H. Chen, K. Guan, Y. Sun, R. Wang, Y. Ma, Identification, inhibitory mechanism and transepithelial transport of xanthine oxidase inhibitory peptides from whey protein after simulated gastrointestinal digestion and intestinal absorption, *Food Res Int*, 2022, **162**, 111959.

15 Y.F. Gao, M.Q. Liu, Z.H. Li, et al., Purification and identification of xanthine oxidase inhibitory peptides from enzymatic hydrolysate of α-lactalbumin and bovine colostrum casein, *Food Res Int*, 2023, **169**, 112882.

16 Z. Yu, Y. Cao, R. Kan, et al., Identification of egg protein-derived peptides as xanthine oxidase inhibitors: virtual hydrolysis, molecular docking, and in vitro activity evaluation, *Food Sci Human Well*, 2022, **11**, 1591-1597.

17 I.T. Jang, S.H. Hyun, J.W. Shin, Y.H. Lee, J.H. Ji, J.S. Lee, Characterization of an anti-gout xanthine oxidase inhibitor from *Pleurotus ostreatus*, *Mycobiology*, 2014, **42**, 296-300.

18 X.D. Pei, F. Li, Y.M. Zhang, et al., Preparation, purification, and identification of novel feather keratin-derived peptides with antioxidative and xanthine oxidase inhibitory activities, *J Agr Food Chem*, 2023, **71**, 8061-8070.

19 P. Zhang, Z. Jiang, J. Lei, Q. Yan, C. Chang, Novel hemoglobin-derived xanthine oxidase inhibitory peptides: Enzymatic preparation and inhibition mechanisms, *J Funct Foods*, 2023, **102**, 105459.

Table S3. Stability of the internal standard in QC samples.

Compounds	Q1 (Da)	RT (min)	CV	CAS	Concentration (mg/L)	Brand
L-Leucine-D7	139.1464	1.14	0.028275	92751-17-2	1	isoreag
L-Tryptophan-d5	210.1291	2.4	0.024499	62595-11-3	0.5	isoreag
Hexanoic Acid-D11	126.145	5.12	0.068963	95348-44-0	1	isoreag
Octanoic acid-13C	144.1106	6.46	0.032438	200-813-2	1	isoreag
benzoic acid d5	126.0614	4.43	0.015013	1079-02-3	1	sigma
caffeine 13C3	198.0982	2.99	0.029074	58-08-2	0.2	Chembind LLC
2-Amino-3-(2-Chlorophenyl) Propanoic Acid	198.0322	2.48	0.025515	103616-89-3	0.2	J&K Scientific
(S)-4-Fluorophenylglycine	170.0617	1.2	0.014454	19883-57-9	1	TCI

Table S4. Differential metabolites between CK and MD.

Compound	KEGG_ID	Fold Change	Log_2 (FC)	P value	VIP
Ala-Pro	C00107	9.861906717	3.301866607	5.50189E-11	2.784896394
Orotic Acid	C00295	4.766573092	2.252952419	4.06596E-05	2.571813748
Inositol 1-phosphate	-	1.845194925	0.883773229	0.000161173	2.465658409
Uracil 5-carboxylic acid	C03030	8.748324936	3.129006807	0.000171837	2.571439336
Gly-Gly-Phe	C00098	0.624733159	-0.67868799	0.000531405	2.359597735
5-hydroxyindole-2-carboxylic acid	-	0.663050985	-0.592808286	0.00200703	2.222379352
7-ketodeoxycholic acid	C04643	32.4165471	5.018658523	0.002933944	2.411885588
Nicotinuric Acid	C05380	0.624668883	-0.678836429	0.003813001	2.147933103
Bovinic acid	C04056	0.373866542	-1.419404728	0.005524117	2.232425639
9,10-Dihydroxyoctadec-12-enoic acid (9,10-DiHOME)	C14828	1.519740449	0.603824952	0.007200387	2.069783004
12,13-DiHOME	-	1.519740449	0.603824952	0.007200387	2.069783004
9,10-Epoxy-18-hydroxyoctadecanoic acid	-	1.519740449	0.603824952	0.007200387	2.069783004
Carnosic acid	C21818	0.099305854	-3.331977422	0.008015601	2.230429532
Ureidosuccinic acid	C00438	2.770083871	1.469929658	0.008121054	2.146182715
Uric acid	C00366	2.25823944	1.175198463	0.008396824	2.002986312
11-Cis-Retinol	C00899	0.282109176	-1.825674505	0.008803528	2.185767622
(R)-2-Hydroxybutyric acid	C05984	1.643575552	0.716837776	0.009068881	2.11162858
2-Methyllactic acid	C21297	1.643575552	0.716837776	0.009068881	2.11162858
2-Pentyl-3-phenyl-2-propenal	C12288	0.58297578	-0.778492147	0.014471274	1.907708765
Lysophosphatidylcholine (17:0) (LPC (17:0/0:0))	C04230	0.635572931	-0.653870413	0.029969296	1.831160243
LPC (0:0/17:0)	C04230	0.635572931	-0.653870413	0.029969296	1.831160243
LPC (O-20:1)	C04230	11.9194885	3.575250421	0.033778355	1.875200246
Histamine	C00388	0.601894752	-0.732416857	0.034832623	1.733000499
Phosphatidylethanolamine lyso alkenyl 18:3	C04438	0.650765035	-0.619791358	0.034917645	1.784018017
Xanthosine	C01762	1.683368955	0.751351416	0.036985999	1.682373577
3-N-Methyl-L-Histidine	C01152	1.525214139	0.60901181	0.046057736	1.666825877

Table S5. Differential metabolites between SCPH and MD.

Compound	KEGG_ID	Fold Change	Log_2 (FC)	P value	VIP
Dl-Glyceraldehyde 3-Phosphate	C00661	26.34720228	4.71957787	1.07681E-07	2.185530465
Phenoxyacetic acid	C02181	0.064182176	-3.961683493	1.35067E-05	2.148152898
Cyclo (Pro-Phe)	C11847	1.603083751	0.6808498	0.000105192	1.973488841
Cyclo (Phe-Pro)	C00107	1.603083751	0.6808498	0.000105192	1.973488841
3-(4-Hydroxyphenyl)-1-propanol	-	0.574793433	-0.798884516	0.000162344	1.921362314
Visnagin	C09049	2.156588672	1.108751037	0.000167767	1.935711867
9, 10-DiHOME	C14828	2.232380539	1.158582975	0.000223651	1.903923615
12, 13-DiHOME	-	2.232380539	1.158582975	0.000223651	1.903923615
9, 10-Epoxy-18-hydroxyoctadecanoic acid	-	2.232380539	1.158582975	0.000223651	1.903923615
Free fatty acids (15:1) (FFA (15:1))	C00162	0.339041143	-1.56046774	0.000297914	1.894316494
9, 10-dihydroxystearic acid	C19622	1.742663977	0.801294414	0.000426697	1.887493615
9(S)-HODE	C14767	2.441145903	1.287558525	0.000542296	1.855502054
Lysophosphatidylcholine (P-16:0) (LPE (P-16:0))	C04438	1.845761839	0.884216412	0.000961826	1.813442749
N'-Methyl-2-pyridone-5-carboxamide	C05842	0.532871272	-0.908141037	0.001060397	1.825061661
Ethylparaben	D01647	0.141759828	-2.818479333	0.001265453	1.812771978
Arg-Val	C00107	0.553214641	-0.854088756	0.001352743	1.810182187
2,4-Dihydroxyacetophenone 5-sulfate	-	0.448056777	-1.158246536	0.001474237	1.859054682
LPC (O-16:1)	C04230	2.02476261	1.017752771	0.001483015	1.861422187
4-Hydroxy-3-methylbenzoic acid	C21167	0.578967381	-0.788446026	0.00153975	1.768804362
2-hydroxyphenylacetic acid	C05852	0.578967381	-0.788446026	0.00153975	1.768804362
Palatinose	C01742	0.629805957	-0.667020692	0.001585215	1.81511244
9-oxoODE	C14766	2.392708249	1.258644495	0.001668635	1.76431364
Cyclo (gly-glu)	C00107	0.598996001	-0.739381723	0.0016852	1.755341437
(R)-(-)-Mandelic acid	C01983	0.500377201	-0.998912037	0.002125312	1.766195359
13(R)-HODE	-	2.511353488	1.328465111	0.0022571	1.72681051
LPE (0:0/16:1)	C04438	0.582860582	-0.778777256	0.002369244	1.726062778
LPE (16:1/0:0)	C04438	0.582860582	-0.778777256	0.002369244	1.726062778
N4-Acetylcytidine	C22293	0.390826891	-1.35539836	0.002393858	1.72570263
Pro-Asn	C00107	0.603686246	-0.728129162	0.002643819	1.713700395
Cyclo (Pro-Val)	C00107	1.554612695	0.636555202	0.002768817	1.718716153
LPC (O-18:0)	C04317	2.539684268	1.344649153	0.002988609	1.789989446
7-ketodeoxycholic acid	C04643	30.25556194	4.919128475	0.003221545	1.868208703
N-Acetylneuraminic Acid	C19910	0.580008524	-0.785853993	0.003590257	1.67817876
cis-9,10-Epoxyoctadecanoic Acid (cis-EODA)	C19418	4.298266077	2.103754793	0.003626175	1.726149661
LPC (O-18:2)	C04230	1.550207043	0.632460912	0.003661707	1.729834352
Proline-Hydroxyproline	C00107	0.633971786	-0.657509458	0.005067047	1.652526343
LPC (0:0/20:2)	C04230	1.984898846	0.989065487	0.005092961	1.716771658
LPC (20:2/0:0)	C04230	1.984898846	0.989065487	0.005092961	1.716771658

Continued Table S5.

Compound	KEGG_ID	Fold Change	Log_2 (FC)	P value	VIP
Biotin	C00120	0.512123604	-0.965436039	0.005153894	1.660963524
Leu-Met	C00107	1.655526603	0.727290194	0.00522895	1.670963187
Ile-Met	C00107	1.655526603	0.727290194	0.00522895	1.670963187
2-Furoylglycine	-	0.48006802	-1.05868926	0.005997194	1.643376254
Genipin	C09780	1.962416902	0.972631564	0.006268132	1.67449582
Pyrroloquinoline Quinone	C00113	1.814593056	0.859646043	0.006643478	1.675262991
N6-Isopentenyladenosine	D05726	1.750092242	0.807430964	0.006681263	1.602608903
2-Naphthol	C11713	0.573420504	-0.802334603	0.006711735	1.634355361
1-Naphthol	C11714	0.573420504	-0.802334603	0.006711735	1.634355361
LPE (P-18:1)	C04438	2.192248084	1.132411069	0.006939872	1.707862464
LPE (0:0/20:5)	C04438	0.63375889	-0.657994015	0.007088083	1.721620939
LPE (20:5/0:0)	C04438	0.63375889	-0.657994015	0.007088083	1.721620939
5-Hydroxy-2'-deoxyuridine	-	0.593105781	-0.753638662	0.007430508	1.585866861
Bovinic acid	C04056	3.306206496	1.725176834	0.007735361	1.644872465
Equol	C14131	3.575008571	1.837946701	0.008178228	1.651830322
2-Pentyl-3-phenyl-2-propenal	C12288	1.698374127	0.764154299	0.00819937	1.598317837
Cholic acid	C00695	2.214825894	1.147193294	0.008700765	1.56485208
Gamma-Mercholic Acid	C17649	2.214825894	1.147193294	0.008700765	1.56485208
3-Methylequuric acid	-	0.579892854	-0.786141735	0.008895684	1.61203895
4-Methylhippuric Acid	-	0.579892854	-0.786141735	0.008895684	1.61203895
Pro-Ile	C00107	2.381767056	1.25203232	0.010892654	1.568880221
Cyclo (Pro-Leu)	C00107	2.234974261	1.160258217	0.0111048	1.547653148
1-Methylguanosine	C04545	0.666278417	-0.585802934	0.011517976	1.576018479
2-Methylguanosine	-	0.666278417	-0.585802934	0.011517976	1.576018479
7-Methylguanosine	-	0.666278417	-0.585802934	0.011517976	1.576018479
Cyclo (pro-pro)	C00107	1.87525059	0.907083396	0.012421833	1.531990793
3-N-Methyl-L-Histidine	C01152	0.647874775	-0.626213108	0.012907718	1.535779126
γ-Glu-Met	C00107	1.578211509	0.658290566	0.015054668	1.500728909
FFA (18:1)	C00712	0.105224326	-3.248459826	0.018390118	1.609772575
Glycerol 3-Phosphate	C03189	1.500459852	0.585404717	0.018934413	1.475887215
Succinic anhydride	C19524	0.578815696	-0.788824051	0.019997149	1.518483353
LPC (17:0/0:0)	C04230	1.892150439	0.920026797	0.020331457	1.496788972
LPC (0:0/17:0)	C04230	1.892150439	0.920026797	0.020331457	1.496788972
Elenaic acid	-	0.665692977	-0.587071146	0.020932775	1.454826961
Dioctyl phthalate	-	3.144093623	1.652644178	0.021356187	1.454996601
Homovanillic Acid sulfate (sodium salt)	-	1.845130745	0.883723049	0.022463544	1.489949954
Cytochalasin H	-	0.573262496	-0.802732198	0.022575066	1.478285784
2-Aminoethanesulfinic Acid	C00519	1.923362065	0.94363037	0.022687166	1.445138429
Phenylacetyl-L-Glutamine	C04148	0.617358507	-0.695819572	0.023912844	1.405924814
Hyodeoxycholic acid	C15517	2.349221763	1.232182908	0.025352843	1.432349792
alpha-Muricholic acid	C17647	2.977534321	1.574118137	0.025933636	1.432055976

Continued Table S5.

Compound	KEGG_ID	Fold Change	Log_2 (FC)	P value	VIP
beta-Muricholic acid	C17726	2.977534321	1.574118137	0.025933636	1.432055976
2,4-Di-tert-butylphenol	-	0.552680073	-0.855483497	0.027200423	1.383914917
4-tert-Octylphenol	C14205	0.552680073	-0.855483497	0.027200423	1.383914917
Scopolamine-methyl-bromide	-	1.566796684	0.64781798	0.028380677	1.413171966
p-Cresol glucuronide	-	0.094572412	-3.402436797	0.030380941	1.49043582
Carnitine C22:3	C02301	1.586850227	0.666165967	0.034297183	1.354083066
Cyclo (his-pro)	C00107	0.428604327	-1.22228168	0.03435057	1.417145001
DL-3-Phenyllactic acid	C01479	1.630521319	0.705333304	0.036129798	1.330840465
Tropate	C01456	1.630521319	0.705333304	0.036129798	1.330840465
1-Aminocyclobutanecarboxylic acid	-	1.562361512	0.643728315	0.038493831	1.334287866
L-Proline	C00148	1.562361512	0.643728315	0.038493831	1.334287866
LPC (16:4/0:0)	C04230	13.91273525	3.798334177	0.038654548	1.462168979
Carnitine C22:4	C02301	1.520787307	0.604818396	0.038794266	1.322689503
Gly-Phe	C00107	4.41437327	2.142208626	0.040550119	1.386215201
Phe-Gly	C00107	4.41437327	2.142208626	0.040550119	1.386215201
Corticosterone	C02140	0.352332862	-1.504989054	0.042313525	1.339300494
21-Deoxycortisol	C05497	0.352332862	-1.504989054	0.042313525	1.339300494
Ureidosuccinic acid	C00438	0.562295753	-0.830598945	0.043020493	1.308375223
3-Guanidinopropionic acid	C03065	1.920572776	0.941536632	0.044412176	1.526372272
Glu-Val-Phe	C00098	1.786344988	0.837010728	0.04737626	1.296524449
Cyclo (leu-phe)	C20519	3.153824658	1.657102453	0.047422069	1.268790194
Dl-3, 4-Dihydroxymandelic Acid	C05580	2.038582849	1.02756659	0.048610623	1.279659907

Table S6. Differential metabolites between LGDF_H and MD.

Compound	KEGG_ID	Fold Change	Log_2 (FC)	P value	VIP
Hypotaurocyamine	C02419	0.128292524	-2.962490992	6.34E-06	2.227440725
Phenoxyacetic acid	C02181	0.067979004	-3.87876697	6.24645E-05	2.181795345
LPC (O-16:1)	C04230	2.38281921	1.252669495	0.000355697	1.925948803
2-Pentyl-3-phenyl-2-propenal	C12288	2.305720555	1.205217674	0.000383571	1.920293963
9,10-dihydroxystearic acid	C19622	1.970371727	0.978467832	0.000518613	1.911369304
Cinnabarinic acid	C05640	70.34810917	6.136439742	0.000935028	2.096072544
9, 10-DiHOME	C14828	1.930588365	0.94904059	0.001264297	1.831499052
12, 13-DiHOME	-	1.930588365	0.94904059	0.001264297	1.831499052
9, 10-Epoxy-18-hydroxyoctadecanoic acid	-	1.930588365	0.94904059	0.001264297	1.831499052
9(S)-HODE	C14767	2.773894379	1.471912855	0.001287344	1.8753609
LPE (P-16:0)	C04438	1.903561829	0.92870143	0.001366753	1.832840099
13(R)-HODE	-	2.754631672	1.461859426	0.001380318	1.826017735
Ethylparaben	D01647	0.171113082	-2.54697803	0.001559525	1.893753783
2-Methylhippuric acid	C01586	0.621985288	-0.685047639	0.001649685	1.800375442
N-Phenylacetylglycine	C05598	0.621985288	-0.685047639	0.001649685	1.800375442
LPC (O-18:0)	C04317	2.704050276	1.435121976	0.001679985	1.836377235
LPC (O-18:3)	C04230	1.608348506	0.685580052	0.00179398	1.935383673
LPE (0:0/20:5)	C04438	0.524808513	-0.930136971	0.001992984	1.818656922
LPE (20:5/0:0)	C04438	0.524808513	-0.930136971	0.001992984	1.818656922
4-Hydroxy-3-methylbenzoic acid	C21167	0.437963267	-1.191118223	0.002152394	1.837708569
2-hydroxyphenylacetic acid	C05852	0.437963267	-1.191118223	0.002152394	1.837708569
Leu-Leu-Leu	C00098	0.651597322	-0.61794742	0.00260684	1.865315032
LPC (0:0/20:2)	C04230	2.188958164	1.130244381	0.002820635	1.868439401
LPC (20:2/0:0)	C04230	2.188958164	1.130244381	0.002820635	1.868439401
p-Cresol glucuronide	-	0.175889655	-2.507257461	0.003151228	1.881328695
3-Guanidinopropionic acid	C03065	3.422789689	1.775172649	0.003477732	1.963883149
LPC (17:0/0:0)	C04230	2.406963683	1.267214374	0.003568455	1.772418346
LPC (0:0/17:0)	C04230	2.406963683	1.267214374	0.003568455	1.772418346
Pantothenol	C05944	0.084473915	-3.565350278	0.003841913	1.806374804
Pyrroloquinoline Quinone	C00113	2.11097063	1.077906507	0.004112483	1.71458065
(10E,12Z)-9-oxooctadeca-10,12-dienoic acid (9-oxoODE)	C14766	2.20952266	1.143734727	0.005671604	1.705606493
3-(4-Hydroxyphenyl)-1-propanol	-	0.457160787	-1.129226433	0.007035934	1.739295931
LPE (P-18:1)	C04438	2.153286055	1.106539988	0.007077657	1.690873389
LPE (0:0/15:0)	C04438	1.529223882	0.612799636	0.008189725	1.812496337
LPC (O-18:2)	C04230	1.601959099	0.679837313	0.008384241	1.627009996
gamma-Glu-Ala	C03740	1.52330943	0.607209027	0.008488554	1.64489861
LPC (O-14:1)	C04230	1.69321382	0.759764169	0.009025508	1.655145486
3-Methylequuric acid	-	0.55451227	-0.850708711	0.010050176	1.59230122
4-Methylhippuric Acid	-	0.55451227	-0.850708711	0.010050176	1.59230122
Polycarbonate (8:0/8:0) (PC (8:0/8:0))	C00157	1.746919657	0.804813258	0.010144471	1.593277889

Continued Table S6.

Compound	KEGG_ID	Fold Change	Log_2 (FC)	P value	VIP
4-Hexylaniline	-	1.681752917	0.74996576	0.01066059	1.598603291
Bovinic acid	C04056	3.090203268	1.627701739	0.011134136	1.650119838
Met-Glu	C00107	1.741578938	0.800395865	0.011625202	1.573904232
LPE (22:3/0:0)	C04438	0.584490704	-0.774748015	0.01364695	1.563149404
Quinic acid	C00296	0.657136146	-0.605735795	0.014114784	1.562577859
(±)17-HDHA	-	2.195105517	1.134290291	0.014454491	1.535913987
Carnitine C22:3	C02301	1.533272819	0.616614422	0.015723707	1.522660145
N6, N6, N6-Trimethyl-L-lysine	C03793	0.620558466	-0.688360955	0.017916123	1.5134899
2-Phenylglycine	-	0.621837203	-0.685391162	0.017998556	1.511910703
Pinolenic acid	-	0.543981993	-0.8783692	0.018459564	1.545844341
FFA (18:3)	C06426	0.543981993	-0.8783692	0.018459564	1.545844341
N-Acetylglucosamine 1-Phosphate	C04256	0.542317907	-0.882789287	0.018983541	1.538187498
5'-Deoxy-5'-(Methylthio) Adenosine	C00170	0.51827735	-0.94820375	0.01910734	1.597429259
2,4-Dihydroxyacetophenone 5-sulfate	-	0.467194719	-1.09790413	0.01987266	1.516846077
Pro-Ile	C00107	2.269487023	1.182366239	0.020089627	1.513612713
Glu-Met	C00107	1.667064126	0.737309601	0.02050197	1.486887312
γ-Glu-Met	C00107	1.76695302	0.821263682	0.022180121	1.479310607
Cholic acid	C00695	2.04587878	1.032720667	0.02319946	1.479626361
Gamma-Mercholic Acid	C17649	2.04587878	1.032720667	0.02319946	1.479626361
Ubiquinone-1	C00399	1.927260598	0.946551661	0.027502049	1.459674531
N'-Methyl-2-pyridone-5-carboxamide	C05842	0.528223206	-0.920780411	0.028233492	1.474269782
Allantoin	C01551	0.549904638	-0.862746641	0.02847004	1.492556695
Ribosyladenosine	-	0.591251354	-0.758156513	0.028698853	1.465220186
Xanthine	C00385	0.375398168	-1.413506487	0.029009502	1.414658672
4-acetoxyphenol	C13636	0.375398168	-1.413506487	0.029009502	1.414658672
FFA (12:0)	C02679	0.647376946	-0.627322104	0.029162566	1.450534259
LPC (18:0/0:0)	C04230	1.649971437	0.72244105	0.029853567	1.446136155
Pro-Asn	C00107	0.623321673	-0.681951217	0.030300645	1.443879593
LPE (0:0/16:1)	C04438	0.635103962	-0.654935325	0.030735895	1.418899142
LPE (16:1/0:0)	C04438	0.635103962	-0.654935325	0.030735895	1.418899142
7-ketodeoxycholic acid	C04643	6.170709327	2.625436338	0.030992743	1.396165175
3-(4-hydroxyphenyl) acrylaldehyde	C05608	0.583205734	-0.77792319	0.032526933	1.416967225
L-Theanine	C01047	1.887245458	0.916282074	0.033472718	1.444097665
N-Alpha-Acetyl-L-Asparagine	-	1.887245458	0.916282074	0.033472718	1.444097665
Hyodeoxycholic acid	C15517	2.219793667	1.150425582	0.033907591	1.441647522
Glycerol 3-Phosphate	C03189	1.567316215	0.648296281	0.034017308	1.439663583
Chrysophanol	C10315	2.232093253	1.158397301	0.037271789	1.391301119
Leu-Leu-Gly	C00098	0.615059363	-0.701202436	0.039136605	1.369976581

Continued Table S6.

Compound	KEGG_ID	Fold Change	Log_2 (FC)	P value	VIP
Hispidol	C08644	2.170790742	1.118220661	0.039315657	1.375698452
N4-Acetylcytidine	C22293	0.519887986	-0.943727279	0.040186136	1.372296006
Cyclo (Pro-Leu)	C00107	2.077412168	1.054787482	0.041890494	1.381997088
Proline-Hydroxyproline	C00107	0.616113179	-0.698732699	0.04454706	1.364296855
Arg-Val	C00107	0.59041879	-0.760189458	0.044712529	1.444066669
Hexaethylene-glycol	-	0.452870441	-1.142829719	0.046162696	1.404058285
(9Z,11E,15Z)-13-hydroxyoctadeca-9,11,15-trienoic acid (13-HOTrE)	-	1.908267632	0.932263522	0.047685576	1.316927736
Glu-Val-Phe	C00098	1.689044476	0.756207318	0.048005867	1.30824753
FFA (14:1)	C08322	0.577274174	-0.792671412	0.049084448	1.295667502
Visnagin	C09049	1.581843703	0.661607059	0.049848601	1.355259569

Table S7. Overall PERMANOVA results.

Distance	Bray-Curtis
Groups_tested	CK, MD, SCPH_H, LGDF_H
N_total	20
Pseudo-F	1.494645
R2	0.2189
P_value	0.0052
df_between	3
df_within	16
SS_between	0.93555
SS_within	3.338318
SS_total	4.273868

Table S8. Pairwise PERMANOVA results.

Group1	**Group2**	**n1**	**n2**	**Pseudo-F**	**R2**	**P_value**	**FDR_BH**
CK	LGDF_H	5	5	1.721252	0.177061	0.0318	0.0477
CK	MD	5	5	1.440232	0.152563	0.0091	0.0273
CK	SCPH_H	5	5	2.525188	0.239919	0.0091	0.0273
LGDF_H	MD	5	5	0.944606	0.105606	0.4467	0.4467
LGDF_H	SCPH_H	5	5	1.083939	0.119325	0.3513	0.42156
MD	SCPH_H	5	5	1.523267	0.159952	0.0176	0.0352

Table S9. Bray–curtis distance results.

Group	**mean**	**std**	**median**	**min**	**max**	**count**
CK	0.571892	0.075633	0.566111	0.403794	0.678542	10
LGDF_H	0.657567	0.099208	0.666227	0.405647	0.739882	10
MD	0.724133	0.114577	0.773647	0.515019	0.865122	10
SCPH_H	0.596013	0.068971	0.613219	0.499908	0.680876	10