Enrichment of polymethoxyflavones from citrus fruit using an optimized enzyme/acid-catalyzed hybrid hydrolysis process and their influence on mice gut microbiota

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Table S1. Factors and levels of Plackett-Burman design (PBD)

Factors	Code	Low (-1)	High (+1)
Material-liquid ratio (mL/mL)	X_1	1:2	1:3
Enzyme concentration (%)	X_2	5	9
Enzymatic hydrolysis time (h)	X_3	12	24
Acid concentration (mol/L)	X_4	0.2	0.3
Acid hydrolysis time (h)	X_5	9	11

Table S2. Factors and levels of Box-Behnken design (BBD)

Factors	Code	Level		
		-1	0	1
Enzyme concentration (%)	X_2	5	7	9
Enzymatic hydrolysis time (h)	X_3	12	18	24
Acid hydrolysis time (h)	X_5	9	10	11

Table S3. Experimental design and respond values of Plackett-Burman.

Run	X_1 （mL/mL）	X_2 （%）	X_3 （h）	X_4 （mol/L）	X_5 （h）	Yield (mg/60 mL sample)
1	1:2	9	24	0.3	9	3.7554
2	1:3	9	24	0.2	9	2.8654
3	1:3	5	12	0.2	11	3.7887
4	1:2	9	12	0.3	11	4.9631
5	1:3	9	12	0.2	9	4.3339
6	1:3	9	12	0.3	11	4.4144
7	1:3	5	24	0.3	9	2.0075
8	1:2	9	24	0.2	11	4.6899
9	1:2	5	12	0.3	9	3.0870
10	1:2	5	12	0.2	9	2.8792
11	1:2	5	24	0.2	11	3.2071
12	1:3	5	24	0.3	11	2.9986

X_1 - material-liquid ratio, X_2 - enzyme concentration, X_3 - enzymatic hydrolysis time, X_4 - acid concentration, and X_5 - acid hydrolysis time.

Table S4. The evaluation of variance for the total yield of nobiletin and tangeretin using PBD.

Factors	Mean squares	F value	P value	Significance
Model	6.0700	8.47	0.0108	*
X_1	0.1816	1.27	0.3035	
X_2	3.3700	23.50	0.0029	**
X_3	0.8772	6.12	0.0482	*
X_4	0.0021	0.0149	0.9070	
X_5	1.6400	11.44	0.0148	*

X_1 - material-liquid ratio, X_2 - enzyme concentration, X_3 - enzymatic hydrolysis time, X_4 - acid concentration, and X_5 - acid hydrolysis time.

Table S5. Characterization of six PMFs in PMFs-rich extracts by UPLC-ESI-MS/MS in positive ion mode.

No	RT (min)	$[M+H]^+$ (*m/z*)	MS^n（*m/z*）	CE/V	Formula	Compound name
1	5.684	373.1	343.1, 315.2, 356.8, 327.5, 181.3	44	$C_{20}H_{20}O_7$	5,7,8,3',4'-pentamethoxyflavone (isosinensetin)
2	5.903	373.1	343.1, 356.8, 312.2, 329.2, 297.3	44	$C_{20}H_{20}O_7$	5,6,7,3',4'-pentamethoxyflavone (sinensetin)
3	6.829	343.0	313.1, 285.4, 326.9, 153.1	46	$C_{19}H_{18}O_6$	5,7,8,4'-tetramethoxyflavone (6-demethoxytangeretin)
4	7.169	403.1	373.1, 326.9, 345.2, 373.1, 211.1	46	$C_{21}H_{22}O_8$	5,6,7,8,3',4'-hexamethoxyflavone (nobiletin)
5	7.642	433.0	403.1, 417.9, 385.1, 375.2, 344.9	46	$C_{22}H_{24}O_9$	3,5,6,7,8,3',4'-heptamethoxyflavone (3-methoxynobiletin)
6	8.573	373.1	343.1, 297.2, 327.9, 211.2	44	$C_{20}H_{20}O_7$	5,6,7,8,4'-pentamethoxyflavone (tangeretin)

Table S6. Standard compounds used for Quantitative analysis of PMFs in PMFs-rich extracts using HPLC-DAD.

Standards	RT (min)	Standard curve	R^2	Linear range (μg/mL)	Precision (RSD) n=6 (%)	Stability (RSD) intraday n=6 (%)	Repeatability (RSD) n = 6 (%)	Recovery (%)
isosinensetin	15.816	y=4.01e+004x-2.49e+003	0.9990	0.10-5.00	1.77	0.85	1.40	97.23
sinensetin	16.380	y=3.45e+004x-7.50e+003	0.9993	1.00-12.50	0.45	0.80	0.87	96.59
6-demethoxytangeretin	18.393	y=2.52e+004x+3.40e+003	0.9996	0.10-4.00	0.87	1.95	1.78	90.21
nobiletin	19.255	y=7.64e+003x+1.68e+004	0.9995	5.00-200.00	1.18	1.47	1.32	98.98
3-methoxynobiletin	19.937	y=4.20e+004x-5.93e+003	0.9995	0.25-5.00	1.18	0.80	1.46	93.48
tangeretin	22.105	y=2.96e+004x-8.78e+004	0.9989	2.50-80.00	0.36	0.40	1.32	99.15

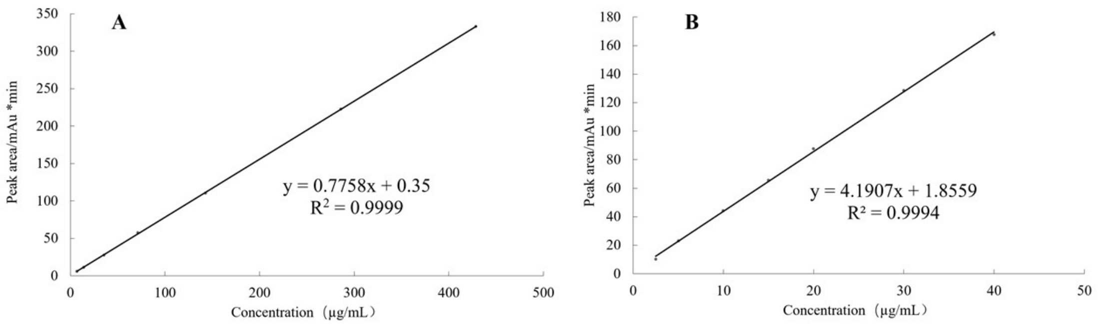

Fig. S1. Standard curve, R^2 and equation for (A) nobiletin and (B) tangeretin analysis. The analysis was performed on an AQ-C18 column (4.6 × 250 mm, 5 µm) using LC-2030C 3D plus HPLC system. The mobile phase was consisted of 0.1% formic acid–water (A) and methanol (B). The gradient elution program was carried out as follows: 0-10 min, 10%-75% B; 10-30 min, 75%-100% B; 30-35 min, 100% B; 35-45 min, 100%-10% B. The column temperature, the injection volume, the flow rate and the detection wavelength were set at 35 °C, 10 µL, 1.0 mL/min and 254 nm, respectively.

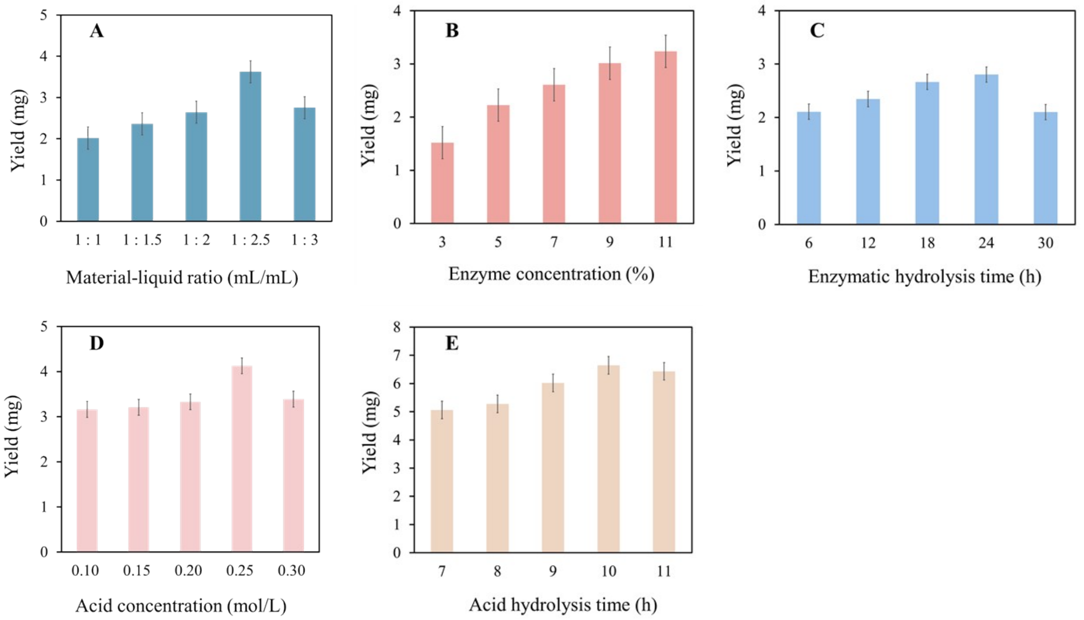

Fig. S2. Effects of extraction parameters on the total yield of nobiletin and tangeretin in Yidu honey orange model. (A) Material-liquid ratio, (B) enzyme concentration, (C) enzymatic hydrolysis time, (D) acid concentration and (E) acid hydrolysis time. The results were presented as mean values ± standard error of the means. All error bars showed standard deviations of the three replicates.

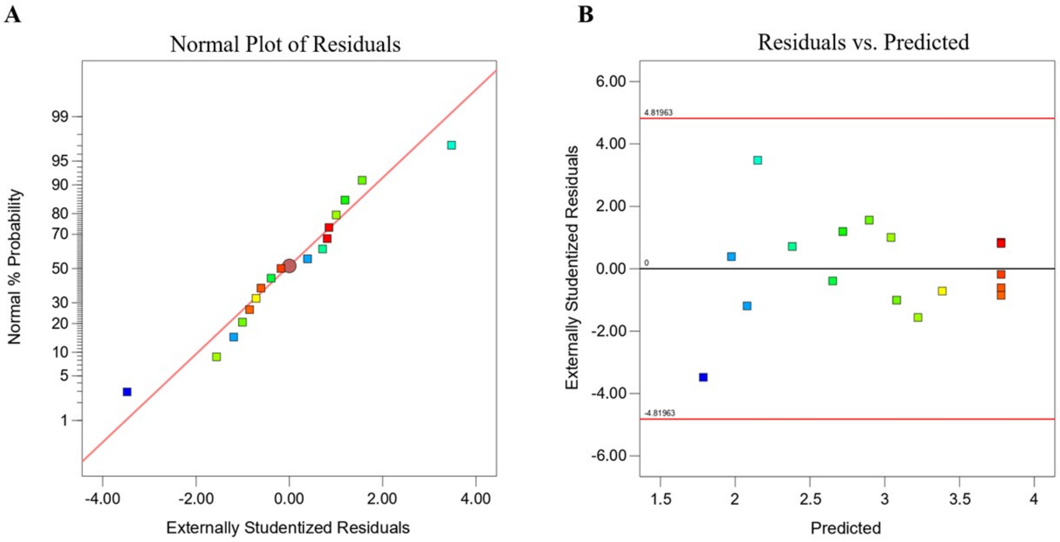

Fig. S3. (A) Normal plot of residuals. (B) Studentized residuals and the predicted response plot.

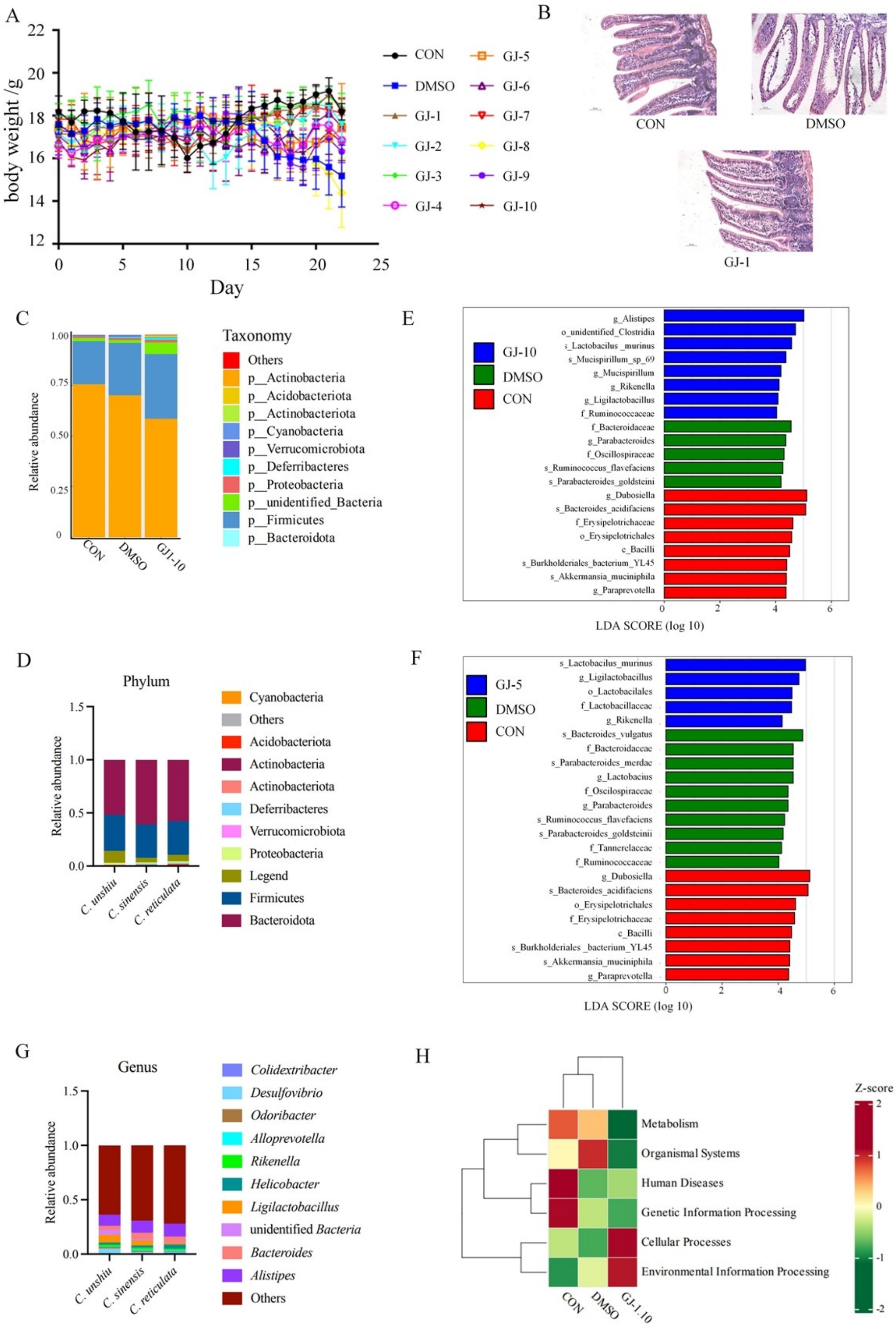

Fig. S4. *In vivo* exanimation of PMFs-extracts. (A) Body weight. (B) H&E staining of CON, DMSO and GJ-1 groups, 200×. (C) Analysis of fecal microbiota at the phylum level of CON, DMSO and GJ-1 to GJ-10 groups. (D) Analysis of fecal microbiota at the phylum level of *C. unshiu* (GJ-1), *C. sinensis* (GJ-4, GJ-5, GJ-6, GJ-7, and GJ-8), and *C. reticulata* (GJ-2, GJ-3, GJ-9, and GJ-10). (E) LEfSe analysis of CON, DMSO and GJ-10. (F) LEfSe analysis of CON, DMSO and GJ-5. (G) Analysis of fecal microbiota at the genus level of *C. unshiu* (GJ-1), *C. sinensis* (GJ-4, GJ-5, GJ-6, GJ-7, and GJ-8), and *C. reticulata* (GJ-2, GJ-3, GJ-9, and GJ-10). (H) Tax4Fun analysis in Level 1.

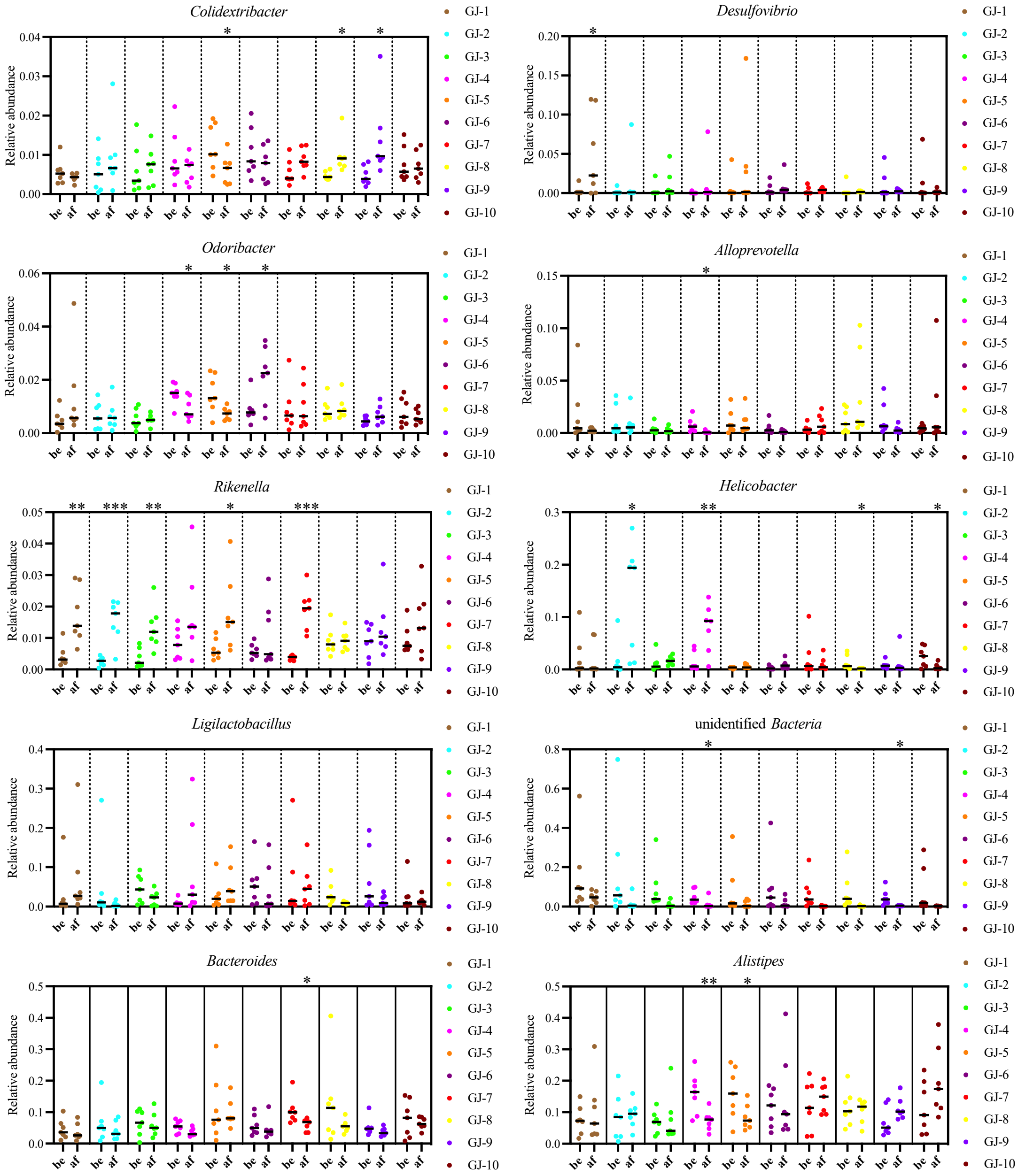

Fig. S5. Relative abundance of main gut bacteria between PMFs-groups at the genus level. be: the gut microbiota before 22-day administration; af: the gut microbiota after 22-day administration. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.