Supplementary Information

Gastrointestinal digestion fate of *Tremella fuciformis* polysaccharide and its effect on intestinal flora: an *in vitro* digestion and fecal fermentation study

Xiangyang Zhu[a], Jing Su[a], Dapeng Li[a], Yang Jiang[a]*, Chen Zhang[a]*

[a] College of Food Science and Engineering, Shandong Agricultural University, Key Laboratory of Food Nutrition and Human Health in Universities of Shandong, Taian 271018, People's Republic of China.

* Corresponding authors:

Dr. Chen Zhang, E-mail: zhangchensdau@163.com;

Dr. Yang Jiang, E-mail: jiangyangsdau@163.com.

Supplementary Methods

2. Materials and methods

2.2 Extraction of polysaccharides from *Tremella fuciformis*

According to the previous research, the hot water extraction of polysaccharides from *Tremella fuciformis* was performed with slight modifications. In short, the liquid-to-material ratio was 1:30, the extraction temperature was 90℃ and the extraction time was 4 h. After the immersion extraction was complete, the supernatant was collected by centrifugation (5000 rpm, 15 min). Then, the precipitate was obtained by ethanol precipitation and centrifugation. After re-dissolving in deionized water, dialysis was performed using a 3500 kDa membrane. The resulting Tremella fuciformis polysaccharide (TFP) was then freeze-dried.

2.3 *In vitro* digestion of TFP

2.3.1 Simulated saliva-gastrointestinal digestion

In vitro simulated digestion of TFP was conducted according to the previous method with slight modifications. The simulated saliva formulation consists of KSCN (14.54 g), NaCl (127.49 g), KCl (65.16 g), $NaHCO_3$ (61.60 g), Na_2SO_4 (41.45 g), NaH_2PO_4 (64.58 g), urea (18.18 g), α-amylase (210.90 mg), distilled water (1.0 L), with a pH of 6.8. A 50 mL solution of TFP (6.67 mg/mL) was mixed with 50 mL of simulated saliva and incubated in a water bath at 37°C with shaking. Digestion samples were collected after 0.25, 0.5 and 1.0 h, followed by inactivation of the enzyme by boiling for 10 minutes. 0.45 g of pepsin was dissolved in HCl (0.1M) and added to the 50 mL saliva digestion

sample to initiate gastric digestion. The pH of the solution was adjusted to 2.0 within 1 h by continuously adding 0.5 mol/L HCl solution. After incubation at 37°C for 0.5, 1.0, 2.0, 4.0 and 6.0 h, a portion of the digestion sample was collected and the enzyme was inactivated by boiling for 10 min. Finally, $NaHCO_3$ solution was added to adjust the pH of the post-gastric digestion sample to pH 6.8. A mixture of pancreatin (3.5 mg/mL) and bile salts (22 mg/mL) was added to the remaining digestion solution in a ratio of 3:10. After incubating at 37°C for 0.5, 1.0, 2.0, 4.0 and 6.0 h, the digestion samples were collected again, treated to inactivate the enzymes, and prepared for subsequent analysis. After centrifugation of the collected samples from each digestion process, the supernatant was collected. Ethanol precipitation, re-dissolution, dialysis, concentration of the dialysate, and freeze-drying were performed to obtain the digestion samples at different stages, named TFP-S, TFP-G, and TFP-I.

2.3.2 Determination of chemical composition of TFP

The chemical composition of TFP was determined according to the previous method with slight modifications. The total polysaccharide content in TFP was determined using the phenol-sulfuric acid method. The total protein content in TFP was determined using the Bradford method with bovine serum albumin as the reference. The total uronic acid content in TFP was measured using the meta-hydroxydiphenyl method with galacturonic acid as the reference.

2.3.3 Determination of the content of reducing sugar

The content of reducing sugars (C_R) in digested TFP was determined according to

previous research with slight modifications. The content of C_R was determined using 3,5-dinitrosalicylic acid (DNS) colorimetric method [21].

2.3.4 Determination of molecular weight

The molecular weight of TFP was determined according to previous research. Detection was performed using a TSKGEL GMPWXL aqueous gel chromatography column (7.8 mm × 300 mm; TSK-gel PWXL G4000, TOSOH (TSK), Japan) and a RID-20 differential refractive index detector. Polyethylene oxide (PEO) was used to calibrate the GPC system.

2.3.5 Determination of monosaccharide composition

Monosaccharide composition of TFP determined following the published procedure. Detection was performed using the HPLC system (U300; Thermo Fisher Scientific, MA, USA) and Xtimate C18 chromatographic column. The area normalization method was used to calculate the peak area ratio to obtain the molar mass of each monosaccharide.

2.3.6 Fourier transform infrared (FT-IR) spectroscopic analyses

The structure of TFP were analyzed and identified by FT-IR according to previous research. Take 2.0 mg of polysaccharide sample and add 150 mg of KBr powder. Grind them together in an agate mortar to obtain a homogeneous fine powder. Then, use a pellet press to compress the powder into thin pellets. Measure the samples at a wavelength range of 4000-400 cm^{-1}, using 32 scans.

2.3.7 Thermogravimetric (TG) analysis

The TG analysis of TFP was measured using the method of Kazemi et al. (2019)

with slight modifications. Weigh approximately 3.0 mg of polysaccharide sample and place them in a crucible. After removing any outer impurities, set the measurement parameters as follows: test temperature range of 25-600 ℃, heating rate of 10 ℃/min, and nitrogen gas as the protective gas.

2.3.8 Congo red assay

The conformation of TFP was confirmed by Congo-red method. The polysaccharide samples TFP, TFP-S, TFP-G and TFP-I were prepared as 0.5 mg/mL solutions. An 80 μmol/L Congo Red reagent and a 1 mol/L NaOH solution were also prepared. To perform the measurement, 2 mL of the polysaccharide sample was mixed with 2 mL of the Congo Red reagent. Then, 2 mL of the NaOH solution was added to the mixture, increasing the NaOH concentration in the system from 0 to 0.5 mol/mL. The maximum absorption wavelength was determined within the range of 200-400 nm using a UV-visible spectrophotometer.

2.3.9 Rheological characterization

The rheological characterization of the solution was measured by using an MCR 102 Rheometer (Anto-Paar Austria). The flow curves of TFP, TFP-S, TFP-G, and TFP-I were measured in the shear rate range of 0.1 s^{-1} to 1000 s^{-1}.

2.3.10 Mean particle size and zeta potential

The mean particle size and zeta potential of the solution were measured by using an adynamic light scattering instrument (Malvern Nano-S90, Malvern Instruments, Worcestershire, UK) at room temperature.

2.4 *In vitro* fecal fermentation of TFP

2.4.1 *In vitro* fermentation using human fecal inoculum

The collection of fresh fecal samples, preparation of fermentation media, and methods for *in vitro* fermentation can be referenced from previous studies with slight modifications.

Fresh fecal samples were collected from 10 healthy volunteers, including 5 males and 5 females, aged between 18 and 26 years old. These volunteers had no digestive system disorders and had not taken antibiotics for at least three months. Their body mass index (BMI) was 21.25±1.79 kg/m^2, which falls within the normal weight range according to the World Health Organization's classification. The BMI categories were as follows: (1) underweight (BMI<18.5 kg/m^2), (2) normal weight (18.5-24.9 kg/m^2), (3) overweight (25-29.9 kg/m^2), (4) obesity class I (30-34.9 kg/m^2), and (5) obesity class II ($\geqslant$35 kg/m^2). All volunteers in this study belonged to the normal weight category.

Fresh fecal samples were collected using disposable fecal collectors. The collected samples were then grouped and mixed to ensure equal weights. Sterile physiological saline solution (NaCl 9.0 g/L, cysteine hydrochloride 0.5 g/L) was prepared and diluted to obtain a 10% fecal slurry. The fecal slurry was homogenized using a vortex mixer and then filtered through sterile gauze (four layers) to remove impurities. The resulting fecal homogenate was used for inoculation.

The carbon source was added at a concentration of 0.5%. The basic culture medium formulation consists of yeast extract (2.0 g), peptone (2.0 g), sodium bicarbonate (2.0 g),

bile salt (0.5 g), cysteine hydrochloride (0.5 g), NaCl (0.1 g), K_2HPO_4 (0.04 g), KH_2PO_4 (0.04 g), hemin chloride (0.02 g), $MgSO_4$ (0.01 g), $CaCl_2$ (0.01 g), crystal violet (0.01 g), vitamin K1 (10 μL), Tween 80 (2.0 mL), and distilled water (1.0 L). The pH of the basic nutrient medium is adjusted to pH 6.8-7.0 using dilute hydrochloric acid. The medium is then sterilized at 121 °C for 30 min and stored for later use.

Using TFP-I as the carbon source, it was added to the culture medium and subjected to *in vitro* fermentation using the collected gut microbiota from healthy individuals, named as TFP group. The no carbon source group was set as the blank control, named as BLANK group. In order to serve as a positive control, inulin (a kind of recognized soluble polysaccharide that can be used as a prebiotic) was used as a substitute for TFP, named as FOS group. The fermentation process was carried out in an anaerobic tube with a working volume of 10 mL. Under an alcohol lamp atmosphere, 9 mL of culture medium was added followed by 1 mL of fecal bacterial solution into a sterile anaerobic tube. The fermentation was conducted in a constant temperature shaking incubator at 37 ℃. Throughout the fermentation process, an anaerobic gas pack was used to maintain an anaerobic environment. Samples are taken at 0 h, 6 h, 12 h, 24 h and 48 h of fermentation.

2.4.2 Determination of pH, C_R, gas produced amount and carbohydrate content during *in vitro* fermentation

Determination of pH, C_R, gas produced amount and carbohydrate content during *in vitro* fermentation were based on previous studies. The content of C_R was determined

using 3,5-dinitrosalicylic acid (DNS) colorimetric method. The total sugar content was determined using the phenol-sulfuric acid method. The pH measurement was conducted following the method described by Kong et al. (2015). The gas produced amount measurement was conducted following the method described by Rycroft et al. (2001).

2.4.3 Gut microbiota analysis during *in vitro* fermentation

The fermentation broth was subjected to low-temperature high-speed centrifugation to collect the bacterial pellet in cryovials, which were then stored at -80°C after liquid nitrogen flash freezing. DNA was extracted from mice fecal samples using an E.Z.N.A.® Soil DNA kit (Omega Bio-Tek, USA). The V3 and V4 regions of bacterial 16S rRNA gene sequences in feces were determined using primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). Purified and quantified, sequencing libraries were constructed, and sequencing analysis was performed. Data were analyzed on the Majorbio cloud platform (http://edu.Majorbio.com).

Figure

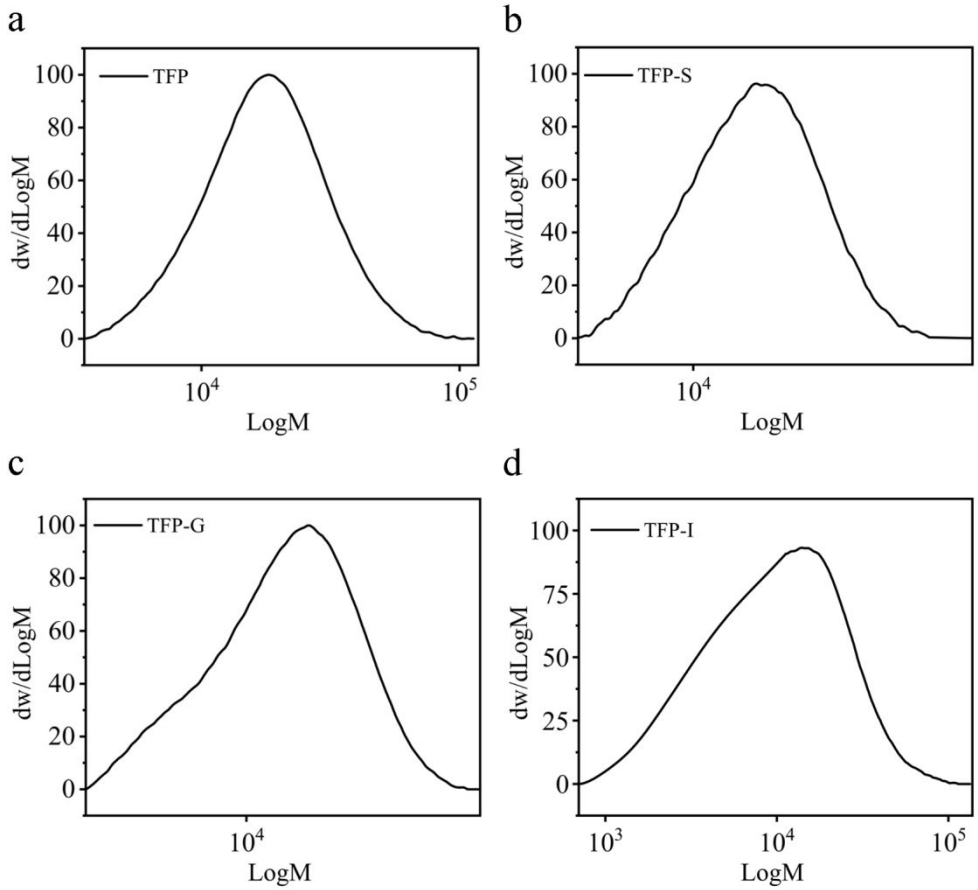

Fig. S1 Changes in molecular weight of TFP during *in vitro* digestion. (a) TFP. (b) TFP-S. (c) TFP-G. (d) TFP-I.