1 **Purification, characterization and bactericidal mechanism of a**
2 **broad spectrum bacteriocin with antimicrobial activity against**
3 **multidrug-resistant strains produced by *Lactobacillus***
4 ***coryniformis* XN8**

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ABSTRACT: The multidrug-resistant strains of food spoilage bacteria and foodborne pathogenic bacteria in food are badly in need of being controlled by effective bio-preservatives. In this study, the XN8 strain was isolated from Jiangshui and identified as *Lactobacillus coryniformis* according to 16S rRNA gene sequence. One of the bacteriocins produced by XN8 was purified by ammonium sulfate precipitation and a series of chromatographic column, and designated as lactocin XN8-A (LXA). The molecular mass of LXA was 3100.0242 Da by MALDI-TOF MS. The LXA showed good heat, pH and storage stabilities. However, it was sensitive to proteases. The LXA was found to have a broad antimicrobial spectrum on both Gram-positive and Gram-negative bacteria including multidrug-resistant strains and *Listeria monocytogenes*. Its minimum inhibitory concentration (MIC) for both *E. coli* and *S. aureus* was 6.85 µg/mL. The LXA had a bactericidal mode without cell lysis by the growth curve and time-kill assay. The results of electron microscope showed that the LXA destroyed membrane permeability and induced pore-formation of target cells. Furthermore, the LXA induced cell cycle arrest at both G1 and G2/M phase by cell cycle analysis. This research suggests that the LXA has promising potential as bio-preservative in food industry.

Keywords: Bacteriocin; *Lactobacillus coryniformis*; multidrug-resistant; purification; characterization; antimicrobial mechanism

1. Introduction

Antibiotics are frequently used in food-producing animals for either therapy or prophylaxis, or growth promotion (feed additives) (Threlfall, 2002). Indiscriminate and inappropriate use of antibiotics in the food industry is one of the largest factors leading to antibiotic resistance (Alanis, 2005). The emergence of multidrug-resistant strains in food is a serious threat to human health. Hence there is urgent necessary to produce new safe and effective antimicrobial substances to conquer these superbugs. In recent years, bacteriocins have attracted much attention because they not only can be used as food preservatives but also are looked as promising alternatives to antibiotics (Paul D. Cotter, Ross, & Hill, 2013; Hammami, Fernandez, Lacroix, & Fliss, 2013; Zendo, 2013). Generally, bacteriocin is a kind of antimicrobial peptide or protein, which is ribosomally synthesized and produced by many kinds of microorganisms (P. D. Cotter, Hill, & Ross, 2005). Lactic acid bacteria (LAB) are an abundant resource of bacteriocins and generally recognized as safe (GRAS). Correspondingly, many bacteriocins produced by LAB have been purified and characterized as promising natural food preservatives which can prolong shelf-life of food (Paul D. Cotter, et al., 2013; Sawa, et al., 2013; Zendo, 2013). However, most bacteriocins are narrow spectrum and can only inhibit or kill the bacteria with close genetic relationship (De Vuyst & Leroy, 2007). Only a small number of bacteriocins are broad spectrum, and bacteriocins owning antibacterial action against multidrug-resistant strains are rare.

Lactobacillus coryniformis, one of the less studied *Lactobacillus* species, is

usually associated to fermented vegetable products (Martin, et al., 2005). Jiangshui is a kind of traditional fermented vegetable which is popular in northwest China. A novel bacteriocin with antimicrobial activity against antibiotic-resistant strains produced by *L. coryniformis* MXJ 32 which was screened from Jiangshui has been purified and characterized in our previous study (Lü, et al., 2014). However, it was generally found that LAB from different sources can produce different bacteriocins in a strain-dependent manner. Thus, the objectives of this study were, firstly, to research whether the different *L. coryniformis* XN8 which was isolated from Jiangshui in Yangling, China can produce different antimicrobial bacteriocin or not; then, to determine if the bacteriocin was broad spectrum and effective to multidrug-resistant strains; last, to investigate what its antimicrobial mechanism was. The ultimate goal was to find a high-efficient bacteriocin to control multidrug-resistant foodborne pathogens.

2. Materials and Methods

2.1. Isolation and identification of antimicrobial LAB strain from Jiangshui

Homemade Jiangshui sample was donated by local people in Yangling district, Shaanxi province, China. LAB were isolated by serial dilution method, in which MRS was used as selective culture medium. The isolated LAB were further screened by double-plate method (Lü, et al., 2014). The size of transparent zone was used to assess antimicrobial activity. In this study, *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 respectively representing Gram-negative and Gram-positive bacteria were used as indicator strains unless stated otherwise.

Strain XN8 with the highest antimicrobial activity was selected and molecular identification was conducted as previous study (Lü, et al., 2014). Subsequently, the 16S rRNA gene was sent to Sangon (Shanghai, China) for cloning and sequencing. The obtained sequence was submitted to NCBI for BLAST search and phylogenetic analysis was carried out by MEGA 5.0 (version 5.0, www.megasoftware.net) as previous study (Lü, et al., 2014).

2.2. Dynamics of growth and bacteriocin production

The XN8 was inoculated in 3 L fresh MRS broth and statically cultivated at 30 °C for 72 h. During this process, 100 mL cell culture was taken every 6 h and cell density was measured at 600 nm. Meanwhile, 70% saturation ammonium sulfate precipitation solution (dissolved in 1 mL pH 6.0 0.1 M citric acid-phosphate buffer) of cell-free supernatant was used to assess the antimicrobial activity of crude bacteriocin by the agar well diffusion method as previous study (Lü, et al., 2014).

2.3. Purification of bacteriocin

The XN8 strain was statically incubated in MRS medium (6 L) at 30 °C for 54 h. Crude bacteriocin was obtained by 70% saturation ammonium sulfate precipitation of the cell-free supernatant.

The crude bacteriocin was applied to an AKTA purifier 100 (GE, Sweden) and the bacteriocin with purity over 95% was obtained by 6 steps. During this process, only the fraction with maximum activity was further purified in the next step: ①crude bacteriocin was loaded in a HIPREP 26/10 DESALTING column to desalt, in this step, ultrapure water was used as mobile phase and only the fractions with conductivity less

than 1 mS/cm were collected; ②after desalting, a HiPrep Q XL 16/10 anion-exchange column was used and linearly eluted with 1 M NaCl in citric acid-phosphate buffer (pH 6.0); ③the desalting column was used again to desalt and all fractions were separately collected based on the cond; ④the active fraction was loaded in a RESOURCE RPC 3 mL column, gradient elution of buffer A (95% water, 5% acetonitrile, 0.1% TFA) and buffer B (100% acetonitrile) was used; ⑤a SP Sepharose Fast Flow cation-exchange column was used and linearly eluted with 2 M NaCl in citric-sodium citrate buffer (pH 3.0); ⑥the RPC column was used again under the same condition as step four without 0.1% TFA. The AKTA system in this work was equipped with an UV detector of 280 nm wavelength and an automatic collector. After each step, collected fractions were concentrated on a lyophilizer (Labogene, Denmark) and the active fraction was determined by the agar well diffusion method.

At last, the purity of the active fraction was verified using an analytical RP-HPLC (Waters1525, USA) equipped with a SunFireTM C₁₈ column (150 × 4.6 mm, 5 μm). Linear gradient elution of acetonitrile from 5% to 100% in 25 min was used for analysis at 0.2 mL/min and monitored at 280 nm. The purified bacteriocin was designated as lactocin XN8-A (LXA).

2.4. Determination of molecular mass

Molecular mass of the LXA was determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using a 4700 MALDI-TOF/TOF instrument (AB SCIEX, USA). Before analysis, a ZipTip C₄ column (Millipore, USA) was used to desalt and concentrate the sample. 1 μL

prepared sample was carefully spotted onto a 123 mm × 81 mm 384 Opti-TOF MALDI target plate (AB SCIEX, USA) and left to dry at room temperature; then 0.6 µL α-cyano-4-hydroxy-cinnamic acid (Sigma, USA) matrix solution was spotted onto the corresponding target plate. Spectrum was recorded in a linear positive mode and exported by 4000 Series Explorer V3.5 Software. The ProteoMass™ Peptide & Protein MALDI-MS Calibration Kit (Sigma, USA) was used as external mass calibration standard.

2.5. Sensitivity to heat, storage, pH, and enzymes

Once used as a bio-preservative in the food industry, the stability of LXA is vital. In this subsection, we studied the sensitivity of LXA to heat, storage, pH and enzymes, where enzymes were used to explore the nature of the bacteriocin. After each treatment, the agar well diffusion method was used to determine the residual inhibitory activity.

To investigate the heat stability of LXA, the purified sample was dissolved in PBS (pH 6.0) at 27.4 µg/mL and treated at 60 °C, 80 °C, 100 °C, 121 °C for 10 min, 20 min, 30 min, respectively. Untreated LXA was used as control.

The preparation of sample used in storage stability analysis was similar to that used in heat stability. Then they were stored at 4 °C for 0 month, 1 month, 3 months and 6 months, respectively in sterile tubes.

In the sensitivity test of LXA to pH, the purified sample powder was added into corresponding pH buffer at a final concentration of 27.4 µg/mL. The buffers were prepared as follows: glycine-HCl (pH 2), citrate-phosphate (pH 3, 4, 5 and 6),

sodium-phosphate (pH 7), Tris-HCl (pH 8 and 9) and glycine-NaOH (pH 10 and 11). Before determining the residual antimicrobial activity, these different pH sample solutions were incubated at 37 °C for 4 h. The same pH buffers without bacteriocin were used as control.

In order to analyze the enzyme sensitivity of LXA, pepsin, papain, trypsin, proteinase K (all from Sigma) were used and dissolved in corresponding optimal buffer at 200 µg/mL: pH 3.0 sodium-citrate buffer, pH 7.0 sodium-phosphate buffer, pH 7.0 sodium-phosphate buffer, pH 7.0 Tris-HCl buffer, respectively. Then sample powder was added into the enzyme solutions at a final concentration of 27.4 µg/mL. After incubated at 37 °C for 4 h, the mixture was boiled for 5 min to inactivate enzymes. Untreated LXA was used as control.

2.6. Antimicrobial spectrum and minimum inhibitory concentrations (MIC)

Table 1

As an antibacterial substance, the scope and the intensity of antimicrobial activity are of great importance. Therefore, antimicrobial spectrum and MIC of the LXA were studied here.

Indicator stains including Gram-positive and Gram-negative bacteria (Table 1) were used to investigate the antimicrobial spectrum of the purified LXA (pH 6.0, 27.4 µg/mL). The inhibitory activity towards different indicators was assayed by the agar well diffusion method.

The sample (about 0.5 mg/mL) was twofold diluted with ddH₂O to 2⁻¹⁰. Equal volumes (50 µL) of diluted sample and indicators (10⁶ CFU/mL) were mixed in 96 well plate (Costar, Corning, USA) and incubated at 37 °C for 24 h. Then OD₆₀₀ was

determined on a microplate reader (M200pro, TECAN, Switzerland). MIC was defined as the lowest concentration without any indicators had grown. Meanwhile, protein concentration of each diluted LXA was measured by bicinchoninic acid (BCA) assay and bovine serum albumin (BSA) was used as standard (Li, Liu, Shang, Yu, & Zhang, 2012).

2.7. Action mechanism

2.7.1. Growth curve and time-kill kinetics

Growth curves of indicators were determined at a concentration of $1 \times \text{MIC}$ of the LXA. The purified sample powder was added into cultures when the indicators were cultivated to exponential phase. Bacterial suspensions were taken each hour for 24 h and absorbance at 600 nm was measured. Control was the growth of indicators without the LXA.

Sample was added into logarithmic-phase (10^8 - 10^9 CFU/mL) indicator cultures with final concentrations of $1 \times \text{MIC}$ and $2 \times \text{MIC}$, then continuously incubated at 37 °C. 0.5 mL aliquots were taken immediately at appointed time (0, 0.5, 1, 1.5, 2, 2.5 and 3 h respectively). With appropriate dilution, bacterial colonies were counted after cultivated at 37 °C for 12 h. Control was count of indicators without the LXA.

2.7.2. Microscopic analysis

The ultrastructure changes of indicators after treated by the LXA were observed using Scanning electron microscope (SEM) and Transmission electron microscopy (TEM) to get visualization of the action mechanism of the LXA.

Exponential-phase indicators were resuspended in fresh LB medium and treated

by the LXA (6.85 $\mu\text{g/mL}$) at 37 °C for 30 min and 2 h, respectively. After treatment, the indicators were collected by centrifugation and washed by PBS (0.1 M, pH 7.2). Then cells were adhered to coated glass slides (6 mm $\times$ 6 mm, Matsunami, Japan) at 4 °C for 30 min and were fixed with 2.5% glutaraldehyde overnight at 4 °C. The target cells were washed again and dehydrated with ethanol, acetone, isoamyl acetate, respectively. Subsequently, the cells were dried by CO₂ for 4 h and coated with gold. The field emission scanning electron microscope S-4800 (Hitachi, Japan) was used to observe cell ultrastructure.

The preparation and treatment of target indicators were the same as those in SEM analysis. After washed by PBS, cells were fixed with 2.5% glutaraldehyde overnight at 4 °C. Then cells were post-fixed in 1% osmium tetroxide at 4 °C for 2 h. After washed again, the cells were dehydrated in a series of ethanol solutions and permeated in white resin. Embedding was done at 60 °C for 48 h. Approximate 70 nm thin sections were prepared on copper grids and stained with 2% uranyl acetate and lead citrate. Ultrastructure observation was performed on a JEM-1230 transmission electron microscope (JEOL, Japan).

2.7.3. Cell-cycle analysis with flow cytometry

Cell cycle of indicators was measured as described by Choi & Kim, (2008) with some modification. The purified LXA was added into indicator cultures (10⁶ CFU/mL) at a final concentration of 6.85 $\mu\text{g/mL}$ and incubated at 37 °C for 30 min. After washed with PBS (pH 7.0), the cells were fixed with 70% ethanol overnight. To eliminate the influence of RNA during measurement, RNase A (dissolved in PBS to

0.2 mg/mL) was added in and incubated at 37 °C for 30 min. Then the cells were resuspended in Propidium Iodide solution (dissolved in PBS to 20 µg/mL with 0.1% Triton X-100) and cultivated without light for 30 min. Subsequently, DNA content of 20000 events for each treatment was measured using a FACS Calibur (BD, USA) equipped with 488 nm argon ion laser. Control was treated under the same condition without the LXA.

2.8. Statistical analysis

All experiments were done in triplicate and the results were expressed as the mean ± standard deviation. Data analysis was performed by ANOVA and Duncan test using the SPSS 19.0. In all analysis, $p < 0.05$ was considered significant.

3. Results and Discussion

3.1. Isolation and identification of LAB

Eight strains with antimicrobial activity to the both indicators were screened from Jiangshui, among which XN8 was selected because it had the highest antibacterial activity. The XN8 was Gram-positive, catalase-negative, facultative anaerobic and short rod-shaped strain. Its 16S rRNA gene sequence was amplified by PCR, and 1562 bp gene fragment was sequenced after cloning (Genbank Accession No. KF994925.1). Nucleotide blast on NCBI revealed that the XN8 had extremely high similarity (identity ≥ 99%, E value = 0) with *L. coryniformis* stains. Additionally, phylogenetic tree was constructed by MEGA 5.0 (Fig. 1).

Fig. 1

It was reported that *L. coryniformis* has produced reuterin (3-hydroxypropionaldehyde) (Martin, et al., 2005), proteinaceous antifungal

compound (Magnusson & Schnurer, 2001) and Lactocin MXJ 32A in our previous study (Lü, et al., 2014), all of which were antibacterial peptide and have enriched its antimicrobial activity.

3.2. Dynamics of growth and bacteriocin production

As shown in Fig. 2, the XN8 entered exponential phase at 12 h, kept stationary phase from 36 to 60 h, and reached decline phase after 60 h. Accordingly, antibacterial activity of crude bacteriocin started at the late exponential phase (24 h), then serially increased to maximum during stationary phase (to 54 h) for the both indicators, which indicated that the bacteriocin produced by XN8 was a secondary metabolite (Bibb, 2005). Similar results were reported for bacteriocin ST8KF (Powell, Witthuhn, Todorov, & Dicks, 2007) and cerein 8A (Bizani & Brandelli, 2004).

Fig. 2

3.3. Purification of bacteriocin

Fig. 3

Crude bacteriocin was purified by chromatographic columns as shown in Fig. 3. Fig. 3A shows that undesirable proteins overlapped with salt (ammonium sulfate) were removed. Then, many unwanted peaks were eluted from anion-exchange column in the second step (Fig. 3B) as only the peak Q1 had antimicrobial activity. The third step (Fig. 3C) had been planned to desalt (NaCl), but actually, the fractions at the late of the elution curve (cond > 1 mS/cm) had higher activity and was used to purify further. Fig. 3D is the graph from RPC column and only the peak R2 had activity, the cohesion of R2 on the column implied that the bacteriocin is hydrophobic. After purified by cation-exchange column in the step five (Fig. 3E), the peak S2 with higher activity was purified further. At the last step (Fig. 3F), the antimicrobial bacteriocin

(peak P2) was purified, and corresponding inhibitory zone to *E. coli* was shown at the top right of this figure. Purity of the P2 was confirmed by analytical RP-HPLC with a single peak (area over 95%).

According to the processes of purification, we deduce that the *L. coryniformis* XN8 strain can produce more than one kind of bacteriocin because the another peak of cation-exchange column (Fig. 3E) also had activity. Thus, *L. coryniformis* XN8 is a multi-bacteriocin producer and has significant exploitation and application value. Production of multiple bacteriocins by one microorganism has also been found in several LAB, such as *Lactobacillus plantarum* C 11 (Diep, Havarstein, & Nes, 1995), *Enterococcus faecium* WHE 81 (Izquierdo, Marchioni, Aoude-Werner, Hasselmann, & Ennahar, 2009).

3.4. Molecular mass

Mass spectrometry based on MALDI-TOF MS indicated that the purified LXA had an exact molecular mass of 3100.0242 Da (Fig. 4), which was different from bacteriocins produced by *L. coryniformis* reported previously (Lü, et al., 2014; Magnusson & Schnurer, 2001) and also different from other bacteriocins produced by LAB.

3.5. Sensitivity of the LXA to heat, time, pH, and enzymes

Table 2 shows that the LXA had excellent heat stability and no significant difference was found in different temperature and time treatments ($p > 0.05$). It still had good antimicrobial activity even treated at 121 °C for 30 min. This indicated that the LXA was more stable than some previously reported bacteriocins (Huang, et al.,

Fig. 4

Table 2

2009; Oguntinyinbo, Sanni, Franz, & Holzapfel, 2007). The good heat stability of LXA makes it possible to be applied in food industry because the sterilization operation in most food industrial processes is usually conducted at 121 °C.

The LXA exhibited good storage stability in 3 months, but its activity decreased when storage time was prolonged further, which may be caused by protein degradation or senior structure destruction.

When the LXA was treated from pH 4.0 to pH 6.0, no significant difference of antimicrobial activity was found ($p > 0.05$). However, its antimicrobial activity decreased significantly ($p < 0.05$) with pH decreasing from 3 to 2 or increasing from 7 to 11, especially $pH \geq 9.0$. It may be owing to strong intramolecular electrostatic interactions caused by the dissociation of amino and carboxyl group in extreme pH conditions (Jaenicke, 1991), which finally causes protein denaturation. However, even at pH 11, the LXA still had about 32% antimicrobial activity and most food has a pH between 4 and 7, which makes it possible to be used in food products for preservation.

For enzyme treatment, the LXA was sensitive to pepsin, papain, trypsin and proteinase K, which was similar to other reported bacteriocin (Kaewklom, Lumlert, Kraikul, & Aunpad, 2013). Thus, the LXA is proteinaceous nature, which contributes to its safe application.

3.6. Antimicrobial spectrum and MIC

Similar to lactocin MXJ 32A (Lü, et al., 2014), the LXA exhibited a broad antimicrobial spectrum against the tested food spoilage strains and foodborne pathogenic strains (Table 1), which included 13 strains of Gram-negative bacteria and

9 strains of Gram-positive bacteria. It is interesting that fifteen multidrug-resistant foodborne pathogenic strains and *Listeria monocytogenes* could also be inhibited by the LXA. What's more, the activity of LXA to multidrug-resistant strains was close to corresponding type strains. Multidrug-resistant strains are huge threat to public health and were largely found in food (Gales, et al., 2001; Petternel, et al., 2014; Threlfall, 2002), for example, multidrug-resistant *Salmonella* in water and retail raw poultry (Threlfall, 2002; Yang, et al., 2014). Thus it is promising for the LXA to be used as bio-preservative to control multidrug-resistant strains existed in food.

The MIC of LXA was 6.85 µg/mL for both indicators, which was close to the well-known nisin (Laukova, Styriak, & Marekova, 2001; Miller, et al., 2010) and was significantly lower than other reported bacteriocins produced by other microorganism strains, such as BLIS (Ruiz, et al., 2012) and bacteriocin PsVP-10 (Padilla, et al., 2006). Its MIC was also much lower than our previous lactocin MXJ 32A (Lü, et al., 2014), which means the LXA is much more effective and its action mechanism may be different from the lactocin MXJ 32A.

3.7. Action mechanism

3.7.1. Growth curve and time-kill kinetics

Fig. 5A and Fig. 5B display the growth curves of the two indicators in 24 h. Controls for both *E. coli* and *S. aureus* had typical growth curves with lag phase, logarithmic phase and stationary phase. However, optical density of *E. coli* almost kept stability and that slightly decreased for *S. aureus* when the LXA was added, which indicated that the bacteriocin did not induce cell lysis. Similar results were

Fig. 5

reported for Plantaricin ZJ008 (Zhu, Zhao, Sun, & Gu, 2014), Weissellin A (Papagianni & Papamichael, 2011), Pediocin N5p (de Nadra, de Lamelas, & de Saad, 1998), etc.

The short time-kill kinetics showed that the LXA significantly cut down the viable count of *E. coli* (Fig. 5C) and *S. aureus* (Fig. 5D) with some time-dependence and dose-dependence. More specifically, the LXA exhibited an instant kill action at 0 h with 0.48 log₁₀ reduction at 1 × MIC and 0.5 log₁₀ reduction at 2 × MIC for *E. coli*, and those for *S. aureus* was 0.99, 1.02 log₁₀ reduction, respectively. Treatment with the LXA for 3 h resulted in 1.5, 2.48 log₁₀ reduction respectively in the viability at 1 × MIC and 2 × MIC for *E. coli*, and those for *S. aureus* was 2.12 and 2.44 log₁₀ reduction. Results indicated that the LXA had potent bactericidal activity to indicators.

3.7.2. Electron microscopy

More details of bactericidal mechanism of the LXA were obtained by ultrastructure observation using electron microscopy as shown in Fig. 6 and Fig. 7. According to SEM, untreated *E. coli* (Fig. 6A) and *S. aureus* (Fig. 6D) had integrated and plump cell structure. However, after treated for 30 min by the LXA, the both indicators (Fig. 6B and Fig. 6E) exhibited pores on the surface of cell. This indicated that the LXA has an effect on cell wall. After treated for 2 h by the LXA, deformations with huge cavities and cell shrink were observed on the both indicators (Fig. 6C and Fig. 6F). Additionally, blebs (arrows in Fig. 6F) protruded from the surface of *S. aureus*, which again suggested the action of LXA on cell wall.

Fig. 6

Fig. 7

TEM of untreated *E. coli* (Fig. 7A) revealed uniform cytoplasm and smooth profile. Untreated *S. aureus* (Fig. 7D) showed a typical Gram-positive structure: uniform cytoplasm, which filled in cytoplasm membrane, and the membrane tightly adhered to a thick wall. After treated for 30 min by the LXA, formation of an electron transparent zone between the cytoplasm membrane and cell wall was observed for *E. coli* (Fig. 7B), which implied a change of cytoplasm membrane. Also cell profile became irregular and cell wall debris can be found around (arrows in Fig. 7B), which indicated the cell wall damage. While different appearance was observed for *S. aureus* (Fig. 7E), it still had regular outline and cytoplasm membrane tightly adhered to cell wall, but the density of cytoplasm rarefied. After treated for 2 h by the LXA, cytoplasm became more tenuous and even blank appeared for the both indicators (Fig. 7C and Fig. 7F1, Fig. 7F2). The leaked cytoplasm material of *E. coli* could be found around cell surrounding (arrows in Fig. 7C). The release of the cytoplasm content was a result of membrane permeability transformation. Moreover, obvious pores were formed for *S. aureus* (arrows in Fig. 7F1 and Fig. 7F2).

Generally, bacteriocins possess bactericidal or bacteriostatic effects on sensitive cells, in which pore formation in cell envelop of sensitive cells is thought to be the key mode of action (Jack, Tagg, & Ray, 1995). The LXA induced progressive damage with time on the ultrastructure and led to death of the both indicators, which was in accordance with the results of time-kill kinetics. Deformation, pore-formation and membrane permeability transformation after treated by the LXA were similar to those caused by other antimicrobial peptides, such as NK-2 (Hashizume, et al., 2011), L10

(Mishra, et al., 2013), housefly pupae peptide mixture (Wang, Dang, Zheng, Wang, & Zhang, 2010), etc. In conclusion, the results suggest that the LXA has a bactericidal effect by the damage of cell wall and membrane of the target microorganisms.

3.7.3. Cell-cycle

Cell cycle distribution of PI stained indicators was measured through flow cytometry and G1, S and G2/M phases were determined as shown in Fig. 8. The LXA induced G1 phase and G2/M phase blocks for the both indicators. For *E. coli*, after treatment, G1 phase and G2/M phase increased from 64.24% to 70.62% and from 11.94% to 21.13% respectively, while S phase decreased from 23.82% to 8.25%. Meanwhile, for *S. aureus*, G1 phase and G2/M phase increased from 65.92% to 72.15% and from 3.29% to 27.80% respectively after treatment and S phase significantly decreased from 30.79% to 0.01%. Cell arrests occurred at G1 phase and G2/M phase for the both indicators with a similar tendency, which demonstrated that the LXA has an analogous mechanism to both Gram-positive and Gram-negative bacteria.

Action mechanism of bacteriocins can be divided into two broad types: one is cell envelope associated mechanism, the other is intracellular regulation. Envelope associated mechanism contains lipopolysaccharide or peptidoglycan synthesis inhibition, such as pyocin L1 (McCaughey, et al., 2014), nisin (Brotz, et al., 1998) and colicin M (Barreteau, et al., 2012); pore formation, such as AS-48 (Gonzalez, et al., 2000); membrane permeability transformation, such as Lacticin Q (Yoneyama, et al., 2009); etc. Intracellular regulation is usually metabolism interference of DNA, RNA,

protein and ribosome (Paul D. Cotter, et al., 2013). Combined with the results of TEM, we infer that metabolism interference of DNA happened on the indicators, which led to a significant reduction in S phase, and cytoplasm release further damaged associated kinases activity required in regulation, all of which led to death of cells at last.

4. Conclusion

The bacteriocin LXA produced by *L. coryniformis* XN8 was purified with a molecular mass of 3100.0242 Da. The advantage of the LXA was broad spectrum and owning antibacterial activity to multidrug-resistant strains. There is a new discovery that bactericidal mechanism of the LXA included metabolic inhibition of DNA, except for usual damage of cell wall and membrane. In addition, the LXA was a novel bacteriocin according to molecular mass, characteristics and antimicrobial mechanism. Hence, the LXA was possible to be used as bio-preservative to control multidrug-resistant strains existed in food. In further, more detailed information about action mechanism of the LXA will be studied, such as binding site, molecular target, etc.

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Captions of Figures

Fig. 1. Phylogenetic tree of XN8 according to 16S rRNA sequence.

Fig. 2. Dynamics of growth and bacteriocin production. Growth curve of XN8 (■); antimicrobial activity of crude bacteriocin to *E. coli* (▨) and to *S. aureu* (▩).

Fig. 3. Purification of bacteriocin produced by XN8 by chromatography column. A, desalting column; B, anion-exchange column; C, desalting column; D, RPC column; E, cation-exchange column; F, RPC colum; (—) 280 nm and (—) cond.

Fig. 4. Molecular mass of LXA by MALDI-TOF MS.

Fig. 5. Growth curve and time-kill kinetics. A, the effect of LXA on *E. coli* growth; B, the effect of LXA on *S. aureus* growth; C, Time-kill kinetics to *E. coli* by LXA; D, Time-kill kinetics to *S. aureus* by LXA; (■) contol, (●) $1 \times \text{MIC}$ and (▲) $2 \times \text{MIC}$.

Fig. 6. SEM images of *E. coli* (A, control; B, treated for 30 min by LXA; C, treated for 2 h by LXA) and *S. aureus* (D, control; E, treated for 30 min by LXA; F, treated for 2 h by LXA).

Fig. 7. TEM images of *E. coli* (A, control; B, treated for 30 min by LXA; C, treated

for 2 h by LXA) and *S. aureus* (D, control; E, treated for 30 min by LXA; F1 and F2, treated for 2 h by LXA).

Fig. 8. Cell cycle of *E. coli* (A1, control; A3, treated for 30 min by LXA) and *S. aureus* (A2, control; A4, treated for 30 min by LXA). Corresponding quantitative results expressed in Materials and Method (B), control of *E. coli* ($\square$), treatment of *E. coli* ($\blacksquare$), control of *S. aureus* ($\boxtimes$), treatment of *S. aureus* ($\boxplus$). $*p < 0.05$, $***p < 0.001$ was considered significantly different in comparison with the control.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Table 1 Antimicrobial spectrum of lactocin XN8-A.

Indicator strains	Isolation source	Diameter of inhibition zone (mm)	Resistant antibiotic
<i>Staphylococcus aureus</i> ATCC25923		22.2 ± 1.7	—
<i>Staphylococcus aureus</i> ATCC29213		22.6 ± 2.2	—
<i>Escherichia coli</i> ATCC25922		25.1 ± 2.4	—
<i>Enterococcus faecalis</i> ATCC29212		26.5 ± 1.3	—
<i>Salmonella</i> CMCC 50071		18.9 ± 1.4	—
<i>Sakazakii</i> ATCC29544		20.4 ± 1.3	—
<i>Listeria monocytogenes</i> ATCC19115		18.6 ± 1.7	—
Antibiotic-resistant <i>Enterobacter sakazakii</i> 14-18 (2)	Milk powder	19.8 ± 1.3	CIP,RA,S
Antibiotic-resistant <i>Enterobacter sakazakii</i> 11-18 (2)	Rice powder	20.5 ± 1.7	CIP,RA,S
Antibiotic-resistant <i>Enterobacter sakazakii</i> 18-15 (2)	Milk powder	19.7 ± 1.7	AM,AMX,C,NA,RA,S,TC
Antibiotic-resistant <i>Enterobacter sakazakii</i> 6-12 (1)	Rice powder	20.2 ± 1.4	FOX,RA
Antibiotic-resistant <i>Enterobacter sakazakii</i> 6-12 (2)	Rice powder	20.6 ± 1.3	C,RA,TC
Antibiotic-resistant <i>Salmonella</i> 36T	Chicken	18.5 ± 1.3	AM,AMX,AN,C,CIP,CFP,CRO,GAT,GM,K,LVX,NA,SXT,TC
Antibiotic-resistant <i>Salmonella</i> 1006D	Chicken	17.7 ± 1.4	AM,AMX,C,GM,K,NA,SXT,TC
Antibiotic-resistant <i>Salmonella</i> 798D	Chicken	17.4 ± 1.7	AM,AMX,AN,C,CIP,CF,CRO,FOX,GAT,GM,K,LVX,NA,S,SXT,TC
Antibiotic-resistant <i>Salmonella</i> 557D	Silkie	18.4 ± 1.3	AM,AMX,AN,C,FOX,GAT,GM,K,LVX,NA,SXT,TC
Antibiotic-resistant <i>Salmonella</i> 87T4	Chicken	18.2 ± 1.7	AM,AMX,AN,C,CIP,CF,CFP,CRO,GAT,GM,K,LVX,NA,S,SXT,TC
Antibiotic-resistant <i>Staphylococcus aureus</i> 1	Pork	18.8 ± 2.2	CF,CIP,CLR,CXM,E,FOX,GM,LVX,NN,OFX,OX,PIP,VA
Antibiotic-resistant <i>Staphylococcus aureus</i> 2	Pork	21.1 ± 1.4	CIP,FOX,GM,LVX,MI,NN,OFX,P,PIP,VA
Antibiotic-resistant <i>Staphylococcus aureus</i> 3	Pork	20.9 ± 1.3	CIP,FOX,GM,LVX,MI,NN,OFX,VA
Antibiotic-resistant <i>Staphylococcus aureus</i> 4	Pork	22.3 ± 1.3	CIP,FOX,GM,LVX,MI,NN,OFX,VA

Antibiotic-resistant <i>Staphylococcus aureus</i> 5	Pork	22.3 ± 1.4	CIP,FOX,GM,LVX,MI,NN,OFX,OX,VA
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AM, ampicillin; AMX, amoxicillin; AN, amikacin; C, chloramphenicol; CIP, ciprofloxacin; CF, cephalothin; CFP, cefoperazone; CLR, clarithromycin; CRO, ceftriaxome; CXM, cefuroxime; FOX, ceftiofur; GAT, gatifloxacin; GM, gentamicin; K, kanamycin; LVX, levofloxacin; MI, minocycline; NA, nalidixic acid; NN, tobramycin; OFX, ofloxacin; OX, oxacillin; P, penicillin; PIP, piperacillin; RA, rifampin; S, streptomycin; SXT, trimethoprim-sulfamethoxazole; TC, tetracycline; VA, vancomycin.

Table 2

Table 2 Sensitivity of lactocin XN8-A to heat, time, pH, and enzymes.

Treatment	Residual inhibitory activity (diameter of inhibition zone mm)	
	<i>E. coli</i> ATCC25922	<i>S. aureus</i> ATCC29213
Heat		
Control	25.4 ± 1.3	22.8 ± 1.7
60 °C, 10 min	25.5 ± 1.5	23.1 ± 2.1
60 °C, 20 min	25.2 ± 1.7	22.6 ± 1.4
60 °C, 30 min	25.5 ± 1.3	22.5 ± 1.8
80 °C, 10 min	25.4 ± 1.3	22.7 ± 1.3
80 °C, 20 min	25.7 ± 1.7	22.5 ± 1.7
80 °C, 30 min	25.1 ± 1.6	22.6 ± 1.5
100 °C, 10 min	25.4 ± 1.3	22.4 ± 1.2
100 °C, 20 min	25.1 ± 1.5	22.2 ± 1.7
100 °C, 30 min	24.9 ± 1.9	22.7 ± 1.9
121 °C, 10 min	25.3 ± 1.4	22.5 ± 1.6
121 °C, 20 min	24.8 ± 1.8	22.3 ± 1.3
121 °C, 30 min	25.1 ± 1.5	21.9 ± 2.2
Storage		
0 month	25.5 ± 1.7	22.6 ± 1.4
1 months	25.3 ± 1.4	21.9 ± 1.8
3 months	24.8 ± 2.3	22.0 ± 2.0
6 months	16.4 ± 1.9	15.7 ± 1.7
pH		
2 (Control 11 ± 1.2 mm)	27.6 ± 1.4	24.3 ± 1.2
3 (Control 0 ± 0 mm)	18.2 ± 1.3	16.5 ± 1.7
4 (Control 0 ± 0 mm)	25.3 ± 1.6	22.4 ± 1.5
5 (Control 0 ± 0 mm)	24.8 ± 1.9	22.1 ± 1.8
6 (Control 0 ± 0 mm)	25.4 ± 2.2	22.7 ± 1.8
7 (Control 0 ± 0 mm)	22.7 ± 1.3	19.6 ± 1.7
8 (Control 0 ± 0 mm)	19.1 ± 1.8	17.5 ± 2.0
9 (Control 0 ± 0 mm)	10.9 ± 1.5	9.4 ± 1.9
10 (Control 0 ± 0 mm)	9.7 ± 1.7	8.2 ± 2.4
11 (Control 0 ± 0 mm)	8.0 ± 1.4	7.1 ± 1.9
Enzymes		
Control	25.5 ± 1.3	22.3 ± 1.6
Pepsin	7.9 ± 1.7	5.6 ± 2.3
Papain	0	0
Trypsin	0	0
Proteinase K	0	0