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Modification and enhanced anti-inflammatory activity by *Bifidobacterial* fermentation of an exopolysaccharide from a medicinal fungus Cs-HK1

Long-Qing Li, Ang-Xin Song, Wing-Tak Wong, Jian-Yong Wu *

Research Institute for Future Food, Department of Applied Biology & Chemical Technology, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong

* *Corresponding author*:

E-mail: jian-yong.wu@polyu.edu.hk (J.Y. Wu).

Abstract

The exopolysaccharide (EPS) from the mycelial fermentation of a medicinal fungus *Cordyceps sinensis* Cs-HK1 had shown significant anti-inflammatory activity previously, and EPS-LM was a highly active fraction with a relatively low molecular weight (MW) isolated from the Cs-HK1 EPS. This study was to assess the effects of *Bifidobacterial* fermentation in anaerobic conditions on the molecular properties and anti-inflammatory activity of EPS-LM. In both *Bifidobacterial* cultures (*B. breve* and *B. longum*), EPS-LM was fractionally consumed as a carbon source, increasing the bacterial growth and acetic acid production. Analytical results from the fermentation digesta (supernatant) suggested that EPS-LM was partially degraded to lower molecular weight (MW) products with modified structures during the *Bifidobacterial* fermentation. More interestingly, the higher MW digesta fraction containing the partially degraded EPS-LM showed even stronger inhibiting activity than the original EPS-LM on the LPS-induced pro-inflammatory responses in THP-1 cell culture, including NF-κB activation, release of NO, TNF-α and IL-8. The study has shown that the fermentation by selected *Bifidobacterial* strains is effective to modify natural polysaccharides with enhanced bioactivities.

Keywords: Exopolysaccharide; *Bifidobacterial* fermentation; Anti-inflammatory activity.

1. Introduction

Fermented foods are increasingly recognized as healthy or functional foods because of their special health benefits such as anti-hypertensive, anti-inflammatory, immunomodulatory and

other activities [1, 2]. These health-beneficial activities of fermented foods are largely attributed to the presence of probiotic bacteria as well as the bioactive products derived from fermentative metabolism of the food components by the probiotic bacteria. SCFAs including acetate, propionate and butyrate are a group of the generally accepted beneficial products from probiotic bacteria metabolism [3], while many other possible metabolites have been suggested but much less abundant or detectable. Lactic acid bacteria, especially the *Lactobacilli* and *Bifidobacteria* species are the most important probiotic species in commercial fermented foods.

Along with the constantly rising concerning about the potential anti-inflammatory properties of gut microbiota and its metabolites [4, 5], some recent studies have attempted to utilize probiotic bacterial fermentation to modify and improve the bioactivities of natural and medicinal products. A previous study that *Bifidobacterium breve* strain could prevent intestinal inflammation through the Tr1 cells [6], and another study demonstrated that *Bifidobacteria* (*B. breve* and *B. longum*) grown on human milk oligosaccharides could downregulate the expression of inflammation-related genes in Caco-2 cells [7]. In a more recently, the water extract of a medicinal fungus *Ganoderma lucidum* showed enhanced inhibiting activity on LPS-induced NO production and pro-inflammatory cytokines in RAW264.7 cells after sequential fermentation with a *Bifidobacterium* followed by a *Lactobacillus* bacterium [8]. However, because of the complex crude extract, it was not clear about the molecules responsible for the higher anti-inflammatory activity. The fermentation with another probiotic *Bacillus* bacterium altered the MW and monosaccharide composition and enhanced the *in vitro* immunostimulatory activity of a heteropolysaccharide extracted from a Chinese herbal plant of *Dendrobium*

officinale [9]. Similarly, the *Lactobacillus* bacterium fermentation of a polysaccharide from vegetable *Asparagus officinalis* yielded polysaccharides with different molecular properties and enhanced antioxidative, and immunomodulatory activities [10].

Natural polysaccharides (non-starch) from various sources have been shown a variety of health benefits and pharmacological activities [11,12]. As most of these polysaccharides are non-digestible as the dietary fibres, they can reach the large intestine to be metabolized by the gut bacteria. It has been suggested that the beneficial activities of natural polysaccharides are acted through the gut microbiota [9, 13]. While the specific mechanisms are very complex and unclear, they can be collectively attributed to the prebiotic effects of the polysaccharides which can be selectively metabolized by certain gut bacteria so as to produce a health benefit [14].

Edible and medicinal fungi have been recognized as the attractive sources of prebiotic polysaccharides [15]. *Cordyceps* (*Ophiocordcyeps*) *sinensis*, an insect-parasitic fungus generally known as the Chinese caterpillar fungus, is a valuable medicinal fungus in traditional Chinese medicine (TCM) [16]. Since natural *C. sinensis* is very rare and expensive, mycelial fermentation is a more feasible means for production of the fungal polysaccharides and other useful components. Cs-HK1 is a *C. sinensis* fungus and an effective producer of exopolysaccharides (EPS) in mycelial fermentation. The crude and partially purified EPS fractions isolated from the Cs-HK1 fermentation liquid have shown notable prebiotic [17,18] and anti-inflammatory activities [19]. Very recently, a lower molecular weight (MW) fraction with an average MW of 360 kDa, designated EPS-LM, was separated from the complex EPS,

which was mainly composed of three monosaccharide residues including glucose, mannose and galactose [20]. This EPS-LM has been found more active than the whole EPS and the higher MW EPS fraction to inhibit the LPS-induced proinflammatory responses in THP-1 cell culture. More interestingly, the *in vitro* anti-inflammatory activity was notably enhanced after the fermentation of EPS with selected *Bifidobacterial* strains (*B. breve* and *B. longum*) in our preliminary experiments.

In view of the attractive potential for the generation of novel functions and enhanced bioactivities from fermented food and natural products, this study was to investigate the effects of probiotic bacterial fermentation on the molecular properties and *in vitro* anti-inflammatory activity of Cs-HK1 EPS. A highly active EPS fraction, EPS-LM found from our previous studies, was chosen in this study and subject to fermentation by two *Bifidobacterial* strains in a liquid culture medium. The fermented EPS-LM liquid medium or digesta and its separated fractions were analysed for the chemical composition, and molecular properties and assessed for their inhibiting activity on LPS-inducted pro-inflammatory responses in THP-1 cell culture.

2. Materials and Methods

2.1 Preparation and analysis of EPS fractions from Cs-HK1 mycelial fermentation

The Cs-HK1 fungus was originally isolated from the fruiting body of a wild *Cordyceps sinensis* and was maintained in mycelial culture as reported previously [21]. For the production of EPS, the Cs-HK1 mycelial fermentation was carried out in a liquid medium in shake-flasks at 20 °C for 7 days. The fermentation liquid was then centrifuged and the supernatant was collected for

the isolation of exopolysaccharide (EPS). As reported previously [20], a two-step ethanol precipitation was performed, firstly adding 40% (v/v) ethanol to the fermentation liquid to precipitate the high-MW EPS (EPS-HM) and 80% (v/v) to the remaining liquid to precipitate the lower-MW EPS (EPS-LM). The precipitate was recovered from the liquid by centrifugation (12,000 rpm, 15 min) and freeze dried as the crude EPS fractions. The EPS-LM fraction was re-dissolved in a small amount of deionized (DI) water and treated repeatedly with the Sevag reagent to remove the protein content. The aqueous solution was then collected and dialyzed against distilled water through a 3500 MW-cut off membrane for 48 h, and then freeze-dried as the final EPS-LM fraction for the following experiments. The deproteinized EPS-LM fraction was mainly composed of a heteroglycan with three monosaccharide residues, Man, Glc and Gal at 7.31: 12.95:1 mole ratio, which were similar to those reported previously [20].

2.2 *Bifidobacterial* fermentation of EPS-LM and digesta collection

Two strains of *Bifidobacteria*, *B. breve* (CICC6079) and *B. longum* (CICC6186), were chosen in this study after preliminary screening. The bacterial cultures were maintained in starch-free Reinforced Clostridium Medium (RCM), which was composed of beef extract (10 g/L), peptone (3 g/L), yeast extract (3 g/L), glucose (5 g/L), cysteine HCl (0.5 g/L), sodium chloride (5 g/L), sodium acetate (3 g/L) and agar (0.5 g/L for broth). Starch-free RCM medium was used in all experiments to avoid the interference of starch with the EPS-LM and its metabolite products. The EPS-LM fraction was dissolved in RCM liquid at 1 g/L final concentration by stirring vigorously for overnight and the solution was sterilized by autoclaving at 121 °C for 20 min. To initiate the liquid culture, a starter culture of each *Bifidobacterial* strain was inoculated

into RCM broth and incubated for about 18 h. The bacterial cell suspension from the starter culture was inoculated at 2% (v/v) into 10-mL centrifuge tubes, each filled with 5 mL of fresh RCM with or without EPS-LM. The culture tubes were all contained in airtight jars enclosed with anaerobic sachets and incubated at 37 °C with constant shaking at 200 rpm for a period of 24 h as reported previously [17, 18].

After the 24 h fermentation, the bacterial suspension was collected for analysis and further experiments. The bacterial concentration was determined by measurement of the optical density (OD) at 600 nm with a spectrophotometer. The bacterial suspension was centrifuged at 12,000 rpm for 20 min and the supernatant was collected as the digesta for analysis of soluble metabolite products and for the following experiments on the *in vitro* anti-inflammatory activity.

2.3 Analysis of short chain fatty acids (SCFAs)

As reported previously [18], SCFAs in the *Bifidobacterial* culture digesta were analyzed by gas chromatograph with flame ionization detection (GC-FID) using an Agilent 7980B GC system equipped with a fused silica capillary column (Agilent Technologies Inc., CA, USA). The digesta was diluted by 5-fold with Milli-Q water and the pH was adjusted to 2-3 with 1 M HCl before injection, with 2-ethyl butyric acid being included as an internal standard. Nitrogen gas was used as the mobile phase at a flow rate of 0.6 mL/min. The major SCFAs including acetic acid, propionic acid and butyric acid were detected and quantified with the standard chromatograms.

2.4 Fractionation of digesta

For detection of the changes in EPS-LM composition and structure from the *Bifidobacterial* fermentation and for identification of the major molecular components contributing to the anti-inflammatory activity, the *Bifidobacterial* digesta as well as the initial RCM medium containing EPS-LM was roughly separated into two MW fractions with 10 kDa MWCO ultrafiltration (UF) membrane. The separation was performed using Amicon® Ultra centrifugal tubes of 10 kDa MWCO (Millipore Amicon Ultra, Germany) at 4,000 rpm for 30 min at 4℃. The filtrate was collected as the lower MW faction <10 kDa, while the retentate was diluted with milliQ water, and centrifuged repeatedly in the UF tubes to remove the small molecules more completely. The final retentate was collected as the higher MW fraction >10 kDa and diluted to the same volume as before separation for the anti-inflammatory test in cell culture.

2.5 Analysis of digesta composition and MW profiles

The chemical composition and molecular weight (MW) of digesta with or without the EPS-LM as well as the liquid medium before the *Bifidobacterial* fermentation were determined by the methods as reported previously [20]. The MW distribution was analyzed by high-pressure gel permeation chromatography (HPGPC). The monosaccharide composition was analyzed by the 1-phenyl-3-methyl-5-pyrazolone high-performance liquid chromatograph (PMP-HPLC) method [22].

Infrared (IR) and NMR spectral analyses were performed of the high MW fractions (>10 kDa) of the *Bifidobacterial* medium attained from above UF separation. In order to eliminate the

interference of glucose and other components, the UF retentate fraction was further purified by chloroform extraction and the aqueous phase was collected, and lyophilized for the spectral analysis. For the IR analysis, the purified samples (1~2 mg) were stacked by potassium bromate precipitation method using a Fourier transform-infrared (FT-IR) spectrophotometer in the range of 600–4000 cm^{-1}. For the NMR analysis, the purified samples were subject to dissolution deuterium oxide and lyophilization for twice, and then re-dissolved in deuterium oxide. The NMR analysis was performed on a Bruker Avance-III 400 MHz instrument.

2.6 Anti-inflammatory assays in THP-1 cell culture

All the methods and conditions for the following assays have been described in detail previously [19,20]. The digesta and other sample solutions were all filter-sterilized through 0.22 μm membrane before being added to the THP-1 cell culture medium.

2.6.1 Cell line and culture conditions

The THP-1-Dual cell line used in this study was from InvivoGen (San Diego, CA, USA), which was originally derived from the human THP-1 monocyte cell line by stable integration of two inducible reporter constructs (NF-kB-SEAP and IRF-Lucia reporter monocytes). The THP-1 cell line is responsive to lipopolysaccharide (LPS) stimulation with proinflammatory responses [23]. As described previously [19,20], the THP-1 cell was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and other components. The cell culture was maintained at 37 °C in a humidified incubator with 5% CO_2 and kept in logarithmic growth at 5–15× 10^5 cells/mL through routine sub-culturing, according to standard ECACC protocol.

177 During the present study, the THP-1 cell culture was sub-cultured every 3 days by inoculating
178 7×10^5 cells/mL and the passage number was below 20.
179
180 **2.6.2 NF-kB assay**
181 NF-κB-induced SEAP activity of THP-1 cells was assessed using Quanti-Blue agent
182 (InvivoGen). The cells from a stable culture were incubated into 96-well flat-bottomed tissue
183 culture plates (2×10^5 cells/well) for 48 h and then subjected to treatment by LPS (Sigma-
184 Aldrich) at 0.2 µg/mL with *Bifidobacterial* digesta (25-100 µg/mL). A normal culture was
185 included as a control which was not subjected to any treatment. After 48 h incubation, 20 µg of
186 culture supernatant was collected from each well and the alkaline phosphatase activity was
187 assayed by adding Quanti-Blue reagent at 1:4 (v/v). The activation of NF-kB was determined
188 by measurement of absorbance at 625 nm relative to that of LPS-stimulated samples.
189
190 **2.6.3 Nitric oxide (NO) assay**
191 The culture supernatant of the THP-1 cells after various treatments was collected from each
192 well and the concentration of nitrite was determined by the Griess assay .
193
194 **2.6.4 Analysis of cytokines by ELISA**
195 After various treatments, the culture supernatant was separated from the THP-1 cells and
196 collected for the cytokine assay. The concentration of cytokines (TNF-α and IL-8) in the
197 supernatant was measured by DuoSet enzyme-linked immune sorbent assay (ELISA) kits
198 (R&D Systems) (Sigma-Aldrich) according to the manufacturer's instructions.

199

200 **2.7 Statistical analysis of experimental data**

201 The relative potency index of the effective doses for 50% inhibition is computed by the AAT
202 Bioquest, Inc. Calculator, 2019 [24], based on the following equation,

203 $Y = d + [(a – d)/(1+(X/50\% \text{ response })^b]$

204 where Y is the response values, X the treatment dose or concentration, and a and d the lower
205 and the upper asymptote, respectively. The steepness of the linear portion of the curve is
206 described by the slope factor, b. The parameter of 50% response was relative to the maximal
207 response achieved by the digesta having a dose–response curve corresponding to the response
208 midway between a and d. All the parameters (a, b, d and 50% response) were calculated by a
209 software for a four-parameter logistic regression model.

210

211 All the microplate assays were performed at least in six replicates and all other experiments
212 were performed in triplicate or more times. Independent samples t-test was performed to
213 determine or compare the statistical significance of the treatment effects. The statistical analysis
214 was performed using the SPSS 16.0 statistical software.

215

216 **3. Results and discussion**

217 **3.1 Effects of EPS-LM on Bifidobacterial growth and acid production**

218 As shown in **Table 1**, the EPS-LM added to the culture medium had significant effects on the
219 bacterial growth (with higher OD values) and the acetic acid production of both *Bifidobacterial*
220 strains, *B. breve* and *B. longum*. The results suggest that EPS-LM was partially utilized by the

221 *Bifidobacteria* as a carbon source for promoting both growth and acetic acid production. Acetic
222 acid is one of the major short chain fatty acids (SCFAs) derived from fermentative metabolism
223 of carbohydrates by gut bacteria. In our previous study, the lower-MW EPS fraction as well as
224 the whole EPS could be well utilized and nearly completely consumed during *in vitro*
225 fermentation of human fecal microflora, resulting in a significant increase in the acetic acid
226 production [25]. In the human GI tract, non-digestible prebiotic polysaccharides can reach the
227 large intestine and be catabolized by certain gut bacteria, generating SCFAs including acetate,
228 propionate and butyrate which are beneficial to the human health [3,26].
229
230 **3.2 Partial consumption and degradation of EPS-LM during *Bifidobacterial* fermentation**
231 **Table 2** shows the analytical results of monosaccharides in the bacterial culture medium before
232 and after fermentation. For both *Bifidobacterial* strains in the RCM culture medium, there was
233 only consumption of glucose but no consumption of mannose or galactose during the
234 fermentation. While in the RCM+EPS-LM medium, the concentrations of mannose and
235 galactose were significantly decreased after fermentation, as well as the glucose concentration
236 of *B. longum*. This set of experimental results provide more convincing evidence for the
237 consumption and utilization of EPS-LM as a carbon source by the two *Bifidobacterial* strains.
238
239 **Table 3** shows the GPC MW profiles of fresh RCM medium and the *Bifidobacterial* digesta
240 (culture supernatants) after 24 h fermentation with or without EPS-LM (Supplemental data
241 **Fig. A1** for the GPC spectra). Because of the highly complex medium composition and limited
242 resolution of GPC analysis, the results are only good for rough and approximate comparison as

follows. The higher MW peaks, e.g. 14.5 kD-620 kDa, may be attributed to the peptides and proteins in the RCM components such as beef extract, peptone and yeast extract. In the RCM+EPS-LM medium before fermentation, the peak area (19.3%) corresponding to the maximum MW (~735.7 kDa) was much larger than that of RCM (5.01% at 620 kDa), which may be attributed to the mixture of EPS-LM and the high-MW medium components with overlapping peaks on the GPC. After the *Bifidobacterial* fermentation, the top two MW peaks in all culture groups were both decreased, but more notably in the EPS-LM culture groups, e.g. from 620.0 and 193.7 kDa in RCM to 480.4 and 126.9 kDa in *B. longum*-RCM without EPS-LM but from 735.7 and 520.4 kDa to 349.0 and 174.7 kDa in *B. longum*-EPS-LM. The overall shifting of the MW distribution to a lower MW range can be attributed to the partial degradation and consumption of EPS-LM and the higher MW medium components by the *Bifidobacterial* fermentation. The more significant decrease in the higher MW peaks of the EPS-LM culture groups was consistent with the enhanced bacterial cell growth and production of acetic acid by EPS-LM in the culture medium.

3.3 Utilization and modification of EPS-LM by *Bifidobacterial* fermentation

Fig. 1 shows the IR spectra of the higher-MW fraction (retentate of 10 kDa UF membrane) of *Bifidobacterial* medium and digesta with or without the EPS-LM in the initial medium. As the spectrum of RCM medium was almost flat after purification, all the major peaks in the spectra of RCM+EPS-LM medium or *Bifidobacterial* digesta can be attributed to the presence of EPS-LM or partially degraded EPS-LM. All EPS-LM medium or digesta exhibited the characteristic absorption peaks of carbohydrates around 3400, 2900, 1650, 1412, 1380, 1250, 1042 and 810 cm^{-1}. For example, the absorption peak around 3400 cm^{-1} was attributed to the hydroxyl

stretching vibration, the peaks around 2900 and 1650 cm^{-1} were attributed to the C-H expansion ratio and bound water on the sugar chains, respectively. Moreover, the absorption bands at around 1400 cm^{-1} could correspond to the carbonyl C-O stretching vibrations, and the absorption peak around 1250 cm^{-1} demonstrated C=O stretching vibration, respectively [27]. In addition, the peak at 1042 cm^{-1} and 810 cm^{-1} indicated the pyranose form of sugar and the α-type glycosidic linkages, respectively [20, 28]. Although the IR spectra can hardly tell the structural changes of EPS-LM from fermentation, it is helpful to confirm the presence of polysaccharides originated from EPS-LM in the digesta.

The structural modifications of EPS-LM by the *Bifidobacterial* fermentation were further detected by comparing the ^{1}H NMR spectra of the RCM medium with or without EPS-LM before and after the fermentation (**Fig. 2**). Before the fermentation, the first peak on the ^{1}H spectra around 5.3 ppm was attributed to the medium components (**Fig. 2a**) and the other two small peaks (peak 1 at 5.11 and peak 2 at 5.13 ppm) were attributed to the C1 position of glucose, both of which overlapped with the C1 position of hexosyl glycosidic residues of EPS-LM fraction with a higher signal intensity (**Fig. 2b**). The RCM+EPS-LM medium also contained another C1 position at 5.04 (peak 3) and 4.97 (peak 4) ppm, respectively **(Fig. 1c**). After the *Bifidobacterial* fermentation for 24 h, the C1 peaks of these glycosidic pyranosyl residues (peaks 1 and 2) were notably suppressed in all digesta samples, indicating the consumption of glucose constituent as well as the pyranosyl residues of EPS-LM. For the RCM+EPS-LM groups, peak 4 split into 3 new peaks (4.95, 4.97 and 4.98 ppm) in the *B. longum* digesta **(Fig. 2d**), while a new peak 5.16 ppm (peak 5) appeared in the *B. breve* digesta (**Fig. 2e**), which all

suggested chain modification of EPS-LM after the fermentation. Moreover, new signals around 4.0-4.1 ppm and 3.6-3.7 ppm conspicuously appeared only in the RCM+EPS-LM digesta (**Figs. 2d&f**), which should assign to the protons of hexosyl glycosidic ring from digested EPS-LM residues, respectively [29, 30]. This also indicated the utilization and modification of the constituents of the EPS-LM fraction.

Taken together, the above analytical results confirmed the structure modification and consumption of EPS-LM during the *Bifidobacterial* fermentation. The ability to catabolize non-digestible complex carbohydrates is a major characteristic of *Bifidobacterial* species, though the activity and specificity on specific chain structures and sugar residues are highly strain dependent [31]. Since EPS-LM was only partially fermented and consumed during the bacterial fermentation, the digesta at the end of fermentation contained partially degraded EPS-LM, which probably contributed to the anti-inflammatory activity in the THP-1 cell culture to be assessed in the following experiments.

3.4 Inhibitory effects of EPS-LM digesta on proinflammatory responses of THP-1 cells

As shown in **Fig. 3**, the EPS-LM digesta of both *Bifidobacterial* strains suppressed the LPS-stimulated proinflammatory responses of THP-1 cells *in vitro* including NF-κB inflammatory activation (**Fig. 3a**), nitric oxide (NO) production (**Fig. 3b**), release of TNF-α (**Fig. 3c**) and IL-8 (**Fig. 3d**). In contrast, the RCM digesta (PC groups) at 100 μg/mL had only slight or insignificant effects on these proinflammatory responses. The results confirmed that the fermented EPS-LM and other products derived from the *Bifidobacterial* fermentation of EPS-

LM were mainly responsible for the significant *in vitro* anti-inflammatory activity of the *Bifidobacterial* digesta. Based on the **Fig. 3**, the effective doses for 50% inhibition were computed and presented **in Table 4**. For most of the proinflammatory markers except TNF-α, the values of both bacterial digesta were much lower than those of the unfermented EPS-LM, implying that the *Bifidobacterial* fermentation of EPS-LM enhanced the *in vitro* anti-inflammatory activity.

3.6 Identification of the active molecular fractions of EPS-LM digesta

For further identification of the active molecular fractions, the *Bifidobacterial* digesta was separate by a 10 kDa UF membrane into two fractions, MW<10 kDa and MW<10 kDa, and **Fig. 4** show their effects on the LPS-stimulated responses in THP-1 cell cultures. While the MW<10 kDa digesta fractions showed little or even negative effect (**Figs. 4a1-a3**). Although it is widely accepted that acetic acid (SCFA) and some other metabolic molecules from *Bifidobacteria* may have some beneficial effects [3,26], the dominated components of unused nutrients in the MW<10 kDa digesta fraction and the acidic condition could even contribute to stimulating the inflammation instead inhibiting. The MW>10 kDa digesta fractions of both *Bifidobacterial* strains showed a remarkable activity on the proinflammatory responses (**Figs. 4b1-b3**). It is noticeable that the MW >10 kDa fraction had a strong and notable inhibiting effect on most of the proinflammatory markers at a very low concentration of 5 μg/mL. As shown in **Table 4** above, the effective doses for 50% inhibition of the proinflammatory markers of the MW>10 kDa fractions were mostly lower than those of the whole EPS-LM digesta.

As predicted from the analytical results of the digesta, MW (**Table 3**), monosaccharide composition (**Table 2**) and NMR spectra (**Fig. 2**), the higher-MW components contained partially degraded EPS-LM and other higher MW metabolite products from the *Bifidobacterial* fermentation. The partially degraded EPS-LM not only had a lower MW but also a modified structure than the original EPS-LM based on the H NMR spectrum analysis. It is envisaged that the partially degraded EPS-LM is a chief contributor to the anti-inflammatory activity of digesta, while contributions from other unknown components are also possible.

A major characteristic of prebiotic fibers including oligosaccharides and polysaccharides is their selective fermentation by certain bacterial species in the gut microbiota which is beneficial to the host health [32]. Furthermore, the beneficial effects of prebiotic polysaccharides can be attributed to the metabolite products derived from the gut bacterial fermentation. A previous study has indicated some fermentable prebiotics allow specific changes both in the composition and/or activity of the gastrointestinal microbiota that confers health benefit [33]. Evidence from animal models also demonstrated that probiotics and their metabolites are contributed to balancing the inflammatory responses via the crosstalk between innate and adaptive immune cells and the intestinal microbiota control the equilibrium between immune tolerance and inflammation [34]. On the other hand, the selective fermentation of prebiotic carbohydrates is not only beneficial to the host health but also favorable to the growth of probiotic bacteria [35]. *Bifidobacterium* is one of the most important probiotic species and also the most active gut bacteria for fermentative metabolism of complex carbohydrates [36, 37]. Therefore, the present results gave the evidence that *Bifidobacterial* fermentation of bioactive polysaccharides may

have distinctive benefits on the modulation of host immune responses and protection against inflammatory diseases.

4. Conclusions

The above experimental results have shown that the EPS from Cs-HK1 mycelial fermentation was partially degraded and consumed as a carbon source by two *Bifidobacterial* strains, *B. breve* and *B. longum*, for growth and acetic acid production during anaerobic fermentation. The *Bifidobacterial* fermentation modified the structure and reduced the MW of EPS-LM and also enhanced the anti-inflammatory activity significantly. It is of significance to isolate, purify and characterize the fermented EPS-LM for the discovery of novel and active polysaccharide structures and for better understanding of the structure modification mechanism. The present study has demonstrated that *Bifidobacterial* fermentation of bioactive polysaccharides is a promising process strategy for generation of new functional food and therapeutic ingredients.

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Table 1 Bacterial cell growth (OD) and acetic acid production in *B. breve* and *B. longum* cultures after 24 h fermentation with or without EPS-LM in the culture medium.

EPS-LM, g/L	Growth profile (OD at 600 nm)		Acetic acid (mM)	
	B. breve	*B. longum*	*B. breve*	*B. longum*
0 (control)	0. 52 ± 0.01	0. 53 ± 0.01	48.2 ± 1.0	60.9 ± 1.1
1.0	0. 63 ± 0.03*	0.60 ± 0.01*	66.7 ± 0.8**	68.0 ± 1.6**

Note: * and **: statistically significant different from the control at $p < 0.05$ and $p < 0.01$, respectively. Each data value is represented by mean ± standard deviation (SD) at $n > 3$.

Table 2 Monosaccharide residues in *Bifidobacterial* medium before and after fermentation (analyzed by HPLC after TFA hydrolysis of the medium samples)

	Glc (g/L)	Man (g/L)	Gal (g/L)
RCM before	4.22 ± 0.10	0.03 ± 0.01	0.18 ± 0.01
RCM after, *B. breve*	1.66 ± 0.01 (consumed 2.56)	0.02 ± 0.01	0.17 ± 0.01
RCM after, *B. longum*	2.20 ± 0.03 (consumed 2.02)	0.04 ± 0.01	0.17 ± 0.01
RCM + EPS-LM before	4.70 ± 0.04	0.29 ± 0.02	0.22 ± 0.01
RCM + EPS-LM, *B. breve*	2.07 ± 0.02 (consumed 2.63)	0.15 ± 0.02*	0.18 ± 0.01*
RCM + EPS-LM, *B. longum*	1.63 ± 0.02** (consumed 3.07)	0.07 ± 0.01*	0.10 ± 0.01*

Note: * and **: statistically significant difference compared with the control at $p < 0.05$ and $p < 0.01$, respectively. Each data value is represented by mean ± SD at $n > 3$.

476 **Table 3** Molecular weight (MW) profiles of Bifidobacterial media before and after fermentation

477 (RT: retention time in HPGPC)

	RT (min)	MW (kDa)	Peak area (%)
RCM before fermentation	28.25	**620.0**	5.01
	39.98	193.7	23.6
	44.66	14.5	28.5
	46.02	2.59	9.29
	47.28	1.81	15.2
RCM after fermentation by *B. breve*	28.70	**523.8**	5.83
	41.45	130.5	33.9
	44.25	11.8	27.3
	45.76	3.64	7.49
	46.65	2.37	14.9
	49.00	1.34	12.9
RCM after fermentation by *B. longum*	28.62	**480.4**	5.53
	40.08	126.9	33.9
	42.69	22.6	13.0
	43.65	7.59	18.7
	45.75	3.56	26.7
	53.03	1.11	2.20
RCM+EPS-LM before fermentation	27.67	**735.7**	19.3
	36.67	520.4	4.84
	43.65	11.5	57.9
	46.96	2.39	15.1
RCM+EPS-LM after fermentation by *B. breve*	29.05	**419.9**	9.54
	40.58	85.4	24.5
	43.57	15.3	20.1
	44.67	4.61	21.7
	47.30	2.73	24.0
RCM+EPS-LM after fermentation by *B. longum*	28.79	**349.0**	13.6
	40.23	174.7	18.8
	43.88	12.0	18.1
	45.38	3.56	16.5
	47.96	2.90	28.6

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Table 4 Relative potency indexes of EPS-LM and the fermented EPS-LM digesta on the major pro-inflammatory markers as represented by the effective doses for 50% inhibition in μg/mL of the LPS-induced response (computed by the AAT Bioquest, Inc. Calculator, 2019 [27]).

Inflammat. markers	EPS-LM	*B. breve* digesta		*B. longum* digesta	
		Whole	MW> 10 kDa	Whole	MW>10 kDa
NF-κB	19.84	12.71	3.58	6.29	3.88
NO	34.74	21.18	n.d.	18.76	n.d.
TNF-α	63.75	73.24	30.25	93.79	19.67
IL-8	n.d.	17.33	10.54	17.96	6.01

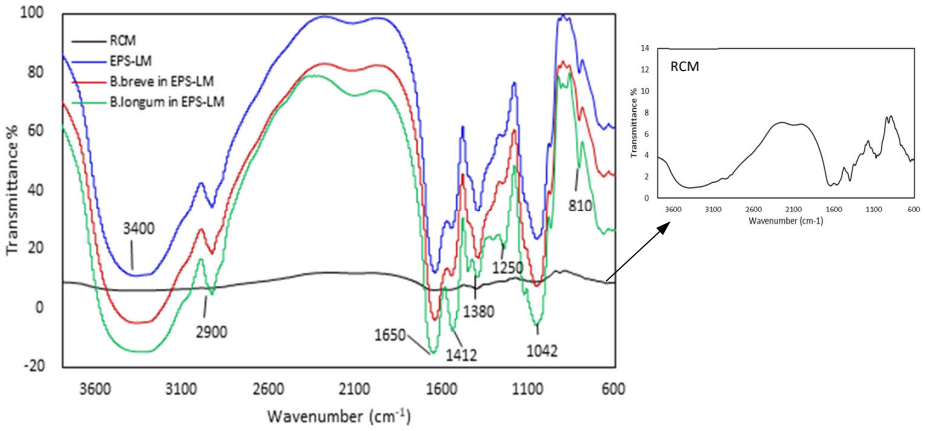
Note: Digesta, the supernatant medium collected after 24 h culture of each bacterial strain in RCM + 1.0 g/l EPS-LM. The values of EPS-LM were from our previous study [20].

Fig.1 FT-IR spectra of RCM and RCM+EPS-LM before, and after fermentation with *B. breve* or *B. longum* bacterium (all medium samples collected from the high MW retentate of 10 kDa UF).

Fig. 2 ^{1}H NMR spectra of Bifidobacterial culture media (>10 kDa): (a) Fresh RCM; (b) RCM + EPS-LM before fermentation; (c) *B.longum* fermented in RCM; (d) *B.longum* fermented in RCM+EPS-LM; (e) *B.breve* fermented in RCM; (f) *B.breve* fermented in RCM+EPS-LM. The signals were assigned according to Agrawal (1992) and Cheong et al (2016) [29, 30].

Fig. 3 Effect of EPS-LM Bifidobacterial digesta on LPS-induced responses in THP-1 cell cultures: (a) NF-κB activation; (b) NO production; (c) TNF-α release; (d) IL-8 release. (LPS at 0.2 μg/mL; EPS-LM at 1.0 g/L; PC: positive control with RCM digesta at 100 μg/mL; * and **: statistically significant differences compared with LPS-treated group at $p < 0.05$ and $p < 0.01$, respectively).

Fig. 4 Effects of lower-MW (**a**) and higher-MW (**b**) digesta fraction separated by a 10 kDa UF membrane on LPS-induced responses in THP-1 cell cultures: NF-κB activation (**a1, b1**), release of TNF-α (**a2, b2**) and IL-8 (**a3, b3**). (1.0 g/L EPS-LM added to RCM medium; * and **: statistically significant difference compared with LPS treated group at $p < 0.05$ and $p < 0.01$, by Student-t test).



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