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Chemical characteristics and anti-*Escherichia coli* mechanism of water-soluble extracts from yeast cell walls

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Abstract

Background Antimicrobial resistance is a threat to global public health and requires the development of new antibiotic alternatives to treat bacterial infection. Infection with *Escherichia coli* (*E. coli*) is the most common cause of diarrhea in pigs, especially in weaning piglets. The objective of this study was to characterize chemical properties of water-soluble extract (WSE) obtained from yeast cell wall, and study its antibacterial activity and antimicrobial mechanisms against *E. coli*.

Results WSE was extracted from yeast cell wall by hot water, followed by anhydrous ethanol precipitation and deproteinization by Sevag method to obtain water-soluble polysaccharides (WSP, accounting for 76.53 ± 5.91% by weight of WSE). WSP was purified by DEAE-52 cellulose column and Sephadex G-100 column to obtain three fractions of polysaccharides, WSNP, WSAP-1 and WSAP-2. Physicochemical properties of them were then characterized. The average molecular weights of WSNP, WSAP-1 and WSAP-2 were 105,130, 94,581 and 91,247 Da, respectively. WSNP was composed of glucosamine (GlcN), glucose (Glc), mannose (Man) and glucuronic acid (GlcA) in a molar ratio of 8.6:55.2:34.4:1.8, WSAP-1 was consisted of GlcN, Glc and Man in a molar ratio of 10.9:42.2:46.9, and WSAP-2 was consisted of GlcN, galactose (Gal), Glc and Man in a molar ratio of 9.0:1.0:55.1:34.9. Spectral analysis indicated that all three polysaccharides had pyran polysaccharides, triple-helix structure, α- and β-glycosidic bond. The minimum inhibitory concentrations of WSE and WSP on *E.coli* were 25 mg/mL and 100 mg/mL, respectively. WSE can not only disrupt the integrity of the cell wall and membrane of *E.coli*, but also increase the permeability of the outer membrane.

Conclusions Our research elucidated chemical composition and structure of WSNP, WSAP-1 and WSAP-2. WSE exerted its bactericidal activity by damaging the bacterial cell wall and membrane and increasing outer membrane permeability. Yeast cell wall products have been recommended as a potential alternative to antibiotics. This work provides more information, regarding chemical properties and anti-*E. coli* mechanism of WSE, and facilitates the application of yeast cell wall products as a promising antibiotic alternative in animal feed.

Keywords Yeast extracts, Water-soluble extracts (WSE), Water-soluble polysaccharides (WSP), *Escherichia coli* (*E. coli*), Chemical characteristics, Antibacterial mechanism

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1 Background

Escherichia coli (*E. coli*) is a major conditional pathogen causing serious diarrhea either in animals or humans worldwide [1, 2]. The incidence of *E. coli* diarrhea in weaning piglets is as high as 80% and mortality rates are over 10% [3], which caused huge economic losses to the swine industry. It is estimated that *E. coli* infections caused 650 million morbidities and approximately 800,000 deaths in children (under 5 years of age) worldwide each year [4]. The unique outer membrane barrier and drug resistance, especially multi-drug resistance, have put the treatment of *E. coli* disease in jeopardy [5].

There is currently no licensed effective vaccine for the prevention of diarrhea caused by *E. coli* and reliance is still placed on medication for prevention and treatment. The long-term misuse and/or abuse, of antibiotics, to prevent microbial infections, especially via the addition of subclinical doses of antibiotics to feed as antibacterial growth promoters, has led to widespread and rapid bacterial resistance [6]. To solve the problem of drug resistance, the EU, China and other countries have banned the use of antibiotics as feed additives [7, 8]. However, the prohibition of feed antibiotics has caused new problems: intestinal bacterial infections have increased, animal industry production levels have declined, and the cost of animal production has increased significantly. Therefore, the development of new and safe alternatives to antibiotics is urgently required. Currently, common antibiotic substitutes include prebiotics [9, 10], probiotics [11], plant extracts [12] and enzyme preparations [12].

Products of yeast cell wall origin have been considered as a potential alternative to antibiotics due to their immunomodulatory [13] and antibacterial activities [14]. Several reports have suggested that WSE can improve the intestinal microenvironment, stimulate innate and acquired immunity, adsorb mycotoxins, promote wound healing and prevent cancer, in addition to possessing antioxidant, blood sugar lowering and blood fat lowering effects [15–18]. Yeast cell wall can be used to replace chlortetracycline, colistin sulfate and other antibiotics in animal production to control necrotic enteritis in chickens [19–21], reduce bacterial infections in aquatic animals [22–24], reduce the diarrhea rate of young animals such as piglets and calves [25, 26], and promote animal growth and development [27, 28]. It has been reported that yeast cell wall polysaccharides (mainly contains glucan and mannan) are the main active components in yeast cell walls [29–31]. Furthermore, studies have found that modification of polysaccharides can improve their biological activities and solubility in water, and researchers have used physical and biological methods to reduce the molecular weights of β-glucan and mannan, as well as chemical methods to change their primary structures [32–35]. However, whether polysaccharides have antibacterial activity, whether they are the main antibacterial components of WSE and the antibacterial mechanism of the active components have not been well elucidated.

According to our previous studies, the antibacterial ability of water-soluble extract from yeast cell walls against common pathogens was studied via inhibition zone testing; however, the potential antibacterial compounds and physicochemical properties of WSE remain unclear. Therefore, this study aimed to investigate compositional properties, antibacterial activity and mode of action of WSE.

2 Methods

2.1 Chemicals and reagents

Yeast (from *Saccharomyces cerevisiae*) cell wall powder used in this study, derived from brewer's yeast FX-2 (deposit number is CCTCC NO: M2016418), was provided by Angel Yeast Co., Ltd (Yichang, China). After the yeast broth was fermented and cultured, the yeast cells were collected and the yeast itself was autolytically broken (yeast endogenous enzyme action), followed by enzymatic hydrolysis of alkaline protease, mannanase, β-glucanase and cellulase in that order, and finally the raw yeast cell wall powder was obtained. The finished product obtained was a spray dried powder after enzymatic digestion. DEAE-52 cellulose and Sephadex G-100 were bought from Wuhan Feiyi Technology Co., Ltd. (Wuhan, China). The D-glucose, D-mannose, dextran standards (1152, 5000, 11,600, 23,800, 48,600, 80,900, 148,000, 273,000, 409,800, 667,800 and 3,693,000 Da) and the propidium iodide (PI) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Monosaccharide standards (mannose (Man), rhamnose (Rha), galactose (Gal), glucose (Glc), arabinose (Ara), xylopyranose (Xyl), fucose (Fuc), guluronic acid (GulA), fructose (Fru), ribose (Rib), amminogalactose (GalN), *N*-acetyl-D glucosamine (GlcNAc), glucosamine (GlcN), glucuronic acid (GlcA), galacturonic acid (GalA) and mannuronic acid (ManA)) were obtained from Yuanye Biotechnology Co., Ltd. (Shanghai, China). Congo red, trifluoroacetic acid (TFA), dialysis bag (500 Da) and 0.45-μm filters were purchased from Beijing Solarbio Science and Technology Co., Ltd. (Beijing, China). Mueller–Hinton (MH) broth and eosin methylene blue (EMB) medium were purchased from Haibo Biotechnology Co., Ltd (Qingdao, China). Colistin sulfate standards were purchased from China Institute of Veterinary Drugs Control (Beijing, China). The alkaline phosphatase (ALP) assay kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). *N*-phenyl-1-naphthylamine (NPN) and *O*-nitrophenyl-β-D-galactoside (ONPG) were purchased from Shanghai Yien Chemical Technology Co., Ltd (Shanghai, China).

Other chemicals and reagents were of analytical grade or equivalent.

2.2 Bacteria

E. coli ATCC 25922 was purchased from American Type Culture Collection (Manassas, USA). *E. coli* 39 (the serotype was O101:K99), isolated from pig with diarrhea, was obtained from the National Reference Laboratory of Veterinary Drug Residues (HZAU) (Wuhan, China). The MIC of ampicillin against *E. coli* 39 is > 128 μg/mL, cefalothin is > 128 μg/mL, streptomycin is 2 μg/mL, doxycycline is 2 μg/mL, florfenicol is 8 μg/mL, and sulfisoxazole is 16 μg/mL.

2.3 Extraction of WSE

WSE was extracted using the hot water extraction method. Yeast cell wall powder was suspended with deionized water in a 1:10 (g/mL) ratio and then magnetically stirred for 1 h at 50 ℃. The suspension was centrifuged at 6000 rpm for 20 min. The supernatant was collected and concentrated with a rotary evaporator at 50 ℃. The concentrated supernatant was then freeze-dried.

2.4 Extraction of WSP

WSP was obtained by anhydrous ethanol precipitate and deproteinization by the Sevag method. So, the supernatant obtained from 2.3 was precipitated overnight with a four fold volume of anhydrous ethanol at 4 °C, and then centrifuged at 4500 rpm for 10 min. The sediment was dissolved in ultrapure water and deproteinized with Sevag reagent (chloroform/n-butyl alcohol, 4:1) [36]. After the mixture was shaken for 30 min, centrifuged at 10,000 rpm for 5 min, the upper water layer was collected. Use Sevag reagent to remove protein repeatedly 4 times, and confirm that no obvious protein layer appears. Add 4 times the volume of absolute ethanol to the upper water layer and allow it to store at 4 ℃ overnight. Afterward, collect the precipitate and freeze-dry it to obtain crude WSP.

2.5 Separation and purification of WSP

Crude WSPs were purified sequentially by DEAE-52 cellulose and Sephadex G-100 chromatography according to the method of Shi with some modifications [37]. WSP was dissolved with ultrapure water at 20 mg/mL and applied to a DEAE-52 cellulose column (2.6 × 30 cm) with a volume of 5 mL. WSP was eluted with four column volumes (200 mL) of ultrapure water and 0.1, 0.3, 0.5 M NaCl solutions at a flow rate of 1.0 mL/min, and the elution fraction was collected for 10 mL/tube. The sample (100 μL) from odd-numbered tubes was detected for the total sugar contents by phenol-concentrated sulfuric acid method. The elution curve was drawn, and combined with the collection liquid of the same eluting peak to concentrate, dialysis (MWCO 500 Da) and freeze-dry. Then, the eluted fraction obtained from ultrapure water named as WSNP (water-soluble neutral polysaccharide) and 0.1 M NaCl named as WSAP (water-soluble acidic polysaccharide) was further purified in a Sephadex G-100 gel column (1.6 × 75 cm) due to its higher yield. The two fractions were eluted with ultrapure water at a flow rate of 1.0 mL/min, respectively, and the elution fraction (5 mL/tube) was collected and the total sugar content was determined. So, we obtained that WSNP contains one component and WSAP contains two components. The major polysaccharides fraction was freeze-dried and named as WSNP, WSAP-1 and WSAP-2.

2.6 Chemical composition of WSE and WSP

Total sugar contents of the WSE and WSP were determined via the phenol sulfuric acid assay using glucose as a standard [38]. Meanwhile, protein contents of the WSE and WSP were determined via the spectrophotometric assay using bovine serum albumin as the standard [39]. Contents of β-glucan and mannan in the WSE and WSP were determined using high-performance liquid chromatography (HPLC). WSE/WSP (400 mg) was hydrolyzed with 6 mL of 37% hydrochloric acid [40, 41] at 30 ℃ for 45 min with vortexing every 15 min. Next, 100 mL of deionized water was added into the suspension and incubated at 121 ℃ for 60 min. After cooling, the pH of the suspension was adjusted to 7.0 using a 50% sodium hydroxide solution and the sample was diluted to 200 mL using deionized water. The aqueous phase was filtered through a 0.45-μm filter for HPLC analysis. The HPLC system consisted of a Chromaster system, a sugar column (6.5 mm × 300 mm waters sugar pak-1, Techcomp, China), and a refractive index detector (Hitachi, Japan). The mobile phase was 5 mmol/L sulfuric acid solution at a flow rate of 0.6 mL/min, the sample volume was 20 μL, and the column temperature was maintained at 65 ℃ during the experiment.

2.7 Determination of water solubility index

The water solubility index was determined by the method of Anderson et al. [42]. The sample was accurately weighed to 2.500 g, added to 20 mL of distilled water, shaken and dispersed, held at 22 ℃ for 30 min, hand shaken for 30 s at 5 min intervals and centrifuged at 3 000 r/min for 15 min. The supernatant was poured into an aluminum box of constant mass and evaporated to a constant mass in an oven at 105 ℃.

2.8 Structural identification of WSP

2.8.1 Monosaccharide composition

WSNP, WSAP-1 and WSAP-2 (10 mg) underwent hydrolysis in 3 M TFA at 120 ℃ for 3 h, followed by drying with nitrogen gas. After drying, a volume of 5 mL ultrapure water was added and vortexed to mix, then 100 µL was withdrawn from it, and 900 µL of ultrapure water was added, and centrifuged at 12,000 rpm for 5 min. The supernatants were identified and quantified with ion chromatograph (IC) (Thermofisher Scientific, China) using a DionexCarbopacTMPA20 (150 mm × 3 mm) and an electrochemical detector (Hitachi, Japan). The mobile phase was A: H_2O, B: 15 mmol/L NaOH, C: 15 mmol/L NaOH and 100 mmol/L NaOAC solutions at a flow rate of 0.3 mL/min, the sample volume was 5 µL, and the column temperature was maintained at 30 ℃ during the experiment. The standard solutions of 16 monosaccharide standards (Man, Rha, Gal, Glc, Ara, Xyl, Fuc, GulA, Fru, Rib, GalN, GlcNAc, GlcN, GlcA, GalA, ManA) were configured with 0.01, 0.1, 0.5, 1, 5, 10, 20 mg/L and processed with the same procedure, respectively.

2.8.2 Molecular weight

The molecular weights of WSNP, WSAP-1 and WSAP-2 were determined by high-performance gel permeation chromatography (HPGPC) using a Chromaster system [43], a BRT105-104-102 series gel column (8 × 300 mm) and a refractive index detector (Hitachi, Japan). The samples were prepared with ultrapure water at 5 mg/mL. The mobile phase was 50 mmol/L NaCl solution at a flow rate of 0.6 mL/min, the sample volume was 20 µL, and the column temperature was maintained at 40 ℃ during the experiment. The molecular weights of the samples were estimated based on the calibration curve obtained from dextran standards with different weight average molecular weights (1152, 5000, 11,600, 23,800, 48,600, 80,900, 148,000, 273,000, 409,800, 667,800, and 3,693,000 Da).

2.8.3 Fourier transform infrared spectra (FT-IR)

Dry potassium bromide was added to 1 mg of WSNP, WSAP-1 and WSAP-2. The pellets were prepared, and their infrared spectra were measured from 400 to 4000 cm^{-1} using a Fourier transform infrared spectrometer (Thermofisher Scientific, China) [44].

2.8.4 Congo red test

WSNP, WSAP-1 and WSAP-2 (4 mg) were dissolved in 2 mL ultrapure water, then mixed with 2 mL Congo red (80 mmol/L) and different volumes of 1 mol/L NaOH solution to obtain the final concentration of NaOH 0, 0.1, 0.2, 0.3, 0.4, 0.5 mol/L, respectively. The max wavelength was recorded in the range of 400–600 nm by an Ultraviolet spectrophotometer (Lab-Spectrum, China) [45].

2.9 Antibacterial susceptibility test

The minimum inhibitory concentrations (MICs) of WSE and WSP against *E. coli* were determined by the microbroth dilution method recommended by Clinical and Laboratory Standardization Institute [46]. WSE/WSP was dissolved in deionized water to prepare stock solutions of 1.6 g/mL. Two fold serial drug dilutions were prepared in MH broth to achieve final concentrations ranging from 1.56 to 400 mg/mL. Colistin sulfate, a narrow-spectrum antibiotic used mainly against Gram-negative bacteria and that can interact with lipopolysaccharide to destroy the bacterial outer membrane (OM), was used as a positive control drug [47]. The stock solutions of colistin sulfate were 1280 µg/mL; two fold serial drug dilutions were prepared in MH broth to achieve final concentrations ranging from 0.06 to 16 µg/mL. Each tube contained approximately 5×10^5 CFU/mL *E. coli*. The MIC was defined as the lowest drug concentration that inhibited bacterial growth for 16–20 h at 37 ℃.

2.10 Antibacterial mechanisms of WSE against *E. coli*

2.10.1 Time kill assay

WSE (5.0 mL) and *E. coli* 39 (1.0 mL) in the logarithmic phase (approximately 10^8–10^9 CFU/mL) were added to 100 mL MH broth, yielding final concentrations of WSE equivalent to 0.25, 0.5, 1, 2 and 4 MICs. Equal amounts of sterile water and colistin sulfate (1 µg/mL) were used as negative and positive controls, respectively. The mixtures were incubated at 37 ℃ at a speed of 220 rpm. Samples were taken at 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 h to count the number of surviving bacteria on MH agar plates.

2.10.2 Cell wall integrity assay

ALP is a phosphodiesterase mostly located between the cell wall and the cell membrane [48]. If the bacterial cell wall is disrupted, ALP will be released into the culture medium, and thus ALP activity in the culture medium can reflect the integrity of the bacterial cell wall [49, 50]. WSE (5.0 mL) and *E. coli* (1.0 mL) in the log phase (approximately 10^8–10^9 CFU/mL) were added to 100 mL MH broth to yield final concentrations of WSE equivalent to 0.25, 0.5, 1, 2 and 4 MICs (6.25, 12.5, 25, 50, 100 mg/mL). Equivalent amounts of sterile water and colistin sulfate (1 µg/mL) were used as negative and positive controls, respectively. The mixtures were incubated at 37 ℃ at a speed of 220 rpm. Samples were taken following incubations of 2, 4, 6, 8 and 12 h and then centrifuged at 5,000 rpm for 10 min. The supernatant was then

collected for ALP activity detection using the ALP assay kit (Nanjing Jiancheng Bioengineering Institute, China).

2.10.3 Cell membrane integrity assay

Damage to the integrity of the cell membrane enables PI, a fluorescent probe, to enter the cell and combine with the DNA and RNA therein [51]. The cultures of *E. coli* (1.0 mL) in the log phase (approximately 10^8–10^9 CFU/mL) were incubated with various concentrations of WSE (0.25, 0.5, 1, 2, or 4 MIC = 6.25, 12.5, 25, 50, 100 mg/mL) at 37 ℃ for 6 h. Equivalent amounts of sterile water and colistin sulfate (1 μg/mL) were used as negative and positive controls, respectively. After centrifugation at 13,000 rpm for 10 min, the cells were washed twice with PBS, and then the final cell suspension was adjusted to obtain an OD_{600} of 0.5. The cell suspension solution (500 μL) was incubated with 1 μL PI (10 mmol/L) at 37 ℃ in the dark for 30 min. The fluorescence intensity was detected by a Flow Cytometer (CytoFLEX LX, USA).

2.10.4 Outer membrane permeability assay

The OM permeability of *E. coli* treated with WSE was determined via the NPN assay according to previous methods [52]. The cultures of *E. coli* (1.0 mL) in the log phase (approximately 10^8–10^9 CFU/mL) were incubated with various concentrations of WSE (0.25, 0.5, 1, 2 and 4 MICs = 6.25, 12.5, 25, 50, 100 mg/mL) at 37 ℃ for 6 h. Equivalent amounts of sterile water and colistin sulfate (1 μg/mL) were used as negative and positive controls, respectively. Following centrifugation at 13,000 rpm for 10 min, the cells were washed twice with PBS and the final cell suspension was adjusted to obtain an OD_{600} of 0.5. 495 μL of *E. coli* culture was mixed with 5 μL of NPN (1 mmol/L). The fluorescence intensity was measured with an RF-5301 (PC) S fluorescence spectrophotometer (Shimadzu Corporation, Japan), using an excitation wavelength of 350 nm and an emission wavelength of 420 nm.

2.10.5 Inner membrane permeability assay

The IM permeability of *E. coli* treated with WSE was determined via the ONPG assay, which was modified for application [53]. The cultures of *E. coli* (1.0 mL) in the log phase (approximately 10^8–10^9 CFU/mL) were incubated with various concentrations of WSE (0.25, 0.5, 1, 2, and 4 MICs = 6.25, 12.5, 25, 50, 100 mg/mL) in MH broth supplemented with 2% lactose at 37 ℃ for 6 h. Equivalent amounts of sterile water and colistin sulfate (1 μg/mL) were used as negative and positive controls, respectively. After incubation, the cells were centrifuged at 13,000 rpm for 10 min to separate the supernatant and precipitate. The precipitate was washed twice with PBS and the final cell suspension was adjusted to obtain an OD_{600} of 0.5. 450 μL of either supernatant or cell suspension was then incubated with 50 μL of ONPG (30 mM) at 37 ℃ for 3 h. The A_{420} was measured by UV spectrophotometer (Agilent, USA).

2.10.6 Morphological analysis

WSE (1.0 mL) and *E. coli* (1.0 mL) in the log phase (approximately 10^8–10^9 CFU/mL) were added to 2 mL MH broth in order to create final concentrations of WSE solution equivalent to 0.25, 0.5 and 1 MIC (6.25, 12.5, 25 mg/mL). Equivalent amounts of sterile water and colistin sulfate (1 μg/mL) were used as negative and positive controls, respectively. The mixtures were then cultured under shaking at 220 rpm at 37 ℃ for 8 h. Bacterial cells were harvested via centrifugation at 5000 rpm at 4 ℃ for 10 min. Cells were then washed three times with PBS (pH 7.4) before morphological analysis.

2.10.6.1 Scanning electron microscope (SEM) The washed cells (approximately 10^6 CFU/mL) were immobilized overnight with 2.5% glutaraldehyde at 4 ℃. After immobilization, the cells were harvested by centrifugation at 5000 rpm for 10 min and then washed with PBS (pH 7.4) three times. Next, the cells were dehydrated using a series of gradually increasing concentrations of ethanol solution (30, 50, 70, 85 and 95%) for 15 min. After dehydration, the cells were washed twice in 100% ethanol for 20 min before being naturally dried for two days, plated with gold and observed under SEM (HITACHI SU-8010, Japan).

2.10.6.2 Transmission electron microscopy (TEM) Washed bacterial cells (approximately 10^6 CFU/mL) were immobilized overnight with 2.5% glutaraldehyde at 4 ℃. The immobilized bacteria were then washed three times with 0.1 M phosphate buffer, immobilized with 1% osmic acid (pH 7.4) for 2 h and washed three times with 0.1 M phosphate buffer. Bacterial cells were then collected by centrifugation and then stained with 1% uranyl acetate for 2 h, dehydrated with graded acetone, immersed and embedded. Sample sections for conventional TEM were created using a microtome (Germany Leica ultra-thin microtome EM UC7) and then stained with lead citrate and uranium acetate. Samples were then observed and recorded using a TEM (Tecnal G^2 20 TWIN, American FEI company, acceleration voltage: 200 kv).

2.11 Statistical analysis

All statistical analyses were performed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA) with all data represented as the mean ± standard deviation (SD) from at least three independent experiments.

3 Results

3.1 Extraction and chemical components analysis of WSE and WSP

The yield of WSE is 44.40 ± 0.60% (*w*/*w*, given as a percentage of the dry matter). The content of WSP in WSE is 76.53 ± 5.91%, indicating that polysaccharides are the main components in WSE. Therefore, the compositional characteristics of WSP were characterized to help clarify the active antimicrobial components in WSE in subsequent research.

After WSP was purified by DEAE-52 cellulose column, two independent peaks of WSP were obtained (Fig. 1A) as follows: WSNP (neutral polysaccharides, eluted with ultrapure water) and WSAP (acid polysaccharides, eluted with 0.1 mol/L NaCl). Sephadex G-100 chromatographic column was used to separate neutral and acid polysaccharides with different molecular weight ranges. In Fig. 1C, WSAP had two independent elution peaks, representing two molecular weight ranges, namely the elution components: WSAP-1 and WSAP-2. However, Fig. 1B shows a rather wide molecular weight distribution, indicating that sub-fraction is present in WSNP.

In Table 1, the total sugar, protein, β-glucan and mannan contents of WSE, WSP, WSNP, WSAP-1 and WSAP-2 were described. The protein content of WSE was 23.18 ± 0.27% and WSPs (WSP, WSNP, WSAP-1 and WSAP-2) were both at 2–3%. A comparison of the protein content shows that the WSPs still contained a small amount of protein after the removal of the free protein, which indicates that WSPs may contain protein bound to the polysaccharides. Additionally, the β-glucan and mannan contents were 7.37 ± 0.12% and 34.50 ± 0.91% in WSE, and 6.83 ± 0.21% and 30.60 ± 1.93% in WSP, respectively, as determined by HPLC.

3.2 Characterization of WSP

3.2.1 Monosaccharide composition

WSNP consisted of GlcN, Glc, Man and GlcA in a molar ratio of 8.6:55.2:34.4:1.8 (Fig. 2B and Table 1), WSAP-1 consisted of GlcN, Glc and Man in a molar ratio of 10.9:42.2:46.9 (Fig. 2C and Table 1), WSAP-2 consisted of GlcN, Gal, Glc and Man in a molar ratio of 9.0:1.0:55.1:34.9 (Fig. 2D and Table 1), indicating that WSNP, WSAP-1 and WSAP-2 are mainly composed of Glc and Man, and the contents of Glc and Man in WSNP and WSAP-2 are similar (Table 1).

3.2.2 Molecular weight

In Fig. 2E and Table 2, the HPGPC elution curve of WSNP displayed a peak, and the average molecular weight (M_w) of WSNP was 105,130 Da, the number-average M_w (M_n) was 65,053 Da and the peak M_w (M_p) was 82,832 Da. In addition, the M_w of WSAP-1 was 94,581 Da, the M_n was 59,232 Da, and the M_p was 75,008 Da (Fig. 2F and Table 2); the M_w of WSAP-1 was 91,247 Da, the M_n was 57,487 Da, and the M_p was 72,524 Da (Fig. 2G and Table 2).

3.2.3 FT-IR

Vibrations in FT-IR analysis indicate the presence of carbohydrates in WSNP, WSAP-1 and WSAP-2 [54]. As shown in Fig. 3A–C, WSNP, WSAP-1 and WSAP-2 presented similar FT-IR spectra, WSNP had a strong and broad signal at 3262 cm^{-1}, 2935 cm^{-1}, 1636 cm^{-1}, 1410 cm^{-1}, 1022 cm^{-1}, 915 cm^{-1}, 810 cm^{-1} and 579 cm^{-1}; WSAP-1 had a signal at 3290 cm^{-1}, 2933 cm^{-1}, 1651 cm^{-1}, 1386 cm^{-1}, 1021 cm^{-1}, 911 cm^{-1}, 811 cm^{-1} and 588 cm^{-1}; WSAP-2 had a signal at 3281 cm^{-1}, 2933 cm^{-1}, 1645 cm^{-1}, 1362 cm^{-1}, 1019 cm^{-1}, 915 cm^{-1}, 808 cm^{-1}. Among them, 3262 cm^{-1}, 3290 cm^{-1} and 3281 cm^{-1} had broad absorption peaks, which are the stretching vibrations of the polysaccharides –OH; the absorption peaks at 2935 cm^{-1} and 2933 cm^{-1} were the C–H stretching vibrations of polysaccharides; the absorption peaks at 1636 cm^{-1}, 1651 cm^{-1} and 1645 cm^{-1} were the bending vibrations of O–H; the absorption peaks at 1386 cm^{-1} and 1362 cm^{-1} were the C–H variable-angle vibrations of polysaccharides; the absorption peaks at 1126 cm^{-1}, 1128 cm^{-1} and 1127 cm^{-1} were the C–O–C stretching vibrations on the pyran ring; the absorption peaks at 1022 cm^{-1}, 1021 cm^{-1} and 1019 cm^{-1} were the C–O–H stretching vibrations on the pyran ring; the absorption peaks at 915 cm^{-1} and 911 cm^{-1} indicated the presence of β-glycosidic bonds; the absorption peaks at 810 cm^{-1}, 811 cm^{-1} and 808 cm^{-1} indicated the existence of α-glycosidic bonds and the absorption peaks at 579 cm^{-1}, 588 cm^{-1} and 599 cm^{-1} were the symmetrical stretching of the pyranose backbone vibration. So, WSNP, WSAP-1 and WSAP-2 are pyranoses, and the monosaccharides were connected by α-glycosidic bonds and β-glycosidic bonds.

3.2.4 Congo red test

Figure 3D shows the variation of λ_{max} for the complexes of Congo Red with WSNP, WSAP-1 and WSAP-2 over the concentration range of 0–0.5 mol/L NaOH. The λ_{max} of WSNP-, WSAP-1- and WSAP-2-Congo red complex was higher than that of Congo red at 0.1 mol/L NaOH, and then gradually decreased as the NaOH concentration increased, which is different from Conge red. Therefore, it could be concluded that WSNP, WSAP-1 and WSAP-2 all exhibited a triple-helix structure.

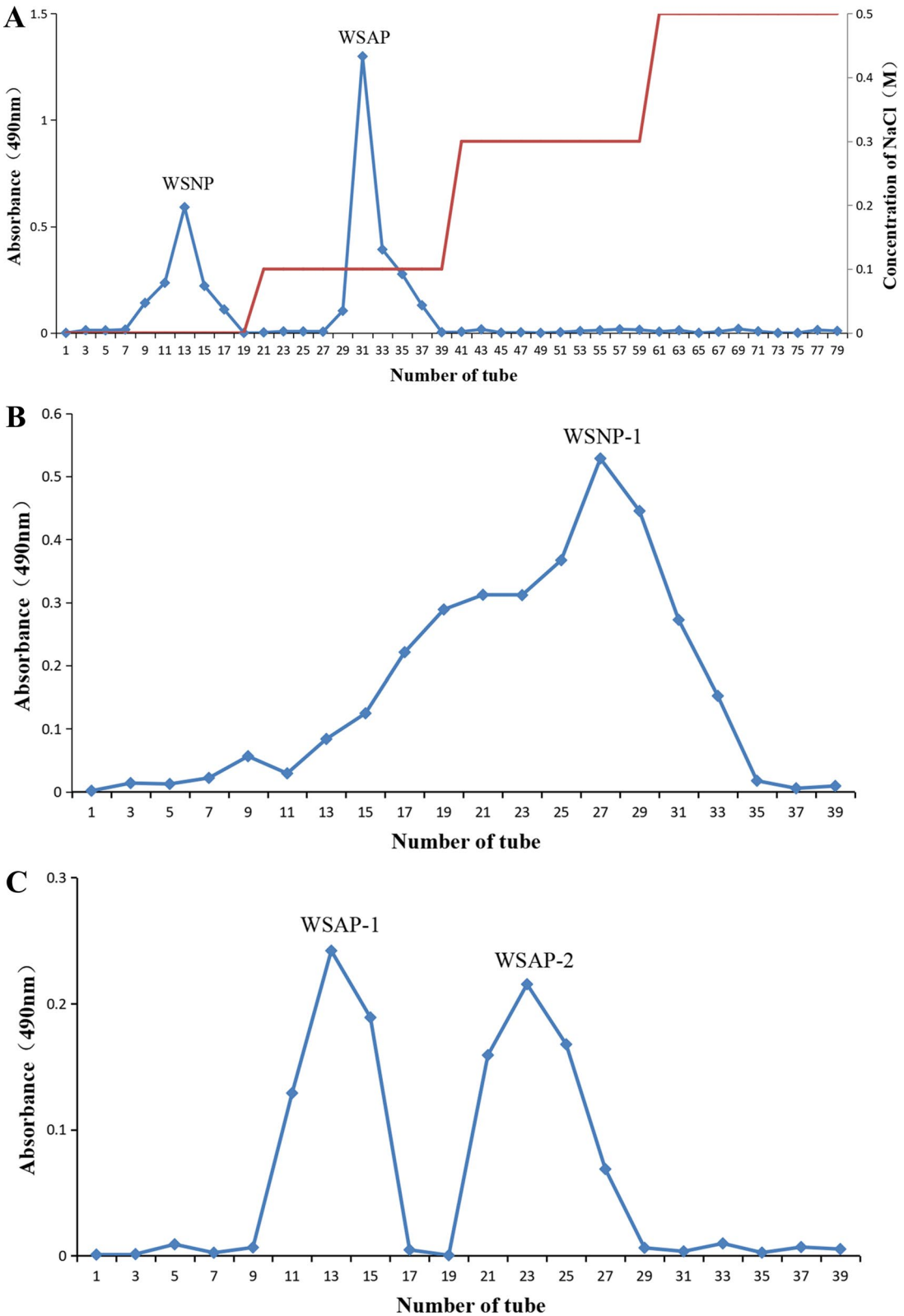

Fig. 1 **A** The elution curve of WSP on DEAE-52 cellulose column; **B** and **C** The elution curve of WSNP and WSAP on Sephadex G-100 column

Table 1 The WSI, yield, total sugar, protein, β-glucan and mannan contents and monosaccharide composition of WSE and WSP

Name	WSE	WSP	WSNP	WSAP-1	WSAP-2
WSI	41.21 ± 0.91	48.73 ± 0.86			
Yield	44.40 ± 0.60	33.98 ± 2.62	–	–	–
Total sugar	42.01 ± 0.94	45.82 ± 0.47	74.04 ± 1.35	84.01 ± 1.46	67.74 ± 1.46
Protein	23.18 ± 0.27	2.58 ± 0.09	2.73 ± 0.37	2.53 ± 0.12	2.35 ± 0.15
β-glucan	7.37 ± 0.12	6.83 ± 0.21	–	–	–
Mannan	34.50 ± 0.91	30.60 ± 1.93	–	–	–
Glucosami	–	–	8.6	10.9	9.0
Galactose	–	–	0	0	1.0
Glucose	–	–	55.2	42.2	55.1
Mannose	–	–	34.4	46.9	34.9
Glucuronic	–	–	1.8	0	0

"WSI" is *w*%. "Yield" refers to the proportion of WSE or WSP in the weight of yeast cell wall powder (*w*/*w*%). "Monosaccharide composition" is mol%. "–" is no determination. $n = 3$. All values are represented as mean ± SD of three independent experiment

3.3 MICs of WSE and WSP against *E. coli*

The results showed that the MICs of WSE and WSPs against *E. coli* ranged from 25 to 100 mg/mL, WSE was 25 mg/mL, and WSPs all against *E. coli* were 100 mg/mL (not a high viscosity solutions). The MICs of colistin sulfate against *E. coli* were 1 μg/mL (Table 2).

3.4 Antibacterial mechanisms of WSE against *E. coli*

3.4.1 Time to kill assay of E. coli

In Fig. 4, the growth curve of the *E. coli* in the control group demonstrates that the bacteria entered the logarithmic phase at 2 h and then the stationary phase after 8 h. The growth curves of *E. coli* treated with 0.25 or 0.5 MICs (6.25 or 12.5 mg/mL) WSE were consistent with that of control group, demonstrating no inhibitory effect on bacterial growth. Importantly, 1 MIC WSE (25 mg/mL) demonstrated significant antibacterial activity from 0 to 8 h, while the bacteria grew slowly after 8 h. In contrast, both 2 and 4 MICs (50 and 100 mg/mL, not a high viscosity solution) WSE, as well as colistin sulfate, restrained growth of the bacteria from 0 to 24 h. These results indicate that the inhibitory activity of WSE against *E. coli* is concentration-dependent.

3.4.2 Effect of WSE on the cell wall integrity of E. coli

In Fig. 5, both high-dose WSE (1, 2 and 4 MICs = 25, 50 and 100 mg/mL) and 1.0 μg/mL colistin sulfate significantly enhanced ALP levels in the *E. coli* culture medium when compared to the control group; notably, differences in ALP activity between the high-dose WSE group and the control group were significant from 2 to 12 h, with the most obvious enhancement seen at 4 h. ALP activity began to decrease after 4 h, which may be due to degradation of the ALP itself or absorption of the ALP by bacteria. The above results indicate that WSE inhibits bacteria by destroying the bacterial cell wall.

3.4.3 Effect of WSE on the cell membrane integrity of E. coli

The percentages of PI permeable cells observed in our studies were: 27.07% for control treatment (Fig. 6A), 31.93% for 0.25 MIC (6.25 mg/mL) WSE (Fig. 6B), 52.26% for 0.5 MIC (12.5 mg/mL) WSE (Fig. 6C), 62.20% for 1 MIC WSE (25 mg/mL) (Fig. 6D), 87.46% for 2 MIC WSE (50 mg/mL) (Fig. 6E), 90.21% for 4 MIC (100 mg/mL) WSE (Fig. 6F) and 64.55% following treatment with 1 μg/mL colistin sulfate (Fig. 6G). Comparing the percentages of PI permeable cells following control treatment or treatment with 0.25–4 MICs (6.25–100 mg/mL) WSE, it was found that higher concentrations of WSE led to higher percentages of PI permeable cells, proving that *E. coli* membrane destruction by WSE is concentration-dependent. There was no significant difference between the percentage of PI permeable cells following treatment with either 2 or 4 MICs (50 or 100 mg/mL) WSE (Fig. 6H).

3.4.4 Outer membrane permeabilization assays

When *E. coli* was treated with 0.25–4 MICs (6.25–100 mg/mL) of WSE, higher concentrations of WSE led to stronger effects on OM permeability (Fig. 7A). There was no significant difference in NPN fluorescence between the 0.25 MIC (6.25 mg/mL) WSE and control treatment groups, while the 4 MIC (100 mg/mL) WSE group displayed the greatest OM permeability. 1 μg/mL colistin sulfate increased OM permeability, while the result was not significantly different from that of 1 MIC (25 mg/mL) WSE. Colistin sulfate acted on the OM to destroy the cell membrane, consistent with Hancock's conclusion that colistin sulfate exerts its antibacterial effect by combining with lipopolysaccharide (LPS) on the OM [55].

3.4.5 Inner membrane permeabilization assays

In Fig. 7C, compared to the control treatment, 0.25–4 MICs (6.25–100 mg/mL) WSE and 1 μg/mL colistin sulfate had no significant effect on β-galactosidase activity in the cell precipitation of *E. coli* 39. In this experiment, there was no significant difference in absorbance in the bacterial precipitates treated with different concentrations of WSP. It was hypothesized that the greater disruption of the inner membrane by WSE may have caused more β-galactosidase to be released into the medium, resulting in a decrease in β-galactosidase activity in the precipitates. Based on

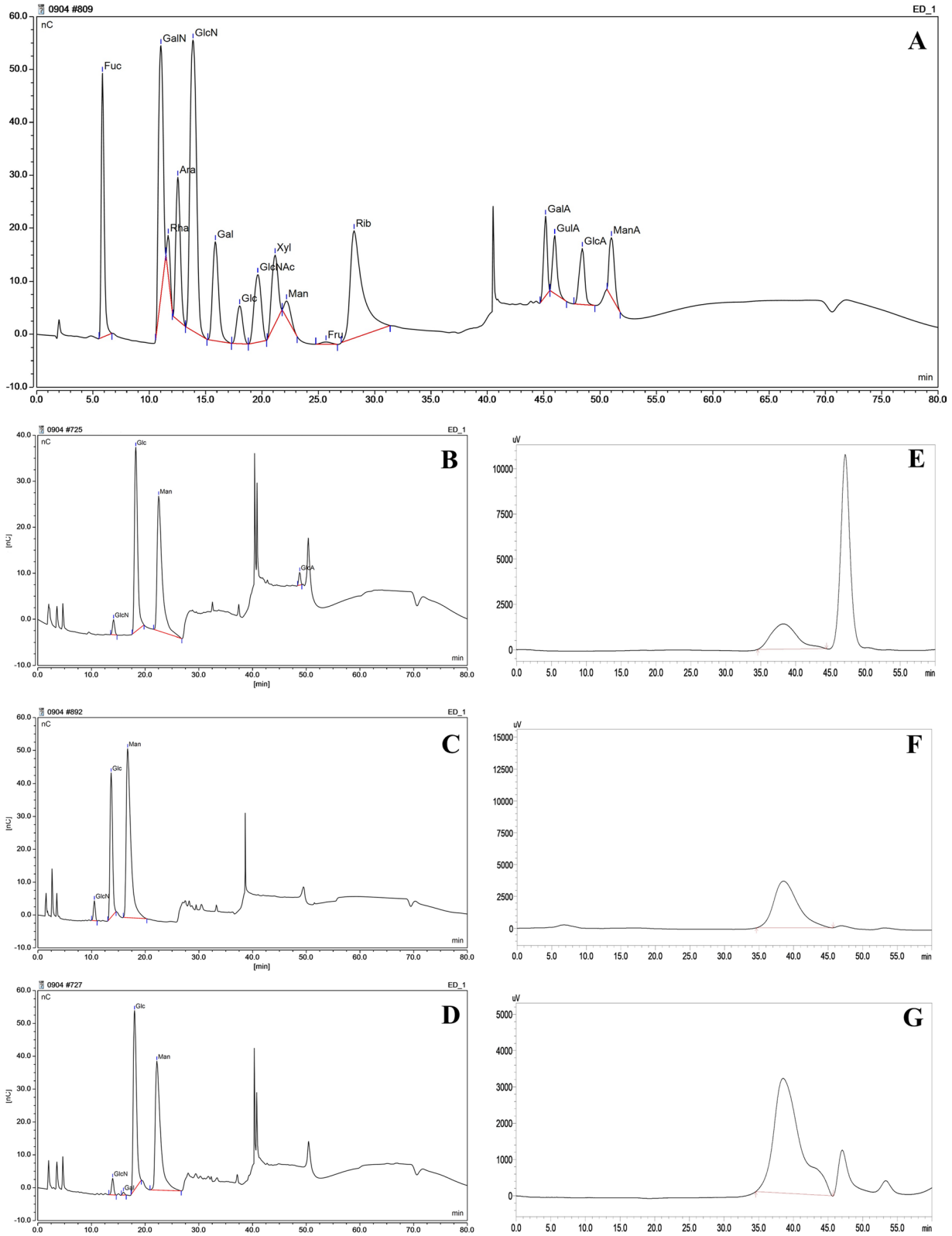

Fig. 2 Ion chromatograms and molecular weight distribution of WSNP, WSAP-1 and WSAP-2. **A** Ion chromatogram of mixed monosaccharide standards; **B** Ion chromatogram of WSNP; **C** Ion chromatogram of WSAP-1; **D** Ion chromatogram of WSAP-2; **E** Molecular weight chart of WSNP; **F**: Molecular weight chart of WSAP-1; **G** Molecular weight chart of WSAP-2

Table 2 MICs of WSE, WSP and colistin sulfate against *E. coli*

Drug	*E. coli* ATCC 25922	*E. coli* 39
WSE (mg/mL)	25	25
WSP (mg/mL)	100	100
WSNP (mg/mL)	100	100
WSAP-1 (mg/mL)	100	100
WSAP-2 (mg/mL)	100	100
Colistin sulfate (μg/mL)	1	1

The MIC of ampicillin against *E. coli* 39 is > 128 μg/mL, cefalothin is > 128 μg/mL, streptomycin is 2 μg/mL, doxycycline is 2 μg/mL, florfenicol is 8 μg/mL, and sulfisoxazole is 16 μg/mL

this hypothesis, we then examined the OD values in the bacterial culture supernatant. As hypothesized, the β-galactosidase activity in the supernatant of the *E. coli* was significantly different when comparing WSE treatment groups to the control group, and the comparison results of 1 μg/mL colistin sulfate treatment group were similar (Fig. 7B). β-galactosidase activity outside of the bacteria increased, indicating that both the OM and IM of *E. coli* were destroyed. Hence, the results indicate that WSE and colistin sulfate can destroy the cell membrane integrity of *E. coli*.

3.4.6 Scanning electron microscopy

Following exposure to 0.25–1 MICs of WSE, scanning electron microscopy (SEM) (Fig. 8) demonstrated that adhesions and aggregations occurred when *E coli* treated with 0.25 MIC (6.25 mg/ml) and 0.5 MIC (12.5 mg/ml) of WSE, while treated with 1 MIC (25 mg/ml) of WSE (Fig. 8D), the morphological structure of *E. coli* was blurred, with almost none single and normal bacterial morphology within view of the observation. After treatment with 1 μg/mL colistin sulfate, *E. coli* became dried out, adherent, blurred in morphological structure and completely dead (Fig. 8E).

3.4.7 Transmission electron microscopy

The TEM images (Fig. 8) showed that control-treated bacteria displayed a smooth and compact surface with regularly distributed cytoplasm and normal periplasmic space (Fig. 8A1, 2). Compared to the control treatment, the cytoplasm of *E. coli* treated with either 0.25–1 MICs (6.25–25 mg/mL) WSE (Fig. 8B1, 2, C1, 2, D1, 2) or

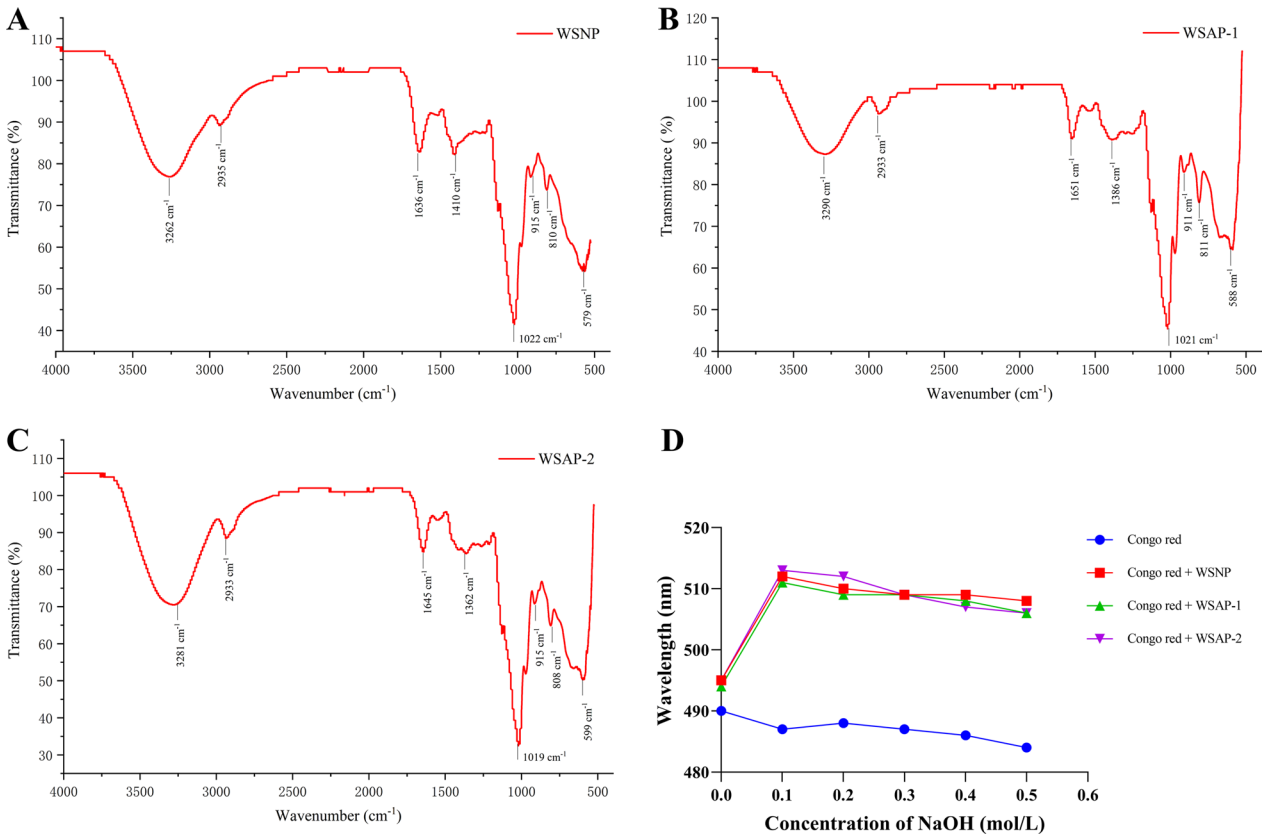

Fig. 3 Infrared spectrum and triple helical conformation analysis of WSNP, WSAP-1 and WSAP-2. **A** Infrared spectrum of WSNP; **B** Infrared spectrum of WSAP-1; **C** Infrared spectrum of WSAP-2; **D** Triple helical conformation analysis

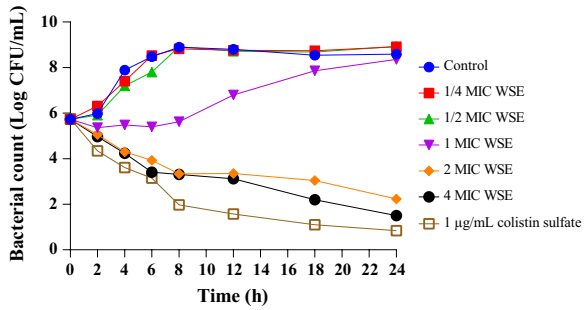

Fig. 4 Changes of antibacterial curve of *E. coli* 39 treated with WSE and colistin sulfate. 1/4 MIC: 6.25 mg/mL; 1/2 MIC: 12.5 mg/mL; 1 MIC: 25 mg/mL; 2 MIC: 50 mg/mL; 4 MIC: 100 mg/mL

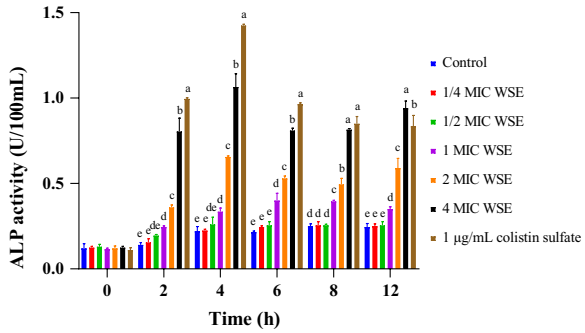

Fig. 5 Effects of WSE and colistin sulfate on the cell wall integrity of *E. coli* 39. 1/4 MIC: 6.25 mg/mL; 1/2 MIC: 12.5 mg/mL; 1 MIC: 25 mg/mL; 2 MIC: 50 mg/mL; 4 MIC: 100 mg/mL. Different letters represent significant differences based on one-way ANOVA ($p < 0.05$). $n = 3$. All values are represented as mean ± SD of three independent experiment

colistin sulfate (Fig. 8E1, 2) demonstrated material leakage and cytoplasmic matrix coagulation. Furthermore, the surfaces of *E. coli* treated with 0.5 MIC (12.5 mg/mL) WSE (Fig. 8C2), 1 MIC (25 mg/mL) WSE (Fig. 8D2) and colistin sulfate (Fig. 8E2) were disrupted and covered by an additional tooth-like layer.

4 Discussion

As a potential alternative to antibiotics, polysaccharides are widely studied, whereas the extraction and purification operations have an impact on the composition and structure, directly affecting polysaccharides activity [56, 57]. Although the acid–base method has been used to purify yeast cell wall polysaccharides in most studies, with the advantage of obtaining high purity β-glucan and mannan, it may disrupt the structure of the polysaccharides and make the polysaccharides chain length unstable. The present work is drawn on the purification methods of plant polysaccharides [58, 59], including aqueous-alcoholic precipitation and a Sevag method for protein removal, which were mild and did not destroy the polysaccharides structure. The characterization showed that WSNP, WSAP-1 and WSAP-2 were all pyranose sugars, linked to each other by α- and β-glycosidic bonds, and all featured a triple-helix structure. Notably, WSNP showed a wide range of molecular weights, inferring that it may contain subcomponents. Further experiments are therefore required to determine the additional subcomponents contained in WSNP. Smith et al. found that mannans in yeast cell walls provide collateral susceptibility to antibiotics (phenotypic resistance) by regulating bacterial cellular respiration [60], suggesting that mannans promote animal health without contributing to the growing problem of antibiotic resistance. In this study, both WSE and WSP contained high levels of mannans, making them promising for use as an alternative to antibiotics and for controlling antibiotic resistance.

The huge economic cost of colibacillosis to animal husbandry and the growing problem of antibiotic resistance has forced us to develop new potential antibiotic alternatives. In this study, both WSE and WSPs showed antibacterial activities. Compared to other water-soluble polysaccharides, such as pumpkin polysaccharides [61] and dandelion polysaccharides [62], both of which had a minimum antibacterial concentration of 100 mg/mL against *Bacillus subtilis*, *Staphylococcus aureus* and *E. coli*, the water-soluble polysaccharides in this work showed better antibacterial activity. Previous research in vivo reported that dietary WSE at 0.2–0.4% addition markedly improved the intestinal health of turbot [63]. Considering the wide availability and low price of yeast cell walls, WSE has obvious advantages in the field of antibiotic alternatives for piglet diarrhea.

Comparing the antibacterial results of the five samples (WSE, WSP, WSNP, WSAP-1 and WSAP-2), WSE (MIC = 25 mg/mL) has the best antibacterial activity, indicating that there were other non-polysaccharides active substance in WSE. As previously described, there are killer yeasts that produce toxins having protein nature [64, 65], which is acknowledged as a promising premise for antimicrobial activity [66]. Of course, the reason for the excellent antibacterial activity of WSE does not exclude that the small molecular weight of the monosaccharides or disaccharides in the water-soluble extract exerts antibacterial activity. It will be interesting to identify the non-polysaccharide antimicrobial components of WSE in the future.

The integrity of the cell wall and membrane, as well as the permeability of the inner and outer membrane of bacteria, are essential for maintaining the intrinsic morphology of the bacterium and ensuring proper growth. WSE exerted its antibacterial activity by disrupting the integrity of the *E. coli* cell wall and membrane, while increasing outer membrane permeability. It has been

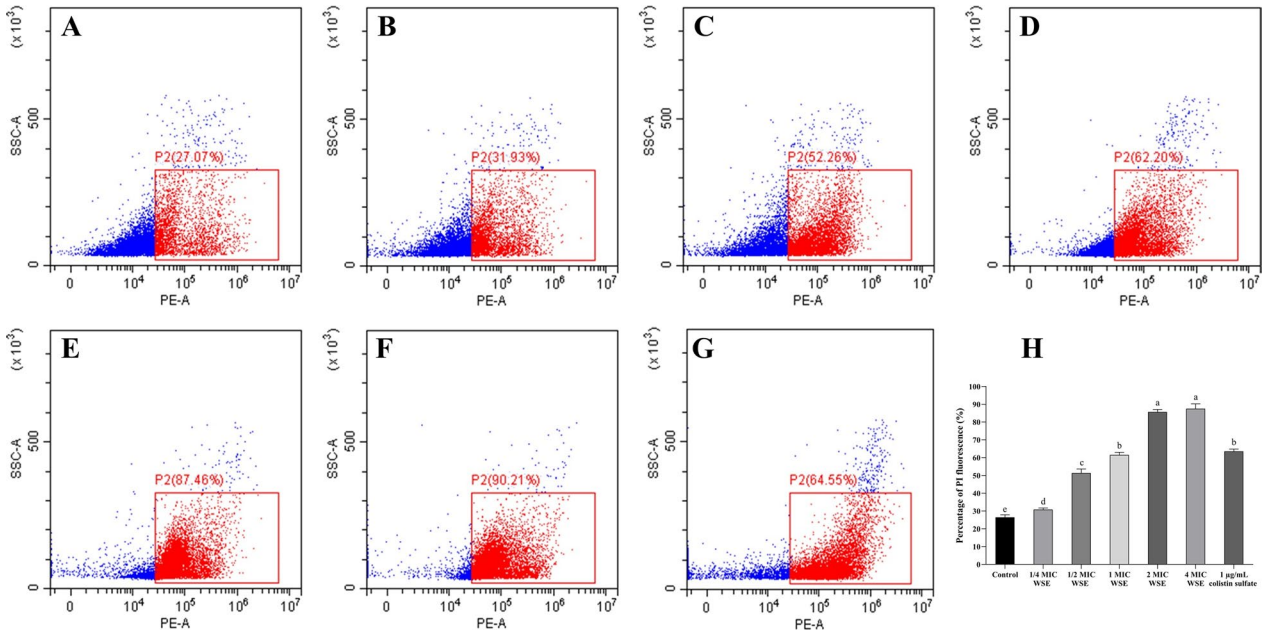

Fig. 6 Effects of WSE and colistin sulfate on the cell membrane integrity of *E. coli* 39. Control (**A**), 1/4 MIC (**B**), 1/2 MIC (**C**), 1 MIC (**D**), 2 MIC (**E**), 4 MIC (**F**) WSE and 1 μg/mL colistin sulfate (**G**). 1/4 MIC: 6.25 mg/mL; 1/2 MIC: 12.5 mg/mL; 1 MIC: 25 mg/mL; 2 MIC: 50 mg/mL; 4 MIC: 100 mg/mL. Proportion of PI permeable cells under drug action (**H**). Bars with different lowercase letters indicate significant difference ($p < 0.05$). $n = 3$. All values are represented as mean ± SD of three independent experiment

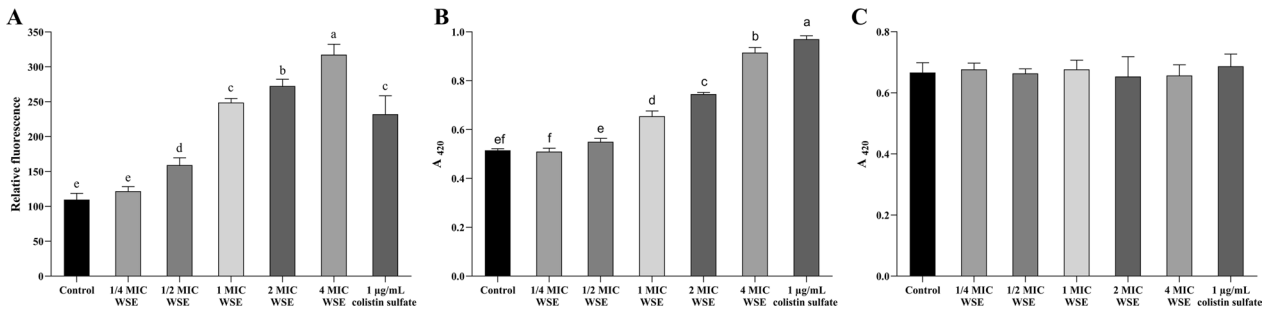

Fig. 7 Effects of WSE and colistin sulfate on the outer membrane permeabilization and the inner membrane permeabilization of *E. coli* 39. **A** The outer membrane permeabilization; **B** the inner membrane permeabilization (β-galactosidase activity in supernatant); **C** the inner membrane permeabilization (β-galactosidase activity in cell precipitation). 1/4 MIC: 6.25 mg/mL; 1/2 MIC: 12.5 mg/mL; 1 MIC: 25 mg/mL; 2 MIC: 50 mg/mL; 4 MIC: 100 mg/mL. Bars with different lowercase letters indicate significant difference ($p < 0.05$). $n = 3$. All values are represented as mean ± SD of three independent experiment

reported that polysaccharides from *Cordyceps cicadae* and *Chaetomium globosum* CGMCC 6882 were capable of acting on the cell wall of *E. coli* to exert antibacterial activity [50, 67]. In this study, colistin sulfate (positive control) acted on the outer membrane of *E. coli*, which is the same conclusion as the antibacterial mechanism of colistin sulfate by disrupting the lipopolysaccharide of the outer cell membrane [55]. Zhou et al. used the same method to study the effects of green tea polysaccharide on the cell membrane of *E. coli*, and the results showed that the proportion of PI permeable cells treated with 0 or 0.6 mg/mL green tea polysaccharides were 8.79% and 15.10%, respectively [51]. This result was similar to our results regarding WSE treatments in those higher drug concentrations led to higher proportions of PI permeable cells. Liu et al. found that depolymerized fucoidans can not only combine with bacterial membrane proteins but also change their structures [26]. However, the antibacterial targets of

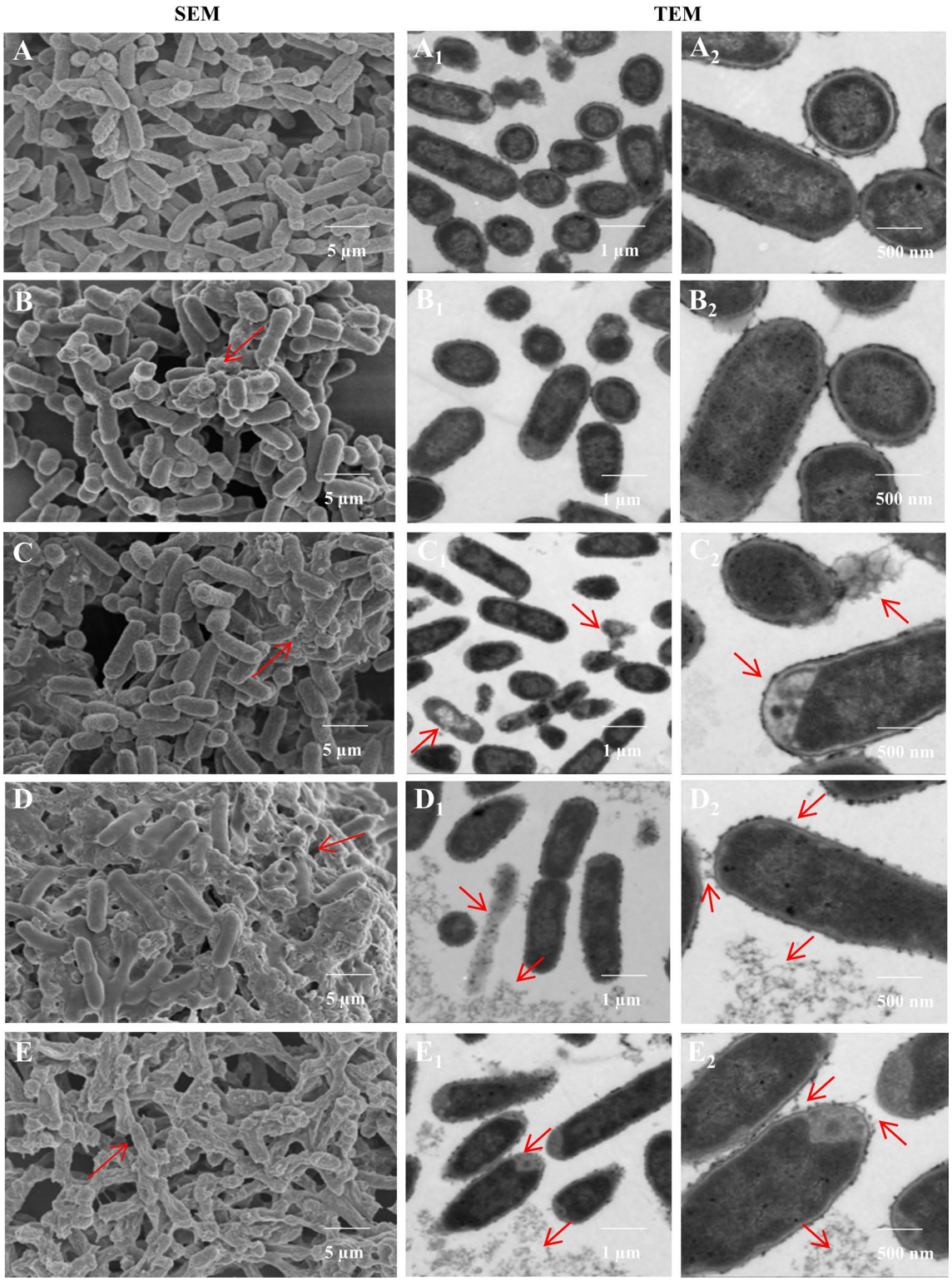

Fig. 8 Scanning electron microscopy and transmission electron microscopy of *E. coli* 39 was treated with Control (**A**, $\mathbf{A}_1$, $\mathbf{A}_2$), 1/4 MIC (**B**, $\mathbf{B}_1$, $\mathbf{B}_2$), 1/2 MIC (**C**, $\mathbf{C}_1$, $\mathbf{C}_2$), 1 MIC (**D**, $\mathbf{D}_1$, $\mathbf{D}_2$) WSE and 1 μg/mL colistin sulfate (**E**, $\mathbf{E}_1$, $\mathbf{E}_2$). 1/4 MIC: 6.25 mg/mL; 1/2 MIC: 12.5 mg/mL; 1 MIC: 25 mg/mL. The red arrows in the figure indicated that the morphology and internal structure of *E. coli* have changed

WSE and the signaling pathways by which they disrupt cell walls and membranes are currently unknown.

SEM results showed that WSE can adsorb and aggregate *E. coli*. The reason behind SEM analysis was that WSE has adhesive properties, enabling it to adhere to and wrap up bacteria. Cai et al. found that the levan-type exopolysaccharide from *Bacillus amylolyticus* JN4 was able to closely adhere to enterotoxigenic *E. coli* [68]. Polysaccharides are similar to the receptor structures on host cells and, thus, can block the binding and adhesion of bacteria to the receptor on the cell, exerting strong antibacterial activities [69]. An additional potential mechanism is that polysaccharides alter bacterial surface charges and enhance the flocculation effect [70]. The TEM results demonstrated that the bacterial cell wall and membrane were broken, which was consistent with the experimental results on the cell wall and cell membrane integrity. In addition, there was a shedding of the bacterial cell wall and outer cell membrane, the same morphological changes were observed in the study of chitosan on *E. coli* 36 and colistin sulfate on polymyxin B-resistant *Pseudomonas aeruginosa* 60 [71]. It was also observed in the TEM results that the material inside the bacteria coagulated when the bacterial cell wall and cell membrane were intact. The possible reasons for this are that WSE inhibits the activity of enzymes associated with the growth and reproduction of *E. coli*, hinders the action of enzymes or alters protein and nucleic acid molecules, so the effect of WSE on bacterial material metabolism needs to be experimentally investigated further.

5 Conclusion

The yeast cell wall was extracted and purified to obtain WSE (water-soluble extract), WSP, WSNP, WSAP-1 and WSAP-2. WSE and WSP both contained β-glucan and mannoprotein, and the mannoprotein content was higher than the β-glucan content. The molecular weight, monosaccharide composition, infrared spectroscopy and Congo red analysis of WSNP, WSAP-1 and WSAP-2 were almost consistent with each other. The comparison of the MIC of WSE, WSP, WSNP, WSAP-1 and WSAP-2 against *E. coli* showed that WSE had the best antibacterial activity. WSE exerted its antibacterial activity by disrupting the integrity of the cell wall and cell membrane of *E. coli*. WSE can not only disrupt the integrity of the cell wall and membrane, but also increase the permeability of the outer membrane, resulting in structural lesions and release of cellular components, which can lead to bacterial death.

In conclusion, this research investigated the extraction, isolation, purification, chemical properties of the active components of yeast cell wall, and elucidated inhibition mechanisms of WSE, laying the foundation for antimicrobial mechanistic studies of yeast cell wall products and facilitating their application in animal production.

Abbreviations

ALP	Alkaline phosphatase
E. coli	*Escherichia coli*
FT-IR	Fourier transform infrared spectroscopy
HPGPC	High-performance gel permeation chromatography
HPLC	High-performance liquid chromatography
IC	Ion chromatography
MIC	Minimal inhibitory concentration
NPN	*N*-Phenyl-1-naphthylamine
ONPG	2-Nitrophenyl β-D-galactopyranoside
PI	Propidium iodide
SEM	Scanning electron microscope
TEM	Transmission electron microscope
WSE	Water-soluble extract
WSAP-1	Water-soluble acidic polysaccharide 1
WSAP-2	Water-soluble acidic polysaccharide 2
WSNP	Water-soluble neutral polysaccharide
WSP	Water-soluble polysaccharide
WSI	Water solubility index

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Author contributions

YL, BD, FG and JH contributed equally to this work. YL performed the experiment, data analyses and wrote the manuscript. BD reviewed and edited the manuscript. FG and JH contributed to the conceptualization, project administration and financial support. MZ, YP, XW, XH, ZX, HH, GC and ZL helped perform the analysis with constructive discussions. Yulian Wang guided the experiment and revised the manuscript.

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Availability of data and materials

All relevant data and materials are contained within the article.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflicts of interest.

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