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Characterization and bioactivities of the exopolysaccharide from a probiotic strain of *Lactobacillus plantarum* WLPL04

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ABSTRACT

Exopolysaccharide (EPS) was extracted and purified from *Lactobacillus plantarum* WLPL04, which has been confirmed previously as a potential probiotic for its antagonistic and immune-modulating activity. It has a molecular weight of 6.61×10^4 Da, consisting of xylose, glucose, and galactose in an approximate molar ratio of 3.4:1.8:1. Microstructural studies demonstrated that the EPS appeared as a smooth sheet structure with many homogeneous rod-shaped lumps. The preliminary in vitro assays indicated that the EPS could significantly inhibit the adhesion of *Escherichia coli* O157:H7 to HT-29 cells in competition, replacement, and inhibition assays at a dose of 1.0 mg/mL, with an inhibition rate of 20.24 ± 2.23, 29.71 ± 1.21, and 30.57 ± 1.73%, respectively. Additionally, the EPS exhibited strong inhibition against biofilm formation by pathogenic bacteria, including *Pseudomonas aeruginosa* CMCC10104, *E. coli* O157:H7, *Salmonella* Typhimurium ATCC13311, and *Staphylococcus aureus* CMCC26003. Furthermore, the EPS showed good inhibitory activity against the proliferation of HT-29 cells. The characteristics and bioactivities of this EPS may make it a promising candidate in developing functional food.

Key words: *Lactobacillus plantarum* WLPL04, exopolysaccharide, characterization, bioactivities

INTRODUCTION

Exopolysaccharides (**EPS**) represent a large group of biopolymers that are produced during the metabolic process of microorganisms (Amjres et al., 2015) and can be classified as homo- or heteropolysaccharides, according to whether they are composed of one or more types of sugar. Among the variety of EPS-producing microorganisms, lactic acid bacteria (**LAB**) have been recognized as having safe status, and have attracted more attention due to their potential probiotic properties (Badel et al., 2011).

The EPS produced by LAB have received a great deal of interest in the fermented food industry (Liu et al., 2010; Serafini et al., 2013) because they are used as natural additives of in situ fermentation to promote physicochemical properties, such as stabilizing, emulsifying, and providing texture and mouthfeel of cheese, yogurt, and so on (Badel et al., 2011; Patel et al., 2012; Ahmed et al., 2013). In addition to applications in the dairy industry, EPS of LAB have several potential applications due to their biological activities [e.g., antioxidant activity (Li et al., 2014a), antitumor activity (Wang et al., 2014), activity against biofilm forming by pathogens (Li et al., 2014b, 2015), cholesterol-lowering activity (London et al., 2014), and immunomodulation (Wu et al., 2010; Li and Shah, 2014)]. Furthermore, EPS have been shown to be important for LAB because of potential roles in stress resistance, adhesion, colonization, and host–bacteria interactions (Fanning et al., 2012; Dertli et al., 2013; Lee et al., 2016).

Recently, *Lactobacillus plantarum* has attracted scientific interest due to its broad distribution in ecological niches (pickles, sausage, sourdough, and so on), beneficial effects on the host, and high commercial value for dairy products (da Silva Sabo et al., 2014; Kwak et al., 2014). The EPS-producing *L. plantarum* strains were considered beneficial microorganisms and widely used as starters for various fermented dairy products (da Silva Sabo et al., 2014; Kwak et al., 2014; Caggianiello et al., 2016), and the structures and bioactivities of EPS have been disclosed in some publications (Li et al., 2013; Fontana et al., 2015; Zhou et al., 2016). However, the molecular weight (**Mw**) and monosaccharide composition are strain dependent, and so are the biological functions (e.g., antioxidant, antitumor, and antibiofilm activity).

In our previous study, an EPS-producing *L. plantarum* WLPL04 from human breast milk has been found with antibacterial and antiinflammatory ability, and it exhibited antiadhesion capability against pathogens

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to the human intestinal epithelial cells (Jiang et al., 2016). Its genome data have also been reported (Tao et al., 2015). The objectives of the present work were to isolate and purify the EPS from *L. plantarum* WLPL04 and determine its molecular and monosaccharide composition using gel-permeation chromatography (**GPC**) and GC-MS, respectively. We also investigated the physicochemical properties of EPS using UV-visible spectrophotometry, Fourier-transform infrared (**FT-IR**) spectroscopy, scanning electron microscopy, and atomic force microscopy (**AFM**). Furthermore, the probiotic properties of EPS were evaluated in vitro, including antiadhesion activity, activity against biofilm formation by pathogens, and its antitumor activities.

Due to the increasing incidence of colon cancer and the side effects of current chemical antitumor drugs, developing natural antitumor compounds is becoming critically important to prevent colon cancer (Zhang et al., 2012). Our work provided a pre-fundamental basis for reasonable use of EPS for human health.

MATERIALS AND METHODS

Bacterial Growth, Cell Culture, and Culture Conditions

The EPS-producing strain *L. plantarum* WLPL04 was previously isolated from healthy female breast milk (Jiang et al., 2016), and cultured in de Man, Rogosa, and Sharpe broth (Beijing Solarbio Science and Technology Co. Ltd., Beijing, China) at 37°C under anaerobic condition. Indicator strains of pathogens (*Pseudomonas aeruginosa* CMCC10104, *Escherichia coli* O157:H7, *Salmonella* Typhimurium ATCC13311, and *Staphylococcus aureus* CMCC26003) were cultured in Luria-Bertani medium at 37°C overnight in a shaker incubator.

Human colon cancer HT-29 cells, purchased from Cell Bank of the Chinese Academy of Sciences (Beijing, China), were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (ExCell Bio Co., Ltd., Shanghai, China) and a standard mixture of antibiotics (100 U/mL of penicillin, 100 μg/mL of streptomycin; Beijing Solarbio Science and Technology Co. Ltd.) in an atmosphere of 5% (vol/vol) CO_2 at 37°C, in 75-cm^2 cell culture flasks, and the medium was replaced every 2 d.

EPS Isolation and Purification

The EPS was isolated and purified as described previously (Zhang et al., 2016). Briefly, after incubation at 37°C for 24 h, culture of *L. plantarum* WLPL04 was centrifuged at 9,000 × *g* for 5 min at 4°C to remove the bacteria; then, 2 volumes of chilled absolute ethyl alcohol were added. The solution was centrifuged at 10,000 × *g* for 20 min at 4°C. The precipitates were collected and dissolved in Milli-Q water (Millipore, Shanghai, China) to dialyze against Milli-Q water for 3 d at 4°C, which was changed twice a day. Crude EPS was obtained by lyophilizing, and then additionally purified to reduce the DNA and protein content. Briefly, the crude EPS powder was dissolved in 50 m*M* Tris-HCl, 10 m*M* $MgSO_4 \cdot 7H_2O$ (pH 7.5) at a final concentration of 5 mg/mL and treated with DNase I (final concentration 2.5 μg/mL, Sigma, St. Louis, MO) at 37°C for 6 h, followed by Pronase E (final concentration 50 μg/mL, Sigma) treatment at 37°C for 18 h. Afterward, trichloroacetic acid was added (12% final concentration) to precipitate enzymes and residual AA or peptides, and the mixture was stirred for 30 min at room temperature. The solution was centrifuged at 10,000 × *g* for 20 min at 4°C to collect supernatant, which was adjusted to pH 4.0 to 5.0 with 10 *M* NaOH. After dialysis at 4°C for 3 d against Milli-Q water (water was changed twice daily), the dialyzed retentate was finally freeze-dried to obtain pure EPS powder for subsequent experiments.

The EPS was suspended in distilled water (1 mg/L) and the concentration was determined by the phenol-sulfuric acid method (DuBois et al., 1956) using glucose as standard, and the purity of EPS was analyzed by a UV-visible spectrophotometer (TU-1901, Persee, China) in the wavelength range of 200 to 600 nm.

Molecular Weight Analysis of EPS

The Mw of the purified EPS was determined by GPC equipped with a TSK G-5000 PWXL column (7.8 × 300 mm, TOSOH, Tokyo, Japan) and TSK G-3000 PWXL column (7.8 × 300 mm, TOSOH) in conjunction with a refractive index detector (Waters 2414, Milford, MA). The samples (2.0 mg/mL, 20 μL) were injected and eluted with 0.02 mol/L KH_2PO_4 solutions at 35°C with a flow rate of 0.6 mL/min. Data were collected and processed using Breeze 1 GPC software (Waters). Molecular weights were calculated according to the relative molecular mass of dextran standards (Sigma).

Determination of Monosaccharide Composition of EPS

A total of 5 mg of the purified EPS was hydrolyzed with 2 mL of 2.0 *M* trifluoroacetic acid at 100°C for 30 min. The hydrolysates were then repeatedly co-concentrated with methanol to dryness, and converted to their trimethylsilyl derivatives by adding 0.2 mL of trimethylchlorosilane, 0.4 mL of hexam-ethyldisilazane,

1 mL of pyridine, and heating at 80°C for 30 min. After cooling, samples were analyzed on HP 7890A GC (Agilent, Santa Clara, CA) equipped with a flame-ionization detector and a HP-5 fused-silica capillary column (30 m × 0.32 mm × 0.25 mm, J&W Scientific Inc., Folsom, CA). The chromatographic conditions were set according to our previous method (Zhang et al., 2016). Rhamnose, arabinose, fucose, xylose, mannose, glucose, and galactose were prepared for comparison and inositol was used as an internal standard.

FT-IR Spectroscopy Analysis

Fourier-transform infrared spectroscopy was used to detect the major structural groups of the purified EPS. The sample of EPS was pressed in KBr pellet and recorded on a FT-IR spectrophotometer (FTIR Nicolet 5700, Thermo Nicolet Co., Waltham, MA) in the region from 400 to 4,000 cm^{-1}.

Microscopic Morphology Analysis of EPS

The microscopic morphology of the purified EPS was evaluated by scanning electron microscopy and AFM. The purified EPS was fixed to the scanning electron microscopy stubs with double-sided tape and then coated with a layer of gold approximately 10 nm thick. The samples were observed in a scanning electron microscope (JSM 6701F, Jeol, Tokyo, Japan) at an acceleration voltage of 3.0 kV.

The purified EPS was dissolved in distilled H_2O at the concentration of 1 mg/mL. The aqueous solution was stirred for 2 h at 40°C in a sealed bottle under a stream of N_2 until the EPS dissolved completely. After cooling to room temperature, the solution was diluted to a final concentration of 10 μg/mL. Five-microliter solutions were dropped onto the surface of a mica sample carrier and allowed to dry at room temperature. Then the AFM images were obtained by PicoView program in the tapping mode (Agilent 5500).

Effects on Adhesion of E. coli O157:H7 to HT-29 Cells

The effects of the purified EPS on adhesion of *E. coli* O157:H7 to HT-29 cells were evaluated as reported by Donnarumma et al. (2014). Briefly, HT-29 cells were incubated in 6-well plates (5.0×10^5 cells per well) under 5% CO_2 at 37°C for 24 h. Then, 100 μL of *E. coli* O157:H7 (1×10^8 cfu/mL) and 10 μL of EPS with different concentrations (0.01, 0.1, and 1.0 mg/mL) were added for 3 types of assays: (1) competition assay (i.e., adding EPS and *E. coli* simultaneously and incubated for 1 h); (2) displacement assay (i.e., pre-incubating HT-29 cells with 100 μL of *E. coli* for 1 h, then adding EPS and incubating for another 1 h); and (3) inhibition assay (i.e., pre-incubating HT-29 cells with EPS for 1, 4, 6, and 18 h, then adding 100 μL of *E. coli* and incubating for another 1 h). After incubation, unbound bacteria were washed off and adhered bacteria were detached by EDTA-trypsin solution (Sigma). The attached *E. coli* cells were counted by plating on Luria-Bertani agar. The ability of EPS to inhibit the adhesion of *E. coli* to HT-29 cells was expressed as the percentage of adhesion of *E. coli* added with EPS relative to the one without EPS (control).

Anti-Biofilm Activity

The inhibition assay of biofilm formation was carried out as described by Li et al. (2014b) with a slight modification. Overnight cultivated pathogens were diluted to 1.0×10^8 cfu/mL and added into a 96-well plate together with aqueous solution of EPS of different concentration (0.2, 0.5, 1.0, 2.0, and 5.0 mg/mL), which was sterilized by filtration through a 0.22-μm membrane filter. Wells without EPS were used as a negative control, and wells adding ampicillin (50 μg/mL) as a positive control. After 24 h incubation at 37°C, the wells were washed with PBS and dried under the room temperature. The biofilms were stained with 2% (wt/vol) crystal violet (Tianjin Kemiou Chemical Reagent Co., Tianjin, China). Then, the dye was solubilized with 0.16 mL of 33% (vol/vol) glacial acetic acid. To determine the biofilm mass, aliquots of 125 μL of the solubilized dye from each well were transferred into a new 96-well plate and the absorbance at 590 nm was measured by a microplate reader (DNM-9602, Perlong Medical Co., Beijing, China). The antibiofilm ability was calculated using the following formula: antibiofilm activity (%) = $[1 - (OD_{sample}/OD_{negative\ control})] \times 100$, where OD is optical density.

Antitumor Activity

The antitumor activity of the purified EPS on HT-29 cells was evaluated by a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (**MTT**) assay as previously reported (Wang et al., 2015b) with slight modification. Briefly, HT-29 cells were incubated in 96-well plates (2.0×10^4 cells/well) under 5% CO_2 at 37°C for 24 h. The cells were then treated with 100 μL of the various concentrations of EPS (50, 100, 200, 400, and 800 μg/mL) and fluorouracil (**5-FU**, 50 μg/mL) for 24, 48, and 72 h. After incubation, 10 μL of MTT (5.0 mg/mL) was added into each well, and the cells were incubated for another 4 h. Then, the supernatant was discarded, and 100 μL of dimethyl sulfoxide was added

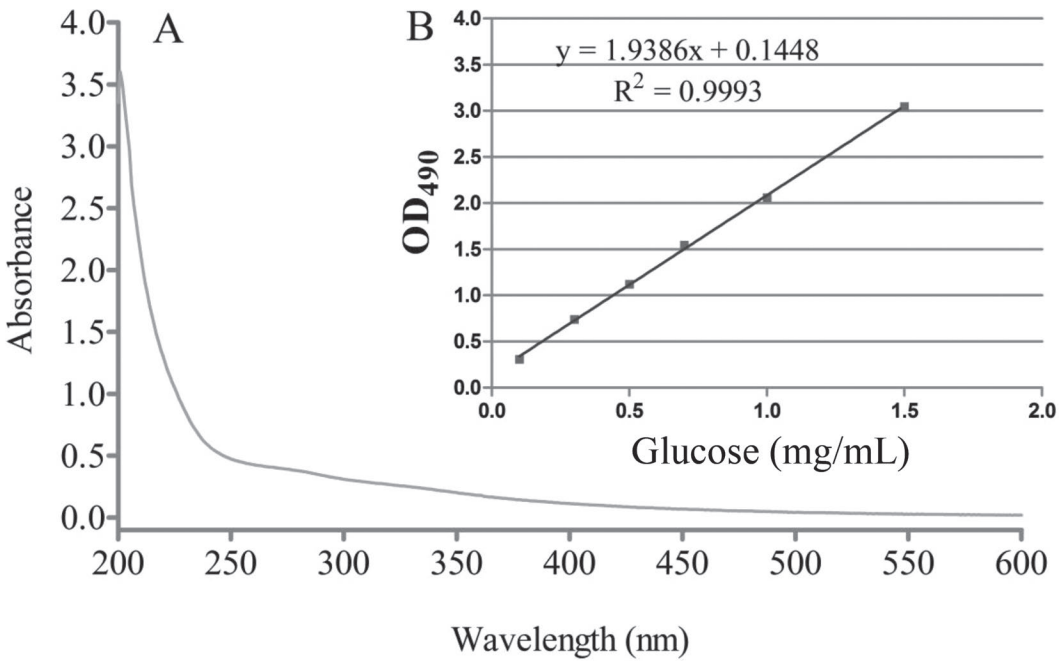

Figure 1. The UV spectrum of the purified exopolysaccharide from *Lactobacillus plantarum* WLPL04 in the range of 200 to 600 nm (A), and the calibration curve of glucose used for the phenol-sulfuric acid method (B). OD_{490} = optical density at 490 nm.

to solubilize the formazan. The absorbance at 570 nm of the plate was measured by a microplate reader (DNM-9602, Perlong Medical Co., Beijing, China). The inhibition rate was calculated as follows: inhibition rate (%) = $[1 - (OD_{sample} - OD_{blank})/(OD_{control} - OD_{blank})] \times 100$, where $OD_{control}$ and OD_{blank} were the absorbance with and without the addition of the EPS and cells, respectively.

Statistical Analysis

Triplicate data were expressed as mean ± SD. The SPSS 13.0 software (SPSS Inc., Chicago, IL) was used to perform statistical analyses by means of independent 1-way ANOVA tests. Differences with $P < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Purification of the EPS

The crude EPS, obtained from the supernatant of *L. plantarum* WLPL04, was shown to have a nonropy phenotype by ethanol precipitation. DNase I and Pronase E were used to remove DNA and protein of the crude EPS. After lyophilizing, some light brown powder was obtained, which could be dissolved into water with a transparency state (data not shown). Our previous study showed that the EPS yielding of *L. plantarum* WLPL04 reached the highest amount of 426.73 ± 65.56 mg/L at 24 h (Jiang et al., 2016); in this study, the purity of the EPS was proved to be 96.48% as determined by phenol-sulfuric acid method (Figure 1B).

The UV-visible spectrum of purity EPS showed no absorption at 260 or 280 nm, indicating that the purified EPS was free from protein and nucleic acid (Figure 1A).

Molecular Weight of EPS

Molecular weight is a basic property of EPS that might influence the probiotic properties (Xu et al., 2010). In this study, the Mw of the EPS was analyzed by GPC. The chromatogram of the EPS appeared as a symmetrical narrow peak at 25.55 min (data not shown), with an elution volume of 15.33 mL. According to the calibration curve of standards [log Mw = $-0.351V_E + 10.109$, $R^2 = 0.9918$; where V_E is the elution volume (mL)], the Mw of the EPS from *L. plantarum* WLPL04 was determined to be 6.61×10^4 Da, which was lower than that of the EPS (1.1×10^5 Da) from *L. plantarum* YW11 (Wang et al., 2015a) and the EPS (1.15×10^6 Da) of *L. plantarum* C88 (Zhang et al., 2013), but higher than that (1.83×10^4 and 1.33×10^4 Da) of the EPS of *L. plantarum* BC-25 (Zhou et al., 2016). In our parallel study, the Mw of *L. plantarum* ZDY2013 was 5.17×10^4 Da by high-performance size exclusion chromatography (Zhang et al., 2016).

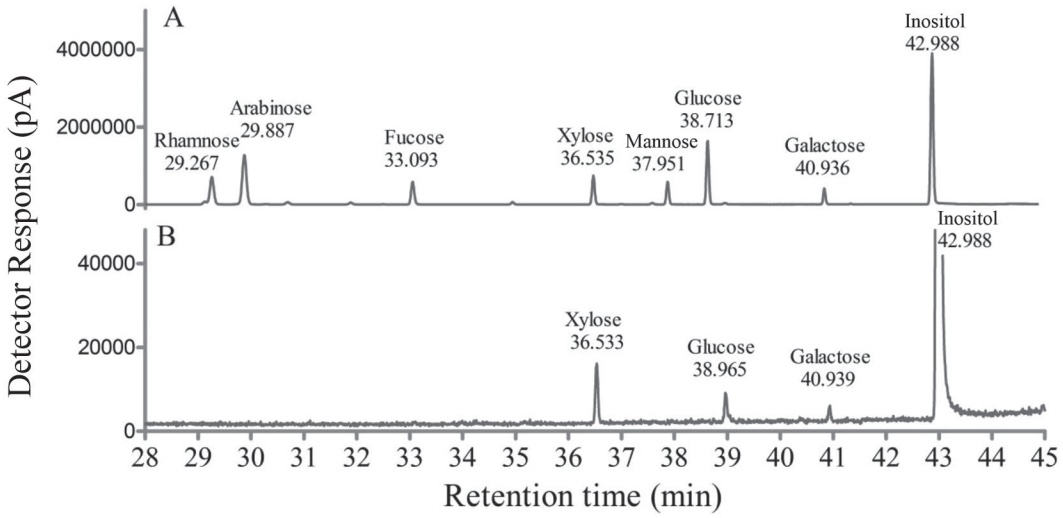

Figure 2. Gas chromatograms of standard sugars (A) and purified exopolysaccharide of *Lactobacillus plantarum* WLPL04 (B). The retention times correspond to rhamnose (29.267), arabinose (29.887), fucose (33.093), xylose (36.535), mannose (37.951), glucose (38.713), galactose (40.936), and inositol (42.988).

The Mw of EPS produced by *L. plantarum* strain is important for understanding its bioactivity and potential structure–function relationship of the EPS in fermented milk (Vaningelgem et al., 2004). A high-Mw EPS could improve the texture of fermented milk and may have greater antitumor activity than low-Mw EPS (Hassan et al., 2003; Peng et al., 2005). Moreover, the differences in Mw of EPS might be associated with its specific functions.

Monosaccharide Composition of EPS

Gas chromatography-mass spectrometry was used to analyze the monosaccharide composition of the EPS from WLPL04. As shown in Figure 2, the EPS was composed of xylose, glucose, and galactose in a molar ratio of 3.4:1.8:1 (Figure 2B), determined by comparing the retention time with the standards (Figure 2A), and xylose was the predominant monosaccharide in the EPS. As previously reported, most EPS produced by other *L. plantarum* strains consists of galactose and glucose (Zhang et al., 2013; Wang et al., 2015a), and additional *N*-acetylgalactosamine (Tallon et al., 2003) or mannose (Ismail and Nampoothiri, 2010; Wang et al., 2010; Zhou et al., 2016). Specially, the EPS produced by *L. plantarum* NTU102 contained arabinose, galactose, glucose, fructose, mannose, and maltose (Liu et al., 2011). Only the EPS produced by *L. plantarum* ZDY2013 contained xylose, but at a very low ratio (1.7% of total monosaccharide; Zhang et al., 2016). These results suggested that the monosaccharide composition of EPS differs among strains, and this composition might be closely related to the varied functions of EPS (Bello et al., 2001; Russo et al., 2012; Das et al., 2014), which was in accordance with some previous research (Wang et al., 2014; Caggianiello et al., 2016).

FT-IR Spectrum Analysis of EPS

To investigate the functional groups of the purified EPS, an FT-IR spectrum was recorded at the absorbance mode from 4,000 to 500 cm^{-1}. As shown in Figure 3, the spectrum of the purified EPS of WLPL04 showed an intense and broad band at around 3,417 cm^{-1}, which was considered the O–H stretching vibration (Wang et al., 2010). The strong absorbance peak around 2,931 cm^{-1} was assigned to C–H stretching vibration (Li et al., 2014b). The band around 1,645 cm^{-1} was due to the stretching vibration of C=O and carboxyl group (Wang et al., 2015a). Two peaks were observed around 1,413 and 1,249 cm^{-1}, which were assigned to C–O stretching vibrations and O–H deformation vibrations (Wang et al., 2012). The bands in the region of 900 to 1,150 cm^{-1} were attributed to the vibration of C–O–C bond (Wang et al., 2015a). Generally, the FT-IR spectrum of *L. plantarum* WLPL04 was similar to *L. plantarum* ZDY2013, as described in our previous report (Zhang et al., 2016).

Microcosmic Morphology of EPS

The microstructure and surface morphology of the purified EPS from *L. plantarum* WLPL04 were observed by scanning electron microscopy (Figure 4), which showed an irregular structure resembling sheets of polysaccharide overlaid with some scattered pieces

(Figure 4A) and that appeared as a smooth surface under 5,000× magnification (Figure 4B), exhibiting sheet and compact structure. A previous study suggested that this smooth surface is a favorable characteristic of the material used to make plasticized biofilms (Wang et al., 2010), and the consistent polymeric matrix of EPS gives mechanical stability to biofilms (Saravanan and Shetty, 2016).

Recently, EPS has been extensively studied by AFM, which is a powerful tool to observe the morphological topographies of polymers. The AFM images of the EPS from WLPL04 are shown in Figures 4C and 4D. The EPS deposited from a 10 μg/mL aqueous solution exhibited many homogeneous rod-shaped lumps with height ranging from 0.25 to 4.51 nm, which was significantly greater than that of a single polysaccharide chain (approximately 0.1–1 nm). This structural property of the EPS was also observed in *Bifidobacterium animalis* RH, and might be involved with inter- or intramolecular aggregation (or both) of polysaccharide chains and branches, and further forming of the interconnecting network structure (Shang et al., 2013).

Effect of the EPS on Adhesion of E. coli O157:H7 to HT-29 Cells

The antiadhesion ability against pathogens is considered one of the most important assays when evaluating probiotic properties. In our previous study, *L. plantarum* WLPL04 has shown a significant inhibitory effect on adhesion of *E. coli* O157:H7 to human intestinal epithelial cells (Jiang et al., 2016). To investigate whether the EPS of WLPL04 had a similar inhibitory effect on adhesion, we assessed the adhesion of *E. coli* O157:H7 to HT-29 cells in the presence of EPS by competition, displacement, and inhibition assays.

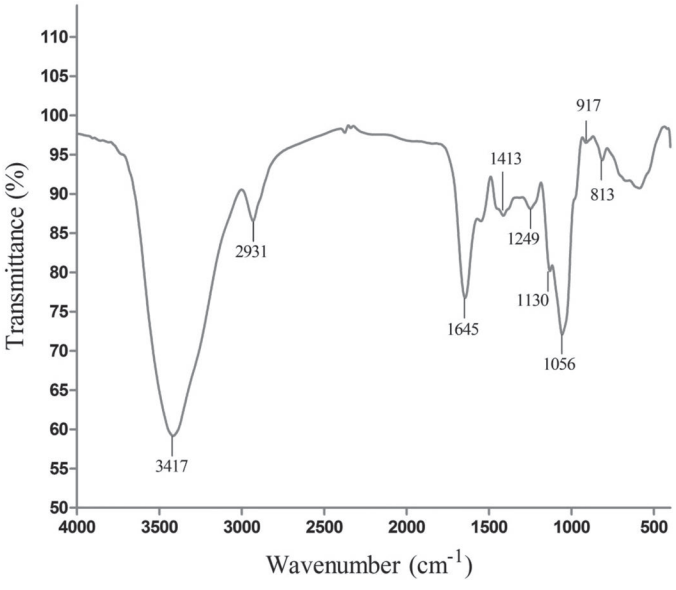

Figure 3. Fourier-transform infrared spectrum of the purified exopolysaccharide from *Lactobacillus plantarum* WLPL04 in the range of 400 to 4,000 cm^{-1}.

As shown in Figure 5, EPS was able to compete with, displace, and inhibit *E. coli* O157:H7 at different levels and the effects were concentration dependent. In competition and displacement assays (Figures 5A and 5B), the inhibition ratio dramatically increased with the concentration of EPS (0.01 to 1.0 mg/mL), with maximal inhibition rates of 20.24 ± 2.23% and 29.71 ± 1.21%, respectively. In the inhibition assay (Figure 5C), when HT-29 cells were pre-incubated for a long period (18 h) with a low concentration of EPS (0.01 mg/mL), the inhibition rate significantly increased up to 21.55 ± 1.89%; however, at the high concentration of EPS (1.0 mg/mL), inhibition rates were not correlated with incubation time, and the highest rate was 31.91 ± 1.87% at 6 h.

According to our previous study, we further proved that not only the viable cells of *L. plantarum* WLPL04 but also its EPS reduced the adhesion of *E. coli* O157:H7 by competition, displacement, and inhibition assays. To our knowledge, few studies have focused on the antiadhesion effect of EPS from lactobacilli strains against pathogens. Donnarumma et al. (2014) used the EPS of *L. crispatus* L1 to reduce the adhesion of *Candida albicans* to Vk2/E6E7 cells by inhibition assay (1.0 mg/mL of EPS, pre-incubated for 18 h), with an inhibition rate of 48%.

Inhibition of Pathogens to Form Biofilm

Except for the antiadhesion effect against pathogens in vitro, probiotics and their metabolic substances (e.g., EPS) face a challenge of in vivo formation of biofilm by pathogens, which is quite critical for their resistance to antibiotics and the host immune system as a result of persistent chronic infections (Jiang et al., 2011). We investigated activity against biofilm formation of the purified EPS from strain WLPL04 to pathogens and the result is shown in Figure 6. The inhibitory effects of the purified EPS (0.2, 0.5, 1.0, 2.0, and 5.0 mg/mL) on 4 pathogens (i.e., *P. aeruginosa* CMCC10104, *E. coli* O157:H7, *Salmonella* Typhimurium ATCC13311, and *S. aureus* CMCC26003) increased gradually with increasing concentrations of EPS (0.2 to 5.0 mg/mL), without a significant difference ($P < 0.05$), and the highest inhibition was for *P. aeruginosa* CMCC10104 (47.02 ± 4.83%) and lowest for *E. coli* O157:H7 (25.82 ± 5.34%).

When compared with the positive group of ampicillin (50 μg/mL), the difference of the effect of inhibition by EPS was nonsignificant at higher concentrations (2.0, 5.0 mg/mL). Previous studies reported that EPS from *L. helveticus* MB2-1 also showed activity against biofilm formation by pathogens, with inhibition rates of 43.58% (against *P. aeruginosa* PAO1), 65.82% (*S. aureus* ATCC 6538), and 33.41% (*E. coli* K-12; Li et al., 2014b); EPS of *L. plantarum* YW32 showed inhibition rates of 45.13% (*S. aureus* AC1), 44.04% (*Salmonella* Typhimurium S50333), and 12.71% (*E. coli* O157:H7; Wang et al., 2015b). These results demonstrated that strains of *L. plantarum* from different resources might have different specificity against pathogens (Garcia-Ruiz et al., 2014).

Plenty of literature has reported the inhibition of pathogens by LAB, mainly focusing on the effect of organic acids (e.g., lactic acid and acetic acid); a few studies have mentioned the antagonism of EPS from *L. plantarum* against pathogens. Our results showed that EPS of WLPL04 had a broad activity againt those biofilm-forming pathogens. Biofilm formation is a problem not only for chronic infection by pathogen in the host, but also for the disinfection procedures

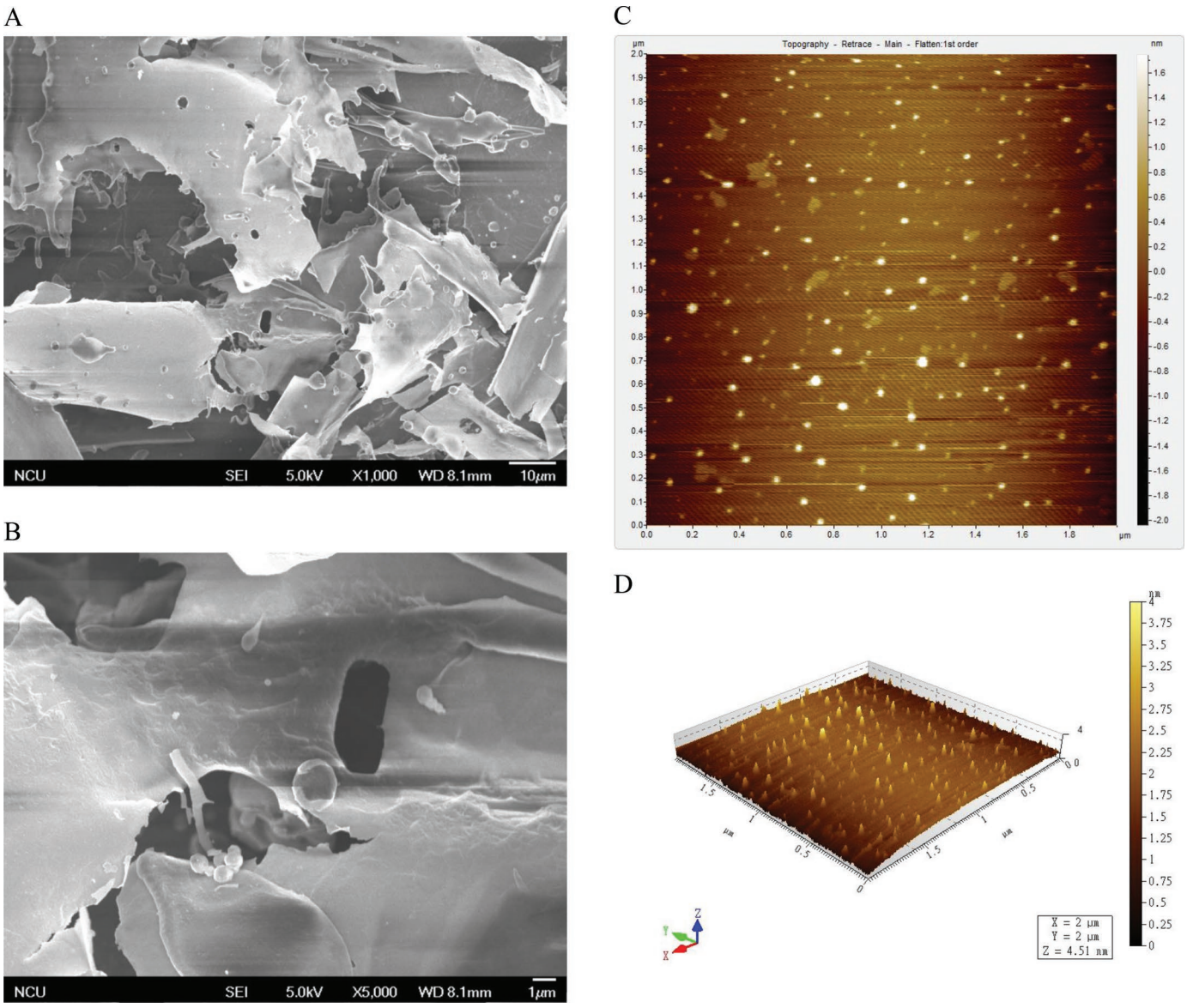

Figure 4. Scanning electron micrograph images of the purified exopolysaccharide (EPS) from *Lactobacillus plantarum* WLPL04 (A: 1,000×, B: 5,000×). Planar (C) and cubic (D) atomic force microscopy images of the purified EPS from *L. plantarum* WLPL04. Color version available online.

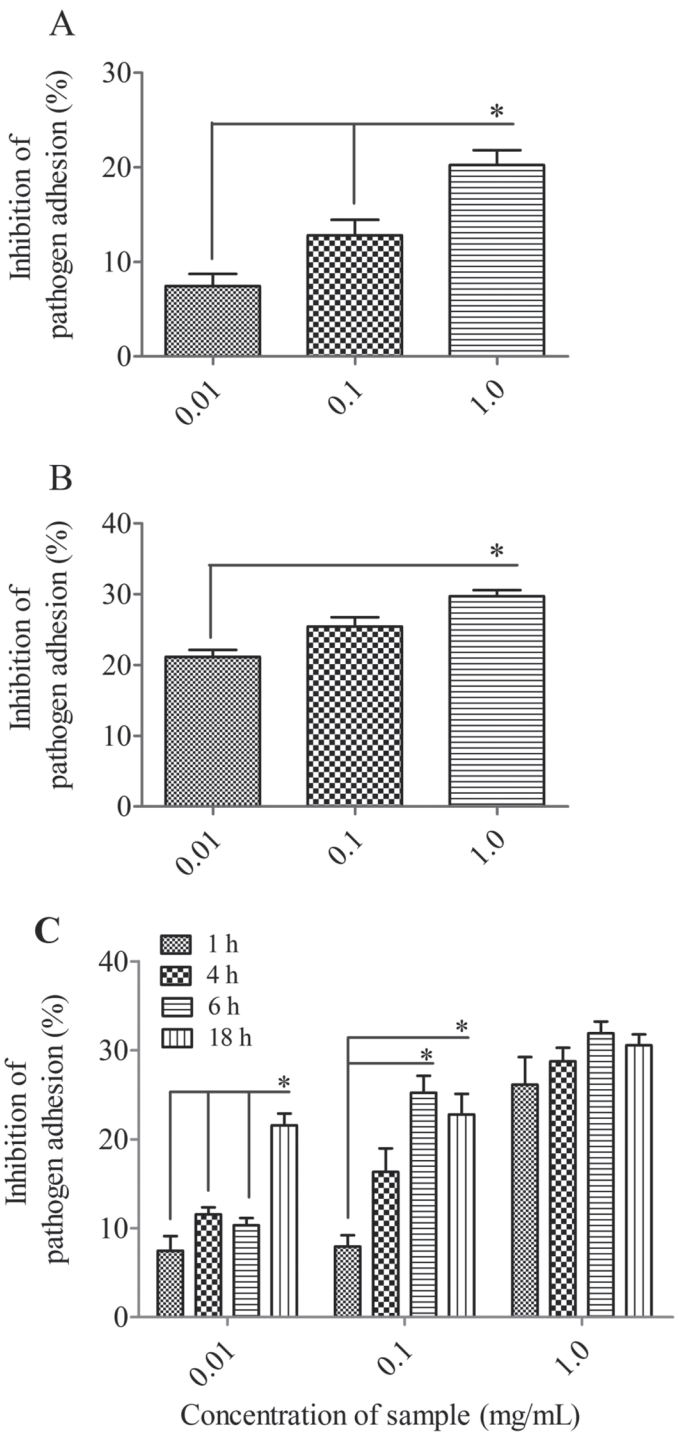

Figure 5. Antiadhesion assays (A: competition, B: displacement, and C: inhibition) of *Escherichia coli* O157:H7 on human colon cancer HT-29 cells in the presence of exopolysaccharide (EPS; 0.01, 0.1, and 1.0 mg/mL) from *Lactobacillus plantarum* WLPL04. The inhibition rate was calculated as the relative reduction percentage of the test group (EPS), using the control group (without EPS) as 100% of adhesion. Data of triplicate trials were expressed as the mean ± SD. $^*P < 0.05$.

in food processing. Although we did not evaluate the mechanism of activity against biofilm formation by the EPS in our study, this effect occurs by inhibiting of the initial attachment of the bacterial cell surface (Valle et al., 2006), by influencing the release of substances associated with biofilm formation (Wu et al., 2016), or by modifying the bacterial cell surfaces (Rendueles et al., 2013).

Antitumor Activity

The EPS from WLPL04 was not only antagonistic against the adhesion of pathogens and formation of biofilm, but it might have antitumor activity. The antitumor activity of EPS at different concentrations (50, 100, 200, 400, and 800 μg/mL) was investigated by using 5-FU (50 μg/mL) as a positive control. As shown in Figure 7, EPS had a nonsignificant difference on antitumor activity compared with 5-FU. The antitumor effect of EPS increased with time and concentration. After incubation for 72 h at 800 μg/mL of EPS, the inhibition rate was the highest (60.77 ± 11.11%). Wang et al. (2014) reported that the inhibition rate of EPS (600 μg/mL) from *L. plantarum* 70810 was 88.34%, when treated for 72 h, whereas Wang et al. (2015b) showed that the inhibition rate of EPS from *L. plantarum* YW32 was 39.24% under the same conditions. The reasons for the difference may be due to the different Mw, monosaccharide composition, and grouped structure of different EPS (Wu et al., 2014).

CONCLUSIONS

In this study, EPS from *L. plantarum* WLPL04 was isolated, purified, and shown to have a molecular mass of 6.61×10^4 Da. The EPS was composed of xylose, glucose, and galactose in an approximate molar ratio of 3.4:1.8:1. Under the microscope, purified EPS showed a smooth sheet structure with many homogeneous rod-shaped lumps. The purified EPS exhibited excellent capacity to inhibit the adhesion of *E. coli* O157:H7 to HT-29 cells, and had probiotic properties such as activity against biofilm formation by several pathogenic bacteria, and antitumor activity against HT-29 cells. These results showed that EPS from *L. plantarum* WLPL04 has obvious biological activities in vitro, which might form the basis for potential application of EPS from *L. plantarum* WLPL04 in developing functional food.

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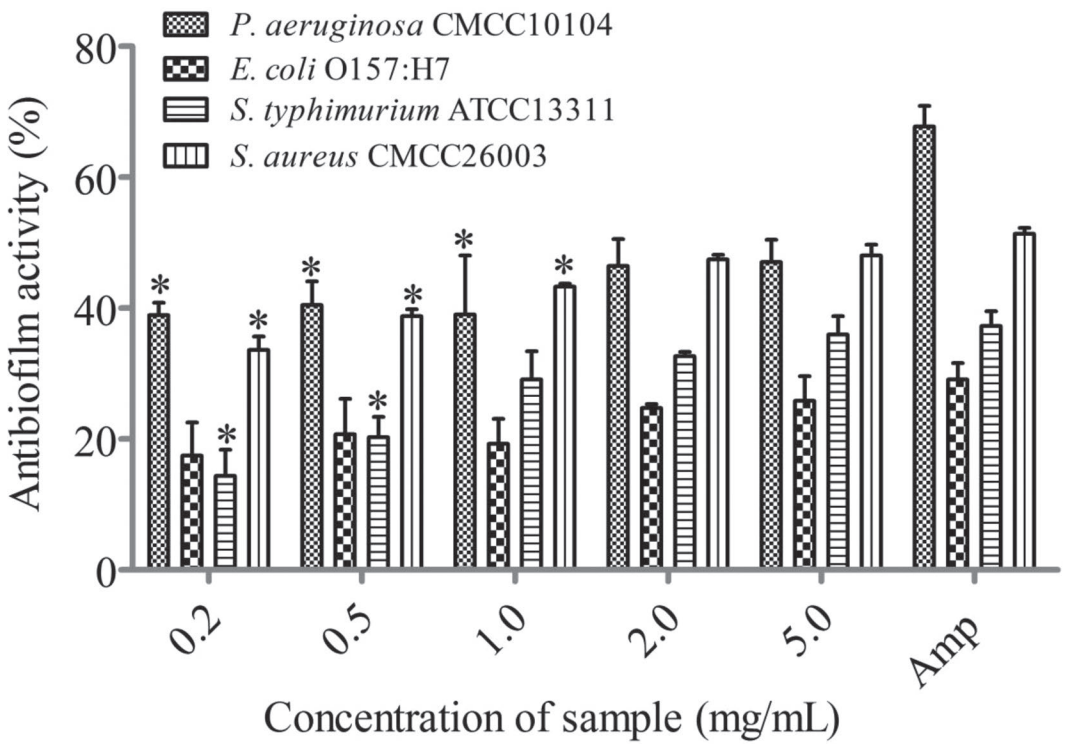

Figure 6. Anti-biofilm-formation assays of the purified exopolysaccharide (EPS; 0.2, 0.5, 1.0, 2.0, and 5.0 mg/mL) from *Lactobacillus plantarum* WLPL04 against *Pseudomonas aeruginosa* CMCC10104, *Escherichia coli* O157:H7, *Salmonella* Typhimurium ATCC13311, and *Staphylococcus aureus* CMCC26003. Ampicillin (Amp; 50 μg/mL) was used as a reference material. Data are presented as the mean ± SD of triplicates. $^*P < 0.05$.

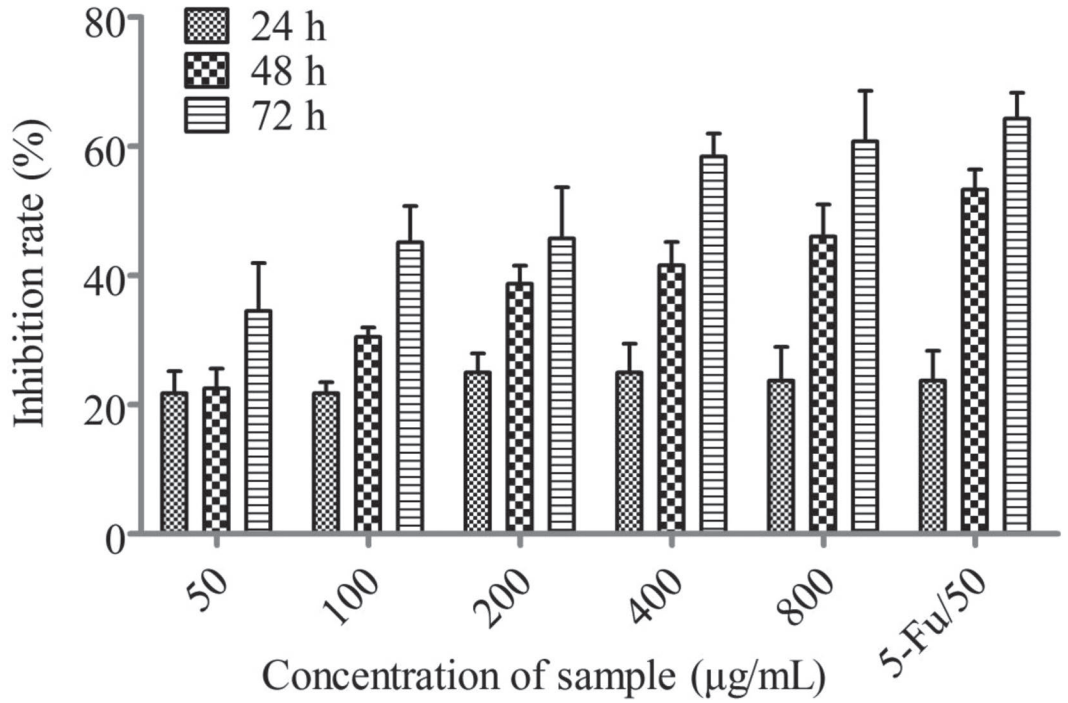

Figure 7. In vitro inhibitory assay of the purified exopolysaccharide from *Lactobacillus plantarum* WLPL04 at different concentrations (50, 100, 200, 400, and 800 μg/mL) against human colon cancer HT-29 cells in 24, 48, and 72 h; fluorouracil (5-Fu; 50 μg/mL) was used as a reference. Data are presented as the mean ± SD of triplicates.

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