https://doi.org/10.1016/j.ijbiomac.2019.12.072

Revised Ms. IJBIOMAC-2019-7678

(Original Research MS)

Effects of exopolysaccharide fractions with different molecular weights and compositions on fecal microflora during *in vitro* fermentation

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Abstract

This study was to investigate the potential prebiotic function of exopolysaccharide (EPS) from a medicinal fungus and the relationship to the molecular properties by *in vitro* human fecal fermentation. The EPS from *Cordyceps sinensis* Cs-HK1 mycelial fermentation was processed into three fractions with different monosaccharide contents, a higher molecular weight (MW) and a lower MW attained by two-step ethanol precipitation, and an intermediate MW by ultrasound-degradation of EPS. All the EPS fractions were well utilized during 24-48 h of fecal fermentation, leading to significant increases in the short chain fatty acid (SCFA) production. The consumption rate and production level of SCFAs varied slightly with the different EPS fractions. The EPS also influenced the composition and diversity of the fecal microflora, increasing the relative abundance of *Firmicutes* but suppressing that of *Proteobacteria*, which may be a beneficial effect for human health. Overall the results have shown that the Cs-HK1 EPS has significant prebiotic activity which is dependent on its molecular properties.

Keywords: *Cordyceps sinensis* fungus; Exopolysaccharides; prebiotic effects; human fecal microflora; *in vitro* fermentation

1. Introduction

The human intestinal microflora is a diverse microbial community which is composed mainly of bacteria [1]. Among the numerous bacterial phyla that have been detected in the human gut, *Firmicutes* and *Bacteroidetes* are most dominant, and Actinobacteria including *Proteobacteria* and *Verrucomicrobia* are also commonly found as the minor components of microflora [2]. It is generally believed that the gut microbial composition and diversity have a close association with the host physiology and pathology [3]. Diet is the most probable factor which can affect the microbial composition and balance [4]. The non-digestible dietary fibers and prebiotics including complex polysaccharides and oligosaccharides can reach the large intestine and be fermented by the gut bacteria [5]. The selective consumption of the non-digestible carbohydrates by different microbial species can directly affect their proliferation and relative abundance in the gut microflora [6]. On the other hand, the fermentative metabolism of carbohydrates in the gut yields short chain fatty acids (SCFAs) as the end products, including acetic acid, propionic acid and butyric acid, which have important and beneficial functions in the host both locally and systemically [7].

Natural polysaccharides from edible plants, fungi, macro-algae and other sources have been

widely explored as functional food ingredients because of their notable health benefits. As most of these complex polysaccharides are non-digestible, their most possible site of utilization and action is in the gut microflora [8]. In this regard, increasing attention in recent studies has been paid to the effects of natural polysaccharides on or associated with the gut microflora [9-11]. Edible mushrooms or fungi which are rich of bioactive polysaccharides have been recognized as the potential source of prebiotics [12]. The Chinese caterpillar fungus *Cordyceps sinensis* is a precious medicinal fungus with a wide range of bioactivities and health benefits [13]. As natural *C. sinensis* is very rare, mycelial fermentation has been widely applied to produce *C. sinensis* fungal materials to meet the high and rising demand for health food and herbal medicine. Cs-HK1 is a fungus species isolated from a natural *C. sinensis* fruiting body and has been applied to mycelial fermentation for production of mycelial biomass and exopolysaccharides (EPS). The EPS attained from the Cs-HK1 mycelial fermentation was mainly composed of heterogenous polysaccharides in a wide molecular weight (MW) range and the major monosaccharide constituents include glucose (Glc), mannose (Man) and galactose (Gal) [14, 15].

Our recent studies performed in pure cultures of bifidobacteria have shown the beneficial and protective effect of the Cs-HK1 EPS on the probiotic bacteria [16]. However, neither the high-MW native EPS nor the partially hydrolyzed low-MW EPS fractions could be well utilized as a carbon source for the bifidobacterial growth. It has been suggested that the degradation and consumption of non-digestible complex carbohydrates by the gut bacteria is accomplished through cross-feeding of different bacterial species in the human colon [17]. The mixture fermentation system is more effective and realistic for assessment of complex polysaccharides in human gut microflora. Fecal fermentations (*in vitro*) have been widely used to study the functions and metabolism fates of food ingredients such as natural polysaccharides [18, 19].

This work was to investigate the fermentability and prebiotic function of the Cs-HK1 EPS in human fecal microflora by *in vitro* fermentation and the dependence on the composition and molecular weight of EPS fractions. The monosaccharide contents of EPS and SCFAs were analyzed quantitatively during the *in vitro* human fecal fermentation to assess the consumption and utilization of EPS by the gut bacteria. The microbial composition and diversity of the fecal microflora was examined by 16S rRNA gene analysis.

2. Materials and methods

2.1. Preparation of Cs-HK1 EPS fractions

As described previously, the liquid medium for Cs-HK1 mycelial fermentation consisted of 40 g/L glucose, 5 g/L peptone, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$ and 10 g/L yeast extract. The Cs-HK1 mycelial fermentation was carried out in shake-flasks at 20 °C for 6 days and exopolysaccharide (EPS) was isolated from the Cs-HK1 liquid fermentation medium by ethanol precipitation (with 80% v/v ethanol) as described in detail previously [16]. The power ultrasound (US) treatment was applied to the liquid fermentation medium before ethanol precipitation to produce EPS-US fraction with a lower MW and a higher water-solubility as reported in previously [16, 20]. Another two EPS fractions were prepared by two-step ethanol precipitation as reported previously [21, 22], firstly using 40% (v/v) ethanol to attain EPS-H with a much higher MW and secondly using 80% (v/v) ethanol to obtain EPS-L with a much lower MW than the EPS attained by single step precipitation using 80% (v/v) ethanol. Both the strategies of using power US and two-step ethanol precipitation have been proven effective in our previous studies for preparation of the desired lower MW EPS fractions. As shown in our previous studies, the whole EPS attained by a single step precipitation with 80% ethanol, the EPS-H and EPS-L fractions by two-step ethanol precipitation and the EPS-US after US treatment were mainly composed of heterogenous polysaccharides with different average MWs. All the EPS precipitates were recovered from the liquid by centrifugation at 12,000 rpm for 15 min, re-dissolved in a small amount of deionized (DI) water and freeze-dried as the final EPS fractions for experiments. The MW of EPS fractions were analyzed by high performance gel permeation chromatography (HPGPC) as reported previously [21].

Methylcellulose (MC) (15 centipoise viscosity with 2% aqueous solution at 25 °C; Sigma, Saint Louis, MO, USA), a non-digestive and non-fermentable carbohydrate polymer [23, 24], was included as a control for comparison with the EPS fractions in the fecal fermentation experiments.

2.2. Fecal sample collection and preparation

Fecal material was collected from three healthy donors (D1, D2 and D3, two females and one male, 26-30 years of age). All donors were free of known gastrointestinal diseases and metabolic disorders and had not received antibiotic probiotic and not taken any prebiotic supplements for at least 3 months prior to the sample collection. Fecal samples were collected in sterile tubes and immediately stored in anaerobic jars containing anaerobic sachets (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan), which were used in the fermentation

experiments within 30 minutes of collection. The fecal samples were 10-fold diluted with the phosphate buffered saline (PBS) and homogenized for 2 min, and then centrifuged at 500 rpm for 5 min to remove large particles. The remaining suspension was used as the initial inoculum in the fecal culture fermentation. All these were carried out in anaerobic atmosphere according to well documented protocol [25].

2.3. Fecal culture fermentation

The basal culture medium was consisted of peptone water (2 g/l), yeast extract (2 g/l), NaCl (0.1 g/l), dipotassium phosphate (0.04 g/l), monopotassium phosphate (0.04 g/l), sodium bicarbonate (2 g/l), magnesium sulfate heptahydrate (0.01 g/l), calcium chloride hexahydrate (0.01 g/l), Tween-80 (2 ml/l), hemin (50 mg/l), phylloquinone (vitamin K1, 10 ml/l), L-cysteine (0.5 g/l), bile salts (0.5 g/l), resazurin (1 mg/l) in distilled water [25]. The medium pH was adjusted to 6.8 with 1 M HCl. The EPS fractions and MC were dissolved in the basal medium at 5 mg/ml with vigorous stirring for overnight and then autoclaved at 121ºC for 20 min. The liquid medium was dispensed aseptically into 10-mL culture tubes at 5.4 mL each. Each tube was inoculated with 0.6 ml freshly prepared fecal slurry (1:10, w/w). The fecal culture tubes were enclosed in anaerobic jars containing anaerobic sachets and incubated with shaking at 200 rpm and 37 °C for 48 h. During the fecal fermentation, samples were collected at 0, 5, 10, 24 and 48 h for enumeration of the bacterial populations, 16S rRNA gene sequencing, determination of carbohydrate utilization profiles, and SCFA production. A new anaerobic gas generating sachet was applied each time when the jar was opened for sample collection.

2.4. Enumeration of bacterial populations on selective agar plates

Cultivation on selective agar plates is a rapid and convenient method for enumeration of some common bacterial groups in mixed cultures by counting the viable cells (CFU/ml). Total anaerobic and aerobic bacteria were counted on BHI agar; *Clostridia, Esherichia coli, Staphylococcus, Enterococcus, Lactobacillus* and *Bifidobacterium* were counted respectively by Tryptose Sulfite Cycloserine (TSC), MacConkey agar, Mannistol Salt Agar (MSA) Enterococcus agar, MRS agar, Bifidobacterium agar (Haibo Biotechnology Co. Ltd., Qingdao, China). The fecal slurry was diluted with PBS sequentially from 10^{-1} to 10^{-12} according to preliminary results, then 100 µl was spread onto each agar plate. After incubation in anaerobic or aerobic atmosphere at 37 °C for 48 h, the number of colonies on the plate was counted.

2.5. DNA extraction, 16S rRNA gene sequencing and microbial composition analysis

153 Since culture-based analysis is limited to enumeration of certain bacterial species [26], genomic
154 analysis was performed for enumeration of the many bacterial species, and for assessment of
155 the microbial composition and diversity of fecal microflora. The fecal culture samples were
156 centrifuged at 12,000 rpm for 5 min and the sediment was collected for the gene sequencing
157 and the supernatant analysis for monosaccharide (1.4 ml) and SCFA analysis (0.2 ml). DNA
158 extraction was carried out of the solid samples using Tiangen stool DNA extraction kit
159 (Tiangen, Beijing, China) according to the manufacturer's manual. The microorganism DNA
160 concentration was determined by NanoDrop 2000 (Thermo Fisher, Massachusetts, USA) to be
161 6-100 ng/μl, total initiation mass ≥ 30 ng. The 16S rRNA gene sequencing was conducted by
162 BGI (Shenzhen, China). The database used for species annotation was Greengene (default):
163 V201305[8]; RDP: Release11_5,20160930.
164
165 2.6. Analysis of monosaccharides in EPS
166 The monosaccharide composition of EPS fractions was analysed by liquid chromatography-
167 mass spectrometry (LC-MS) as reported previously [27] with minor modifications. The
168 monosaccharide standards (d-Arabinose, d-fructose, d-galactose, d-glucose, L-rhamnose
169 monohydrate, d-mannose, d-glucose $13C_1$) and trifluoroacetic acid (TFA), HPLC grade
170 acetonitrile (ACN), and methanol (MeOH) were purchased from Sigma-Aldrich (St. Louis,
171 MO, USA). Stock solutions of monosaccharides and internal standard d-glucose $13C_1$ were
172 prepared at 10 mg/mL in Milli-Q water. Each of the stock solutions was diluted with the LC
173 solvent ACN/H_2O (50:50 v/v) before being injected into the LC system. External calibration
174 standard solutions were prepared in the range of 0.5 to 50 μg/mL, each containing 1 μg/ml d-
175 glucose $13C_1$. All these solutions were stored at 4 °C before use.
176
177 The EPS samples (5 mg) were dissolved in 1.4 ml of Milli-Q water with stirring for overnight.
178 The supernatant (1.4 ml) collected from the fecal fermentation samples in section 2.5 was also
179 analysed. Each of the sample solutions was mixed with 0.6 ml of TFA (4 M TFA final
180 concentration) and hydrolysed at 100 °C for 8 h. Excessive TFA was removed by vacuum
181 evaporation and the residue was redissolved in 2 mL of water. Further clean-up was
182 accomplished by solid phase extraction (SPE). The SPE cartridge was preconditioned by 3 ml
183 of methanol and then 3 ml of ultrapure water. The sample solution was loaded onto the cartridge
184 and the eluent was collected, evaporated to dryness under vacuum, and then redissolved in
185 about 2 mL or more of ACN/ H_2O (50:50 v/v) for the LC/MS/MS analysis. D-glucose 13C1
186 (0.01 mg) as an internal standard was added to the solution. For evaluation of matrix effects,

the vacuum-dried residue was redissolved in 2 mL of ACN/H_2O (80:20 v/v) and was used to prepare matrix-containing standard solution. The treated samples were diluted to desired concentrations by ACN/H_2O (80:20 v/v) before injection.

The LC separation was achieved with a normal phase method on an ACQUITY UPLC® BEH amide column (2.1mm×100mm, 1.7 μm particles; Waters, Milford, Massachusetts, USA) and a mobile phase flow rate of 0.3 ml/min. The mobile phase was consisted of water (A) and acetonitrile (B), MS Grade (Honeywell, Burdick and Jackson, Morristown, NJ, USA). The elution was carried out primarily by a linear gradient over 30 min from 95 to 60 % (v/v) mobile phase B, followed by a column wash and re-equilibration. Column was maintained at 20 °C and auto-sampler plate set at 4 °C. An Agilent 6460 HPLC system (Santa Clara, Foster City, CA, USA) was coupled in-line with an electrospray ionisation triple quadrupole mass spectrometer (Santa Clara, Foster City, CA, USA) equipped with an Agilent Jet Stream electrospray ionisation (AJS ESI) source. Ions were detected by multiple-reaction monitoring (MRM) method in negative polarity with a dwell time of 200 ms and the following source conditions: gas temperature at 300 °C, gas flow at 8 l/min, nebulizer pressure at 45 psi, sheath gas temperature at 320 °C, sheath gas flow at 11 l/min, capillary positive voltage at 3500 V (positive) and 3000 V (negative), nozzle voltage at 500 V. Data acquisition was performed using MassHunter Quantitative Analysis software B.07.00.

2.7. Analysis of short chain fatty acids

Short chain fatty acids (SCFAs) in the fecal cultures were analysed by Gas Chromatograph-Flame Ionization Detection (GC-FID) using an Agilent 7980B GC system and a fused silica capillary column (Agilent Technologies Inc., Palo Alto, CA, USA) as described previously [16]. In brief, 0.2 ml of fecal culture supernatant was diluted by 0.8 ml of Milli-Q water and the pH was adjusted to 2-3 with 1 M HCl. An internal standard (2-ethylbutyric acid) was added at 1 nM final concentration before injection into the GC system. Nitrogen gas was applied as the mobile phase flowing at 0.6 ml/min. The initial oven temperature was maintained at 80 °C for 2 min and raised gradually to 180 °C at 6 °C/min, and maintained for 4 min. The injection volume was 1 μl and the temperature was controlled at 200 °C, and the detector temperature at 220 °C. Six SCFA standards (Aladdin®, Shanghai, China) were used for identification and quantification, including acetic acid, propionic acid, *n*-butyric acid, *i*-butyric acid, *n*-valeric acid, and *i*-valeric acid. The *n*-butyric and *i*-butyric acid were combined in the analysis.

221 2.8. Statistical analysis

222 One-way ANOVA was used to compare the monosaccharide composition of EPS fractions
223 after fecal fermentation. Two-way ANOVA with Tukey post-hoc test was used for the
224 comparison of cfu among different groups. Kruskal-Wallis test was used to compare the
225 operational taxonomic unit (OTU) difference between MC and experimental groups. The
226 paired *t*-test was used to compare different taxonomic compositions between experimental and
227 control groups [28]. The p value < 0.05 indicated a significant difference. SPSS 23.0 was
228 applied for statistical analysis.

229

230 **3. Results and discussion**

231 3.1. Monosaccharide composition of different EPS fractions

232 Table 1 shows the molar ratios of constituent monosaccharides in the different EPS fractions.
233 All EPS fractions were composed mainly of mannose, glucose, galactose and fructose with
234 different molar ratios. EPS and EPS-US had the similar molar composition of four
235 monosaccharides, suggesting that ultrasonic degradation did not cause chemical or structural
236 change in the EPS molecule. Compared with EPS and EPS-US, EPS-H had a much higher ratio
237 of glucose (6.83 to mannose) and EPS-L had a much lower ratio of glucose and a much higher
238 ratio of galactose (0.34 and 2.02 to mannose, respectively).

239

240 As the Cs-HK1 EPS was resistant to hydrolysis by digestive enzymes and simulated gastric
241 acid [29], the undigested EPS fractions were used in the following experiments.

242

243 3.2. Consumption of EPS during fecal fermentation

244 Fig. 1 shows the changes in the total concentration of monosaccharides in the culture media
245 containing MC and EPS fractions after complete TFA hydrolysis during the *in vitro* fecal
246 fermentation. The total monosaccharide concentration in the MC medium did not show any
247 change (ANOVA, $p = 0.880$), implying that MC was not utilized during the fermentation and
248 confirming its role as a non-fermentable carbohydrate polymer control. In contrast, the total
249 monosaccharide concentrations in the cultures with the four EPS fractions all dropped sharply,
250 to about 20% or below 5%, from 10-24 hours during the fecal fermentation, suggesting their
251 consumption by the gut flora. The consumption of most EPS fractions except EPS-H took place
252 mainly between 10-24 h after the inoculation, due probably to that the period of 0-10 h was in
253 the lag period and the period of 24-48 h was in the stationary phase of the fecal fermentation
254 [16]. As for EPS-H, the consumption or reduction of total monosaccharides remained steady

from 10 to 48 h, suggesting a slower fermentation rate as well as a longer growth period than the fecal cultures with other three EPS fractions. The slower consumption of EPS-H than the other EPS fractions was probably attributed to its higher MW, causing a higher viscosity in the culture medium and slower nutrient uptake and slower metabolism. This was in good agreement with the finding in our previous study that the higher MW EPS was more effective to extend the growth and survival of bifidobacteria [29]. Moreover, the EPS-US was consumed more rapidly than EPS, due probably to the increased bioavailability by the ultrasonic degradation of EPS [21].

Table 2 shows the molar ratios of monosaccharides in the EPS fractions before and after 48 h of fecal fermentation. The complete data (5, 10 and 24 h) was shown in Table S2. For EPS-US, the molar ratio of glucose decreased more dramatically, while that of galactose increased significantly over the fermentation period. This suggests that the majority of fecal bacterial community preferred glucose as the major carbon source than galactose or mannose. In contrast, for the EPS-H and EPS-L fractions, galactose was consumed more during the fermentation. It is possible that the EPS-H and EPS-L was taken up as a whole and consumed by the fecal bacteria. It has been suggested that polysaccharides are mostly transported into and metabolized within probiotic bacterial cells [30, 31], and the metabolism of polysaccharides in the gut microflora is dependent on their molecular properties [32].

3.3. Medium pH change and SCFA production during fecal fermentation

Compared with the control MC fermentation, all the fecal fermentation with the EPS fractions experienced an accelerated pH drop from 10 h to 24 h (Fig. 2A). In addition, the fermentation fed with EPS-H exhibited a slower pH drop between 10-24 h and a much faster drop between 24-48 h than the cultures with the three low MW EPS fractions. In association with the pH drop in the fecal cultures, the concentrations of three SCFAs, including acetic acid (Fig. 2B), propionic acid (Fig. 2C) and butyric acid (Fig. 2D) showed a notable ($p < 0.05$) increase from 10 to 24 h and a further, and smaller increase from 24 to 48 h. The SCFAs in the cultures fed with the EPS fractions increased to a much higher level than in the MC culture. Among the three SCFAs, acetic acid was the most abundant and butyric acid was the lowest in the cultures with the four EPS fractions. Another observation was that the butyric acid in the culture fed with EPS-H was notably higher (8.67 mM) than in the other three EPS cultures (3.38-4.58 mM). Compared with other EPS fractions, EPS-H had a much higher glucose content (Table 1), which may be a contributing factor to the high rate of butyric acid production as suggested

289 previously that β-glucans composed of glucose increased the butyric acid formation in intestine
290 [33, 34]. The relative level of pH changes in the fermentation medium with various EPS
291 fractions did not match with the level of EPS consumption, due probably to that the
292 consumption of EPS by the fecal bacteria was not all used for acid production. A practical point
293 is that the batch fecal fermentation is one of the most convenient and economic models for
294 studying the fermentability and metabolism of dietary compounds by gut microflora [35].
295
296 3.4. Bacterial population distributions in fecal microflora
297 Fig. 3 shows the cfu values in the control (with MC) and test groups (with EPS fractions) which
298 was enumerated by selective agar cultivation during fecal fermentation. The cfu values in all
299 groups increased with culture time and the change showed no obvious dependence on the
300 different EPS fractions. However, the cfu values of *Bifidobacterium* (Fig. 3.A), *Clostridium*
301 (Fig. 3.C), the total anaerobic (Fig. 3.F) and aerobic bacteria (Fig. 3.G) at 10 h were notably
302 higher in most of the EPS groups than the MC group, though the difference did not last long
303 and diminished at 24 or 48 h. The cfu values indicated the viability of bacteria. In our previous
304 studies, the higher MW EPS fractions including EPS and EPS-US could significantly protect
305 the viability or survival of several bifidobacterial strains in pure cultures based on the cfu
306 values, though they were not well utilized as a carbon source for the growth (based on the
307 bacterial cell concentration quantified by optical density (OD)) [29]. In contrast, although the
308 EPS fractions were well utilized in the fecal cultures, leading to a significant increase in the
309 SCFA production, they did not offer any protective effect on the viability and survival, with
310 little or no increase in the cfu in the later culture stage (24 - 48 h), especially the EPS-L (nearly
311 no difference compared with MC). The ability of mixed fecal cultures to ferment the complex
312 EPS can be attributed to the availability of all the required enzymes in various bacteria for the
313 degradation of the polysaccharide bonds. On the other hand, the degradation and utilization of
314 EPS by the bacteria resulted in the lack of protective effect in the mixed fecal cultures.
315
316 3.5. Effect of EPS on diversity of fecal microflora
317 There was no shift in the microbial diversity observed in the fecal microflora with MC or any
318 of the EPS fractions (Kruskal-Wallis test, p-value = 0.556) (Supplemental data Fig. S1). In
319 contrast, the fecal materials from different donors affected the results of *in vitro* fecal
320 fermentation (Kruskal-Wallis test, p-value = 0.022) (Fig. S2), which contributed to the reduced
321 richness in D3 incubations (Dunn's test, p-value < 0.1). Although the diversity or richness of
322 bacterial populations is generally regarded as an indicator for the healthy balance and resilience

of gut microflora [36], some previous studies have observed that the high microbiome richness indices were associated with gastrointestinal disorders including prolonged transit times [37], hard stools [38], and increased proteolytic fermentation [39]. This signifies a complex relationship between community richness and gut health.

3.6. Effect of EPS on composition of fecal microflora

Fig. 4 shows the taxa relative abundance after 24 h of fecal fermentation with EPS and MC in the matching donor fecal slurries at different levels for identifying substrate specific responsive genera. On the phylum level (Fig. 4A), the Cs-HK1 EPS fractions resulted in increased *Firmicutes* relative abundance (paired *t*-test, EPS-H, $p = 0.042$; EPS-L, $p = 0.006$) at the expense of *Proteobacteria* relative abundance (EPS, $p = 0.031$; EPS-H, $p = 0.013$; EPS-L, $p = 0.014$) (Supplemental data Table S2). At the family level (Fig. 4B), the EPS fractions decreased the relative abundance of *Desulfovibrionaceae* (EPS, $p = 0.043$; EPS-US, $p=0.034$; EPS-H, $p = 0.045$; EPS-L, $p = 0.033$) and *Enterobacteriaceae* (EPS, $p = 0.029$; EPS-H, $p = 0.008$; EPS-L, $p = 0.017$). At the genus level (Fig. 4C), the EPS fractions induced specific changes in fecal microbial composition. The genera *Escherichia* of *Enterobacteriaceae* family (EPS, $p = 0.018$; EPS-H, $p = 0.015$; EPS-L, $p = 0.012$), *Bilophila* of *Desulfovibrionaceae* (EPS-US, $p = 0.048$; EPS-L, $p = 0.049$), *Parabacteroides* (EPS-US, $p = 0.026$) and *Phascolarctobacterium* family (EPS-US, $p = 0.029$) were reduced after fermentation with the EPS fractions (Table S3). In contrast, only one significant increase ($p = 0.029$) was observed with the low MW EPS-L in the *Phascolarctobacterium* relative abundance. In general, the EPS fractions reduced the relative abundance of the *Proteobacteria* phylum and had an insignificant effect on the *Bifidobacterium* relative abundance ($p > 0.05$). In comparison, EPS-US and EPS-L had a broader impact on the compositions of different microbial populations, causing more significant changes on relative abundances of three genera. The different effects of EPS fractions on gut microflora may be due to their different molecular properties.

The alternation of gut microbial composition is widely believed to be linked with numerous local and systemic disorders [40], though the mechanisms are far from being understood. One of the most common ecological patterns observed during intestinal microbial dysbiosis is an expansion of bacterial population of the phylum *Proteobacteria* [41], which is the most diverse phylum of bacteria associated with many diseases [42]. For example, there is mounting evidence for the association of *E. coli* with gastrointestinal disorders, particularly in inflammatory diseases such as Crohn's disease and ulcerative colitis [43, 44]. Therefore, the

significant reduction of *Proteobacteria* (particularly *E. coli*) ratio with the EPS fractions observed in this study may lead to a beneficial effect of EPS on human health through their action on the gut microflora.

There was no significant change ($p > 0.05$) in the relative abundance of *Bifidobacterium* with the addition of EPS fractions (Table S4). This may be due to the very low abundance of this genus in the fecal material of the three donors (0.24%, 0.26% and 1.01%, respectively), which could not compete with other abundant genera to utilize the polysaccharides [45]. Additionally, although the butyrate content was increased by the EPS, there was no significant change in the relative abundance of the butyrate-producing bacteria (including *Eubacterium*, *Faecalibacterium* and *Roseburia*) (Table S4). Indeed, the effect on microbial composition alone is insufficient to tell if the EPS are beneficial or not on the gut health [46]. To minimize the variation among donors, the enterotype classification may also be considered in future studies [47, 48].

4. Conclusions

Our present study has demonstrated the fermentability and consumption in fecal microflora of a complex, high-MW EPS produced by a medicinal fungus Cs-HK1 and the dependence on the molecular properties of different EPS fractions. The EPS fractions with different MWs and compositions derived from the EPS were all well utilized to produce SCFAs during fecal fermentation. Despite the heterogeneous composition of the fecal microflora among different donors and the limitations of in vitro fermentation model, the fecal fermentation experiments were simple and effective for evaluation of various EPS fractions for their effects on the diversity and composition of fecal microflora. Although no statistically significant changes were found on the presumably beneficial genera, the results still suggested the beneficial effect of EPS toward a healthier gut microbial ecosystem. Further studies through animal experiments are warranted to assess and verify the prebiotic function and related health benefits of EPS.

Acknowledgements

This work was funded by The Hong Kong Polytechnic University and the Hong Kong Research Grants Council Collaborative Research Fund (RGC CRF No. C5031-14E). Authors are grateful to Dr. So Pui Kin for help with the LC-MS analysis.

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553 **Table 1** Properties and composition of EPS fractions

EPS fractions	MW (Da)	[η] dl/g	Molar ratio of monosaccharides			
			Man	Fru	Glc	Gal
EPS	1.93×10^8	6.03	1.00 *	0.03	2.57	0.97
EPS-US	2.30×10^7	4.65	1.00	0.03	3.32	1.16
EPS-H	2.57×10^8	12.47	1.00	0.07	6.83	1.04
EPS-L	1.76×10^7	ND	1.00	0.01	0.34	2.02

554 * Standard variation (SD) < 5% for all data; ND: not detectable

555 MC: methylcellulose, EPS: exopolysaccharides, EPS-US: exopolysaccharides treated with
556 ultrasound; EPS-H: exopolysaccharides with high molecular weight, EPS-L:
557 exopolysaccharides with low molecular weight.

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Table 2 The change of monosaccharide composition (molar ratio) of MC and EPS fractions over 48 hours of fecal fermentation (total molar ratio 100%)

	Time (h)	mannose	fructose	glucose	galactose
MC	0	ND	0.30 ± 0.02 c	99.7 ± 1.03 a	ND
	48	ND	0.56 ± 0.02 ab	99.4 ± 1.87 a	ND
EPS	0	22.2 ± 5.00 b	0.59 ± 0.10 b	54.5 ± 3.00 ab	22.7 ± 3.00 b
	48	34.6 ± 3.77 a	1.57 ± 0.19 a	24.8 ± 2.57 c	39.0 ± 4.82 a
EPS-US	0	18.9 ± 1.00 b	0.61 ± 0.05 a	59.2 ± 1.00 a	21.3 ± 1.00 c
	48	13.0 ± 0.22 c	0.16 ± 0.01 c	23.9 ± 0.29 c	62.9 ± 2.30 a
EPS-H	0	5.32 ± 0.90 a	0.61 ± 0.10 a	88.0 ± 7.00 a	6.12 ± 0.50 a
	48	6.88 ± 0.12 a	ND b	93.1 ± 0.26 a	ND d
EPS-L	0	31.7 ± 1.00 a	0.34 ± 0.10 a	5.50 ± 0.50 c	62.5 ± 5.00 a
	48	36.8 ± 0.89 a	ND b	16.6 ± 0.03 a	46.6 ± 0.68 b

MC: methylcellulose, EPS: exopolysaccharides, EPS-US: exopolysaccharides treated with ultrasound; EPS-H: exopolysaccharides with high molecular weight, EPS-L: exopolysaccharides with low molecular weight. ND: not detectable. ANOVA was used. Different letters a, b and c mean significant difference ($p < 0.05$) from each other in the same column with the same substrate fermentation. (Average of three fecal fermentations, error bars representing standard deviation at $n = 3$).

Fig. 1. The changes of total monosaccharide concentration during *in vitro* fermentation of fecal samples with MC and different EPS fractions. Total monosaccharides at 0 h was normalized to 100%. Average of three fecal fermentations, error bars representing standard deviation at n = 3. MC: methylcellulose, EPS: exopolysaccharides, EPS-US: exopolysaccharides treated with ultrasound; EPS-H: exopolysaccharides with high molecular weight, EPS-L: exopolysaccharides with low molecular weight.

Fig. 2. The changes of pH (A) and the concentrations (mM) of acetic acid (B), propionic acid (C) and butyric acid (D) during *in vitro* fermentation of fecal samples with MC and different EPS fractions. MC: methylcellulose, EPS: exopolysaccharides, EPS-US: exopolysaccharides treated with ultrasound; EPS-H: exopolysaccharides with high molecular weight, EPS-L: exopolysaccharides with low molecular weight. (Average of three fecal fermentations, error bars representing standard deviation at $n = 3$)

Fig. 3. Colony form units (cfu) of different groups of bacteria at 0-48 h during *in vitro* fermentation of fecal samples with MC and different EPS fractions: (A) *Bifidobacterium*; (B) *Lactobacillus*; (C) *Clostridium*; (D) *Escherichia coli*; (E) *Enterococcus*; (F) Total anaerobic bacteria; (G) Total aerobic bacteria. Different letters indicating significant differences between substrate at the same time point; Asterisks indicating significant difference compared to 0 h within the same substrate *: $p < 0.05$, **: $p < 0.01$; ***: $p < 0.001$ by two-way multivariate analysis of variance with Tukey post-hoc tests. MC: methylcellulose, EPS: exopolysaccharides, EPS-US: exopolysaccharides treated with ultrasound; EPS-H: exopolysaccharides with high molecular weight, EPS-L: exopolysaccharides with low molecular weight. (Average of three fecal fermentations, error bars representing standard deviation at $n = 3$).

Fig. 4. The taxonomic distribution of fecal microflora at the phylum (A), family (B) and genus level (C). MC: methylcellulose, EPS: exopolysaccharides, EPS-US: exopolysaccharides

596 treated with ultrasound; EPS-H: exopolysaccharides with high molecular weight, EPS-L:
597 exopolysaccharides with low molecular weight. D1/2/3: donor 1/2/3.
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Revised Ms. IJBIOMAC-2019-7678

Submitted to: *International Journal of Biological Macromolecules*

(Original Research MS)

Effects of exopolysaccharide fractions with different molecular weights and compositions on fecal microflora during *in vitro* fermentation

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Abstract

This study was to investigate the potential prebiotic function of exopolysaccharide (EPS) from a medicinal fungus and the relationship to the molecular properties by *in vitro* human fecal fermentation. The EPS from *Cordyceps sinensis* Cs-HK1 mycelial fermentation was processed into three fractions with different monosaccharide contents, a higher molecular weight (MW) and a lower MW attained by two-step ethanol precipitation, and an intermediate MW by ultrasound-degradation of EPS. All the EPS fractions were well utilized during 24-48 h of fecal fermentation, leading to significant increases in the short chain fatty acid (SCFA) production. The consumption rate and production level of SCFAs varied slightly with the different EPS fractions. The EPS also influenced the composition and diversity of the fecal microflora, increasing the relative abundance of *Firmicutes* but suppressing that of *Proteobacteria*, which may be a beneficial effect for human health. Overall the results have shown that the Cs-HK1 EPS has significant prebiotic activity which is dependent on its molecular properties.

Keywords: *Cordyceps sinensis* fungus; exopolysaccharides; human fecal fermentation

1. Introduction

The human intestinal microflora is a diverse microbial community which is composed mainly of bacteria [1]. Among the numerous bacterial phyla that have been detected in the human gut, *Firmicutes* and *Bacteroidetes* are most dominant, and Actinobacteria including *Proteobacteria* and *Verrucomicrobia* are also commonly found as the minor components of microflora [2]. It is generally believed that the gut microbial composition and diversity have a close association with the host physiology and pathology [3]. Diet is the most probable factor which can affect the microbial composition and balance [4]. The non-digestible dietary fibers and prebiotics including complex polysaccharides and oligosaccharides can reach the large intestine and be fermented by the gut bacteria [5]. The selective consumption of the non-digestible carbohydrates by different microbial species can directly affect their proliferation and relative abundance in the gut microflora [6]. On the other hand, the fermentative metabolism of carbohydrates in the gut yields short chain fatty acids (SCFAs) as the end products, including acetic acid, propionic acid and butyric acid, which have important and beneficial functions in the host both locally and systemically [7].

Natural polysaccharides from edible plants, fungi, macro-algae and other sources have been widely explored as functional food ingredients because of their notable health benefits. As most

of these complex polysaccharides are non-digestible, their most possible site of utilization and action is in the gut microflora [8]. In this regard, increasing attention in recent studies has been paid to the effects of natural polysaccharides on or associated with the gut microflora [9-11]. Edible mushrooms or fungi which are rich of bioactive polysaccharides have been recognized as the potential source of prebiotics [12]. The Chinese caterpillar fungus *Cordyceps sinensis* is a precious medicinal fungus with a wide range of bioactivities and health benefits [13]. As natural *C. sinensis* is very rare, mycelial fermentation has been widely applied to produce *C. sinensis* fungal materials to meet the high and rising demand for health food and herbal medicine. Cs-HK1 is a fungus species isolated from a natural *C. sinensis* fruiting body and has been applied to mycelial fermentation for production of mycelial biomass and exopolysaccharides (EPS). The EPS attained from the Cs-HK1 mycelial fermentation was mainly composed of heterogenous polysaccharides in a wide molecular weight (MW) range and the major monosaccharide constituents include glucose (Glc), mannose (Man) and galactose (Gal) [14, 15].

Our recent studies performed in pure cultures of bifidobacteria have shown the beneficial and protective effect of the Cs-HK1 EPS on the probiotic bacteria [16]. However, neither the high-MW native EPS nor the partially hydrolyzed low-MW EPS fractions could be well utilized as a carbon source for the bifidobacterial growth. It has been suggested that the degradation and consumption of non-digestible complex carbohydrates by the gut bacteria is accomplished through cross-feeding of different bacterial species in the human colon [17]. The mixture fermentation system is more effective and realistic for assessment of complex polysaccharides in human gut microflora. Fecal fermentations (*in vitro*) have been widely used to study the functions and metabolism fates of food ingredients such as natural polysaccharides [18, 19].

This work was to investigate the fermentability and prebiotic function of the Cs-HK1 EPS in human fecal microflora by *in vitro* fermentation and the dependence on the composition and molecular weight of EPS fractions. The monosaccharide contents of EPS and SCFAs were analyzed quantitatively during the *in vitro* human fecal fermentation to assess the consumption and utilization of EPS by the gut bacteria. The microbial composition and diversity of the fecal microflora was examined by 16S rRNA gene analysis.

2. Materials and methods

2.1. Preparation of Cs-HK1 EPS fractions

As described previously, the liquid medium for Cs-HK1 mycelial fermentation consisted of 40 g/L glucose, 5 g/L peptone, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$ and 10 g/L yeast extract. The Cs-HK1 mycelial fermentation was carried out in shake-flasks at 20 °C for 6 days and exopolysaccharide (EPS) was isolated from the Cs-HK1 liquid fermentation medium by ethanol precipitation (with 80% v/v ethanol) as described in detail previously [16]. The power ultrasound (US) treatment was applied to the liquid fermentation medium before ethanol precipitation to produce EPS-US fraction with a lower MW and a higher water-solubility as reported in previously [16, 20]. Another two EPS fractions were prepared by two-step ethanol precipitation as reported previously [21, 22], firstly using 40% (v/v) ethanol to attain EPS-H with a much higher MW and secondly using 80% (v/v) ethanol to obtain EPS-L with a much lower MW than the EPS attained by single step precipitation using 80% (v/v) ethanol. Both the strategies of using power US and two-step ethanol precipitation have been proven effective in our previous studies for preparation of the desired lower MW EPS fractions. As shown in our previous studies, the whole EPS attained by a single step precipitation with 80% ethanol, the EPS-H and EPS-L fractions by two-step ethanol precipitation and the EPS-US after US treatment were mainly composed of heterogenous polysaccharides with different average MWs. All the EPS precipitates were recovered from the liquid by centrifugation at 12,000 rpm for 15 min, re-dissolved in a small amount of deionized (DI) water and freeze-dried as the final EPS fractions for experiments. The MW of EPS fractions were analyzed by high performance gel permeation chromatography (HPGPC) as reported previously [21].

Methylcellulose (MC) (15 centipoise viscosity with 2% aqueous solution at 25 °C; Sigma, Saint Louis, MO, USA), a non-digestive and non-fermentable carbohydrate polymer [23, 24], was included as a control for comparison with the EPS fractions in the fecal fermentation experiments.

2.2. Fecal sample collection and preparation

Fecal material was collected from three healthy donors (D1, D2 and D3, two females and one male, 26-30 years of age). All donors were free of known gastrointestinal diseases and metabolic disorders and had not received antibiotic probiotic and not taken any prebiotic supplements for at least 3 months prior to the sample collection. Fecal samples were collected in sterile tubes and immediately stored in anaerobic jars containing anaerobic sachets (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan), which were used in the fermentation experiments within 30 minutes of collection. The fecal samples were 10-fold diluted with the

phosphate buffered saline (PBS) and homogenized for 2 min, and then centrifuged at 500 rpm for 5 min to remove large particles. The remaining suspension was used as the initial inoculum in the fecal culture fermentation. All these were carried out in anaerobic atmosphere according to well documented protocol [25].

2.3. Fecal culture fermentation

The basal culture medium was consisted of peptone water (2 g/l), yeast extract (2 g/l), NaCl (0.1 g/l), dipotassium phosphate (0.04 g/l), monopotassium phosphate (0.04 g/l), sodium bicarbonate (2 g/l), magnesium sulfate heptahydrate (0.01 g/l), calcium chloride hexahydrate (0.01 g/l), Tween-80 (2 ml/l), hemin (50 mg/l), phylloquinone (vitamin K1, 10 ml/l), L-cysteine (0.5 g/l), bile salts (0.5 g/l), resazurin (1 mg/l) in distilled water [25]. The medium pH was adjusted to 6.8 with 1 M HCl. The EPS fractions and MC were dissolved in the basal medium at 5 mg/ml with vigorous stirring for overnight and then autoclaved at 121ºC for 20 min. The liquid medium was dispensed aseptically into 10-mL culture tubes at 5.4 mL each. Each tube was inoculated with 0.6 ml freshly prepared fecal slurry (1:10, w/w). The fecal culture tubes were enclosed in anaerobic jars containing anaerobic sachets and incubated with shaking at 200 rpm and 37 °C for 48 h. During the fecal fermentation, samples were collected at 0, 5, 10, 24 and 48 h for enumeration of the bacterial populations, 16S rRNA gene sequencing, determination of carbohydrate utilization profiles, and SCFA production. A new anaerobic gas generating sachet was applied each time when the jar was opened for sample collection.

2.4. Enumeration of bacterial populations on selective agar plates

Cultivation on selective agar plates is a rapid and convenient method for enumeration of some common bacterial groups in mixed cultures by counting the viable cells (CFU/ml). Total anaerobic and aerobic bacteria were counted on BHI agar; *Clostridia, Esherichia coli, Staphylococcus, Enterococcus, Lactobacillus* and *Bifidobacterium* were counted respectively by Tryptose Sulfite Cycloserine (TSC), MacConkey agar, Mannistol Salt Agar (MSA) Enterococcus agar, MRS agar, Bifidobacterium agar (Haibo Biotechnology Co. Ltd., Qingdao, China). The fecal slurry was diluted with PBS sequentially from 10^{-1} to 10^{-12} according to preliminary results, then 100 µl was spread onto each agar plate. After incubation in anaerobic or aerobic atmosphere at 37 °C for 48 h, the number of colonies on the plate was counted.

2.5. DNA extraction, 16S rRNA gene sequencing and microbial composition analysis

Since culture-based analysis is limited to enumeration of certain bacterial species [26], genomic

analysis was performed for enumeration of the many bacterial species, and for assessment of the microbial composition and diversity of fecal microflora. The fecal culture samples were centrifuged at 12,000 rpm for 5 min and the sediment was collected for the gene sequencing and the supernatant analysis for monosaccharide (1.4 ml) and SCFA analysis (0.2 ml). DNA extraction was carried out of the solid samples using Tiangen stool DNA extraction kit (Tiangen, Beijing, China) according to the manufacturer's manual. The microorganism DNA concentration was determined by NanoDrop 2000 (Thermo Fisher, Massachusetts, USA) to be 6-100 ng/μl, total initiation mass ≥ 30 ng. The 16S rRNA gene sequencing was conducted by BGI (Shenzhen, China). The database used for species annotation was Greengene (default): V201305[8]; RDP: Release11_5,20160930.

2.6. Analysis of monosaccharides in EPS

The monosaccharide composition of EPS fractions was analysed by liquid chromatography-mass spectrometry (LC-MS) as reported previously [27] with minor modifications. The monosaccharide standards (d-Arabinose, d-fructose, d-galactose, d-glucose, L-rhamnose monohydrate, d-mannose, d-glucose $13C_1$) and trifluoroacetic acid (TFA), HPLC grade acetonitrile (ACN), and methanol (MeOH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of monosaccharides and internal standard d-glucose $13C_1$ were prepared at 10 mg/mL in Milli-Q water. Each of the stock solutions was diluted with the LC solvent ACN/H_2O (50:50 v/v) before being injected into the LC system. External calibration standard solutions were prepared in the range of 0.5 to 50 μg/mL, each containing 1 μg/ml d-glucose $13C_1$. All these solutions were stored at 4 °C before use.

The EPS samples (5 mg) were dissolved in 1.4 ml of Milli-Q water with stirring for overnight. The supernatant (1.4 ml) collected from the fecal fermentation samples in section 2.5 was also analysed. Each of the sample solutions was mixed with 0.6 ml of TFA (4 M TFA final concentration) and hydrolysed at 100 °C for 8 h. Excessive TFA was removed by vacuum evaporation and the residue was redissolved in 2 mL of water. Further clean-up was accomplished by solid phase extraction (SPE). The SPE cartridge was preconditioned by 3 ml of methanol and then 3 ml of ultrapure water. The sample solution was loaded onto the cartridge and the eluent was collected, evaporated to dryness under vacuum, and then redissolved in about 2 mL or more of ACN/ H_2O (50:50 v/v) for the LC/MS/MS analysis. D-glucose 13C1 (0.01 mg) as an internal standard was added to the solution. For evaluation of matrix effects, the vacuum-dried residue was redissolved in 2 mL of ACN/H_2O (80:20 v/v) and was used to

prepare matrix-containing standard solution. The treated samples were diluted to desired concentrations by ACN/H_2O (80:20 v/v) before injection.

The LC separation was achieved with a normal phase method on an ACQUITY UPLC® BEH amide column (2.1mm×100mm, 1.7 µm particles; Waters, Milford, Massachusetts, USA) and a mobile phase flow rate of 0.3 ml/min. The mobile phase was consisted of water (A) and acetonitrile (B), MS Grade (Honeywell, Burdick and Jackson, Morristown, NJ, USA). The elution was carried out primarily by a linear gradient over 30 min from 95 to 60 % (v/v) mobile phase B, followed by a column wash and re-equilibration. Column was maintained at 20 °C and auto-sampler plate set at 4 °C. An Agilent 6460 HPLC system (Santa Clara, Foster City, CA, USA) was coupled in-line with an electrospray ionisation triple quadrupole mass spectrometer (Santa Clara, Foster City, CA, USA) equipped with an Agilent Jet Stream electrospray ionisation (AJS ESI) source. Ions were detected by multiple-reaction monitoring (MRM) method in negative polarity with a dwell time of 200 ms and the following source conditions: gas temperature at 300 °C, gas flow at 8 l/min, nebulizer pressure at 45 psi, sheath gas temperature at 320 °C, sheath gas flow at 11 l/min, capillary positive voltage at 3500 V (positive) and 3000 V (negative), nozzle voltage at 500 V. Data acquisition was performed using MassHunter Quantitative Analysis software B.07.00.

2.7. Analysis of short chain fatty acids

Short chain fatty acids (SCFAs) in the fecal cultures were analysed by Gas Chromatograph-Flame Ionization Detection (GC-FID) using an Agilent 7980B GC system and a fused silica capillary column (Agilent Technologies Inc., Palo Alto, CA, USA) as described previously [16]. In brief, 0.2 ml of fecal culture supernatant was diluted by 0.8 ml of Milli-Q water and the pH was adjusted to 2-3 with 1 M HCl. An internal standard (2-ethylbutyric acid) was added at 1 nM final concentration before injection into the GC system. Nitrogen gas was applied as the mobile phase flowing at 0.6 ml/min. The initial oven temperature was maintained at 80 °C for 2 min and raised gradually to 180 °C at 6 °C/min, and maintained for 4 min. The injection volume was 1 µl and the temperature was controlled at 200 °C, and the detector temperature at 220 °C. Six SCFA standards (Aladdin®, Shanghai, China) were used for identification and quantification, including acetic acid, propionic acid, *n*-butyric acid, *i*-butyric acid, *n*-valeric acid, and *i*-valeric acid. The *n*-butyric and *i*-butyric acid were combined in the analysis.

2.8. Statistical analysis

One-way ANOVA was used to compare the monosaccharide composition of EPS fractions after fecal fermentation. Two-way ANOVA with Tukey post-hoc test was used for the comparison of cfu among different groups. Kruskal-Wallis test was used to compare the operational taxonomic unit (OTU) difference between MC and experimental groups. The paired *t*-test was used to compare different taxonomic compositions between experimental and control groups [28]. The p value < 0.05 indicated a significant difference. SPSS 23.0 was applied for statistical analysis.

3. Results and discussion

3.1. Monosaccharide composition of different EPS fractions

Table 1 shows the molar ratios of constituent monosaccharides in the different EPS fractions. All EPS fractions were composed mainly of mannose, glucose, galactose and fructose with different molar ratios. EPS and EPS-US had the similar molar composition of four monosaccharides, suggesting that ultrasonic degradation did not cause chemical or structural change in the EPS molecule. Compared with EPS and EPS-US, EPS-H had a much higher ratio of glucose (6.83 to mannose) and EPS-L had a much lower ratio of glucose and a much higher ratio of galactose (0.34 and 2.02 to mannose, respectively).

As the Cs-HK1 EPS was resistant to hydrolysis by digestive enzymes and simulated gastric acid [29], the undigested EPS fractions were used in the following experiments.

3.2. Consumption of EPS during fecal fermentation

Fig. 1 shows the changes in the total concentration of monosaccharides in the culture media containing MC and EPS fractions after complete TFA hydrolysis during the *in vitro* fecal fermentation. The total monosaccharide concentration in the MC medium did not show any change (ANOVA, $p = 0.880$), implying that MC was not utilized during the fermentation and confirming its role as a non-fermentable carbohydrate polymer control. In contrast, the total monosaccharide concentrations in the cultures with the four EPS fractions all dropped sharply, to about 20% or below 5%, from 10-24 hours during the fecal fermentation, suggesting their consumption by the gut flora. The consumption of most EPS fractions except EPS-H took place mainly between 10-24 h after the inoculation, due probably to that the period of 0-10 h was in the lag period and the period of 24-48 h was in the stationary phase of the fecal fermentation [16]. As for EPS-H, the consumption or reduction of total monosaccharides remained steady from 10 to 48 h, suggesting a slower fermentation rate as well as a longer growth period than

the fecal cultures with other three EPS fractions. The slower consumption of EPS-H than the other EPS fractions was probably attributed to its higher MW, causing a higher viscosity in the culture medium and slower nutrient uptake and slower metabolism. This was in good agreement with the finding in our previous study that the higher MW EPS was more effective to extend the growth and survival of bifidobacteria [29]. Moreover, the EPS-US was consumed more rapidly than EPS, due probably to the increased bioavailability by the ultrasonic degradation of EPS [21].

Table 2 shows the molar ratios of monosaccharides in the EPS fractions before and after 48 h of fecal fermentation. The complete data (5, 10 and 24 h) was shown in Table S2. For EPS-US, the molar ratio of glucose decreased more dramatically, while that of galactose increased significantly over the fermentation period. This suggests that the majority of fecal bacterial community preferred glucose as the major carbon source than galactose or mannose. In contrast, for the EPS-H and EPS-L fractions, galactose was consumed more during the fermentation. It is possible that the EPS-H and EPS-L was taken up as a whole and consumed by the fecal bacteria. It has been suggested that polysaccharides are mostly transported into and metabolized within probiotic bacterial cells [30, 31], and the metabolism of polysaccharides in the gut microflora is dependent on their molecular properties [32].

3.3. Medium pH change and SCFA production during fecal fermentation

Compared with the control MC fermentation, all the fecal fermentation with the EPS fractions experienced an accelerated pH drop from 10 h to 24 h (Fig. 2A). In addition, the fermentation fed with EPS-H exhibited a slower pH drop between 10-24 h and a much faster drop between 24-48 h than the cultures with the three low MW EPS fractions. In association with the pH drop in the fecal cultures, the concentrations of three SCFAs, including acetic acid (Fig. 2B), propionic acid (Fig. 2C) and butyric acid (Fig. 2D) showed a notable ($p < 0.05$) increase from 10 to 24 h and a further, and smaller increase from 24 to 48 h. The SCFAs in the cultures fed with the EPS fractions increased to a much higher level than in the MC culture. Among the three SCFAs, acetic acid was the most abundant and butyric acid was the lowest in the cultures with the four EPS fractions. Another observation was that the butyric acid in the culture fed with EPS-H was notably higher (8.67 mM) than in the other three EPS cultures (3.38-4.58 mM). Compared with other EPS fractions, EPS-H had a much higher glucose content (Table 1), which may be a contributing factor to the high rate of butyric acid production as suggested previously that β-glucans composed of glucose increased the butyric acid formation in intestine

[33, 34]. The relative level of pH changes in the fermentation medium with various EPS fractions did not match with the level of EPS consumption, due probably to that the consumption of EPS by the fecal bacteria was not all used for acid production. A practical point is that the batch fecal fermentation is one of the most convenient and economic models for studying the fermentability and metabolism of dietary compounds by gut microflora [35].

3.4. Bacterial population distributions in fecal microflora

Fig. 3 shows the cfu values in the control (with MC) and test groups (with EPS fractions) which was enumerated by selective agar cultivation during fecal fermentation. The cfu values in all groups increased with culture time and the change showed no obvious dependence on the different EPS fractions. However, the cfu values of *Bifidobacterium* (Fig. 3.A), *Clostridium* (Fig. 3.C), the total anaerobic (Fig. 3.F) and aerobic bacteria (Fig. 3.G) at 10 h were notably higher in most of the EPS groups than the MC group, though the difference did not last long and diminished at 24 or 48 h. The cfu values indicated the viability of bacteria. In our previous studies, the higher MW EPS fractions including EPS and EPS-US could significantly protect the viability or survival of several bifidobacterial strains in pure cultures based on the cfu values, though they were not well utilized as a carbon source for the growth (based on the bacterial cell concentration quantified by optical density (OD)) [29]. In contrast, although the EPS fractions were well utilized in the fecal cultures, leading to a significant increase in the SCFA production, they did not offer any protective effect on the viability and survival, with little or no increase in the cfu in the later culture stage (24 - 48 h), especially the EPS-L (nearly no difference compared with MC). The ability of mixed fecal cultures to ferment the complex EPS can be attributed to the availability of all the required enzymes in various bacteria for the degradation of the polysaccharide bonds. On the other hand, the degradation and utilization of EPS by the bacteria resulted in the lack of protective effect in the mixed fecal cultures.

3.5. Effect of EPS on diversity of fecal microflora

There was no shift in the microbial diversity observed in the fecal microflora with MC or any of the EPS fractions (Kruskal-Wallis test, p-value = 0.556) (Supplemental data Fig. S1). In contrast, the fecal materials from different donors affected the results of *in vitro* fecal fermentation (Kruskal-Wallis test, p-value = 0.022) (Fig. S2), which contributed to the reduced richness in D3 incubations (Dunn's test, p-value < 0.1). Although the diversity or richness of bacterial populations is generally regarded as an indicator for the healthy balance and resilience of gut microflora [36], some previous studies have observed that the high microbiome richness

indices were associated with gastrointestinal disorders including prolonged transit times [37], hard stools [38], and increased proteolytic fermentation [39]. This signifies a complex relationship between community richness and gut health.

3.6. Effect of EPS on composition of fecal microflora

Fig. 4 shows the taxa relative abundance after 24 h of fecal fermentation with EPS and MC in the matching donor fecal slurries at different levels for identifying substrate specific responsive genera. On the phylum level (Fig. 4A), the Cs-HK1 EPS fractions resulted in increased *Firmicutes* relative abundance (paired *t*-test, EPS-H, $p = 0.042$; EPS-L, $p = 0.006$) at the expense of *Proteobacteria* relative abundance (EPS, $p = 0.031$; EPS-H, $p = 0.013$; EPS-L, $p = 0.014$) (Supplemental data Table S2). At the family level (Fig. 4B), the EPS fractions decreased the relative abundance of *Desulfovibrionaceae* (EPS, $p = 0.043$; EPS-US, $p=0.034$; EPS-H, $p = 0.045$; EPS-L, $p = 0.033$) and *Enterobacteriaceae* (EPS, $p = 0.029$; EPS-H, $p = 0.008$; EPS-L, $p = 0.017$). At the genus level (Fig. 4C), the EPS fractions induced specific changes in fecal microbial composition. The genera *Escherichia* of *Enterobacteriaceae* family (EPS, $p = 0.018$; EPS-H, $p = 0.015$; EPS-L, $p = 0.012$), *Bilophila* of *Desulfovibrionaceae* (EPS-US, $p = 0.048$; EPS-L, $p = 0.049$), *Parabacteroides* (EPS-US, $p = 0.026$) and *Phascolarctobacterium* family (EPS-US, $p = 0.029$) were reduced after fermentation with the EPS fractions (Table S3). In contrast, only one significant increase ($p = 0.029$) was observed with the low MW EPS-L in the *Phascolarctobacterium* relative abundance. In general, the EPS fractions reduced the relative abundance of the *Proteobacteria* phylum and had an insignificant effect on the *Bifidobacterium* relative abundance ($p > 0.05$). In comparison, EPS-US and EPS-L had a broader impact on the compositions of different microbial populations, causing more significant changes on relative abundances of three genera. The different effects of EPS fractions on gut microflora may be due to their different molecular properties.

The alternation of gut microbial composition is widely believed to be linked with numerous local and systemic disorders [40], though the mechanisms are far from being understood. One of the most common ecological patterns observed during intestinal microbial dysbiosis is an expansion of bacterial population of the phylum *Proteobacteria* [41], which is the most diverse phylum of bacteria associated with many diseases [42]. For example, there is mounting evidence for the association of *E. coli* with gastrointestinal disorders, particularly in inflammatory diseases such as Crohn's disease and ulcerative colitis [43, 44]. Therefore, the significant reduction of *Proteobacteria* (particularly *E. coli*) ratio with the EPS fractions

observed in this study may lead to a beneficial effect of EPS on human health through their action on the gut microflora.

There was no significant change ($p > 0.05$) in the relative abundance of *Bifidobacterium* with the addition of EPS fractions (Table S4). This may be due to the very low abundance of this genus in the fecal material of the three donors (0.24%, 0.26% and 1.01%, respectively), which could not compete with other abundant genera to utilize the polysaccharides [45]. Additionally, although the butyrate content was increased by the EPS, there was no significant change in the relative abundance of the butyrate-producing bacteria (including *Eubacterium*, *Faecalibacterium* and *Roseburia*) (Table S4). Indeed, the effect on microbial composition alone is insufficient to tell if the EPS are beneficial or not on the gut health [46]. To minimize the variation among donors, the enterotype classification may also be considered in future studies [47, 48].

4. Conclusions

Our present study has demonstrated the fermentability and consumption in fecal microflora of a complex, high-MW EPS produced by a medicinal fungus Cs-HK1 and the dependence on the molecular properties of different EPS fractions. The EPS fractions with different MWs and compositions derived from the EPS were all well utilized to produce SCFAs during fecal fermentation. Despite the heterogeneous composition of the fecal microflora among different donors and the limitations of in vitro fermentation model, the fecal fermentation experiments were simple and effective for evaluation of various EPS fractions for their effects on the diversity and composition of fecal microflora. Although no statistically significant changes were found on the presumably beneficial genera, the results still suggested the beneficial effect of EPS toward a healthier gut microbial ecosystem. Further studies through animal experiments are warranted to assess and verify the prebiotic function and related health benefits of EPS.

Acknowledgements

This work was funded by The Hong Kong Polytechnic University and the Hong Kong Research Grants Council Collaborative Research Fund (RGC CRF No. C5031-14E). Authors are grateful to Dr. So Pui Kin for help with the LC-MS analysis.

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552 **Table 1** Properties and composition of EPS fractions

EPS fractions	MW (Da)	[η] dl/g	Molar ratio of monosaccharides			
			Man	Fru	Glc	Gal
EPS	1.93×10^8	6.03	1.00 *	0.03	2.57	0.97
EPS-US	2.30×10^7	4.65	1.00	0.03	3.32	1.16
EPS-H	2.57×10^8	12.47	1.00	0.07	6.83	1.04
EPS-L	1.76×10^7	ND	1.00	0.01	0.34	2.02

553 * Standard variation (SD) < 5% for all data; ND: not detectable

554 MC: methylcellulose, EPS: exopolysaccharides, EPS-US: exopolysaccharides treated with
555 ultrasound; EPS-H: exopolysaccharides with high molecular weight, EPS-L:
556 exopolysaccharides with low molecular weight.

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559 **Table 2** The change of monosaccharide composition (molar ratio) of MC and EPS fractions
560 over 48 hours of fecal fermentation (total molar ratio 100%)

	Time (h)	mannose	fructose	glucose	galactose
MC	0	ND	0.30 ± 0.02 c	99.7 ± 1.03 a	ND
	48	ND	0.56 ± 0.02 ab	99.4 ± 1.87 a	ND
EPS	0	22.2 ± 5.00 b	0.59 ± 0.10 b	54.5 ± 3.00 ab	22.7 ± 3.00 b
	48	34.6 ± 3.77 a	1.57 ± 0.19 a	24.8 ± 2.57 c	39.0 ± 4.82 a
EPS-US	0	18.9 ± 1.00 b	0.61 ± 0.05 a	59.2 ± 1.00 a	21.3 ± 1.00 c
	48	13.0 ± 0.22 c	0.16 ± 0.01 c	23.9 ± 0.29 c	62.9 ± 2.30 a
EPS-H	0	5.32 ± 0.90 a	0.61 ± 0.10 a	88.0 ± 7.00 a	6.12 ± 0.50 a
	48	6.88 ± 0.12 a	ND b	93.1 ± 0.26 a	ND d
EPS-L	0	31.7 ± 1.00 a	0.34 ± 0.10 a	5.50 ± 0.50 c	62.5 ± 5.00 a
	48	36.8 ± 0.89 a	ND b	16.6 ± 0.03 a	46.6 ± 0.68 b

561 MC: methylcellulose, EPS: exopolysaccharides, EPS-US: exopolysaccharides treated with
562 ultrasound; EPS-H: exopolysaccharides with high molecular weight, EPS-L:
563 exopolysaccharides with low molecular weight. ND: not detectable. ANOVA was used.
564 Different letters a, b and c mean significant difference ($p < 0.05$) from each other in the same
565 column with the same substrate fermentation. (Average of three fecal fermentations, error bars
566 representing standard deviation at $n = 3$).
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Fig. 1. The changes of total monosaccharide concentration during *in vitro* fermentation of fecal samples with MC and different EPS fractions. Total monosaccharides at 0 h was normalized to 100%. Average of three fecal fermentations, error bars representing standard deviation at n = 3. MC: methylcellulose, EPS: exopolysaccharides, EPS-US: exopolysaccharides treated with ultrasound; EPS-H: exopolysaccharides with high molecular weight, EPS-L: exopolysaccharides with low molecular weight.

Fig. 2. The changes of pH (A) and the concentrations (mM) of acetic acid (B), propionic acid (C) and butyric acid (D) during *in vitro* fermentation of fecal samples with MC and different EPS fractions. MC: methylcellulose, EPS: exopolysaccharides, EPS-US: exopolysaccharides treated with ultrasound; EPS-H: exopolysaccharides with high molecular weight, EPS-L: exopolysaccharides with low molecular weight. (Average of three fecal fermentations, error bars representing standard deviation at $n = 3$)

Fig. 3. Colony form units (cfu) of different groups of bacteria at 0-48 h during *in vitro* fermentation of fecal samples with MC and different EPS fractions: (A) *Bifidobacterium*; (B) *Lactobacillus*; (C) *Clostridium*; (D) *Escherichia coli*; (E) *Enterococcus*; (F) Total anaerobic bacteria; (G) Total aerobic bacteria. Different letters indicating significant differences between substrate at the same time point; Asterisks indicating significant difference compared to 0 h within the same substrate *: $p < 0.05$, **: $p < 0.01$; ***: $p < 0.001$ by two-way multivariate analysis of variance with Tukey post-hoc tests. MC: methylcellulose, EPS: exopolysaccharides, EPS-US: exopolysaccharides treated with ultrasound; EPS-H: exopolysaccharides with high molecular weight, EPS-L: exopolysaccharides with low molecular weight. (Average of three fecal fermentations, error bars representing standard deviation at $n = 3$).

Fig. 4. The taxonomic distribution of fecal microflora at the phylum (A), family (B) and genus level (C). MC: methylcellulose, EPS: exopolysaccharides, EPS-US: exopolysaccharides

595 treated with ultrasound; EPS-H: exopolysaccharides with high molecular weight, EPS-L:
596 exopolysaccharides with low molecular weight. D1/2/3: donor 1/2/3.
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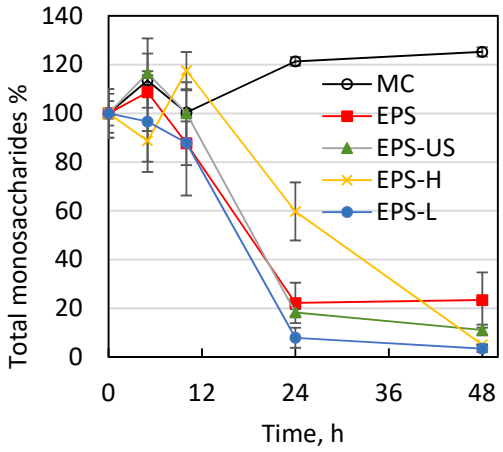

600 <Fig. 1 Int. J. Biol. Macromol, Mao et al>

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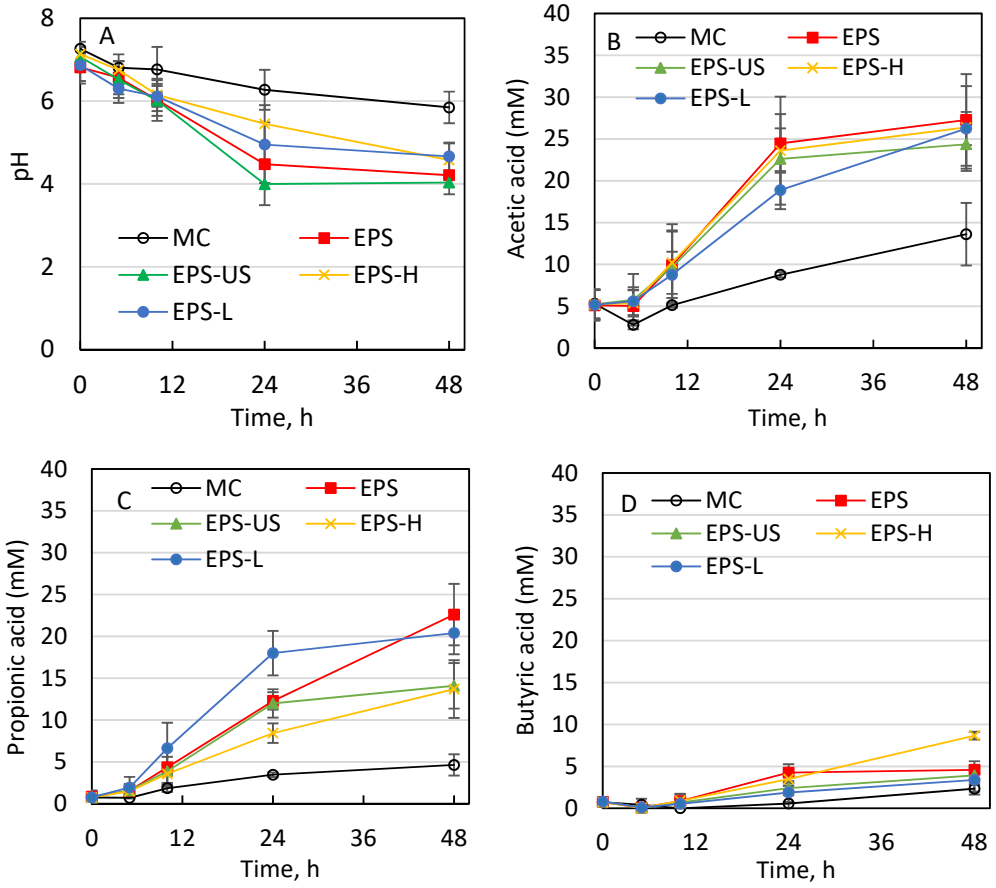

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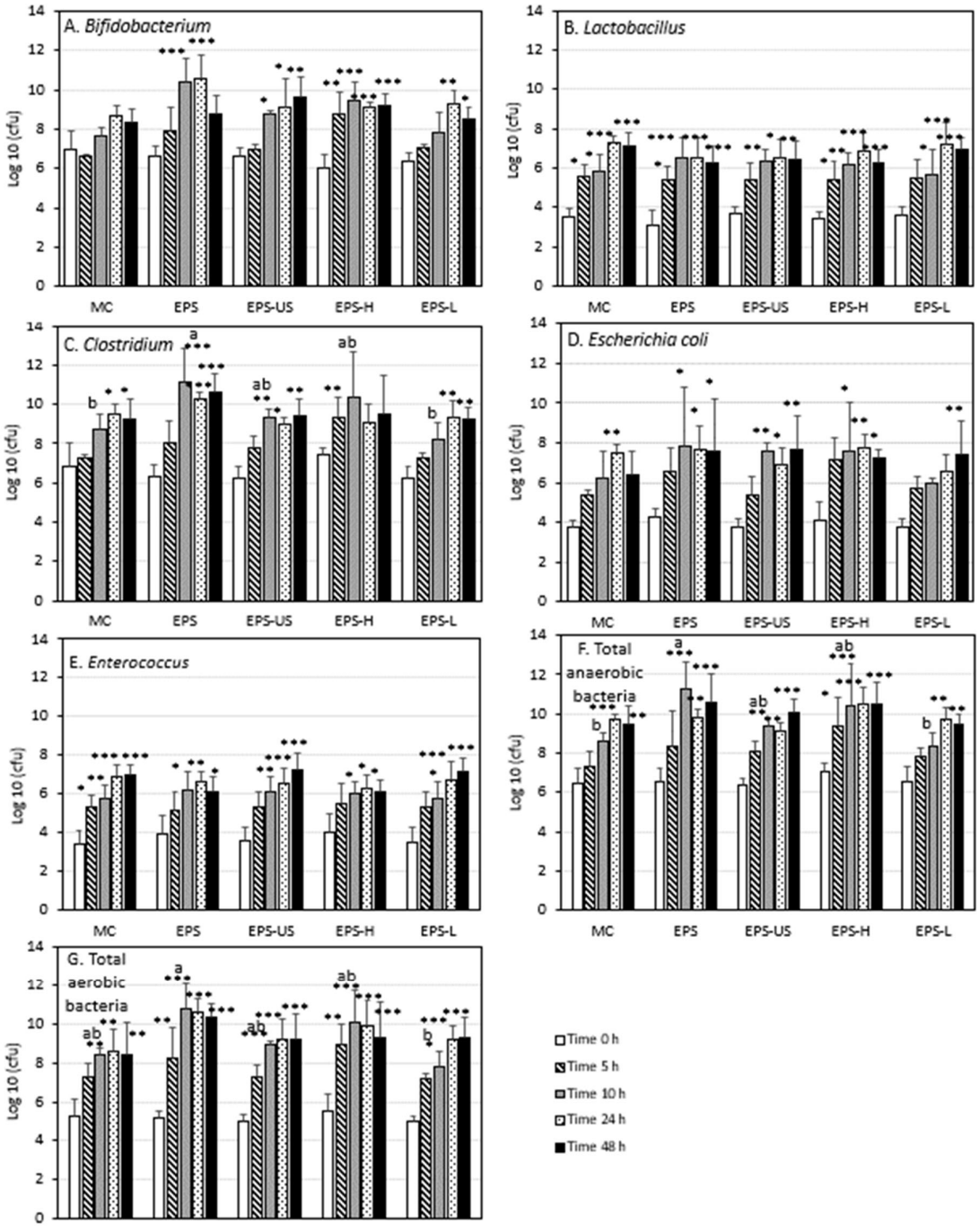

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609 <Fig. 3 Int. J. Biol. Macromol, Mao et al>

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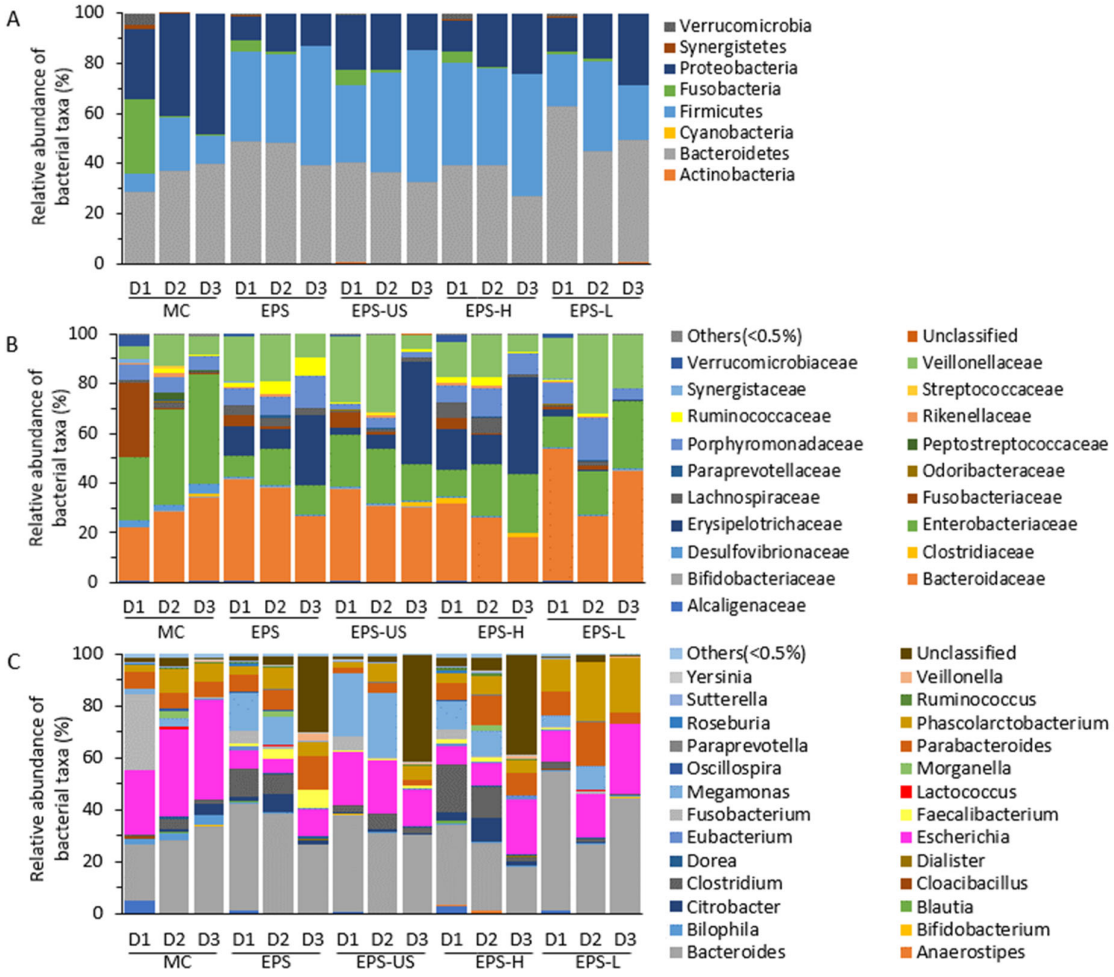

<Fig. 4 Int. J. Biol. Macromol, Mao et al>