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Protection of Bifidobacterial cells against antibiotics by a high molecular weight exopolysaccharide of a medicinal fungus Cs-HK1 through physical interactions

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Abstract

This study was to assess the protective effect of exopolysaccharide (EPS) produced by a medicinal fungus *Cordyceps sinensis* Cs-HK1 on *Bifidobacteria* against antibiotic inhibition. The high-molecular weight EPS fractions showed significant protective effect on all five bifidobacterial strains against four common antibiotics, leading to a dramatic increase in the minimal inhibitory and minimal bactericidal concentrations. The protective effect of EPS on the bacteria was probably attributed to the formation of a viscous layer around the bacterial cell resisting

the access by the antibiotics. The EPS layer surrounding the bacteria cell also promoted the aggregation of bacteria and formation of biofilm as observed by microscopy. EPS also enhanced the bifidobacterial adhesion to Caco-2 cell monolayer. In general, the protective effect as well as biofilm formation due to EPS was significantly correlated with the molecular weigjt of EPS fractions.

Keywords: Exopolysaccharide; Molecular weight; Bifidobacteria; Antibiotics; Biofilm

1. Introduction

Antibiotics are widely used in clinical therapy for human infectious diseases and also in poultry farming and aquaculture for decades [1]. Penicillin, enrofloxacin, tetracycline and streptomycin are among the antibiotics that are most commonly used as veterinary medicine and growth promotors [2-5]. However, the excessive intake of antibiotics from contaminated food products has become a human health threat due to the development of antimicrobial resistance [6]. The human gut microbiota is very sensitive to antibiotics and other food contaminants. Antibiotics may cause disruption of the healthy balance of gut microflora [7]. The imbalanced gut microbiota may be implicated in a number of diseases, such as inflammatory bowel disease (IBD), rheumatoid arthritis (RA), and other chronic metabolic diseases [7]. On the other hand, dietary carbohydrate fibres are beneficial for growth and colonization of Firmicutes in the intestine [8].

Cordyceps sinensis (Berk.) Sacc., generally known as Dong-Chong-Xia-Cao in Chinese, is a valuable medicinal fungus with a wide range of health promoting effects including antioxidant [9], anticancer [10, 11], antifatigue [12] and protective effects on liver, kidney and cardiovas-

cular system [13]. Since natural *C. sinensis* is very limited and cannot meet the increasing demand, mycelial fermentation has become the major source of the fungal products [14, 15]. Cs-HK1 is a fungus species isolated from a natural *C. sinensis* fruiting body and Cs-HK1 mycelial culture has been shown effective for production of exopolysaccharides (EPS) [16].

In a recent study from our group, a high-molecular weight (MW) (7.066×10^7-1.679×10^8 Da) food polysaccharide, konjac glucomannan (KGM) has been shown to offer significant protective effect on the survival of *Bifidobacteria* against specific antibiotics, while the acid-degraded low-MW fraction of KGM lost the protective effect [17]. In another study, the higher-MW fractions of KGM and EPS from Cs-HK1 mycelial fermentation showed more significant protective effect on Bifidobacteria in liquid culture [18]. According to these studies, it is hypothesised that the Cs-HK1 EPS can have a MW-dependant protective effect on Bifidobacteria against antibiotic inhibition through physical interaction with the bacterial cells.

This work was to investigate the protective effect of EPS from Cs-HK1 mycelial liquid fermentation and its partially degraded products against the inhibition of antibiotics with respect to the relative MW and the physical mechanisms. The enhanced biofilm formation as a possible mechanism for the protective effect was directly observed by scanning electronic microscopy (SEM) and atomic force microscopy (AFM), and confocal laser scanning microscopy (CLSM). Moreover, the Caco-2 cell monolayer was applied as a model to detect the effect of EPS on bifidobacterial adhesion on the intestinal mucosa [19].

2. Materials and methods

2.1. Chemicals and biologicals

Reinforced Clostridium Medium (RCM) and four antibiotics (enrofloxacin, streptomycin,

77 penicillin and tetracycline) were purchased from Guangdong Huankai Bio-Technology Co.,
78 Ltd. (Guangzhou, China). Five strains of Bifidobacteria were attained originally from China
79 Center of Industrial Culture Collection (CICC, Beijing, China). Cs-HK1 fungus for the pro-
80 duction of exopolysaccharide (EPS) was originally isolated from the fruiting body of a wild
81 *Cordyceps sinensis* and preserved at China General Microbiological Culture Collection Center
82 (Reg. No. 6004).
83 Reagents for the confocal laser scanning microscope (CLSM), phosphate buffer solution
84 (PBS) glutaraldehyde solution, and propidium iodide (PI) were from Shanghai Yuanye Bio-
85 Technology Co., Ltd., (Shanghai, China), and fluorescein isothiocyanate conjugated conca-
86 navalin A (FITC-ConA) solution from Sigma (USA). Caco-2 cell line (human colonic adeno-
87 carcinoma cell) was purchased from American Type Culture Collection (ATCC) [20]. The cul-
88 ture medium and supplements, Dulbecco's modified Eagle Medium (DMEM) from Gibco™,
89 Invitrogen, fetal bovine serum was attained from Biosera (France), 1% penicillin-streptomycin
90 from Sigma (USA).
91
92 *2.2. Bifodobacterial culture conditions*
93 Five strains of Bifidobacteria were used in this study including *B. adolescentis* (CICC6067),
94 *B. bifidum* (CICC6071), *B. breve* (CICC6079), *B. infantis* (CICC6069) and *B. longum*
95 (CICC6186). The bacterial cultures were maintained in Reinforced Clostridium Medium
96 (RCM), which was composed of beef extract (10 g/l), peptone (3 g/l), yeast extract (3 g/l),
97 soluble starch (1 g/l), glucose (5 g/l), cysteine HCl (0.5 g/l), sodium chloride (5 g/l), sodium
98 acetate (3 g/l), agar (0.5 g/l for broth, 15 g/l for agar plate). Experiments were carried out in
99 bacterial suspension cultures at 37°C for 24 h under anaerobic condition in airtight jars with
100 anaerobic sachets [21]. Details of the storage and culture conditions have been described pre-
101 viously [17, 18].

2.3. Preparation and analysis of Cs-HK1 EPS fractions

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 as reported in detail previously [18]. Two lower-MW EPS fractions were prepared as follows. EPS-US was attained by high-intensity ultrasound treatment of the liquid fermentation medium before ethanol precipitation as described previously [22]. An even lower-MW EPS fraction, EPS-LM was prepared through a two-step ethanol precipitation, firstly using 40% (v/v) ethanol to precipitate and remove the high EPS portion, and secondly using 80% (v/v) ethanol to precipitate the low-MW EPS from the remaining liquid medium. 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 the following experiments.

The chemical composition and MW of EPS fractions were measured by the methods as reported previously [17]. In brief, the total carbohydrate content of EPS was determined by Anthrone test (absorbance at 620 nm); the total protein content was determined by Lowry method (absorbance at 750 nm). The MW profiles of EPS fractions were analysed by high-pressure gel permeation chromatography (HPGPC). The monosaccharide composition was analysed by PMP-HPLC method.

2.4. Test of EPS on bifidobacterial growth

The EPS fractions and inulin as a prebiotic reference were dissolved in DI water at the desired final concentrations with vigorous stirring for overnight. After the addition of 38 g/l RCM powder into each sample solution, the mixture solution was sterilized at 121 °C for 20 min. The EPS fractions were added to RCM at a final concentration of 5 g/l, and then 2-fold

diluted into a series of concentration from 5 to 0.0782 g/l. The liquid medium was dispensed into a 96-well microtiter plate and each well was inoculated with 4×10^5 colony forming units (CFUs) of the Bifidobacteria (pre-cultured for 24 hours as described in 2.1) to a total liquid volume 200 µl per well. The microtiter plate was incubated at 37 °C under anaerobic condition for 48 h. RCM inoculated with bacteria was included as the control and RCM with EPS but no bacteria as the blank. The effect of EPS fractions on the bifidobacterial growth was represented by $(OD_{test} - OD_{blank})/OD_{control} \times 100\%$, where the optical density (OD) was measured at 600 nm.

2.5. Treatment of bifidobacteria with selected antibiotics and the EPS fractions

Four antibiotics that are commonly applied to livestock animals and fishery (enrofloxacin and streptomycin) and human medicine (penicillin and tetracycline) were chosen for this study. Antibiotic solutions were prepared freshly in culture medium at an initial concentration 2.048 mg/mL and used within 24 h. Two assays were applied to evaluate the sensitivity/tolerance of bifidobacteria to antibiotics, minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC), which were performed in microtiter plates as described previously [17]. In brief, each antibiotic solution was subject to serial two-fold dilution and the diluted antibiotic solution was transferred at 100 µl aliquots into a 96-well microtiter plate containing 100 µL of fresh bacterial culture medium in each well. The optical density at 600 nm of bifidobacterial cell suspension in the mid-log growth stage was adjusted to 0.1 with fresh RCM medium and the EPS tested, and 100 µL of the standardized bacterial suspension was inoculated into each plate well and incubated for 48 h. Antibiotic-free RCM medium was included as a control and bacterium-free RCM medium as a blank for each plate test. For the MBC assay, 20 µL of the bacterial suspension from each plate well was inoculated on RCM agar and the lowest concentration of a tested antibiotic with no visible growth after 48 h incubation was taken as the MBC value. All plate assays were performed in four replicates and the median

MIC and MBC values were recorded for each antibiotic–bacterium combination.

2.6. Microscopic examination of bacteria and EPS interactions

Microscopic experiments were performed for direct observation of the microstructures of the bifidobacteria and EPS fractions and their interactions. Three microscopy techniques, scanning electron microscope (SEM), atomic force microscope (AFM) and confocal laser scanning microscope (CLSM) were employed to capture the fiber structures of EPS fractions, the cell surface and biofilm formation of bifidobacteria. Since the effects of EPS fractions on the different bifidobacterial strains showed a similar trend, only one of the bacterial strains, *B. adolescentis*, was taken for the microscopic assessment.

SEM was performed with a JSM 6701F SEM (JEOL Ltd., Tokyo, Japan) to examine the surface and morphological structures of *B. adolescentis* and EPS fibers. The *B. adolescentis* was cultured in RCM for 24 h as described in 2.1. Based on preliminary experiments, 400 μL of the bacterial suspension was washed by sterilized saline and then centrifuged twice. The bacterial pellet was re-suspended in 2 ml of 1 g/l of EPS solution and was immediately frozen in liquid nitrogen, and then freeze-dried for SEM imaging.

AFM imaging was performed on a Bruker Nanoscope-8 device (Bruke, Billerica, Massachusetts, USA) equipped with a J-type scanner to examine EPS surrounding the bacterial cells. Forty microliters of the bacterial suspension in liquid medium containing the EPS fractions (1 and 5 g/L) was loaded onto a clean glass slide and then air-dried. The loaded glass slide was gently rinsed with DI water to remove salt crystals and air dried again for the AFM analysis [23]. The AFM was performed in tapping mode in air, using silicon cantilevers with a resonant frequency about 150 kHz (MikroMasch, Tallinn, Estonia). For each EPS sample, three independently prepared specimens were analyzed, and several areas were captured, but only the characteristic images are presented in the results.

CLSM was performed with a Leica DMi8 Confocal Microscope (Leica Microsystems GmbH, Wetzlar, Germany), to visualize the biofilms formed by the bifidobacteria in the presence of EPS in the culture medium. Glass cover slides were cleaned and autoclaved, and then suspended in the RCM broth with EPS fractions in petri dishes and RCM without EPS as a control. The *B. adolescentis* bacterial suspension from above liquid culture was inoculated at 1% (v/v) into the petri dishes and incubated for 48 h with gentle shaking at 30 rpm. The glass covers were carefully and aseptically removed, and treated as the follows. One side of the slide was scrubbed with isopropyl alcohol cotton swab, rinsed with phosphate buffer solution (PBS) three times, fixed with 100 μL of 2.5% (w/v) glutaraldehyde solution at 4°C for 1.5 h. After rinsing with PBS twice, the slide was stained with 100 μL of 100 μg/ml fluorescein isothiocyanate conjugated concanavalin A (FITC-ConA) solution for 30 min at 4°C in dark [24]. After PBS rinsing, it was stained with 100 μL of 50 μg/ml propidium iodide (PI) solution for 15 min at 4°C in dark, and then rinsed with PBS and dried at 20°C in dark [25]. It was finally treated with the antifade solution and sealed with nail polish, and ready for CLSM. The FITC-ConA combined with EPS exhibited green fluorescence under the laser at 488 nm, while the PI combined with the DNA of bacterial cell exhibited red fluorescence under the laser at 543 nm. The bacteria exhibited orange fluorescence when the two images overlapped.

2.7. Test of bacterial adhesion on Caco-2 monolayer

Caco-2 cell monolayer is commonly used as an in vitro model for intestinal epithelium to screen for adhesive strains [26]. The Caco-2 cell culture was maintained in Dulbecco's modified Eagle Medium (DMEM) supplemented with 4.5 g/l glucose, 1% non-essential amino acids, 20% inactivated (30 min, 56°C) fetal bovine serum and 1% penicillin-streptomycin. The Caco-2 cells were seeded at 4.5 ×103 cells per well in a 96-well culture plates or 5 ×104 cells per well in 24-well plates and incubated at 37 °C with 5% CO2 in the atmosphere. The medium

was replenished every 48 h and the cells maintained for 14 d to obtain a differentiated intestinal epithelial cell monolayer for the assay [27]. For bacterial adhesion assays, MEM without antibiotics was used in the last two rounds of medium replacement.

At 1 hour after the last medium replacement, each of the EPS fractions was added in triplicate to the microplate wells at a final 1 or 5 g/l final concentration. After 1 h incubation, *B. adolescentis* suspension (10 µl) was added to all the wells, maintaining a multiplicity of infection ratio of 100 bacteria to one Caco-2 cell. Prior to the test, the *B. adolescentis* bacterial suspension had been centrifuged at 1000 g for 10 min, washed three times with phosphate buffered saline (PBS), and then diluted with DMEM to 5 $\times 10^9$ colony forming units (cfu)/ml before the following assy. After incubation for 2 h under 5% CO2 at 37 °C, the Caco-2 cells were washed thrice with pre-warmed PBS to remove loosely adherent bacteria and lysed for 10 min with 1% Triton X- 100. Total viable bacteria adherent to or internalized by the cells as well as the initial number of bacteria added were quantified by serial dilution and plating on RCM agar. Control cells were treated with the medium alone. The percentage change in the number of viable adherent bacteria was represented by the average cfu in the treated cells/average cfu in the untreated control × 100.

2.8. Determination of Caco-2 cell viability

Caco-2 cells grown in the 96-well plates for 14 d (see Section 2.8) were used for this assay. Fresh DMEM was added 1 h before the assay. The carbohydrates, EPS and inulin, were then added to the wells at final concentrations of 5, 2.5, 1.25, 0.625, 0.3125, 0.1563, 0.0783 mg/ml in triplicate. After incubation for 2 h under 5% CO2 at 37 °C, all the wells were aspirated and refilled with 100 µl DMEM and 10 µl of MTT reagent. After incubation for 4 h, all wells were aspirated and added with 100 µl of DMSO reagent followed by gentle shake for 10 min. Finally, the culture plate was read at 570 nm by a microplate reader.

2.9. Statistical analysis

The microplate assays were performed in four replicates and all other experiments performed in triplicate and the results were averaged. Kruskal-Wallis test and Nemenyi test were applied in the analysis of MIC and MBC data. Student's *t* test was performed to compare the statistical significance of treatment effects on the bacterial adhesion.

3. Results and discussion

3.1. Molecular properties of EPS

Table 1 shows the molecular weight (MW) data of EPS, EPS-US and EPS-LM attained from HPGPC analysis. Compared with the native EPS, the EPS-US attained after 30 min US treatment of the native had overall shift of the average MW to a lower range with the major MW fraction decreased from 2.252×10^{8} to 2.699×10^{7} Da. The MW reduction caused by the US treatment was similar to that reported previously [22]. The EPS-LM had an even lower MW distribution with a major MW fraction (58%) at 3.346×10^{6}.

According to composition analysis, the total carbohydrate content was 71% for the native EPS, 70% for EPS-US, and 28% for EPS-LM and the total protein content was 6.97% for EPS, 18.4% for EPS-US, and 40% for EPS-LM. The native EPS consisted of three monosaccharide constituents, mannose, glucose and galactose at a molar ratio of 1.73:1.81:1.

3.2. Effect of EPS on bifidobacterial growth

As shown in Fig. 1, the native EPS and EPS-US at 1.25 g/L or a higher concentration suppressed the growth of most bifidobacteria strains while EPS-LM with relatively low MW had a slightly positive effect on some of the bacterial strains but negative effect on others. Among the three EPS fractions, the native EPS with the highest MW caused most notable inhibition

on the growth of the five bacteria strains, especially at higher concentrations, and EPS-LM with the lowest MW caused slight or not growth inhibition. As the major difference among the three EPS fractions was the MW, the inhibitory effect became more severe as the MW was higher. A plausible explanation for the inhibitory effect of high MW EPS fractions on the bacterial growth is the resistance to nutrient transport to the bacteria cell created by the highly viscous EPS in the liquid medium. The increased resistance to molecular transport by the high MW EPS may also contribute to the protective effect on the bacteria against the harmful chemicals such as the antibiotics as shown below [19].

3.3. Protective effects of EPS against antibiotic inhibition of bifidobacteria

Table 1 presents the MIC and MBC values of four antibiotics on the five bifidobacterial strains cultured with or without various EPS fractions. In the control cultures, penicillin was the most potent (with lowest MIC and MBC values) to inhibit the five strains of bifidobacteria compared with the other three antibiotics. On the other hand, most of the bifidobacteria were more resistant or tolerant to streptomycin than the three antibiotics with lower MIC and MBC values ranged from 16 to >512 μg/ml. Additionally, the MIC and MBC values of enrofloxacin and tetracycline were widely varied with the different bacterial strains. The prebiotic reference inulin added to the bacterial medium (at 5 g/L) caused little or no change in the MIC or MBC values of antibiotics as compared with the control.

Both EPS and EPS-US fractions at 5 g/L significantly increased the MIC and MBC values to over 512 μg/ml of four antibiotics on most of the bacterial strains. The relative significance of the effects between EPS and EPS-US varied with the different antibiotics and bacterial species.

With a lower concentration (1 g/L) of EPS and EPS-US, the MIC and MBC values of four antibiotics were increased less significantly than with 5 g/l in most cases. The lower MW EPS

fraction EPS-LM at both concentrations also increased the MIC and MBC values of antibiotics on most bacterial species, but less significantly than EPS and EPS-US.

The MIC and MBC results indicate that the high-MW EPS fractions had a significant, concentration-dependent protective effect against antibiotic inhibition of the bifidobacteria, and the lower MW EPS-LM had a less significant protective effect. The results suggest that a relatively high MW is an important factor for the protective effect of EPS fractions on the bacteria. In this regard, the lack of protective effect by the prebiotic inulin was due probably to the low MW. Similarly, konjac glucomannan (KGM) and US-degraded KGM with high MW showed a significant protective effect on bifidobacteria against antibiotics but the low-MW acid-hydrolyzed KGM had a significantly lower or no protective effect (Mao et al., 2017). In general, EPS and EPS-US showed more stable protective effect than KGM on all four antibiotics due probably to the relatively high MW and different polymer structure of EPS compared with KGM.

In our previous study [17], three possible mechanisms have been proposed for the protective effects of KGM on the bifidobacteria, the physical adsorption of antibiotic molecules, the formation of a viscous layer surrounding the bacteria, and the increased formation of biofilms by the bacteria by the polysaccharides. However, the EPS fractions showed no significant adsorption to any of the four antibiotics (data not shown) and the other two mechanisms were investigated in the following experiments.

3.4 Microstructures and interactions of bacterial cells and EPS

Fig. 2 shows the SEM images of *B. adolescentis* bacteria and EPS fractions. The native EPS and US-treated EPS-US appeared as a relatively smooth and planner sheet (Fig. 2a; Fig. 2b), the lower EPS fraction EPS-LM as an irregular and distorted sheet (Fig. 2c), and inulin

appeared as aggregates. Many bacteria were attached on the sheet surface of EPS and EPS-US, and much fewer were found on the surface of EPS-LM and inulin.

Fig. 3 shows the AFM images of *B. adolescentis* samples in absence or presence of the EPS fractions. In the absence of any EPS (Fig. 3a), few or no *B. adolescentis* cells were attached to the glass slide. In the presence of different EPS fractions (Fig. 3b-g), the bacteria cells had similar size and morphology, but their surface roughness varied, increasing in the order EPS>EPS-US>EPS-LM, and with the concentration. The increased cell surface roughness with the high-MW EPS suggests the presence of a thin coat or layer with the viscous EPS on the bacterial cell. This viscous EPS layer surrounding the bacterial cell may contribute to the protective effect against the antibiotics and also to the inhibitory effect on the bacterial growth as seen above (Fig. 3), by creating a barrier to the antibiotic and nutrient transport. Similarly, in previous studies the inhibitory effect of chitoligosaccharides at a high concentration (10 g/l) on *Bacillus cereus* growth was attributed to the formation of a film layer on the bacterial cell surface, although no direct observation or experimental evidence was provided [23, 28].

3.5 Biofilm formation of B. adolescentis in presence of EPS

Fig. 4 shows the CLSM images of bifidobacteria on glass slides stained with the fluoresce marker. In the control sample without any EPS (Fig. 4a), few biofilm spots (green spots) were present on the glass slide. In the samples with 1 g/l of EPS (Fig.4a) or EPS-US (Fig.4c), some small pieces of biofilms appeared. With 5 g/L EPS (Fig. 4b) or EPS-US (Fig.4d), many small pieces of biofilms were formed. No biofilms appeared in the sample with EPS-LM at 1 g/L or 5 g/L (Fig. 4f-Fig. 4g). Therefore, EPS and EPS-US promoted the formation of biofilms by the bifidobacterial cells in a dose dependant manner. The effects of various EPS fractions on biofilm formation were consistent or correlated with their protective effects against antibiotic inhibition as shown above (Table 1). The results suggest that the protective effect of EPS and

EPS-US on the bifidobacteria was attributable to the promotion of biofilm formation. The similar relationship has been suggested for the protective effect of KGM on the bifodobacteria [17]. Formation of biofilms by bacterial cells can usually increase their resistance to antimicrobial agents [29].

A previous study has shown that biofilm formation of bifidobacteria was enhanced under bile stress due probably to the increase in the surface hydrophobicity of bacterial cell [30]. In *E. coli*, RpoS protein (σ^S) as a chief factor for response to general stresses was produced at higher concentration when the bacterial growth was terminated due to starvation or the lack of major nutrients such as carbon, nitrogen, phosphorus and amino acids [31]. It has been found that RpoS plays a crucial role in the biofilm formation by *E. coli* [32]. These previous research findings suggest that biofilm formation by bacteria cells is enhanced in response to environment stress and nutrient limitation. In the present study, the high-MW EPS and EPS-US probably retarded the transfer of the nutrients to the bifidobacterial cell leading to slower growth rate. In addition to nutrient limitation, the increase in the liquid viscosity or formation of a viscous layer surrounding the bacterial cell may also promote the aggregation of bacterial cells and formation biofilm.

3.6 Effect of EPS on adhesion of B. adolescentis to Caco-2 monolayer

As shown in Fig. 5, both EPS and EPS-US increased the adhesion of bifidobacteria to Caco-2 cell monolayer, especially at the higher concentration of 5 g/L, by about 35% compared to the control. The prebiotic reference inulin at 5 g/l also increased the adhesion by 15.6%. However, EPS-LM had no statistically significant effect on the adhesion. The increase in bacterial adhesion to the Caco-2 monolayer has a positive correlation with the molecular weight and concentration of polysaccharides. Additionally, most of the Caco-2 cells remained alive with

359 the three EPS fractions. The result suggests that the high-MW EPS fractions have the ability to
360 bifidobacteria to intestinal epithelium. Similarly, a previous study has shown based on molec-
361 ular techniques that the EPS produced by a well-known probiotic, *B. animal subsp.* Lactis in-
362 fluence bacterial surface properties so as to increase the adhesion to the intestinal epithelial cell
363 HT29 monolayer [33]. The enhanced adhesion of probiotic bacteria to the intestinal epithelium
364 may facilitate their colonization in the intestine.
365
366 **4. Conclusions**
367 The high-MW EPS produced by the Cs-HK1 fungus exhibited a significant protective ef-
368 fect on several strains of bifidobacterial against four commonly used antibiotics. The protective
369 effect of different EPS fractions was generally more significant with a higher MW and higher
370 concentration, which proved the initial hypothesis of MW-dependent protection. Microscopic
371 observation suggests the formation of a viscous coat on the bacterial cell to by the high MW
372 EPS. This viscous coat not only retarded the access of bacteria cell to the antibiotics but also
373 to useful nutrients, leading to slower growth rate. The physical and biological effects of EPS
374 on the bacterial cells also enhanced the formation of biofilm and adhesion to epithelia cell layer
375 by the bifidobacteria. Overall, the results suggest that Cs-HK1 EPS was beneficial to the
376 bifidobacteria by protecting against the antibiotics and by promoting their adhesion to and col-
377 onization in the intestine epithelium. Our preliminary experiments have shown that the Cs-
378 HK1 EPS are resistant to degradation by simulated gastric acid and digestive enzymes, and not
379 significantly protective for *E. coli* or *Staphylococcus aureus* against the antibiotics (un-
380 published results). Further work is needed to verify the potential benefits of EPS to the gut
381 microbiota in animal experiments and to investigate the mechanism for the EPS effects on the
382 bifidobacterial cells such as the expression of RpoS protein during stress conditions and biofilm
383 formation.

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488 **Table 1** The intrinsic viscosity and molecular weight of different EPS fractions

Sample	MW (Da)	% Area
EPS	2.252×10^{8}	85.8
	1.858×10^{5}	4.61
EPS-US	2.699×10^{7}	85.1
	1.492×10^{4}	3.49
EPS-LM	5.784×10^{7}	26.9
	3.346×10^{6}	58.9

489 **Table 2** MIC and MBC (μg/ml) of antibiotics against five strains of *Bifdobacterium* in RCM
490 supplemented with different carbohydrates (Control in TCM medium).

OS/PS (5 g/l or specified)	Enrofloxacin		Penicllin		Tetracycline		Streptomycin	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
B. adolescentis								
Control (none)	1-2	2	<1	<1	<1	32-128	256	256-512
EPS (1 g/l)	8	64	<1	4	2	16	256	512
EPS	32	>512	>512	>512	2	>512	256-512	>512
EPS-US (1 g/l)	1	32	<1	1	<1	32	128	256
EPS-US	>512	>512	>512	>512	>512	>512	>512	>512
EPS-LM (1 g/l)	8	32	<1	1	4	16	256	256
EPS-LM	128	128	<1	4	32	32	256	256
B. bifidum								
Control (none)	64-128	32	<1	8	8	128	128	128
EPS (1 g/l)	512	512	1	8	16	128	128	256
EPS	>512	>512	>512	>512	>512	>512	>512	>512
EPS-US (1 g/l)	128	64	<1	2-4	1-2	64	16-64	32
EPS-US	>512	>512	>512	>512	64	64	>512	>512
EPS-LM (1 g/l)	256	128-256	2	8	8	128	128	64-128
EPS-LM	64	128	2	8	16	128	128	128
B. breve								
Control (none)	64	512	1	8	8	4-16	16	16-32
EPS (1 g/l)	256	>512	4	32	128	128	512	>512
EPS	64-128	>512	>512	>512	>512	>512	64	>512
EPS-US (1 g/l)	8	32	<1	8-16	<1	16	16	32
EPS-US	>512	>512	>512	>512	256	256	>512	>512
EPS-LM (1 g/l)	256	256-512	8	32	128	128	32	32
EPS-LM	128-256	256-512	16	32-64	512	256-512	256	256-512
B. infantis								
Control (none)	4-8	32	<1	8-16	32	16	>512	>512
EPS (1 g/l)	64	64	<1	16	16	32	>512	>512
EPS	>512	>512	>512	>512	>512	>512	>512	>512
EPS-US (1 g/l)	32	64	<1	8	8	32	>512	>512
EPS-US	256	256-512	>512	>512	64	64	>512	>512
EPS-LM (1 g/l)	64	128	<1	16	32	32	>512	>512
EPS-LM	64	128	<1	16	32	64	>512	>512
B. longum								
Control (none)	4	4-16	<1	8-16	1-2	32-128	64	32-512
EPS (1 g/l)	16	16	<1	32	2	32	256	256
EPS	>512	>512	>512	>512	512	>512	128-256	>512
EPS-US (1 g/l)	4	16-32	<1	4-8	1	32	128	>512
EPS-US	64-512	64-512	>512	>512	64	64	>512	>512
EPS-LM (1 g/l)	8	8	8	4-8	2	32-64	64	64-128
EPS-LM	16	64	4-8	16	2	16-32	128	128

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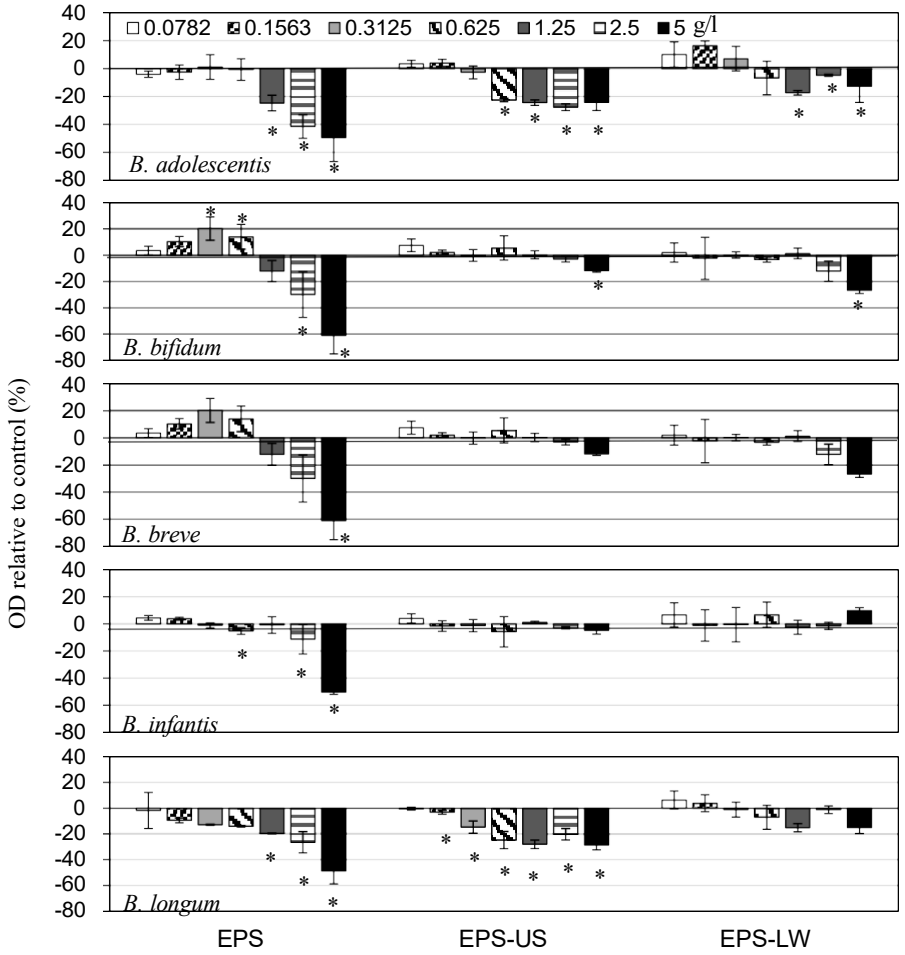
Fig. 1. Effects of different EPS fractions on proliferation of five strains of *Bifidobacteria*. Inoculum concentration: 4×10^5 colony forming unites (cfu) in 200 µl; incubation 48 h. Error bars for standard deviations (SD, n =3); *: significant difference ($p < 0.05$) compared with the control.

Fig. 2. SEM images of *B. adolescentis* in different EPS fractions: (a) EPS; (b) EPS-US; (c) EPS-LM; (d) Inulin. Arrows indicating the bacterial cells.

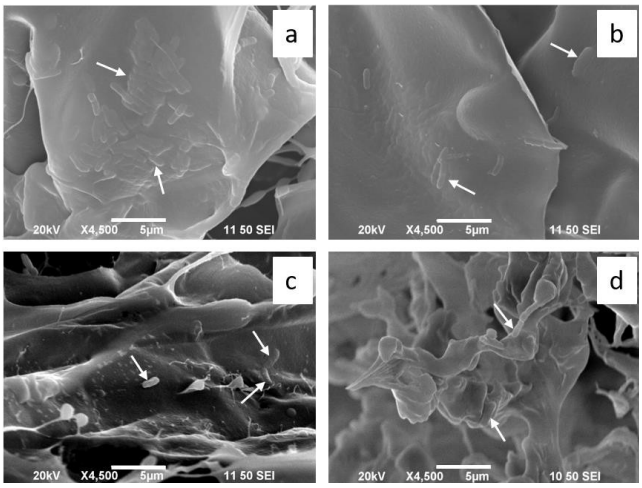
Fig. 3. AFM images (tapping mode) of the effect of different EPS fractions upon *B. adolescentis* morphology: (a) Control with no EPS; (b) EPS 5 g/l; (e) EPS-US 5 g/l; (g) EPS-LM 5 g/l.

Fig. 4. Confocal laser scanning microscopy (CLSM) images of *B. adolescentis* biofilms on glass slides in RCM with existence of different polysaccharides. Inoculation with 1% (v/v) bifidobacterial suspension. Cultured for 2 days at 37 ℃, 30 rpm. (a) Control with no EPS (b) EPS 1 g/l, (c) EPS 5 g/l, (d) EPS-US 1 g/l, (e) EPS-US 5 g/l, (f) EPS-LM 1 g/l, (g) EPS-LM 5 g/l. Bacteria were stained by PI and emitted red light, while biofilms were stained by FITC-ConA and emitted green light. The overlap parts showed orange.

Fig. 5. Effect of inulin and different Cs-HK1 EPS on adhesion and survival of *Bifidobacterium adolescentis* to Caco -2 cells. The cells were pretreated with EPS for one hour and then incubated with bacteria for two hours. The number of adherent bacteria was counted by the plate count method. The values are the mean of triplicates ± SD. The control is untreated Caco-2 monolayer incubated with bacteria, and the adhesion increase of bacteria = (average log10(cfu in treated cells)/average log10(cfu in untreated control) - 1) × 100. The asterisks denote the significance of $p < 0.05$, in comparison with the control.



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



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525 <Fig. 2 Int. J. Biol. Macromol, Mao et al>

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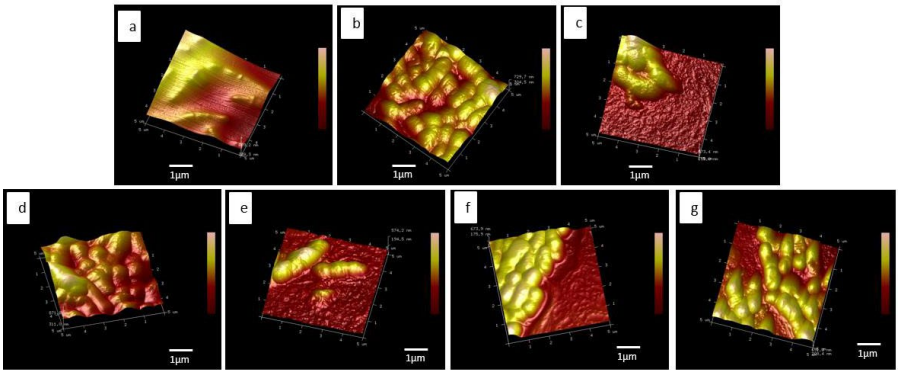

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529 <Fig. 3 Int. J. Biol. Macromol, Mao et al>

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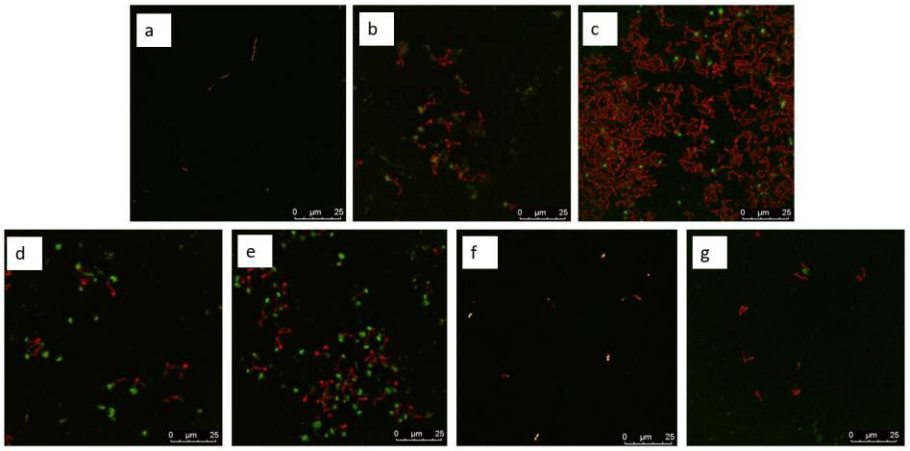

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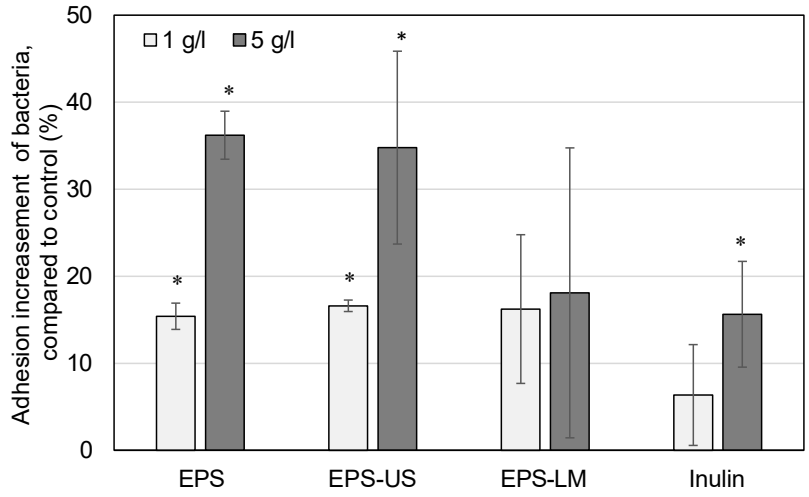
533 <Fig. 4 Int. J. Biol. Macromol, Mao et al>



<Fig. 5 Int. J. Biol. Macromol, Mao et al>