Inhibiting *in vitro* colonic fermentation of cooked gluten by dietary fibers with individual fermentability

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Section 1

1.1 *In vitro* digestion of gluten-dietary fiber mixtures

The simulated digestion experiments were carried on a pH–stat model (907 Titrino, Metrohm, Switzerland) described previously [1, 2]. A 5 mL of simulated gastric-digestive juice (SGJ) was prepared containing 35 mmol/L NaCl, 2.5 mM $CaCl_2$, and pepsin (4 units/mg of protein) and then it was adjusted to pH 3.0 using 1 mol/L HCl. A 5 mL of simulated intestinal-digestive juice (SIJ) was prepared containing 7.4 mmol/L bile salts, 46.0 mmol/L NaCl 7.6 mmol/L $CaCl_2$, 20.3 mmol/L Tris, trypsin (40 units/mg of protein) and then it was adjusted to pH 7.0 using 1 mol/L NaOH. Gluten (250 mg), gluten-alginate mixture (250 mg gluten + 250 mg and 500 mg alginate), gluten-cellulose mixture (250 mg gluten + 250 mg and 500 mg cellulose), and gluten-FOS mixture (250 mg gluten + 250 mg and 500 mg FOS) were dispersed in SGJ and the pH was kept at 3.0 with 1 mmol/L HCL, followed by addition of pepsin to an enzyme activity of 4 U/mg pepsin/gluten, and incubated for 2 h at 37 ℃. The reaction was stopped by heating for 5 min in a boiling water bath. After that, gastric-digested gluten was dispersed in SIJ and the pH was kept at 7.0 with 1 mmol/L NaOH, followed by addition of pancreatin to an enzyme activity of 40 U/mg pancreatin/gluten, and incubated for 2 h at 37 ℃. The reaction was stopped by heating for 5 min in a boiling water bath. Digested dispersions were centrifuged, and the supernatants were used to quantify the degree of hydrolysis (DH).

1.2 *In vitro* fecal fermentation procedure

First, stool samples from eight donors were collected. The donors were required to have no gastrointestinal diseases, probiotic use, or antibiotic use within the last 3 months. All the procedures were approved by the Research Ethics Committee of the Hubei University of Technology (Approval No.: HBUTLL20240051). To eliminate individual microbiota differences and to ensure that vital microbes were included, fresh fecal samples were instantly transferred to an anaerobic chamber (Long Yue LAI-3, Shanghai, China) constantly filled with 85 % N_2, 10 % H_2, and 5 % CO_2, where they were mixed in sterilized phosphate-buffered saline. Subsequently, ten grams of blended stool samples were

dispersed in saline at 2:8 w/v. The slurries were filtered with a filter bag divided into 5 mL aliquots in an anaerobic chamber and kept in the anaerobic chamber at 37 ℃ for subsequent usage.

The fermentation media was prepared according to the basal medium, and the ingredient was listed in **Table S1** [3,4]. A 50 mL fermentation broth comprised three contents: 10 mL fecal inoculum, 40 mL culture medium, and 500 mg (w/v) gluten in a gluten-dietary fiber mixture. A gluten-dietary fiber mixture contained 500 mg (w/v) gluten and 500 mg or 1 g dietary fiber. The fermentation broth of gluten only was set as the negative control, and the fermentation broth without any samples was set as the blank control. The fermentation profile of FOS, cellulose, and alginate including SCFA production was tested before the start of formal research. Fermentation was carried out in an anaerobic incubator vibrating at 120 r/min and 37 ℃.

A 50 mL fermentation broth comprised three components: 10 mL fecal inoculum, 40 mL culture medium, and 500 mg (w/v) gluten in a gluten-dietary fiber mixture. The gluten concentration was set at 1.20 g N/L, based on the nitrogen content in gluten (**Table S2**) and the ranges typically used in *in vitro* fermentation studies (0.32-2.40 g N/L) [5]. A gluten-dietary fiber mixture contained 500 mg (w/v) gluten and 500 mg or 1 g dietary fiber. Samples naming was explained in Fig. legends. The fermentation broth containing only gluten was used as the negative control, and the fermentation broth without any samples was used as the blank control. The fermentation profiles of FOS, cellulose, and alginate, including SCFA production, were tested prior to the commencement of formal research. Fermentation was carried out in an anaerobic incubator vibrating at 120 r/min and 37 ℃. Samples were collected after 0, 6, 12, 24, and 48 hours of incubation and were immediately frozen in liquid nitrogen for subsequent analytical testing. Samples were acquired after 0, 6, 12, 24, and 48 h of incubation and were immediately frozen in liquid nitrogen for analytical testing.

1.3 LC-MS method

Accurately weigh 20 mg of dry matter followed by adding 141 μL of water, 100 μL of 0.15% DOC, and 4 μL of 100 μg/mL of the three-phase mixture Standard solution (Lys-d4/Try-d5/Gln-d4).

After mixing, the mixtures were treated with an ultrasonic probe for 10 min (4 ℃, 40 kHz) followed by the addition of 5 μL 10 mol/L trichloroacetic acid (TCA) addition mixing well. The samples were settled for 10 min by freezing, and then were centrifuged at 14000 g at 4 ℃ for 10 min. Adding 375 μL of water into each of 25 μL of supernatant and then subjected to vortex mixing. The fine samples were passed through the 0.2 μm PTFE membrane (Biotage, Shanghai, China).

LC-ESI-MS/MS (UHPLC QTRAP, SCIEX, Washington DC, USA) was used for qualitative and quantitative detection of the target substance in the sample. The specific parameters are as follows: chromatographic conditions: ACQUITY UPLC BEH amide (2.1 × 100 mm, 1.7 μm), column temperature 40 ℃, and injection volume 1 μL; Mobile phase A (0.1% 10 mm formic acid-ammonium formate 95% aqueous solution) and mobile phase B (0.1% formic acid 10 mm ammonium formate 95% acetonitrile solution). Mass spectrometry conditions: SCIEX QTRAP 6500+, positive/negative mode detection; Curtain gas is 35 while Collision gas is medium; Ion-Spray voltage is +5500/-4500 and temperature is 550; Ion Source gas1 is 50, and Ion Source gas2 is 50. Scheduled MRM is basic, and the MRM detection window is 120 sec [6].

1.4 Gas chromatography method

The detection protocol was conducted as follows: an initial temperature of 40 ℃ was run for 5 min and increased to 120 ℃ at a rate of 20 ℃/min. A final temperature of 220 ℃ was reached at a rate of 10 ℃/min and was then run for 3 min. The carrier gas of helium flowed with a split ratio of 10/1 at a rate of 5 mL/min. The inlet temperature was 250 ℃, and the detecting temperature was 280 ℃ [7].

References:

1 M. Hu, Y. Wu, J. Wang, W. Lu, Z. Gao, L. Xu, S. Cui, Y. Fang, and K. Nishinari, Emulsions stabilization and lipid digestion profiles of sodium alginate microgels: Effect of the crosslink density. *Food Biophys.* **2021,** *16*, 346-354.

2 W. Wen, S. Li, Y. Gu, S. Wang, and J. Wang,. Effects of starch on the digestibility of gluten under different thermal processing conditions. *J. J. Agric. Food Chem.* **2019**, *67*, 7120-7127.

3 F. Rubino, C. Carberry, S. M. Waters, D. Kenny, M. S. McCabe, and C. J. Creevey, Divergent functional isoforms drive niche specialisation for nutrient acquisition and use in rumen microbiome. *The ISME. Journal,* **2017,** *11*, 932-944.

4 S. Pérez-Burillo, S. Molino, B. Navajas-Porras, Á. J. Valverde-Moya, D. Hinojosa-Nogueira, A. López-Maldonado, S. Pastoriza, and J. Á. Rufián-Henares, An *in vitro* batch fermentation protocol for studying the contribution of food to gut microbiota composition and functionality. *Nat. Protoc.* **2021,** *16*, 3186-3209.

5 M. Behounek, D. Cochran, H. A. Motta-Romero, Q. Yang, W. Ding, M. Morton, K. Majumder, R. Powers, and D. J. Rose, *In vitro* fermentation of animal and plant protein isolates by the human gut microbiota under high and low carbohydrate conditions. *Mol. Nutr. Food Res.* **2024,** *68*, 2300555.

6 S. Pérez-Burillo, S. Molino, B. Navajas-Porras, Á. J. Valverde-Moya, D. Hinojosa-Nogueira, A. López-Maldonado, S. Pastoriza, and J. Á. Rufián-Henares, An *in vitro* batch fermentation protocol for studying the contribution of food to gut microbiota composition and functionality. *Nat. Protoc.* **2021,** *16*, 3186-3209.

7 D. Yuan, W. Xiao, X. Tao, Z. Gao, Y. Wu, W. Jiang, Y. Li, X. Ni, and M. Zhou Formation of dimers delayed alginate degradation in fecal microbiota fermentation. *Carbohyd. Polym.* **2025,** *358*, 123524.

Section 2

Table S1. Protein content of each sample

Sample	Nitrogen proportion (%)	Weight (g)	Protein content (%)
Gluten	12.87	0.0143	80.48
Alginate	0.58	0.0114	3.62
Cellulose	0.73	0.0135	4.56
FOS	0.60	0.0122	3.75

Table S2. The composition of *in vitro* culture medium

Class	Compound	Content (per 1 L)
Nitrogen source	Yeast extracts	2.0 g
Nitrogen source	L-cysteine	0.50 g
Salts & Minerals	$MgSO_4$	0.01g
Salts & Minerals	NaCl	0.1g
Salts & Minerals	$CaCl_2$	0.01g
Salts & Minerals	$NaHCO_3$	1.0 g
Vitamins	Vitamin k_1	0.002 g
Buffer compounds	K_2HPO_4	0.04 g
Buffer compounds	KH_2PO_4	0.04 g
Others	Resazurin	0.001 g
Others	Hemin	0.05 g
Others	Bile acids	0.5 g
Others	Tween 80	2.0 mL

Table S3. Degree of gluten hydrolysis in each mixture

Sample	Serine-NH_2	h_f	DH (%)
Gluten	3.77	3.37	39.44
G+Alg-0.5	1.42	1.02	12.29
G+Alg-1	1.27	0.87	10.48
G+Cel-0.5	2.34	1.94	23.37
G+Cel-1	2.18	1.78	21.52
G+FOS-0.5	2.24	1.84	22.17
G+FOS-1	1.92	1.52	18.33

h_f = (serine-NH_2–β)/α; α=1; β=0.4; DH= h_f/8.3×100%

Raw SDS-PAGE

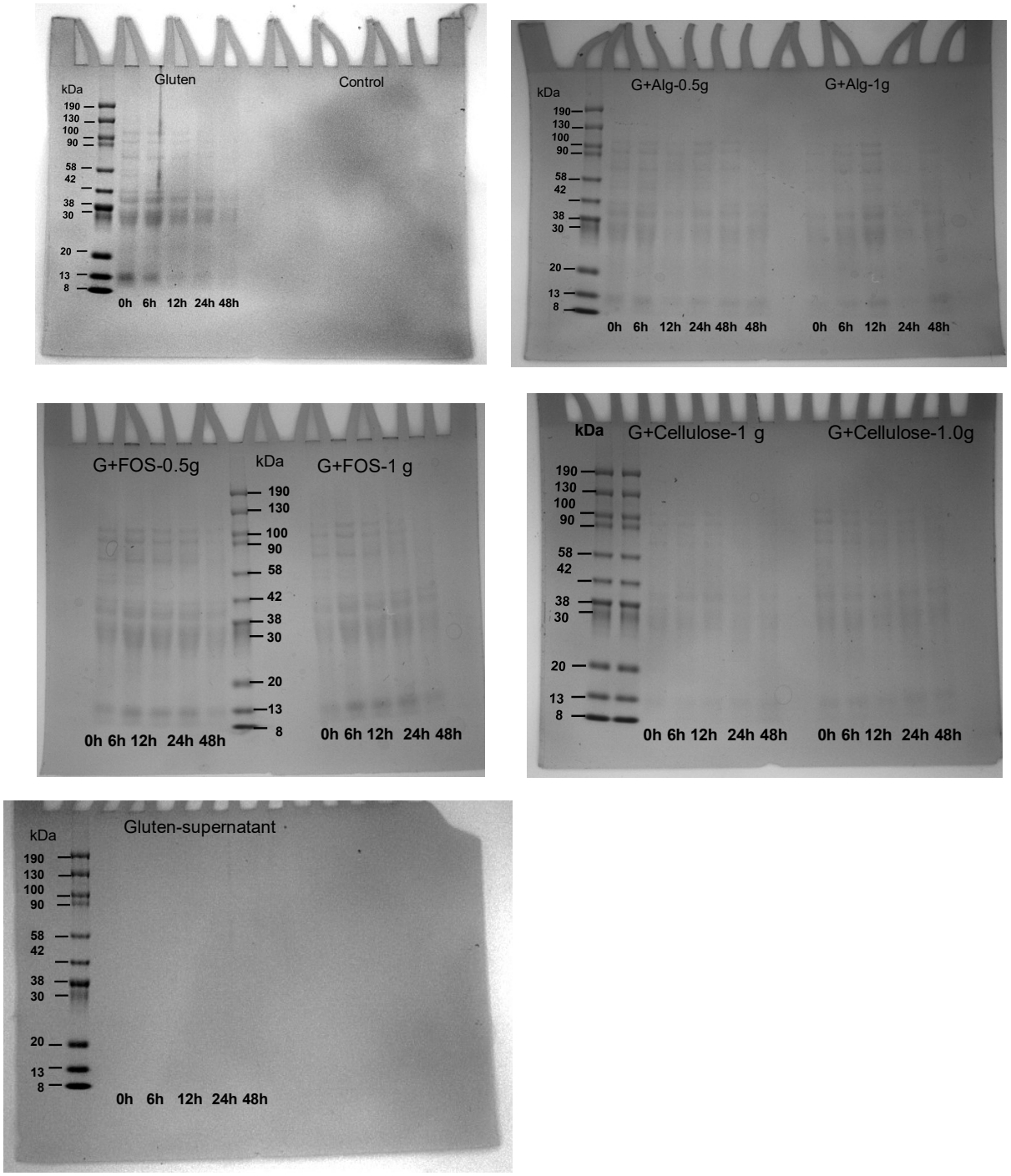

Figure S1. Raw DS-PAGE results, and gray value and molecular analysis are listed in **Supporting Information.xlsx**. There was no gluten stained for the supernatant of the gluten groups. Note: the gluten mixed with a type of dietary fiber were loaded in a gel page, and the gluten mixed with a type of dietary fiber at different time were compared with a same marker in the same page.

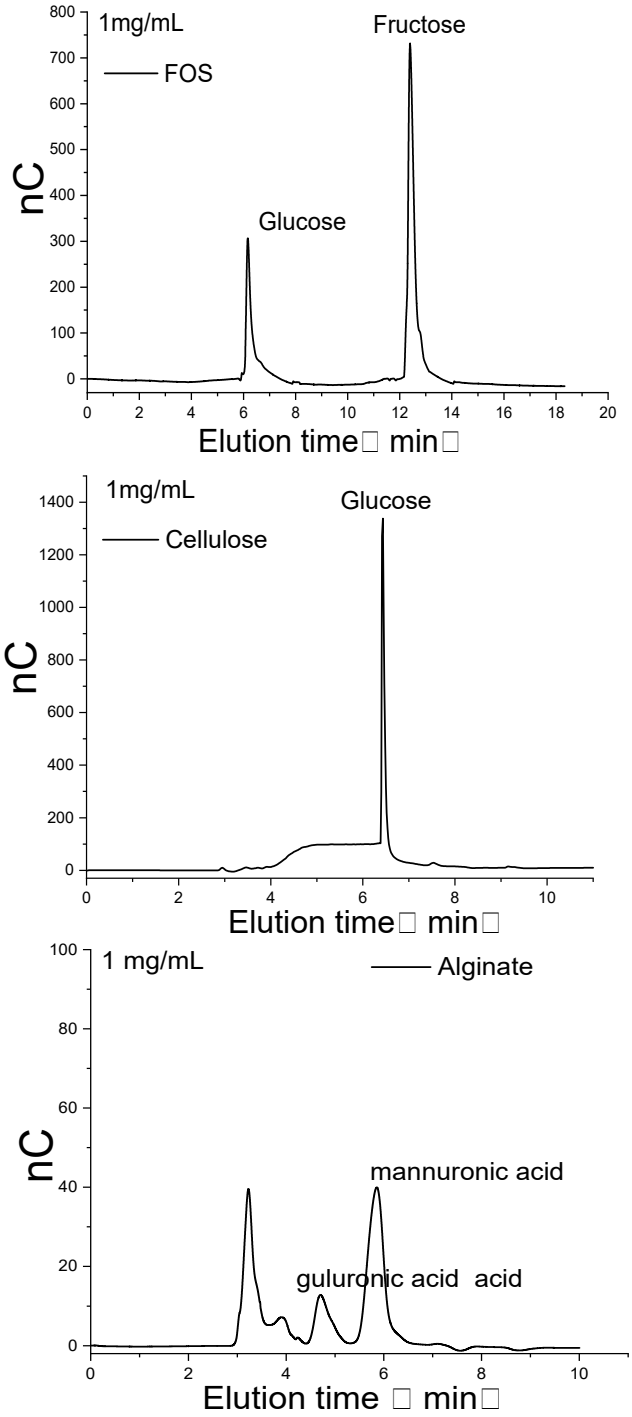

Figure S2. Monosaccharides profiles of FOS, cellulose, and alginate at 1 mg/mL after trifluoroacetic acid hydrolysis.

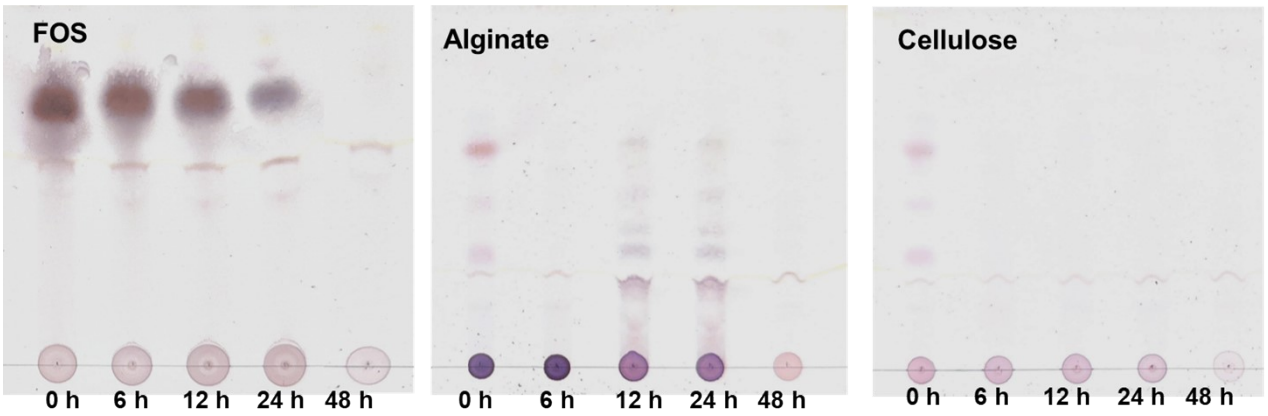

Figure S3. TLC test of fermented broth after 0, 6, 12, 24, and 48 h fermenting 500 mg of FOS, Alginate, and Cellulose, respectively.

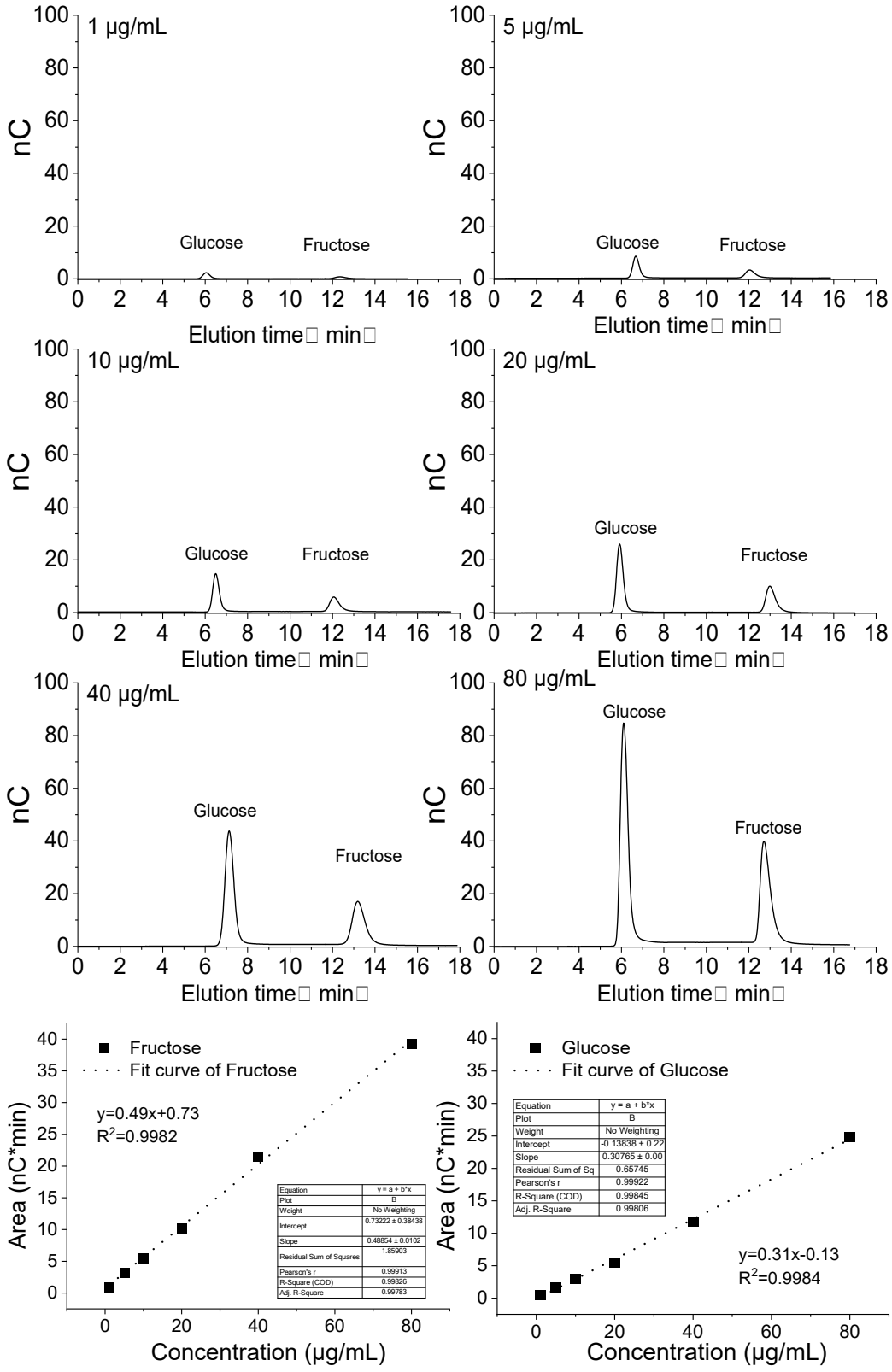

Figure S4 Ion-exchange chromatograms of the glucose and fructose standards (1, 5, 10, 20, 40, and 80 μg/mL), and fitting curves of standards.

Table S4. Development of free fructose from FOS during fermentation

	Peak Area (nC*min)	
Time	Fructose (G+FOS-0.5g)	Fructose (G+FOS-1.0g)
0 h	17.63±0.84	13.05±0.80
6 h	24.61±2.19	14.77±0.32
12 h	12.98±1.05	16.75±0.54
24 h	6.50±0.94	7.27±0.92
48 h	1.58±0.50	2.80±0.35
	Concentration (μg/mL)	
0 h	$14.58±0.82^{b}$	$15.21±0.15^{c}$
6 h	$48.87±5.04^{a\,**}$	$28.75±0.82^{b}$
12 h	$25.08±0.66^{c}$	$32.79±0.37^{a\,**}$
24 h	$11.81±0.43^{d}$	$13.39±0.38^{d*}$
48 h	$1.72±0.48^{e}$	$4.25±0.78^{e*}$

Numerical values marked asterisk represented significances of different samples against the same time node (*$p < 0.05$, **$p < 0.01$), and numerical values marked letters represented a sample at different time node ($p < 0.05$).

Table S5. Development of free glucose from cellulose during fermentation

	Peak Area (nC*min)	
Time	Glucose (G+Cel-0.5g)	Glucose (G+Cel-1.0g)
0 h	0	0
6 h	0	0
12 h	0.330±0.079	0.213±0.025
24 h	0.502±0.024	0.331±0.065
48 h	0.801±0.027	0.462±0.026
	Concentration (μg/mL)	
0 h	-	-
6 h	-	-
12 h	$1.52±0.041^{c\,*}$	$1.14±0.051^{c}$
24 h	$2.08±0.053^{b\,*}$	$1.52±0.064^{b}$
48 h	$3.05±0.055^{a\,*}$	$1.95±0.054^{a}$

Numerical values marked asterisk represented significances of different samples against the same time node (*$p < 0.05$), and numerical values marked letters represented a sample at different time node ($p < 0.05$).

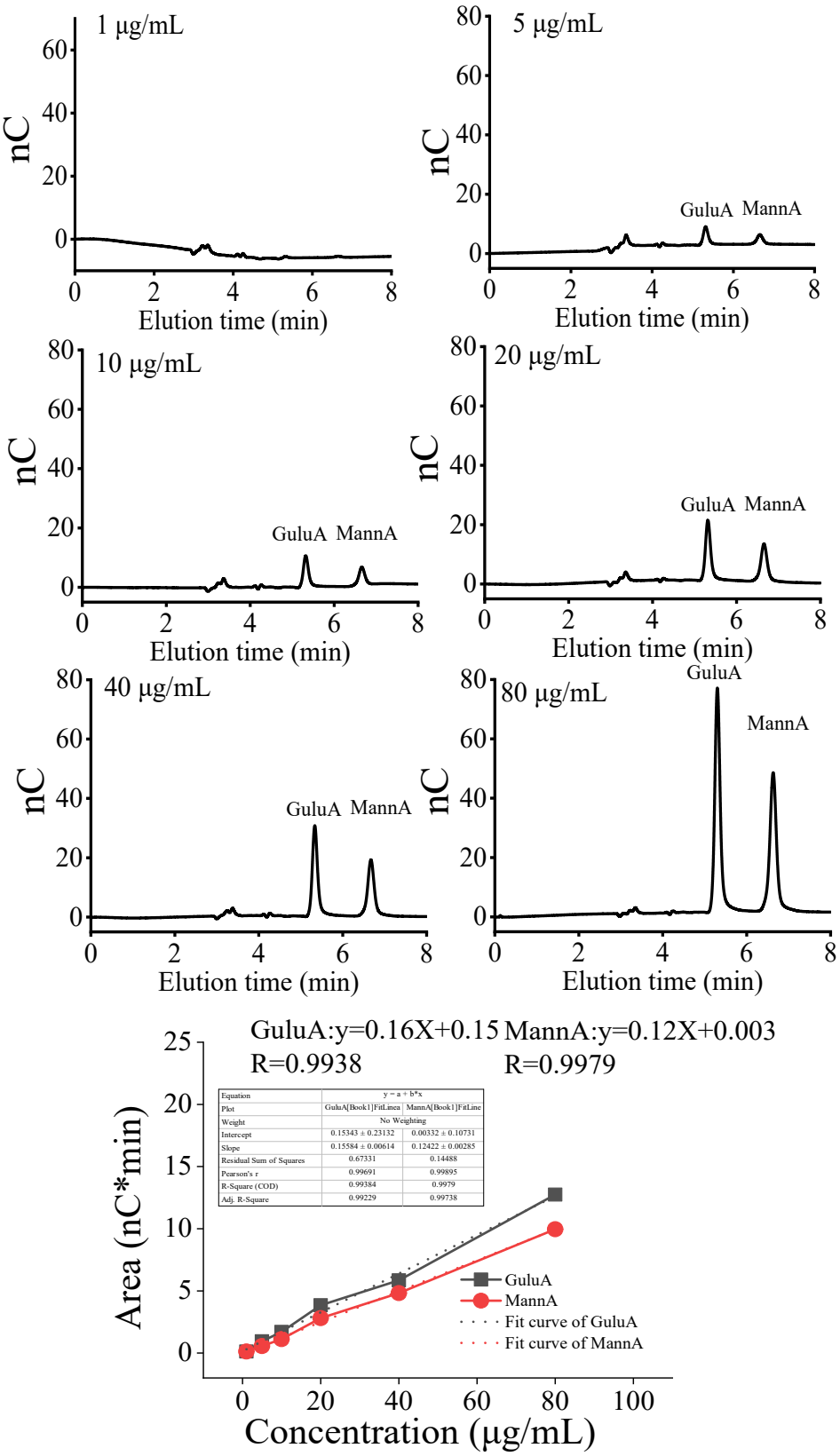

Figure S5. Ion-exchange chromatograms of the guluronic and mannuronic acid standards (1, 5, 10, 20, 40, and 80 μg/mL), and Fitting curves of standards.

Table S6. Development of free guluronic and mannuronic acid from alginate during fermentation

Time	Area (nC*min)			
	G+Alg-0.5g		G+Alg-1.0g	
	GuluA	MannA	GuluA	MannA
0 h	-	-	-	-
6 h	0.093±0.007	0.587±0.020	0.101±0.014	0.641±0.096
12 h	0.129±0.006	0.757±0.012	0.119±0.051	0.729±0.081
24 h	0.254±0.029	0.726±0.027	0.103±0.018	0.634±0.075
48 h	0.055±0.006	0.078±0.007	0.075±0.013	0.165±0.042
	Concentration (μg/mL)			
0 h	-	-	-	-
6 h	0.301 ± 0.028^{c}	4.869 ± 0.143^{b}	0.352 ± 0.024^{b}	5.322 ± 0.782^{a}
12 h	0.545 ± 0.030^{b}	6.289 ± 0.076^{a}	0.476 ± 0.009^{a}	6.050 ± 0.653^{a}
24 h	$1.410\pm0.140^{a**}$	6.028 ± 0.200^{a}	0.359 ± 0.021^{b}	5.258 ± 0.608^{a}
48 h	0.035 ± 0.041^{d}	0.625 ± 0.03^{c}	$0.174\pm0.025^{c**}$	$1.350\pm0.329^{b*}$

Numerical values marked asterisk represented significances of different samples against the same time node (*$p < 0.05$, **$p < 0.01$), and numerical values marked letters represented a sample at different time node ($p < 0.05$).

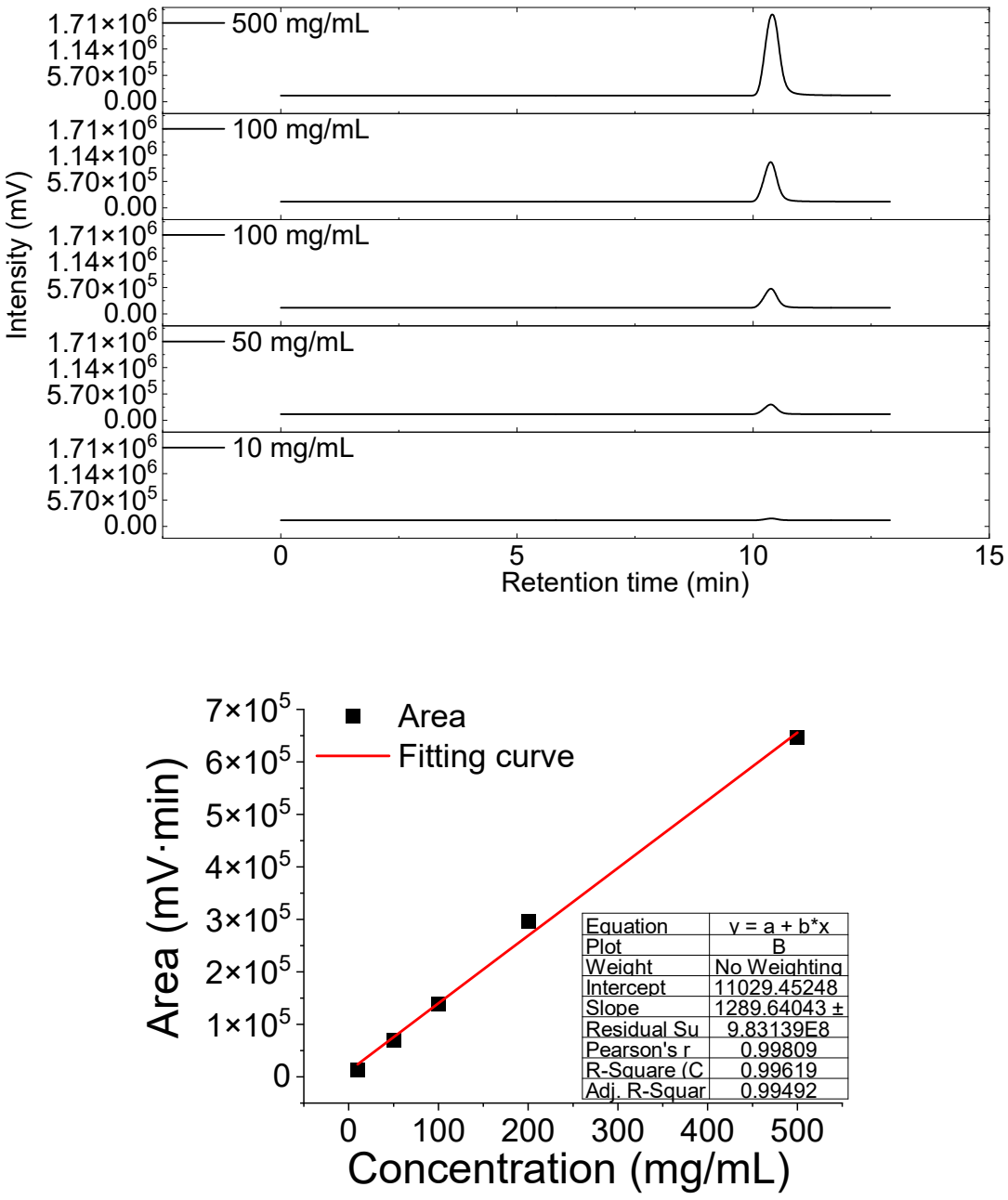

Figure S6. Liquid chromatograms and its fitting curve of the indole standard (10, 50, 100, 200, and 500 mg/mL).

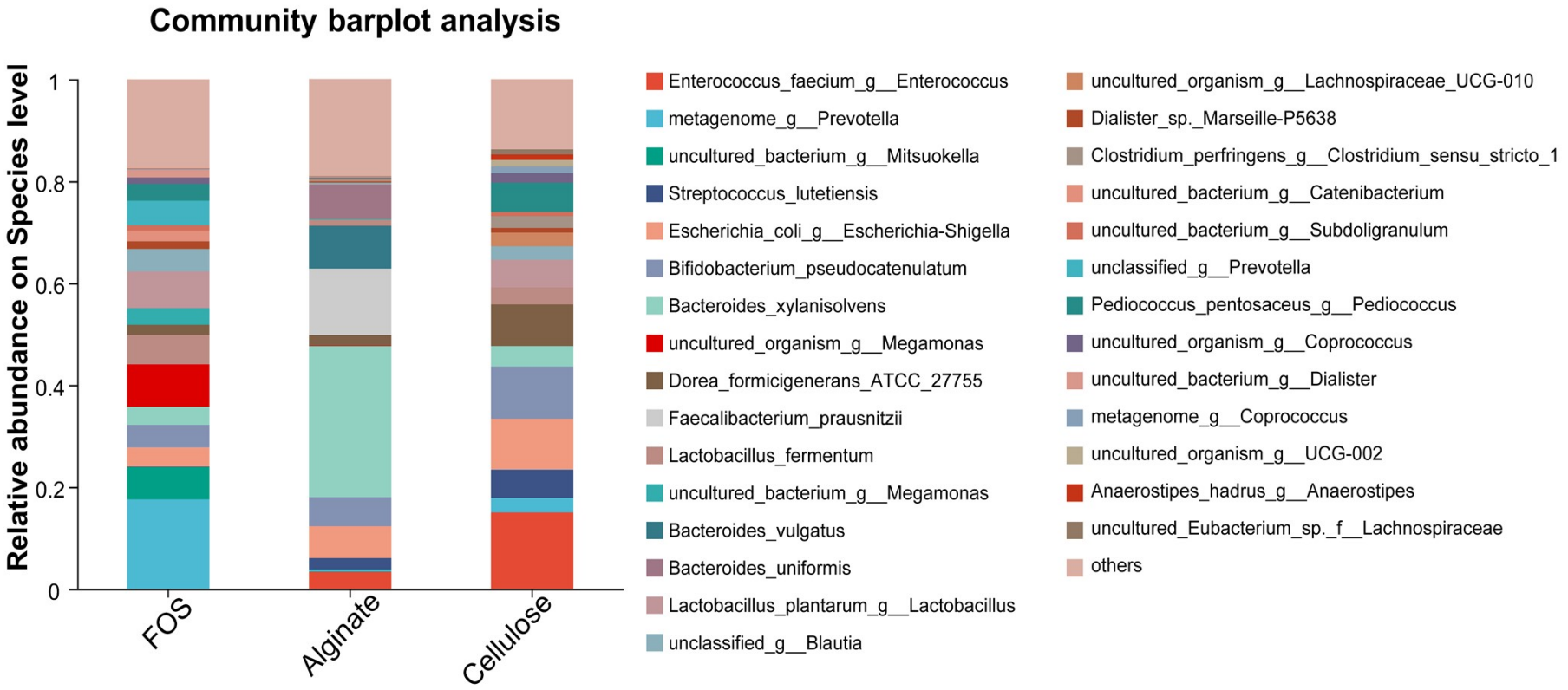

Figure S7. Gut microbiota composition at the species level after fermenting FOS, alginate, and cellulose.

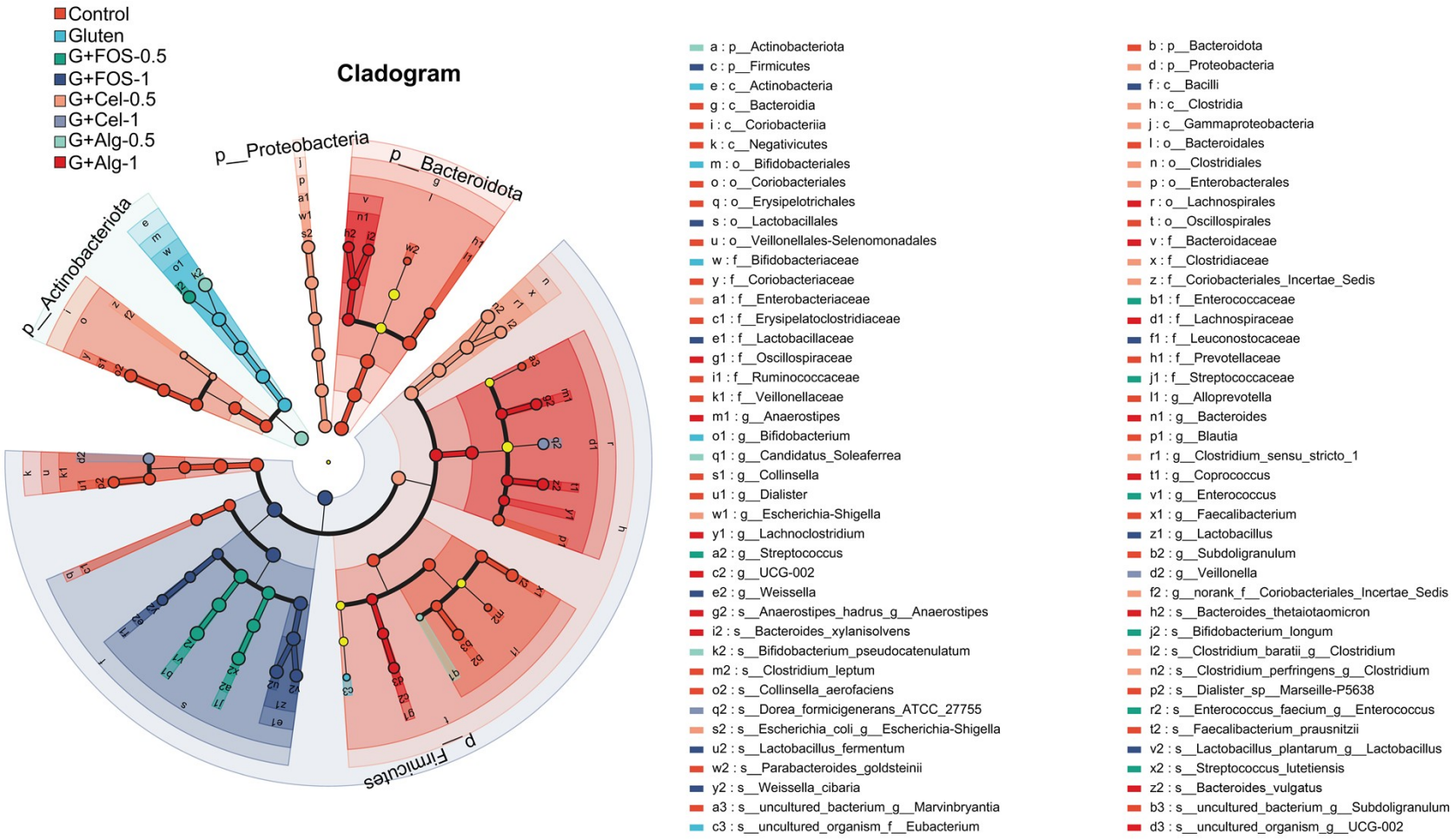

Figure S8. LEfSe analysis at the OTU level of the fecal microbiota structural composition of each group.