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Inhibitory effects of a novel antimicrobial peptide from kefir against *Escherichia coli*

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ABSTRACT

Antimicrobial peptide F1, a novel antimicrobial peptide from Tibetan kefir, have shown strong antimicrobial activity against several bacteria and fungi. We identified the amino acid sequence and studied the antimicrobial mechanism of peptide F1 against *Escherichia coli*. Our results showed that antimicrobial peptide F1 contained 18 amino acids (Thr-DAP-Asn-Thr-PEA-His-Pro-Asn-Thr-His-Leu-Ile- PEA-CySH-Val-Asn-PEA-Tau), which increased the outer and inner membrane permeability of *E. coli*, and the leakage of the cytoplasmic β-galactosidase and potassium ions was detected in the process. Morphologies of *E. coli* were observed by confocal laser scanning microscopy and transmission electron microscopy, which visually showed that antimicrobial peptide F1 could penetrate and accumulate into cell causing disruption of cell membrane functions. Furthermore, we elucidated the DNA binding ability of antimicrobial peptide F1 by agarose gel retardation and atomic force microscopy. Our findings indicated that antimicrobial peptide F1 has multiple targets in the killing of *E. coli*.

1. Introduction

Food safety is an important worldwide issue for both food industry and public health. The growing concern on the use of synthetic preservatives, the discovery of foodborne pathogens with resistance to classical antimicrobial agents (Teuber, 1999), and the increased consumer demand for natural and minimally processed foods have created many technological challenges in the food industry. The novel bio-preservatives, such as bacteriocins, have been considered as one of the most promising candidates to combat these obstacles (Cleveland, Montville, Nes, & Chikindas, 2001).

Bacteriocins are a group of antimicrobial peptides that are produced by a variety of bacteria, and they can kill and/or inhibit the growth of other bacteria (Bendjeddou, Fons, Strocker, & Sadoun, 2012; Gálvez, Abriouel, López, & Omar, 2007; Martinez et al., 2015; Milioni et al., 2015; Sonsa-Ard, Rodtong, Chikindas, & Yongsawatdigul, 2015). In addition, bacteriocins usually possess a strong and broad-spectrum of antimicrobial activity and microorganismal eradication with minimum resistance development. The bacteriocins have been isolated from a variety of bacteria that predominantly consist of lactic acid bacteria (Anastasiadou, Papagianni, Filiousis, Ambrosiadis, & Koidis, 2008; Kruger et al., 2013; Riley & Wertz, 2002). Nisin was the first bacteriocin approved for food use, which was produced by *Lactococcuslactis* subsp. *lactis* and has been used in more than 40 countries for over 50 years.

In our previous research, we found that antimicrobial peptide F1, a novel bacteriocin produced by *Lactobacillus paracasei* subsp. *Tolerans* FX-6 isolated from Tibetan kefir, has a wide antimicrobial spectrum and stability to heat, pH and protease (Miao et al., 2014). Elucidating the mechanism of antimicrobial peptide F1 is particularly important for enhancing its application in food preservation. In our current study, we chose *Escherichia coli* ATCC 25922, a major gram-negative foodborne pathogen, as a target strain to determine the antimicrobial mechanism of antimicrobial peptide F1.

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2. Materials and methods

2.1. Materials

E. coli ATCC 25922 was stored in the microbial culture laboratory in South China Agricultural University, College of Food Science (Guangzhou, China). *E. coli* was cultured in Luria–Bertani broth, and the overnight *E. coli* cultures (10^8 CFU/mL) were used in subsequent experiments. Fluorescein isothiocyanate (FITC), o-nitrophenyl-β-D-galactopyranoside (ONPG) and erythromycin were all purchased from Sigma–Aldrich (Shanghai, China).

2.2. Purification and identification peptide

Antimicrobial peptide F1 was purified from the cell-free supernatant of 72-h fermented pure milk by *L. paracasei* subsp. *tolerans* FX-6 according to a three-step purification procedure. First, cell-free supernatant was separated by a reversed-phase (RP) C18 column (20 mm × 450 mm, 10 μm, Macherey Nagel, France). Then, the active fraction was further purified by a RP Shim-pack PRC-ODS(K) column (30 mm × 250 mm, 15 μm, Shimadzu). Lastly, the antimicrobial peptide F1 was obtained by a RP ECOSIL C18 column (4.6 mm × 250 mm, 5 μm, Lubex, Japan). *E. coli* was used as an indicator strain during the process of purification. The full purification method of antimicrobial peptide F1 was explained in our previous paper (Miao et al., 2014).

The molecular mass of antimicrobial peptide F1 was determined in our previous work using an ABI 4800 MALDI-TOF-MS (Shanghai Applied Protein Technology Co. Ltd, Shanghai, China) (Miao et al., 2014). The amino acid sequence was performed on an LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Electron, Bremen, Germany) equipped with an ESI ion source and coupled to a Surveyor solvent delivery pump and a Surveyor autosampler (College of Veterinary Medicine, South China Agricultural University, China).

2.3. Growth curve assay

After overnight incubation at 37 °C in Luria–Bertani broth, the culture of *E. coli* was diluted to an optical density (OD) of 0.2 at 600 nm and 50 mL of dilution was placed in a 100 mL conical flask. Antimicrobial peptide F1 was added to a final concentration of 1 MIC. The culture without antimicrobial peptide F1 was used as negative control. The cultures were grown for 20 h and the OD at 600 nm was recorded at different time intervals (0, 1, 2, 3, 4, 6, 8, 10, 12, 14, 16, 18 and 20 h). All the testing points were measured in triplicate.

2.4. Outer membrane permeabilization assay

Erythromycin has a weak effect on gram-negative bacteria and penetrates poorly with the intact enterobacterial outer membrane, but it can easily traverse the damaged outer membrane (Vaara & Porro, 1996). The efficacy of antimicrobial peptide F1 in enhancing the outer membrane permeability was determined in *E. coli* with four experimental groups. *E. coli* (10^8 CFU/mL) in all groups was incubated in Luria–Bertani broth. Before the test, erythromycin or antibacterial peptide F1 were dissolved in sterile water. In the first group, antimicrobial peptide F1 (the final concentration 0.5 MIC, namely 31.25 μg/mL) was added with different concentrations of erythromycin (the final concentration 0.4 μg/mL, 0.6 μg/mL, 0.8 μg/mL, 1 μg/mL, and 2 μg/mL, respectively). In the second group, the erythromycin in different concentrations (the final concentration 0.4 μg/mL, 0.6 μg/mL, 0.8 μg/mL, 1 μg/mL, and 2 μg/mL, respectively) was added. In the third group, antimicrobial peptide F1 (the final concentration 0.5 MIC, namely 31.25 μg/mL) was added. The fourth group was the negative control group, without erythromycin and antimicrobial peptide F1 being added, and the same volume of sterile water was added instead. All the groups were incubated at 37 °C for 10 h. The bacterial growth was measured by a microplate reader (Multiskan MK3, Thermo, USA) at 630 nm. All assays were carried out in triplicate.

2.5. Inner membrane permeabilization assay

Inner membrane permeabilization assay was performed by measuring the release of cytoplasmic β-galactosidase activity from *E. coli* in a culture medium using ONPG as the substrate. It was determined by the previous methods with slight modifications (Marri, Dallai, & Marchini, 1996; Tsuji et al., 2001). After overnight incubation at 37 °C in Luria–Bertani broth, *E. coli* cultures were centrifuged at 3000 × g, and the harvested cells were induced in M9 lactose medium (1.28 g Na_2HPO_4, 0.3 g KH_2PO_4, 0.05 g NaCl, 0.1 g NH_4Cl, 0.05 g $MgSO_4$, 0.001 g $CaCl_2$, 0.5 g lactose and dissolved in 100 mL double-distilled water) at 37 °C for 8 h. Then the cells were centrifuged at 3000 × g for 1 min. The centrifuged cells were washed twice in sterile saline and resuspended to an optical density (OD) of 0.2 at 600 nm by the assay buffer (0.8 g NaCl, 0.02 g KCl, 0.29 g Na_2HPO_4, 0.024 g KH_2PO_4, 0.025 g $MgSO_4$, 0.39 g β-mercaptoethanol and dissolved in 100 mL double-distilled water). In addition, ONPG and antimicrobial peptide F1 were added to a final concentration of 0.1 mg/L and 1 MIC (62.5 μg/mL), respectively. The resuspended cell solution was incubated at 37 °C. The production of o-nitrophenol over time was measured using a microplate reader (Multiskan MK3, Thermo, USA) at 420 nm.

2.6. Potassium ion release assay

The cell membrane integrity was assessed by measuring potassium ions released from *E. coli* treated with antimicrobial peptide F1. The exponential phase *E. coli* cells in Luria–Bertani broth were centrifuged, washed and resuspended in 0.9% sterile saline (10^8 CFU/mL). The cell suspensions were incubated with antimicrobial peptide F1 (final concentration 1 MIC) at 37 °C. At multiple time intervals (30, 60, 90, 120 and 150 min) the cell suspensions were centrifuged at 10,000 rpm for 10 min then the supernatants were measured using atomic absorption spectrometer (S7-AA-7000, Shimadzu, Japan) (Hao, Shi, Tang, & Le, 2009). A control was carried out with 0.9% sterile saline. All assays were carried out in triplicate.

2.7. Confocal laser scanning microscopy

Antimicrobial peptide F1 was labeled with fluorescein isothiocyanate (FITC) as previously reported with some modifications (Helmerhorst et al., 1999). In summary, 1 mg/mL of FITC dissolved by Me_2SO was added to 2 mg/mL of antimicrobial peptide F1 in 50 mM potassium phosphate buffer (final pH 7.6), and the final concentration of FITC was 25 mg/mL. The mixture of FITC and antimicrobial peptide F1 was incubated for 16 h in the dark at 4 °C. Then 50 mM NH_4Cl was added to inactivate the residual FITC. The mixed solution was placed in a dialysis bag with a molecular mass cutoff of 500 Da and dialyzed for 24 h in 0.01 mol/L phosphate buffer. The FITC-conjugated antimicrobial peptide F1 was finally obtained by freeze-drying the mixed solution in the dialysis bag. The exponential phase *E. coli* cell suspension (10^8 CFU/mL) was mixed with a 2 mg/mL stock solution of FITC-conjugated antimicrobial peptide F1 to a final concentration equal to 2MIC (125 μg/mL). Samples were kept in the dark for 1 h at 37 °C. At multiple time intervals (10, 30, and 60 min) the cells were washed with the PBS buffer three times and observed using an LSM 710 Zeiss confocal laser-scanning microscope (Zeiss, Germany).

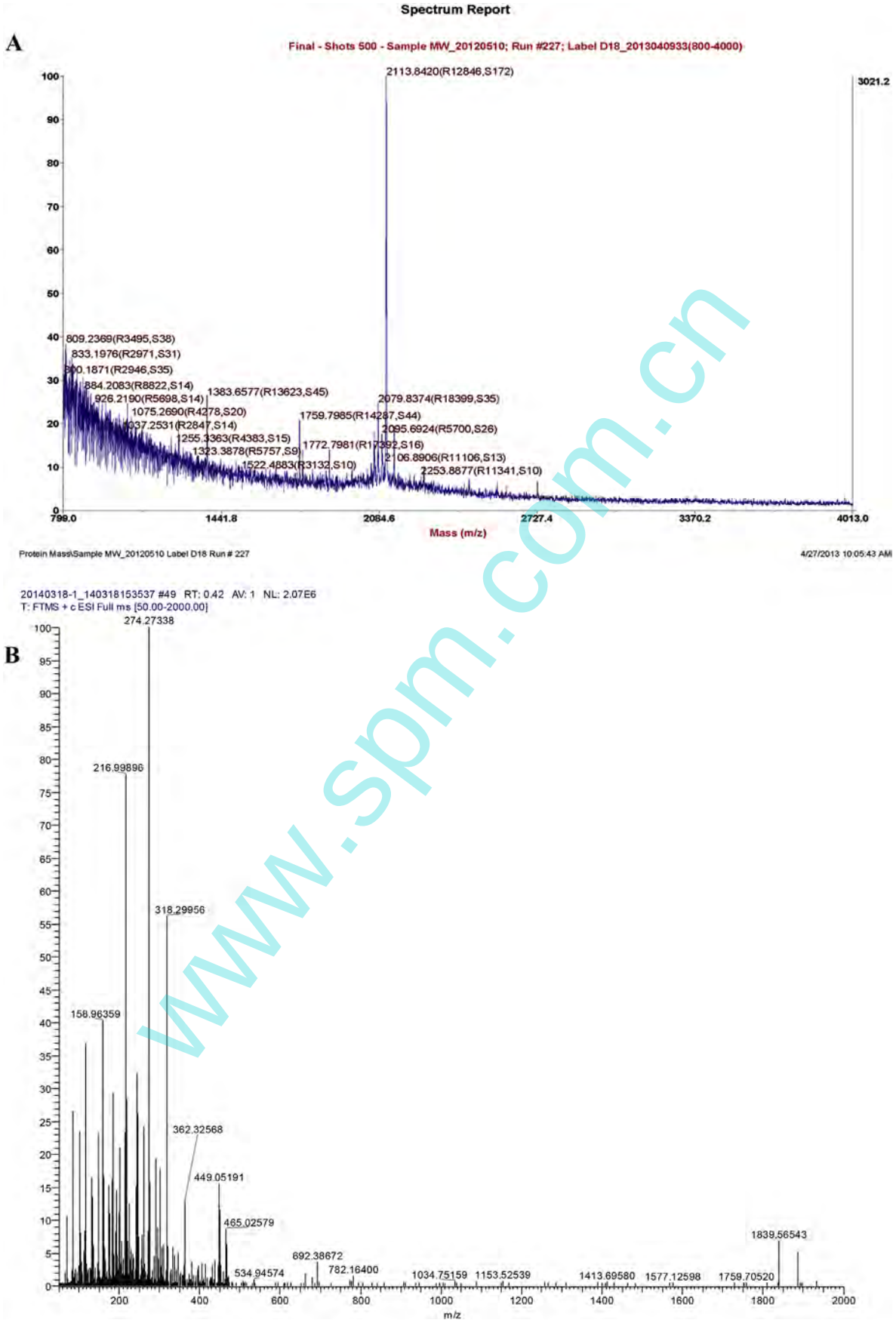

Fig. 1. The molecular mass and amino acid sequence analysis of antimicrobial peptide F1. (A) The molecular mass analysis of antimicrobial peptide F1 by MALDI-TOF-MS (Miao et al., 2014); (B) ESI-MS spectrum of antimicrobial peptide F1; (C) ESI-MS/MS spectrum of ion *m*/*z* 274.27338; and (D) ESI-MS/MS spectrum of ion *m*/*z* 1839.56543.

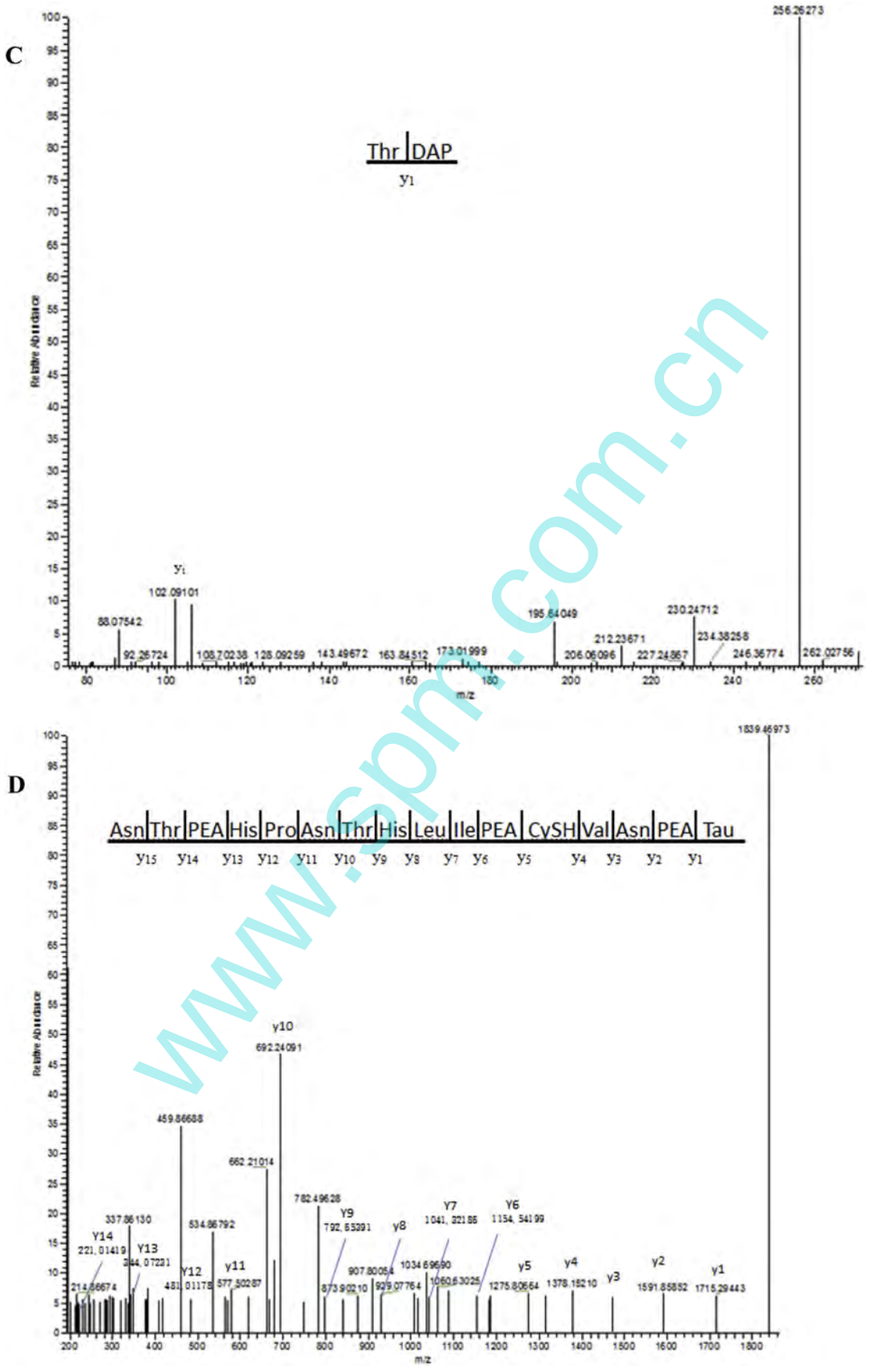

Fig. 1. *(continued)*

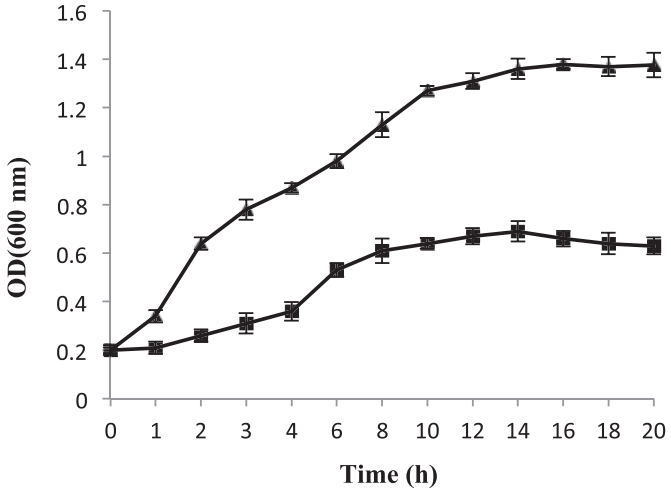

Fig. 2. Growth curve of *Escherichia coli* cells cultured in Luria–Bertani broth in the absence (▲) or presence of 1 MIC of antimicrobial peptide F1 (■).

2.8. Transmission electron microscopy

5 MIC (312.5 μg/mL) antimicrobial peptide F1 was added to the exponential phase *E. coli* (10^8 CFU/mL). The mixtures were incubated at 37 °C for multiple time periods (0, 30, 60 and 120 min). The cells were pelleted by centrifugation at 3000 × g for 10 min. The transmission electron microscopy processing of the pellets was taken according to a previously published method (Duan, Jin, Zhang, Li, & Xiang, 2014). In brief, the pelleted cells were placed in a 2.5% glutaraldehyde in phosphate buffer (pH 7.4) and stored overnight at 4 °C. Then transferred to 1% osmium tetroxide in the same buffer for 2 h. The pelleted cells were then washed in double distilled water and dehydrated by ethanol, propylene oxide, and acetone. The dehydrated cells were finally cut and double-stained for ultramicro observation. The ultrathin sections were examined by a transmission electron microscope (Hitachi H-7000, Japan) at magnification of 6800×.

2.9. DNA binding assay

The interaction between antimicrobial peptide F1 and genomic DNA of *E. coli* was determined by DNA gel retardation assay and atomic force microscopy imaging analysis. The *E. coli* cultures were centrifuged at 3000 × g for 10 min at 4 °C, and the obtained cells were used to extract genomic DNA by a bacterial genomic DNA extraction kit (Sangon Biotech Co., Ltd, Shanghai, China). The extracted DNA was dissolved in TE buffer (10 mM Tris–HCl, and 1 mM EDTA; pH 8.0) for further usage.

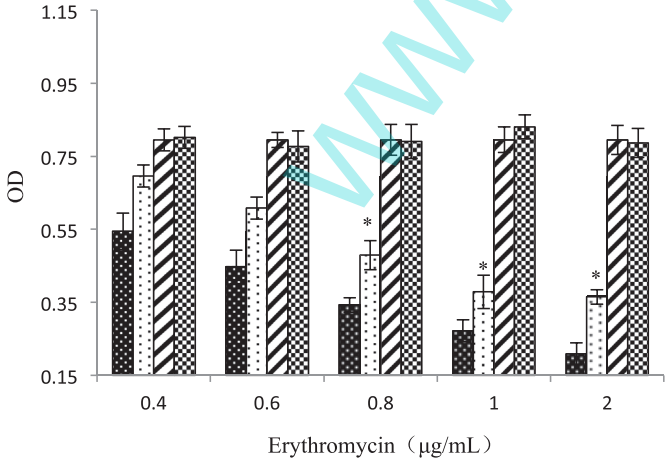

Fig. 3. Effects of antimicrobial peptide F1 on the outer membrane permeability of *Escherichia coli* cells. *Escherichia coli* cells were incubated with 0.5 MIC of antimicrobial peptide F1 and erythromycin (▩), erythromycin (⊡), 0.5 MIC of antimicrobial peptide F1 (◪), or sterile water (⊠), respectively. The optical density (OD) of *Escherichia coli* cultures were measured at 630 nm * indicates statistical significance between 0.5 MIC of antimicrobial peptide F1 and erythromycin and erythromycin ($p < 0.01$, $n = 3$).

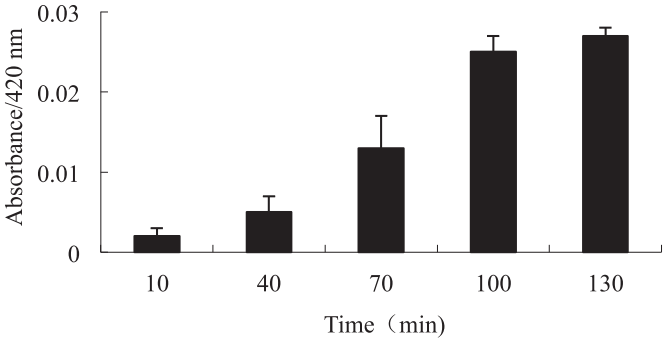

Fig. 4. The effects of antimicrobial peptide F1on the release of cytoplasmic β-galactosidase in the *Escherichia coli* cells.

For the DNA gel retardation assay, the genomic DNA (3 mg/mL) was incubated with antimicrobial peptide F1 (0, 0.25, 2.5, 5, 12.5, 25, 50 and 100 mg/mL) at a volume ratio of 1:1 at 30 °C for 10 min. Then the mixture was analyzed on a 0.8% agarose gel to detect DNA binding. Gel retardation was visualized under UV illumination using a Gel Doc XR gel imaging system (Bio-Rad, USA).

For the atomic force microscopy experiments, the genomic DNA (3 mg/mL) was incubated with antimicrobial peptide F1 (50 mg/mL) at a volume ratio of 1:1 at 30 °C for 10 min. Then the mixture was imaged by CSPM5500 scanning probe microscope (Guangzhou Primitive Nano Instrument, China).

2.10. Statistical analysis

All assays were carried out in triplicate and the results were expressed as mean values ± standard deviation (SD). The data were analyzed by using SPSS 18.0 statistical software.

3. Results and discussion

3.1. Purification and Amino acid sequence analysis of antimicrobial peptide F1

The purification procedure of antimicrobial peptide F1 was performed by a combination of a three-step reverse-phase

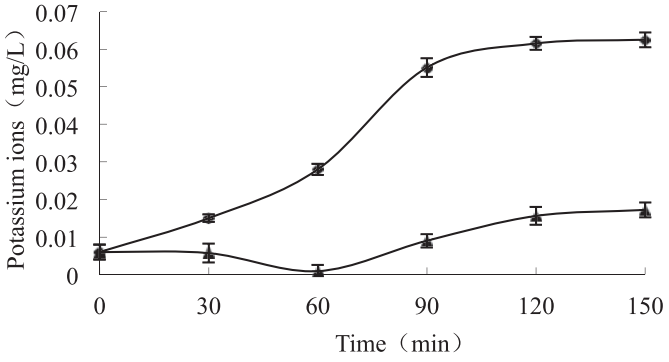

Fig. 5. Relative amount of potassium ions released by antimicrobial peptide F1 from *Escherichia coli* cells treated with antimicrobial peptide F1 (◆) or 0.9% sterile saline water (▲) over time.

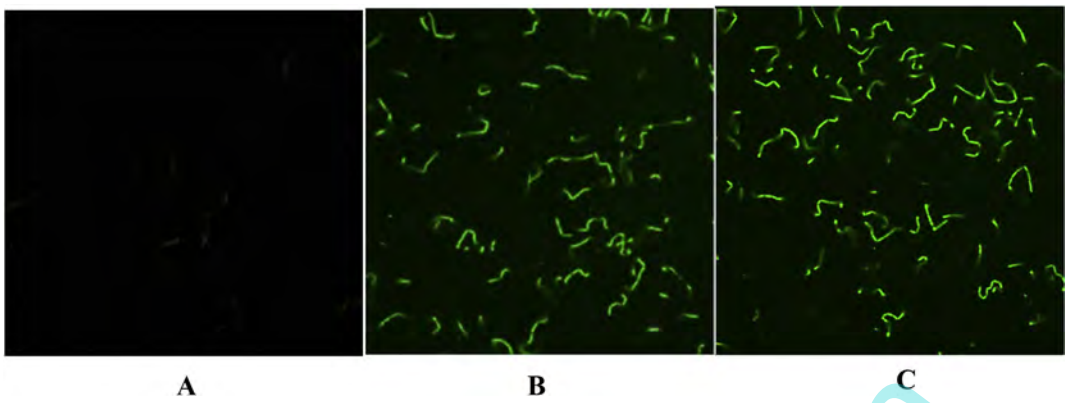

Fig. 6. Localization of FITC-conjugated antimicrobial peptide F1 in *Escherichia coli* cells by confocal laser scanning microscopy. (A) *Escherichia coli* cells treated with FITC-conjugated antimicrobial peptide F1 for 10 min; (B) *Escherichia coli* cells treated with FITC-conjugated antimicrobial peptide F1 for 30 min; and (C) *Escherichia coli* cells treated with FITC-conjugated antimicrobial peptide F1 for 60 min. Representative pictures were shown (selected from at least 6 replicates).

chromatography, guided by monitoring antimicrobial activity against *E. coli*. The minimal inhibition concentration (MIC) of antimicrobial peptide F1 was 62.5 μg/mL (0.03 mol/mL) against *E. coli* ATCC25922 (Miao et al., 2014).

The molecular mass of antimicrobial peptide F1 was 2112.842 Da by MALDI-TOF-MS analyses (Fig. 1A) (Miao et al., 2014). To determine the amino acid sequence, antimicrobial peptide F1 was subjected to ESI-MS/MS analysis. The results showed that antibacterial peptide F1 was fractured into two major fragment ions, the fragment ions *m*/*z* 274.27338 and 1839.56543 (Fig. 1B). These two major fragment ions were further analyzed by ESI-MS/MS to determine the amino acid sequence. The amino acid sequence was proposed to be Thr-DAP- (Fig. 1C) and -Asn-Thr-PEA-His-Pro-Asn-Thr-His-Leu-Ile-PEA-CySH-Val-Asn-PEA-Tau (Fig. 1D). Altogether, the whole amino acid sequence of antimicrobial peptide F1 was preliminarily identified as Thr (Threonine)-DAP (Meso-2,6-diaminopimelic acid)-Asn (Asparagine)-Thr (Threonine)-PEA (Phosphoethanolamine)-His (Histidine)-Pro (Proline)-Asn (Asparagine)-Thr (Threonine)-His (Histidine)-Leu (Leucine)-Ile (Isoleucine)-PEA (Phosphoethanolamine)-CySH (Cysteine)-Val (Valine)-Asn (Asparagine)-PEA (Phosphoethanolamine)-Tau (Taurine). After our analysis, we noticed this sequence contained three unusual amino acids, which included DAP (Meso-2, 6-diaminopimelic acid), PEA (Phosphoethanolamine), and Tau (Taurine). When the antibacterial peptide F1 was searched in the APD and NCBI databases, there was no match found, which suggested that it was a novel peptide.

The amino acid sequence of antibacterial peptide F1 was determined by Edman degradation analysis in our previous research, and it was found that the reaction could not proceed. It was proposed that antibacterial peptide F1 might contain a blocked N-termini that prevent the reaction from occurring (Miao et al., 2014). However, from the result of the LC-MS/MS analysis in this study, it was found that antibacterial peptide F1 does not contain a blocked N-termini, but the N-terminal threonine (Thr) is connected with the Meso-2,6-diaminopimelic acid (DAP, a unconventional amino acids). This special connection between threonine (Thr) and Meso-2, 6-diaminopimelic acid (DAP) may be responsible for the failed N-terminal sequencing, which warrants future research.

3.2. Growth curve of *E. coli* exposed to antimicrobial peptide F1

The growth curves of *E. coli* in the absence or presence of 1 MIC of antimicrobial peptide F1 are shown in Fig. 2 by measuring optical density at 600 nm. *E. coli* had rapid growth in the absence of antimicrobial peptide F1 and the highest optical density reached about 1.4 within 20 h. While exposed to 1 MIC of antimicrobial peptide F1, the growth of *E. coli* was significantly inhibited.

3.3. Antimicrobial peptide F1 increased outer membrane permeability

The results on the effects of antimicrobial peptide F1 against the outer membrane of *E. coli* are given in Fig. 3. It was found that erythromycin showed dose-dependent inhibition on the growth of *E. coli*, while 0.5 MIC of antimicrobial peptide F1 did not cause any significant inhibition. Interestingly, when 0.5 MIC of antimicrobial peptide F1 was added to the cell culture together with erythromycin, an enhanced inhibitory effect was observed on the growth of *E. coli*. For example, the inhibitory effect of erythromycin (at 2 μg/mL) was increased more than 3-fold by the presence of antimicrobial peptide F1.

Membrane binding and permeability is the first step of the interaction between antimicrobial peptides and bacterial cells (Anderson & Hancock, 2004; Tang, Hui, Li, & Qian, 2014), which is also a key link of antimicrobial mechanism (Epand & Vogel, 1999; Hawrani, Howe, Walsh, & Dempseyd, 2010). The outer membrane of gram-negative bacterium is composed of lipopolysaccharide (LPS), and this is the first permeability barrier to prevent entry of harmful agents such as antibiotics (Sampson, Misra, & Benson, 1989; Sutterlin, Zhang, & Silhavy, 2014). Erythromycin is less effective against gram-negative bacteria because it poorly penetrates the intact enterobacterial outer membrane. However, erythromycin can easily penetrate damaged outer membrane to reach the cytoplasm causing increased antimicrobial activities (Vaara & Porro, 1996). Our results showed that the presence of antimicrobial peptide F1 (0.5 MIC) had no inhibitory effect against *E. coli*; however, it can significantly enhance the antimicrobial activity of erythromycin. The results indicate antimicrobial peptide F1 may damage the outer membrane and increase cellular permeability, which facilitates higher erythromycin uptake.

3.4. Antimicrobial peptide F1 permeabilized inner membrane

As shown in Fig. 4, extracellular β-galactosidase increased in a time-dependent manner after treatment with antimicrobial peptide F1 (1 MIC). In less than 10 min, treatment with antimicrobial peptide F1 led to release of β-galactosidase to the extracellular compartment. At about 130 min, the extracellular levels of β-galactosidase reached a steady state.

Generally, the cytoplasmic β-galactosidase cannot pass through the integrated inner membrane of *E. coli*, but it can be detected

extracellularly if the inner cell membrane was damaged. ONPG can be hydrolyzed by the released of β-galactosidase to o-nitrophenol, which causes a color change that can be measured spectrophotometrically. Our results supported that the inner membrane of *E. coli* was permeabilized by antimicrobial peptide F1. Additionally, the ability to permeabilize inner the cellular membrane of bacteria has been observed for other antimicrobial peptides including buforin 2 (Berglund, Piggot, & Khalid, 2014), cecropin P1 (Arcidiacono, Soares, Meehan, Marek, & Kirby, 2009), insect cecropin A (Silvestro, Weiser, & Axelsen, 2000), defensins (Morgera et al., 2008), and tachyplesin I (Imura, Nishida, Ogawa, Takakura, & Matsuzaki, 2007).

3.5. Antimicrobial peptide F1 caused potassium ion release

The action of antimicrobial peptide F1 against *E. coli* cell membrane was further examined by the potassium ion release assay. Fig. 5 shows that the addition of antimicrobial peptide F1 to *E. coli* cells induced a time-dependent potassium ion release efflux from the cells. The amount of potassium ion released reached a steady state at 120–150 min, which was consistent with the pattern of β-galactosidase release. The increase amount of potassium ion efflux from *E. coli* cells provided further evidence that antimicrobial peptide F1 caused damage to the cell membrane. Measuring the efflux of potassium ions from bacterial cells is a classical method to investigate the membrane damage caused by antimicrobial agents (Codling, Maillard, & Russell, 2003; Hao et al., 2009; Lee et al., 2002, 2014; Morgan & Connor, 2005; Orlov, Nguyen, & Lehrer, 2002; Riazi, Dover, & Chikindas, 2012).

3.6. Visualization of the interaction between antimicrobial peptide F1 and E. coli cells by confocal laser scanning microscopy

For more precise observation of the dynamic interaction and the cellular localization of antimicrobial peptide F1 in *E. coli*, FITC was used as a fluorescent probe to label antimicrobial peptide F1 and visualize its localization in the cells by confocal laser scanning microscopy. Using FITC-conjugated antimicrobial peptide F1, we observed that antimicrobial peptide F1 was localized and accumulated in the cytoplasm of bacterial cells, and this process is time dependent (Fig. 6). After mixing the FITC-conjugated antimicrobial peptide F1 and *E. coli* cells for 10 min, only a few cells showed cellular accumulation of antimicrobial peptide F1 (Fig. 6A). At 30 min and 60 min, antimicrobial peptide F1 penetrated into most of the cells and accumulated in their cytoplasm (Fig. 6B and C). Furthermore, the results also showed that the florescent intensity increased from 30 to 60 min of treatment, which suggested the increased accumulation of antimicrobial peptide F1. It is noteworthy that FITC alone could not penetrate *E. coli* cells (no fluoresce), and it had no effect on the antimicrobial activity of antimicrobial peptide F1 (data not shown), which is consistent with previous findings (Mangoni et al., 2004). FITC has been used as an effective probe in previous research to evaluate the membrane permeabilization of antimicrobial peptides, as it can only penetrate into bacterial cells when the cell membrane is disrupted (Liao et al., 2010; Mangoni et al., 2004; Pagán & Mackey, 2000). Overall, our results showed that the cytoplasm of bacterial cells maybe a major site of action of antimicrobial peptide F1, which was able to enter bacterial cells and accumulate in the cytoplasm.

3.7. Transmission electron microscopy

To gain insight on the direct effects of antimicrobial peptide F1 on the morphology of bacterial cells, transmission electron microscopy was used to exam the ultrastructure of *E. coli* cells exposed to 5 MIC of antimicrobial peptide F1 for 0, 0.5, 1, and 2 h (Fig. 7). The untreated *E. coli* (Fig. 7A) were intact cells with a distinct and smooth cell membrane, and uniform cytoplasm region. After treatment with antimicrobial peptide F1 for 0.5 h (Fig. 7B) and 1 h (Fig. 7C), the cells suffered cellular shrinkage, and the smooth and

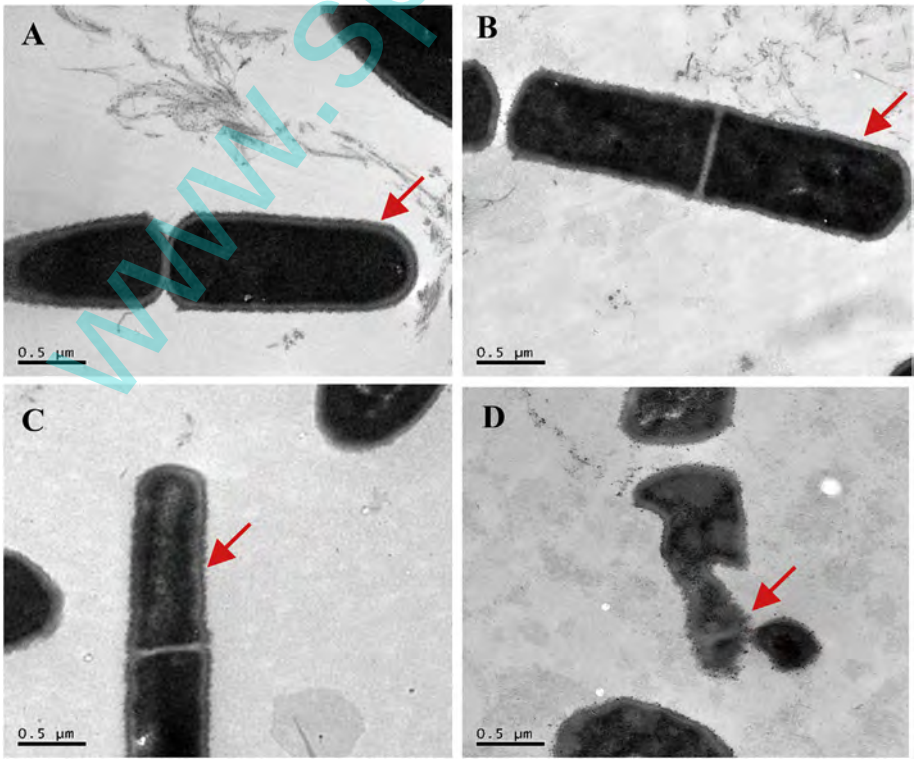

Fig. 7. Transmission electron microscopic analysis of *Escherichia coli* treated by 5MIC of antimicrobial peptide F1 for 0 h (A), 0.5 h (B), 1 h (C) and 2 h (D), respectively.

regular surface of *E. coli* became flaccid, suggesting that bacterial contents had exuded from the damaged membrane. The disruption of cell wall and membrane was clearly observed in *E. coli* cells after 2 h of treatment with antimicrobial peptide F1 (Fig. 7D). The results on the membrane damages by antimicrobial peptide F1 on *E. coli* was in accordance with the results obtained from the outer and inner membrane permeability assays (Figs. 3 and 4), the potassium ion release assay (Fig. 5), and the confocal laser scanning microscopy observation (Fig. 6). Many previous transmission electron microscopy analyses showed that the effects of antimicrobial peptides on bacteria cell membrane are different. Antimicrobial peptide temporin L did not lyse *E. coli* cells but rather forms ghost-like bacteria (Mangoni et al., 2004). Melittin caused the cell membrane of *Staphylococcus aureus* to form pores (Park et al., 2006). In the case of moricin or JCpep8, a ghost-like appearance and many lysed cells were observed (Hu et al., 2013; Xiao & Zhang, 2012).

3.8. Interaction of antimicrobial peptide F1 with cellular DNA

Previously, it was reported that antimicrobial peptides had the ability to inhibit the intracellular biopolymer synthesis and functions (del Castillo, del Castillo, & Moreno, 2001; Patrzykat, Friedrich, Zhang, Mendoza, & Hancock, 2002), especially the ones that showed the specific inhibition of DNA synthesis by direct binding to bacterial DNA (Rotem et al., 2008; Subbalakshmi & Sitaram, 1998). To assess the interaction between antimicrobial peptide F1 and genomic DNA of *E. coli*, the gel retardation assay and the atomic force microscopic analysis were employed in the present study. Increasing amounts of antimicrobial peptide F1 were mixed with the bacterial DNA on an agarose gel (Fig. 8A). The result showed that antimicrobial peptide F1 had DNA binding activity in a concentration-dependent manner. These results are similar with those of antimicrobial peptide Lasioglossin II as previously reported (Bandyopadhyay, Lee, Sivaraman, & Chatterjee, 2013). At the weight ratio (antimicrobial peptide F1/DNA) of 0.5/3, 5/3 and 10/3, the bacterial DNA could migrate into the gel. When the weight ratio was at 25/3 and 50/3, only a small fraction of the bacterial DNA was able to migrate to the gel; whereas, the weight ratio of 100/3 and 200/3 had complete retardation of DNA migration and no migration of bacterial DNA was observed in the gel. In order to have a direct visualization of antimicrobial peptide F1 binding with bacterial DNA, we used atomic force microscopy to determine the binding effect. As shown in the Fig. 8B1 and B2, no aggregation was

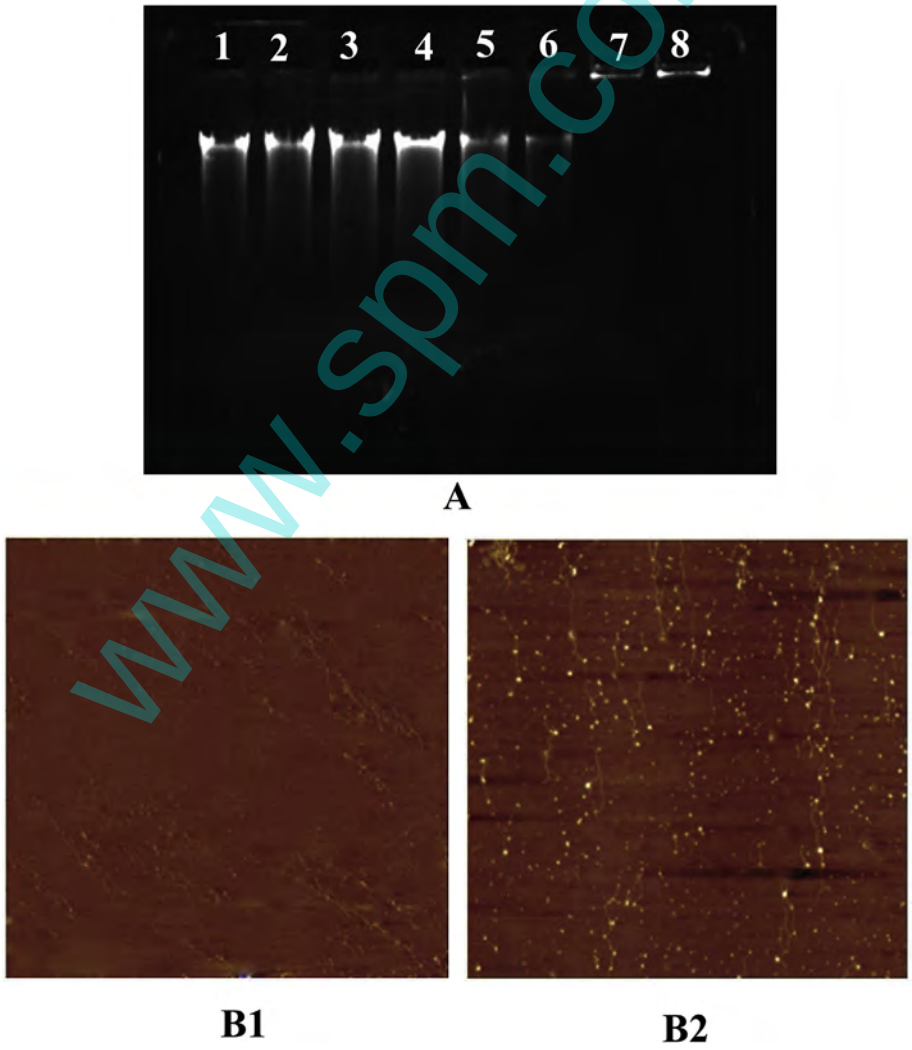

Fig. 8. DNA binding analysis of antimicrobial peptide F1 to *Escherichia coli* DNA. (A) the gel retardation analysis. Banks 1–8, the weight ratio (antimicrobial peptide F1/DNA) was 0, 0.5/3, 5/3, 10/3, 25/3, 50/3, 100/3, and 200/3, respectively. The atomic force microscopy images of genomic DNA in the absence (B1) and presence (B2) of the antimicrobial peptide F1.

observed in the bacterial DNA samples in the absence of antimicrobial peptide F1, whereas, significant amount of aggregation was observed in the presence of antimicrobial peptide F1. These results further confirmed the direct binding between antimicrobial peptide F1 and bacterial DNA.

4. Conclusions

In conclusion, our results clearly demonstrated that peptide F1 is a novel antimicrobial peptide exerting its antimicrobial effects by increasing the outer and inner membrane permeability of *E. coli* causing damage to the cell membrane and promoting intracellular material leakage. Moreover, antimicrobial peptide F1 was able to bind to genomic DNA, which may interfere with important cellular functions and lead to cell death. These findings indicate that antimicrobial peptide F1 has multiple targets against *E. coli*, which suggests that antimicrobial peptide F1 is a versatile inhibitor against bacterial pathogens and have promising applications in a variety of fields, such as the agriculture, food and medical industry.

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