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Antimicrobial property of recombinant Lactolisterin BU in vitro and its initial application in pork refrigerated storage

Bin Dong*, Guowen Zhou, Yanjun Lin, Cailing Yu, Jun Wang, Chunlong Sun and Tao Wu

Abstract

Lactolisterin BU is a novel bacteriocin identified from *Lactococcus lactis* in 2017. It exhibits antimicrobial activity against food spoilage and foodborne pathogens. In this study, Lactolisterin BU was expressed in *Pichia pastoris* (*P. pastoris*) and isolated from the supernatant of yeast culture for the first time. It was found to exhibit a broad antimicrobial spectrum and rapid bactericidal activity against foodborne bacterial pathogens, both gram-positive and gram-negative ones, with minimum inhibition concentrations ranging within 10–60 μg/mL. The recombinant Lactolisterin BU (rLactolisterin BU) also had an antioxidant effect and was resistant to heating, acid–base, and high-dose-saline treatments and barely had any hemolytic activity or cytotoxicity. Moreover, rLactolisterin BU effectively suppressed the growth of bacterial pathogens; suppressed the increases in pH, total volatile basic nitrogen (TVB-N), and thiobarbituric acid reactive substances (TBARS) of pork samples; and maintained a high quality of fresh pork during storage at 4 ℃. Furthermore, rLactolisterin BU effectively inhibited the growth of three kinds of bacteria in a pork-spoilage model. Taken together, rLactolisterin BU could be a promising preservative for food storage.

Keywords: Lactolisterin BU, Antimicrobial peptides (AMPs), Spoilage bacteria, Bio-preservation, Storage

Introduction

Pork and pork products are essential foods for people around the world. They provide high-value nutrition, including all the essential amino acids, lipids, vitamins, and minerals. However, microbial-pathogen contamination always occurs during processing, storage, transportation, distribution, and retailing [1]. Notably, consumers are paying increased attention to food quality and safety [2], especially to nutritional values, food-processing procedures, storage conditions, and shelf-lives. Although the traditional storage methods such as using low temperature, vacuum packaging, and chemical preservatives can prolong the storage time of pork to some extent, they may damage the sensory properties or introduce food-hazard risks during storage [3]. Thus, new storage methods and antimicrobial agents are urgent to develop for meat preservation. In recent years, bio-preservation technology has emerged as a potential method with high efficiency and no chemical preservatives. It uses natural metabolic products from various organisms in the earth to ensure food security by preventing food-pathogen reproduction [4]. In particular, antimicrobial peptides (AMPs) are gaining widespread attention in related to food preservation owing to its low molecular weight, extensive bactericidal performance, stable bioactivity, and lack of residue and drug resistance [5]. AMPs are generally spread in various organisms, including microorganisms, plants, invertebrates, and vertebrates [6]. At present, more than 2700 types of AMPs have been isolated, and their sequences can be found in the Antimicrobial Peptide Database (http://aps.unmc.edu/AP/). Many AMPs including nisin [7], mytichitin-CB [8], and

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MccJ25(G12Y) [9], which originate from *Lactococcus lactis, Mytilus coruscu*, and *E. coli*, respectively, reportedly have the potential to be substituted for chemical preservatives for inhibiting the growth of foodborne pathogens in various foods, including meat, yogurt, and fruit drinks. Therefore, the application of these AMPs provided a promising way to improve food safety and quality by inhibiting food pathogens/spoilage microorganisms.

Lactolisterin BU with 42 amino acids was identified from *Lactococcus lactis* in 2017 and found to exhibit potent antimicrobial activity against food-spoilage bacteria, such as *Staphylococcus aureus*, *Bacillus* spp., *Listeria monocytogenes*, and *Streptococci*. These features suggest that Lactolisterin BU may be applied as a new type of food preservative in the food industry [10]. However, natural Lactolisterin BU AMPs exist in low abundance, which is a drawback for its massive production and application. The application of Lactolisterin BU in food has also not yet been investigated.

To further explore the function of Lactolisterin BU, the current work aimed to generate rLactolisterin BU from *Pichia pastoris* system by recombinant-DNA engineering method, to evaluate its in vitro antimicrobial activity and biostability, and to investigate the effect of rLactolisterin BU when used as a food preservative in pork-preservation models.

Materials and methods

Strains, plasmids, and reagents

P. pastoris strain X-33, *E. coli* strain DH5α, and pPICZα-A plasmid were bought from Invitrogen Corporation (Carlsbad, CA, USA). They were used for gene manipulation and heterologous protein expression. *E. coli* American Type Culture Collection (ATCC) 25922, *E. coli* H7: O157 ATCC 35150, *Bacillus subtilis* AHU 1035, *S. aureus* ATCC 25923, *L. monocytogenes* ATCC 21633, *Pseudomonas aeruginosa* ATCC 27853, and *Salmonella enteriditis* ATCC 10467 were obtained from the ATCC (http://www.atcc.org/). Restriction enzymes and T4 ligase for DNA fragments cloning were purchased from Life Technologies Corporation (Carlsbad, CA, USA) and Ni–NTA resin for protein purification were bought from GE Healthcare Corporation (Chicago, IL, USA). All other chemicals were bought from Solarbio (Beijing, China).

Production of rLactolisterin BU from *P. pastoris*

Vector construction and positive-transformant screening

The Lactolisterin BU DNA encoding sequences were synthesized by Genewiz company (Suzhou, China) and then ligated into pPICZα-A vector, resulting in the pPICZα-A-Lactolisterin BU vector. The *Sac*I restriction enzyme linearized pPICZα-A- Lactolisterin BU vectors were electroporated (1200 V, 200 μF, and 50 Ω) into *P. pastoris* competent cells and spread onto YPDS plates (1% yeast extract, 2% peptone, 2% glucose, 1 M sorbitol, and 2% agar) containing 100 μg/mL of Zeocin (Invitrogen, Carlsbad, CA, USA). pPICZαA vectors served as negative controls. After 3 days of incubation, positive transformants were collected and identified by colony PCR with designed primers.

Expression and purification of 6 × His-rLactolisterin BU

Positive transformants were collected, placed in BMGY medium, and cultured at 28 °C and 250 rpm/min for 24 h. The yeast culture was then inoculated into fresh BMGY medium at a ratio of 5% and cultured at 28 °C and 250 rpm/min. When the optical density (OD) of 600 nm reached 8–10, the cultured cells were collected by centrifugation at 4000 rpm/min and washed twice with sterilized deionization water before culturing in fresh BMGY medium at 28 °C and 250 rpm/min. Then, 0.22 μm-membrane-filtered 100% methanol was added into cultures every 24 h until reaching 144 h of induction with a final concentration of 1% methanol. The cultured suspension was collected by centrifugation at 6000 rpm/min, and the supernatant was collected for affinity purification and Tricine-SDS-PAGE analysis. Then, the 6 × His-Lactolisterin BU was bound to the Ni–NTA column after equilibration with binding buffer (20 mM NaH_2PO_4, 500 mM NaCl, and 5 mM imidazole; pH 7.4), and then the unbound proteins were washed away by washing buffer (20 mM NaH_2PO_4, 500 mM NaCl, and 60 mM imidazole, pH 7.4). The purified 6 × His-rLactolisterin BU was separated using elution buffer (20 mM NaH_2PO_4, 500 mM NaCl, and 500 mM imidazole; pH 7.4) and then stored at − 80 °C after lyophilization.

Tricine-SDS-PAGE and silver staining

Tricine-SDS-PAGE assay was carried out as previously described with minor modifications [11]. We used 20.0% of separating gels and 4.0% stacking gels to separate the small-molecular-weight proteins in the samples, with the voltage set to 60 V before all samples entered the separating gels. Then, the voltage was increased to 120 V until the proteins ran to the gel bottom. Afterwards, the gels were stained with a silver staining kit (G7210, Solarbio, Beijing, China) following the manufacturer's instructions and captured with a gel imager (CLINX GenoSens 1800, Shanghai, China).

Minimum inhibition concentration (MIC) assay

The bacterial growth-inhibition effect was determined as previously described [12]. In a typical procedure, rLactolisterin BU from *P. pastoris* was adjusted with PBS to a series of concentrations ranging within 0–60 μg/mL and incubated with tested bacterial cultures (*S. aureus*

ATCC 25923, *S. enteriditis* ATCC 10467, *E. coli* H7:O157 ATCC 35150, *E.coli* ATCC 25922, *B. subtilis* AHU 1035, *P. aeruginosa* ATCC 27853 and *L. monocytogenes* ATCC 21633) at mid-log phase with concentrations of about 5×10^5 CFU/mL for 14 h at 37 °C. PBS buffer served as a negative control. The purified Lactolistrin BU from *Lactococcus lactis* were diluted with PBS to a serious of concentrations as described in the previous study [10], which were used to perform the comparative trial with the rLactolisterin BU from *P. pastoris*. Then, the optical value at 600 nm was measured with a microplate reader (HBS-1096A, Detie, Nanking, Jiangshu, China). All experiments were performed three times.

Time-killing kinetic assay

To determine the time-killing kinetics of rLactolisterin BU, *E. coli* H7:O157 ATCC 35150 was used to count the colonies with different treatments and to create the time-killing curve as previously described [13, 14]. In a typical procedure, the *E. coli* colony was collected and initially cultured in LB medium at 37 °C and 200 rpm/min for 12 h. After incubating the cell suspensions in LB medium at a ratio of 1:100 (v/v) and in various concentrations of rLactolisterin BU (0×, 0.5×, 1×, 2×, and 4×MIC) until the bacterial cell cycle reached the mid-log growth phase. The post-incubation samples, collected at different time points (0, 30, 60, 90, 120, 150, and 180 min), were spread onto the solid LB medium and cultured at 37 °C for 12 h before counting the viable colonies. The LB medium and gentamicin (50 μg/mL) treated bacteria suspensions served as negative and positive controls, respectively. All assays were performed in triplicate.

Biofilm formation assay

Crystal violet staining assay was used to investigate the biofilm inhibition effect of rLactolisterin BU as described previously [15], and *E. coli* H7:O157 ATCC 35150 was applied as the tested strain. Gentamicin was used as positive control, and PBS was used as blank control.

Cell culture and cytotoxicity assay

The cytotoxicity of rLactolisterin BU on mammalian cells was determined using MTT method as previously described [14]. In a standard procedure, mouse RAW264.7 cells were cultured in different concentrations of rLactolisterin BU at 37 °C in 5% CO_2 atmosphere for 24 h. The post-incubation cell suspensions were mixed with MTT solutions and incubated for 3 h before removing the supernatant. Then, the formazan crystals were dissolved in DMSO, and the OD at 570 nm was measured using a microplate reader (HBS-1096A, Detie, Nanking, China). The percentage of cell viability was calculated according to the formula: cell viability (%) = OD sample/OD control. All experiments were performed in triplicate.

Hemolytic assay

The hemolytic activity of rLactolisterin BU was measured as previously described [14]. Blank and 100% positive controls were prepared with rabbit erythrocytes suspended in PBS or 1% Triton X-100, respectively. All assays were performed in triplicate.

Inhibition-zone assay

Inhibition-zone assay was applied to determine the antimicrobial activity of rLactolisterin BU under various treatment conditions through the growth-inhibition effect on *E. coli* as previously described [14]. In a typical procedure, a single colony of *E. coli* H7:O157 ATCC 35150 was picked, cultured in LB medium at 37 ℃ and 200 rpm/min for 12 h, and transferred into fresh LB medium at a ratio of 1:100. The bacterial cultures were then spread on solid LB-medium plates when cell growth reached the mid-log phase (1×10^5 to 5×10^5 CFU/mL). Then, the rLactolisterin BU samples dissolved in PBS buffer at various pH values (2, 4, 6, 8, and 10) or salt concentrations (20, 40, 60, 80, and 100 mM), heated at various temperatures (4 ℃, 25 ℃, 37 ℃, 65 ℃, and 90 ℃), or digested with different proteinases (papain, pepsin, trypsin, and proteinase K) were prepared and added to the punched wells of the plates. After 14–16 h of incubation, the incubation zones were captured and analyzed with image J software. All assays were performed in three times.

Antioxidant-activity assay

The hydroxyl and superoxide anion radical-scavenging effects were used to determine the antioxidant ability of rLactolisterin BU by using a Hydroxyl Free-Radical Scavenging Capacity Assay Kit (BC1325, Solarbio, Beijing, China) and Superoxide Anion Detection Kit (BC1290, Solarbio, Beijing, China) as described by the manufacturer's instructions. Furthermore, the DPPH and $ABTS^+$ test methods were performed as described in our previous study [13]. All assays were carried out in triplicate.

Application of rLactolisterin BU in pork models

Treatment with various concentrations of rLactolisterin BU in pork models

Fresh pork legs were purchased from a local supermarket of Binzhou, China. The lean-pork meat slices (5 ± 0.1 g) were prepared as previously described with minor modifications [8]. In a typical procedure, the pork meat was handled using a knife to remove the fat, tissues, and porcine cortical skins aseptically. Then, the slices were soaked in rLactolisterin BU with different concentrations

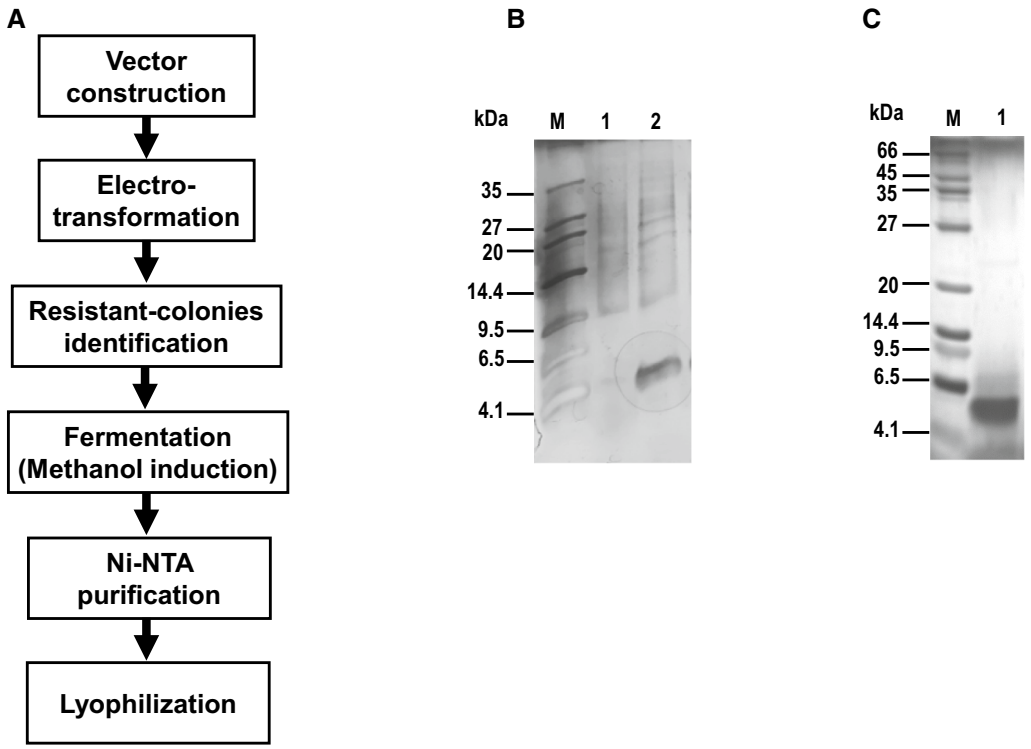

Fig. 1 Production of Lactolisterin BU from *P. pastoris*. **A** The production process of rLactolisterin BU from *P. pastoris*. **B** Silver staining of Tricine-SDS-PAGE gel of the rLactolisterin BU secreted in the supernatant of the *P. pastoris* containing pPICZα-A-Lactolisterin BU vector after induction with methanol. Lane M: protein marker; Lane 1: supernatant of the *P. pastoris* containing pPICZα-A empty vector as a negative control; Lane 2: supernatant of the *P. pastoris* containing pPICZα-A-Lactolisterin BU vector. **C** Silver staining of Tricine-SDS-PAGE gel of the purified rLactolisterin BU. Lane M: protein marker; Lane 1: the purified rLactolisterin BU

(0, 20, 40, and 60 μg/mL) for 10 min before 10-day storage experiments at 4 ℃. The sensory properties and bacterial count were then determined every 24 h. All experiments were carried out in triplicate.

Microbiological analysis

The slices with various treatments were diluted with saline solution (1% *w*/w) at a ratio of 1:10 and homogenized for 5 min every 24 h for 10 days to determine the bacterial viability. For total viable count (TVC), plate-count agar was used to incubate the decimal homogenates' dilutions at 37 ℃ for 48 h. For *S. aureus* count, mannitol-salt agar was used to incubate the decimal homogenates' dilutions at 37 ℃ for 48 h. Similarly, Violet Red bile agar was used for *E. coli* count at 37 ℃ for 48 h. For *L. monocytogenes* count, McBride agar base was used to incubate the decimal homogenates' dilutions at 30 ℃ for 48 h. All assays were carried out in triplicate.

Sensory quality

Quality-index analysis was applied to determine the sensory qualities as previously described [15]. The key factors affecting pork-meat quality including appearance, odor, texture, and overall acceptance were evaluated by nine experienced assessors (approved by the Institutional Review Board (IRB), No. IRB-BZXYSP20210501) within a score scale ranging from 1 to 10, in which the score range of 8–10 represents the best sensory quality, 6–8 represents ordinary good sensory quality, 4–6 represents poor sensory quality with slight spoilage, and 0–4 represents disgusting sensory quality with obvious spoilage.

Measurement of pH and total volatile basic nitrogen (TVB-N)

About 4 g of the pork-meat samples in each treatment group were homogenized with 25 mL of sterile distilled water and thoroughly mixed with a shaker at 200 rpm for 40 min. The pH values and TVB-N of the samples were determined as the National Standard of People's Republic of China (GB 5009.237–2016) illustrated by a pH meter (REX PHSJ-5, Shanghai, China) and automatic Kjelda-happaratus (Kjeltec 8400, Foss, Denmark) individually.

Thiobarbituric acid reactive substance (TBARS) assay

The TBARS method is extensively applied to determine lipid-oxidation products (malondialdehyde, MDA) in meat as previously described [8]. In a typical procedure,

Table 1 Antibacterial activity of the purified Lactolisterin BU

Bacterial species	MIC (μg/mL)	
	Lactolisterin BU derived from *P. pastoris*	Lactolisterin BU derived from *L. lactis*
Gram-positive		
Listeria monocytogenes ATCC 221633	6	8
Bacillus subtilis AHU 1035	10	35
Staphylococcus aureus ATCC 25923	20	5
Gram-negative		
Escherichia coli ATCC 25922	12	–
Escherichia coli H7:O157 ATCC 35150	12	–
Salmonella enteriditis ATCC 10467	20	–
Pseudomonas aeruginosa ATCC 27853	60	–

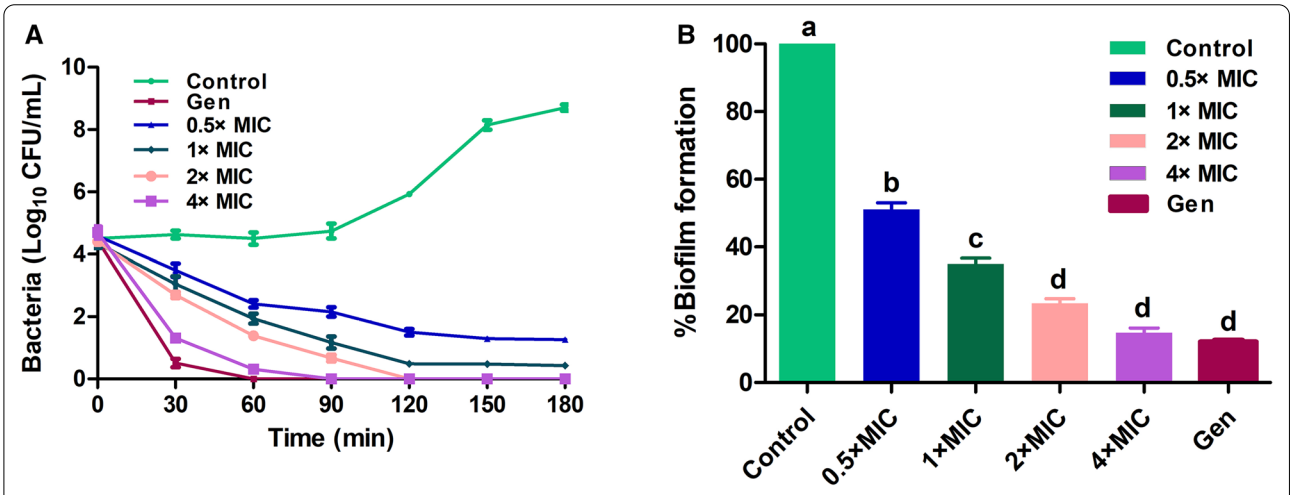

Fig. 2 Antimicrobial activity and efficiency of rLactolisterin BU. Time-killing kinetic curve (**A**) and biofilm formation inhibition curve (**B**) of rLactolisterin BU against *E. coli* H7:O157 ATCC 35150. Data are shown as the mean ± S.D. of triplicate measurements. Different letters represent significant difference ($P < 0.05$)

2 g of meat sample was homogenized with 20 mL of 5% trichloroacetic acid and passed through a filter paper to discard the insoluble residues. Then, 5 mL of the filtered solution was mixed with 5 mL of 2-thiobarbituric acid (2.88 g/L) by using a shaker at 200 rpm for 10 min before treatment in a 100 ℃ water bath for 15 min. After cooling to room temperature, the OD of the solution at 532 nm was evaluated with a spectrophotometer (T-6 series UV–Vis spectrophotometer, Feile, Nanking, China). TBARS values were measured from a standard curve of MDA solution.

Microbiological-challenge tests

Pork-meat spoilage models were constructed independently with three types of foodborne pathogens including *S. aureus* ATCC 25923, *E. coli* H7:O157 ATCC 35150, and *L. monocytogenes* ATCC 221633 to investigate the potential of rLactolisterin BU as a bio-preservative during meat storage. For individual bacteria, the prepared meat slices (5 ± 0.1 g) were inoculated with 100 μL of 2.0×10^5 CFU/piece of bacteria by infusion, and then various concentrations of rLactolisterin BU (0, 20, 40, and 60 μg/mL) were used to analyze the preservative effect. Slices inoculated with bacteria but without rLactolisterin BU served as a control. Bacterial count was then calculated as described in section "Microbiological analysis". All assays were carried out in triplicate.

Statistical analysis

GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) was used to analyze the data by one-way analysis of variance (ANOVA), and Tukey's test. $P < 0.05$ was

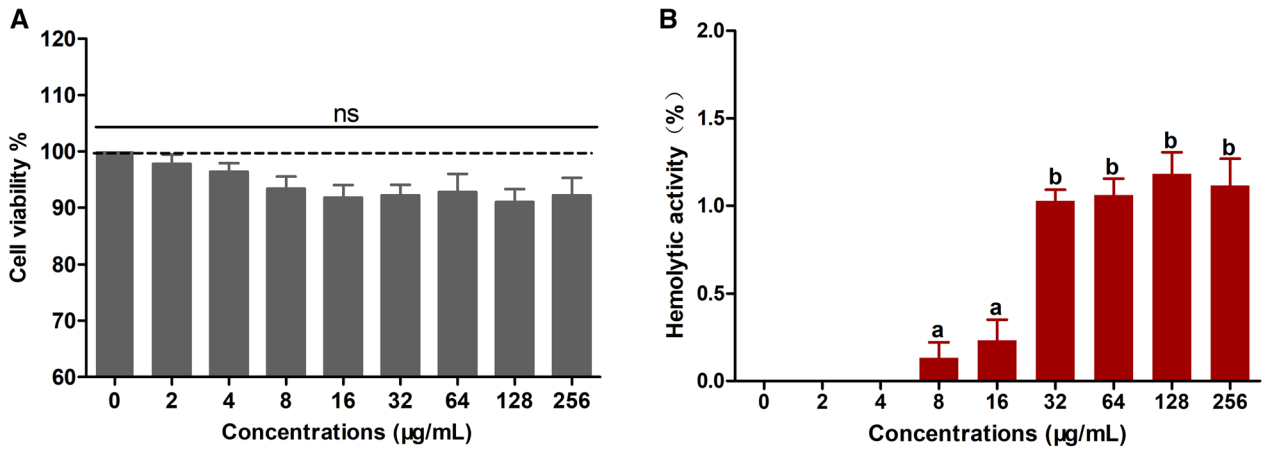

Fig. 3 Mammalian cell cytotoxicity and hemolytic activity of rLactolisterin BU. **A** Survival rate of RAW264.7 cells analyzed through MTT assay method. **B** Hemolytic effect of rLactolisterin BU against rabbit erythrocytes. All the data are shown as the mean ± S.D. of triplicate measurements. ns: non-significance. Different letters represent significant difference ($P < 0.05$)

considered statistically significant. All experiments were performed in triplicate, and each independent assay was performed in duplicate.

Results and discussion

Expression and purification of rLactolisterin BU from *P. pastoris*

The production scheme of rLactolisterin BU from *P. pastoris* is shown in Fig. 1A. The rLactolisterin BU were secreted in the supernatant of the *P. pastoris* containing pPICZα-A-Lactolisterin BU vector after induction with methanol, which was analyzed by using Tricine-SDS-PAGE assay as shown in Fig. 1B. The purified rLactolisterin BU was confirmed by Tricine-SDS-PAGE analysis as shown in Fig. 1C. Lactolisterin BU, a type of bacteriocin, was initially isolated from *L. lactis* subsp. *lactis* bv. diacetylactis BGBU1-4 in 2017 [14]. Lactolisterin BU offers various metabolites with potent antimicrobial activity and potential applications in the food industry, but its purification from *L. lactis* is not economical and efficient. Accordingly, *P. pastoris* aroused our interest because yeast could produce large amounts of functional recombinant proteins and perform many eukaryotic post-translational modifications, including protein folding and glycation, and the DNA manipulation, transformation and identification were easily to be achieved [11, 12]. Moreover, many AMPs including bovine lactoferricin peptide [16], Microcin J25 [17], Mytichitin-A [11], Hispidalin [18], and mytichitin-CB [8], have been successfully expressed in *P. pastoris* and exhibited potent antimicrobial activity and high yields. In a previous study, Lactolisterin BU has been purified by reversed-phase high-performance liquid chromatography, but the production rate is unclear [10].

To further improve the efficiency of Lactolisterin BU preparation, we used protein-affinity purification method, which is generally considered as low cost and high throughput. Meanwhile, His-tag is widely used for protein expression because it has a low molecular weight and does not easily affect the protein structure and characters compared with MBP-tag and GST-tag. The requirement of His-tag fusion protein purification was also easy to meet. Therefore, in the current study, rLactolisterin BU was produced from *P. pastoris* and purified with a Ni–NTA column for subsequent experiments.

Characterization of the antibacterial peptide

Antimicrobial activity

The MIC values are listed in Table 1. *P. pastoris* derived rLactolisterin BU showed a broad and enhanced antibacterial effect against gram-negative and gram-positive strains, with values ranging within 6–40 μg/mL. Of note, *L. lactis* derived Lactolisterin BU showed antimicrobial effect against gram-positive strains including *L. monocytogenes* ATCC 221633, *B. subtilis* AHU 1035 and *S. aureus* ATCC 25923 with values ranging within 5–35 μg/mL, but *P. pastoris* derived rLactolisterin BU exhibited antimicrobial activity against gram-negative strains including *E. coli* H7:O157 ATCC 35150 (MIC = 12 μg/mL), *S. enteritidis* ATCC 10467 (MIC = 20 μg/mL), and *P. aeruginosa* ATCC 27853 (MIC = 60 μg/mL), whereas *L. lactis* derived Lactolisterin BU did not show any antimicrobial effect against those strains. The antibacterial rate of *P. pastoris* derived rLactolisterin BU was determined by a time-killing kinetic assay against *E. coli* H7:O157

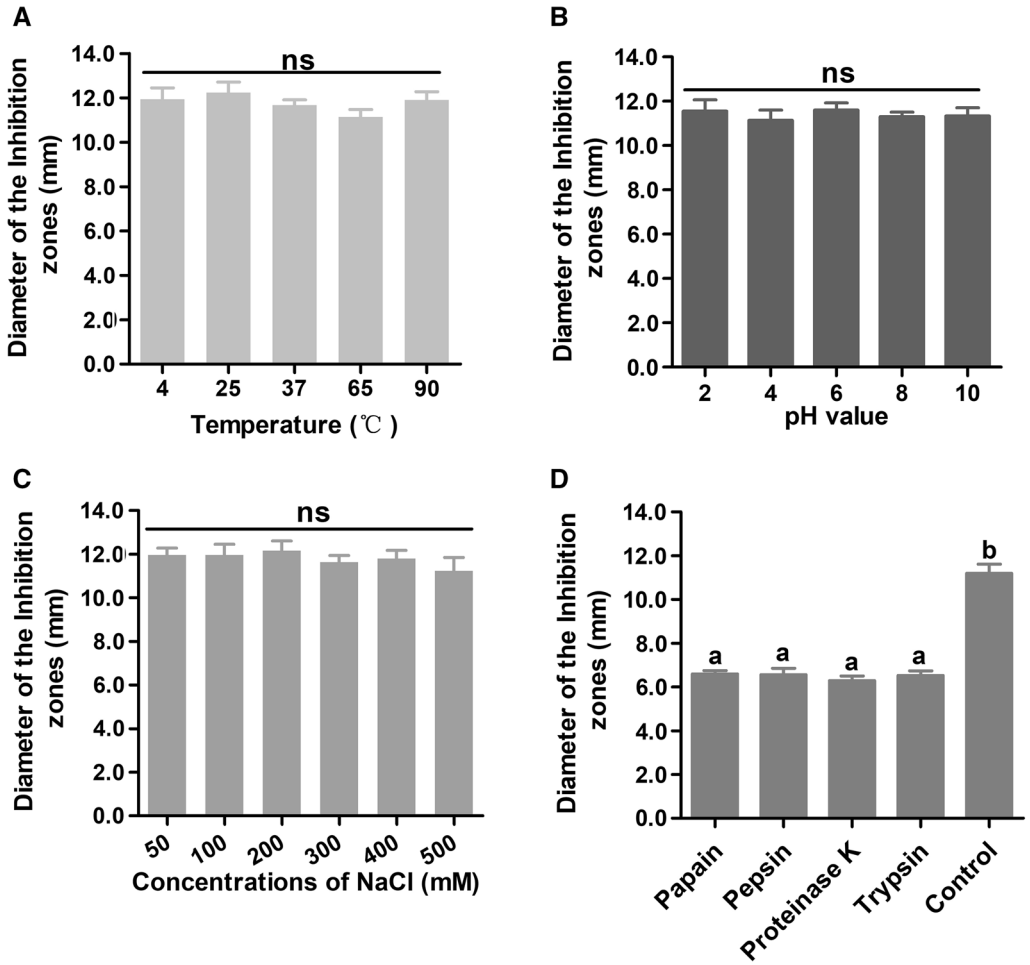

Fig. 4 Antibacterial effect and stability of rLactolisterin BU against *E. coli* H7:O157 ATCC 35150. The thermostability (**A**), pH resistance (**B**), saline resistance (**C**), and proteinase resistance (**D**) of rLactolisterin BU on antibacterial activity. All the data are shown as the mean ± S.D. of triplicate measurements. ns: non-significance. Different letters represent significant difference ($P < 0.05$)

ATCC 35150. As illustrated in Fig. 2A, rLactoliserin BU demonstrated rapid antimicrobial activity within 1.5 and 2.0 h at concentrations of 4 × and 2 × MIC, respectively. Nevertheless, rLactoliserin BU at 0.5 × and 1 × MIC showed that bacterial counts of *E. coli* H7:O157 ATCC 35150 decreased by 58.3% and 72.2%, respectively. Results of biofilm-formation assay (Fig. 2B) indicated that various concentrations of rLactoliserin BU dramatically decreased the biofilm formation in relation to the antibiotic resistance of bacteria. Furthermore, Lactoliserin BU originating from *L. lactis* was not sensitive to gram-negative bacteria, including *E. coli*, *S. enteritidis*, and *P. aeruginosa*. By contrast, rLactoliserin BU expressed in *P. pastoris* displayed a broad antibacterial spectrum. These results were in accordance with some recombinant peptides obtained from *P. pastoris* such as EntP::EntHF peptide [19], Hispidalin [18], Mytichitin-A [11], and defensins (including NZ2114 and its derivatives) [20]. They showed improved antimicrobial activity because the correct post-translational modifications such as O- and N-linked glycosylation and disulfide-bond formation were easy to achieve in the *P. pastoris* system [21]. Additionally, the results of antibacterial rate indicated that the antibacterial effects of rLactoliserin BU were similar to those of gentamycin with bactericidal rather than bacteriostatic effects in time- and dose-dependent manners. Thus, rLactoliserin BU was potential to replace antibiotics or other chemical preservatives for food preservation.

Cytotoxicity and hemolytic activity

The safety properties such as the cytotoxicity and hemolytic activity of rLactoliserin BU should be examined before its application as a food preservative. The MTT assay was used to evaluate the cytotoxicity of rLactoliserin BU, and results suggested that rLactoliserin BU (0–256 μg/mL) had no cytotoxicity to RAW264.7 cells,

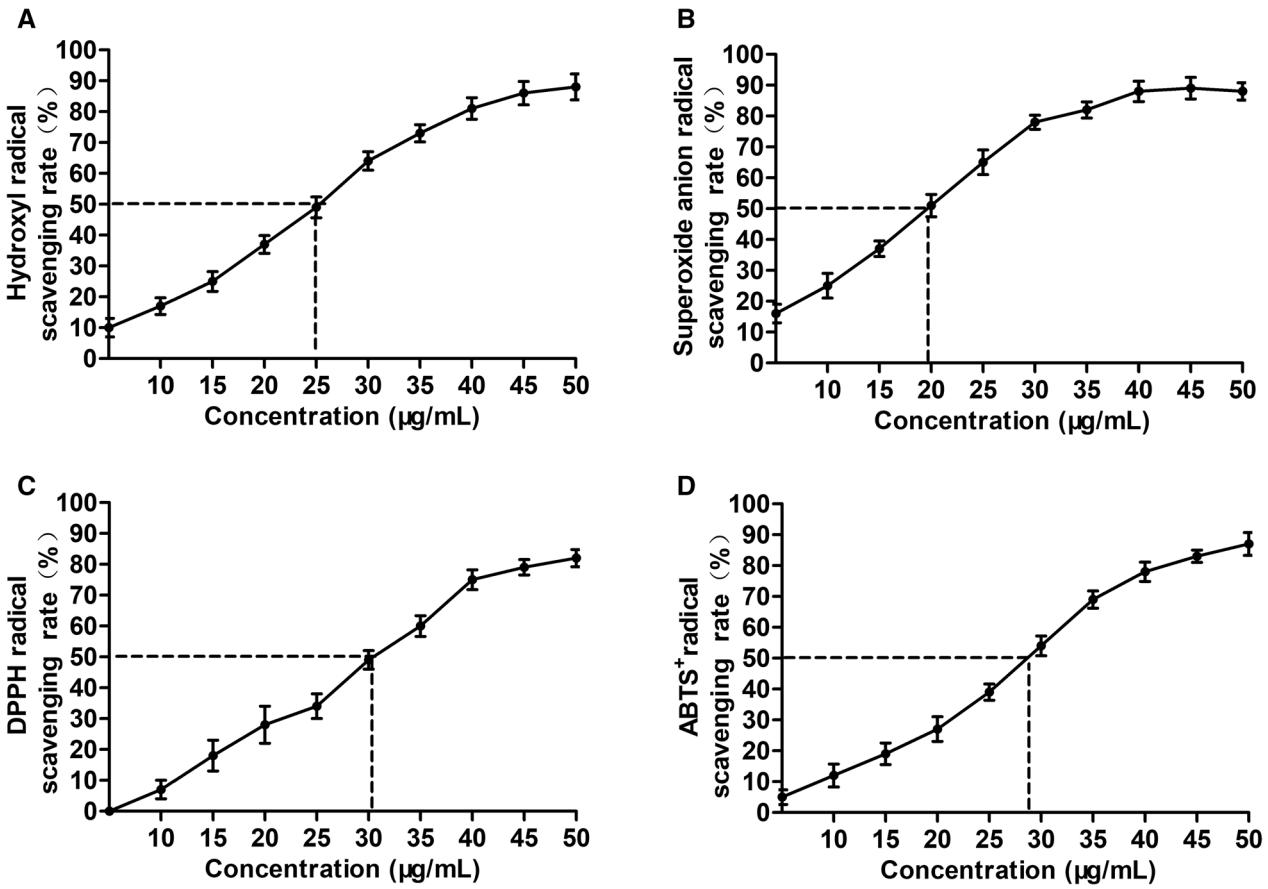

Fig. 5 Antioxidant activity of rLactolisterin BU. Hydroxyl radical (**A**), Superoxide anion radical (**B**), DPPH radical (**C**), and $ABTS^+$ radical (**D**) scavenging assay. All the data are shown as the mean ± S.D. of triplicate measurements

which are a classical type of macrophages related to immunoreaction in mouse (Fig. 3A). Moreover, hemolytic assay of rLactoliserin BU (0–256 μg/mL) was performed to determine its hemolytic activity. Results (Fig. 3B) indicated that rLactoliserin BU had low hemolytic activity and could be used in fresh meat with a small amount of blood. Therefore, rLactoliserin BU can replace chemical preservatives as a bio-preservative for meat storage.

Stability of rLactoliserin BU

To further assess the application of rLactolisterin BU in the food industry, the thermostability, pH resistance, proteinase resistance, and saline resistance of rLactoliserin BU in relation to its antibacterial activity were determined by inhibition-zone assay. As shown in Fig. 4A–C, the inhibition diameters of rLactolisterin BU treated with heat (4–90 ℃), extreme pH (2–10), and various concentrations of NaCl (50–500 mM) were similar to those of the control, suggesting that antibacterial activity was not affected. However, rLactolisterin BU was not tolerant to proteinase digestion, as shown in Fig. 4D, and antibacterial activity decreased compared with that of the control. These results agreed with the *L. lactis* derived Lactolisterin BU and most bacteriocins, including BAC-IB17 [22], BaCf3 [23], and plantacyclin B21AG [24], which were thermostable. These results showed that rLactolisterin BU could be used in food products processed with high temperature, addition of acid or base, and salinization.

Antioxidant activity

To determine the antioxidant effect of rLactolisterin BU, the scavenging rates of hydroxyl, superoxide anion, DPPH, and $ABTS^+$ free radicals were measured. As shown in Fig. 5A–D, the IC_{50} values of rLactolisterin BU on scavenging radicals of hydroxyl, superoxide anion, DPPH, and $ABTS^+$ were 25.0, 19.8, 30.6, and 29.0 μg/mL, respectively. A specific food preservative with antibacterial effects and antioxidant activity may show synergetic effect when used in food storage. Many AMPs including lactoferrin [25], cathelicidin [26], and mytichitin-CB [8]

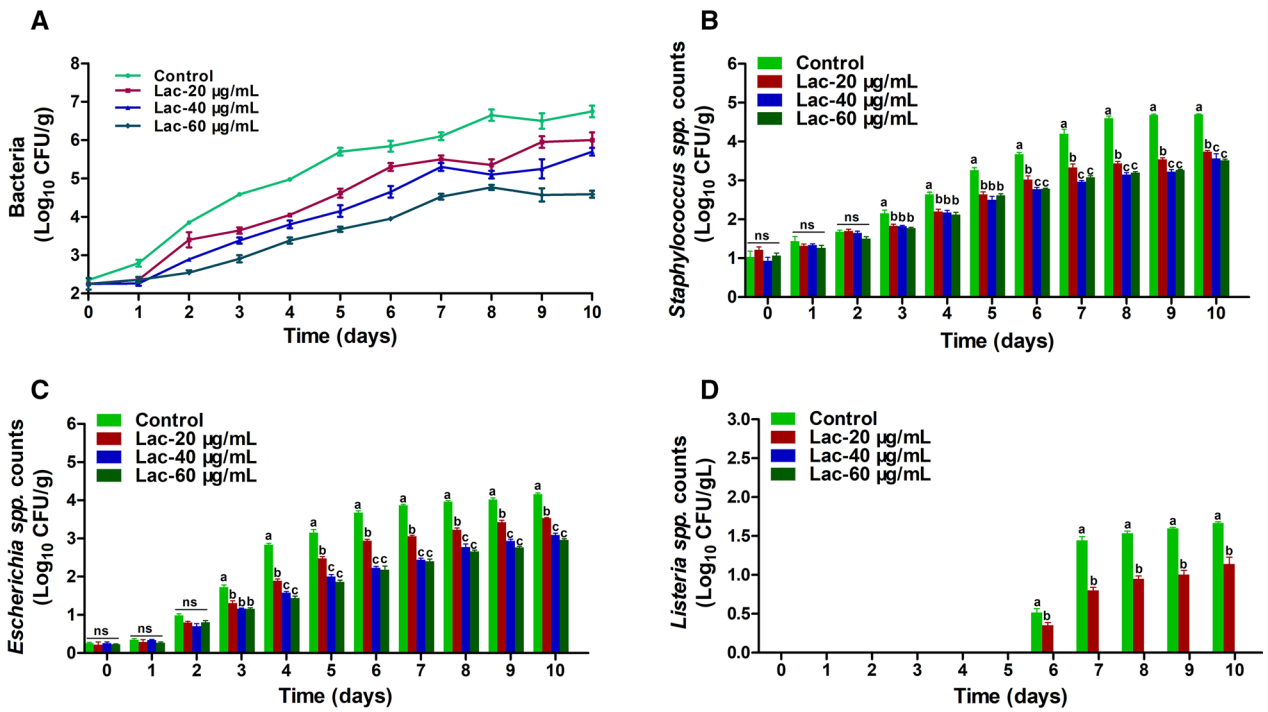

Fig. 6 Counts of microorganisms in fresh meat during storage at 4 ℃. Total viable count (TVC) (**A**), *Staphylococcus* spp. (**B**), *Escherichia* spp. (**C**), and *Listeria* spp. (**D**) of pork samples treated with various concentrations of rLactolisterin BU during storage. Different letters represent significant difference ($P < 0.05$)

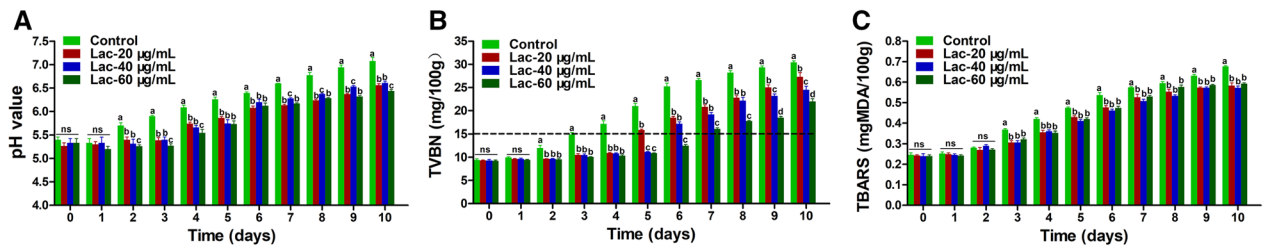

Fig. 7 Freshness and physicochemical performance of meat during storage. The pH (**A**), total volatile basic nitrogen (TVB-N) content (**B**), and thiobarbituric acid-reactive substances (TBARS) content (**C**) of pork samples treated with various concentrations of rLactolisterin BU during storage. Different letters represent significant difference ($P < 0.05$)

are good preservatives having antibacterial and antioxidant activities, and they exert a synergetic effect as a food preservative. Therefore, the application of rLactolisterin BU as a food preservative might be show synergetic effect to enhance the applicability.

Application of rLactoliserin BU in pork models

Bacteriological analyses

To examine the application of rLactoliserin BU in porkmeat storage, the TVC of bacteria contaminated naturally in meat samples were used to assess meat quality and safety during storage. Once the TVC of microorganisms in fresh pork meat exceeded a certain quantity, they became pathogenic microorganisms, causing deterioration during pork storage. Various concentrations (0, 20, 40, and 60 μg/mL) of rLactoliserin BU were used to evaluate the TVC of bacteria contaminated naturally during pork storage, and results are shown in Fig. 6A. The total bacterial growth was obviously inhibited by rLactoliserin BU in a concentration-dependent manner during storage compared with the control. However, the TVC in each treatment group exhibited a gentle growing trend during storage. Notably, the TVC in the control group was

Table 2 Sensory scores of the pork with different treatments

Storage days	Treatment groups			
	Control	20 μg/mL	40 μg/mL	60 μg/mL
0	9.65 ± 0.12^{a}	9.65 ± 0.12^{a}	9.65 ± 0.12^{a}	9.65 ± 0.12^{a}
1	9.43 ± 0.18^{a}	9.52 ± 0.10^{a}	9.57 ± 0.05^{a}	9.60 ± 0.08^{a}
2	8.12 ± 0.25^{b}	8.57 ± 0.35^{b}	8.68 ± 0.15^{b}	8.89 ± 0.24^{b}
3	7.15 ± 0.16^{a}	7.45 ± 0.21^{b}	7.76 ± 0.12^{b}	7.92 ± 0.18^{b}
4	5.20 ± 0.34^{c}	6.52 ± 0.41^{c}	7.12 ± 0.37^{b}	7.23 ± 0.27^{b}
5	3.50 ± 0.25^{c}	5.86 ± 0.24^{b}	6.58 ± 0.12^{a}	6.83 ± 0.18^{a}
6	2.87 ± 0.36^{c}	5.42 ± 0.16^{b}	6.22 ± 0.14^{a}	6.54 ± 0.21^{a}
7	2.01 ± 0.42^{c}	4.85 ± 0.15^{b}	5.86 ± 0.26^{a}	5.95 ± 0.18^{a}
8	1.76 ± 0.26^{c}	3.86 ± 0.21^{b}	4.56 ± 0.30^{a}	4.75 ± 0.26^{a}
9	1.21 ± 0.18^{c}	2.25 ± 0.12^{b}	2.87 ± 0.20^{a}	2.92 ± 0.32^{a}
10	0.87 ± 0.16^{c}	1.52 ± 0.08^{b}	1.68 ± 0.12^{b}	1.85 ± 0.15^{b}

Values are mean ± standard deviation of 3 replicates, different lowercase letters mean significantly different in the same row ($P < 0.05$)

nearly 6.5 Log_{10} CFU/g on the 10th day, 60 μg/mL rLactoliserin BU was effective in inhibiting bacterial growth during storage, and the TVC was only about 4.5 Log_{10} CFU/g on the 10th day. The TVC of 20 μg/mL and 40 μg/mL rLactoliserin BU treatment groups were 6.0 Log_{10} CFU/g and 5.7 Log_{10} CFU/g on the 10th day, respectively. To further investigate the antibacterial effect of rLactoliserin BU on common foodborne bacteria (*Staphylococcus* spp., *Escherichia* spp., and *Listeria* spp.) contaminated naturally during pork storage, bacterial counts were evaluated with different special selective media as described in the Material and methods section. As shown in Fig. 6B, the bacterial counts of *Staphylococcus* spp. in the control group were nearly 5.0 Log_{10} CFU/g on the 10th day, but the rLactoliserin BU treatment groups (20, 40, and 60 μg/mL) had significantly decreased bacterial counts of 3.8 Log_{10} CFU/g, 3.6 Log_{10} CFU/g, and 3.5 Log_{10} CFU/g on the 10th day. Similarly, the bacterial counts of *Escherichia* spp. in the control groups were nearly 4.0 Log_{10} CFU/g on the 10th day as shown in Fig. 6C, but the rLactoliserin BU treatment groups (20, 40, and 60 μg/mL) had significantly decreased bacterial counts of 3.5 Log_{10} CFU/g, 2.9 Log_{10} CFU/g, and 2.7 Log_{10} CFU/g on the 10th day. For the *Listeria* spp. count (Fig. 6D), bacteria did not appear until the 6th day in the control and 20 μg/mL rLactoliserin BU treatment groups, but the 40 and 60 μg/mL rLactoliserin BU groups did not show any *Listeria* spp. colonies during the storage period. The meat-storage application of many AMPs including peptide from housefly pupae [27], mytichitin-CB [8], and Mcc J25(G12Y) [9] have been investigated. The results in this study are in agreement with those peptides and exhibited prominent efficiency in pork storage.

Physicochemical analyses

To evaluate the freshness and physicochemical performance of meat, we considered pH, lipid oxidation, and TVB-N as the key parameters to determine the meat's physicochemical attributes. When proteins in meat are degraded, they generate volatile alkaline nitrogen molecules that cause the pH of pork meat to easily increase during storage [28–30]. In the present study, the pH of all pork-meat samples increased from the beginning to the end of the storage period. Compared with the control, the pH increase of pork-meat samples was effectively retarded in the rLactoliserin BU treatment groups (20, 40, and 60 μg/mL) from the 2nd to the 10th day (Fig. 7A), and various concentrations of rLactoliserin BU did not show any difference throughout the storage period. These results may be related to the antibacterial effect of rLactoliserin BU, which delayed and attenuated the production of amines.

TVB-N refers to compounds such as trimethylamine, ammonia, and dimethylamine that are generated through the protein degradation caused by microorganisms during meat storage [31]. TVB-N is viewed as one of the most vital freshness indices to reveal the quality and safety of meat products, as stated by Chinese National Standard GB 2707–2016. As shown in Fig. 7B, the TVB-N of all pork-meat samples slowly increased in the

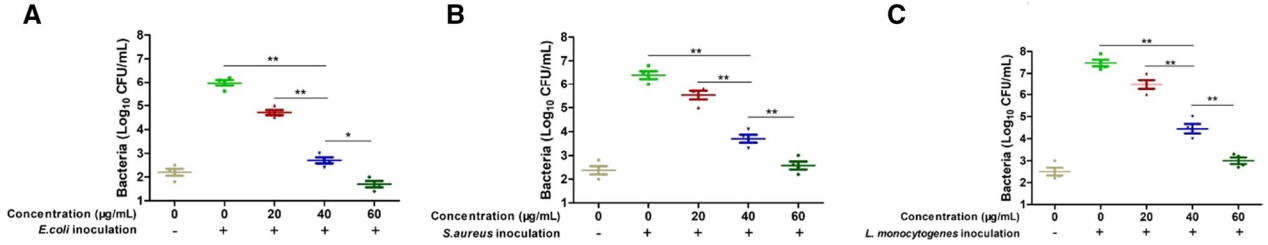

Fig. 8 Counts of microorganisms of meat artificially contaminated with bacteria treated with rLactolisterin BU. *E. coli* H7:O157 ATCC 35150 (**A**), *S. aureus* ATCC 25923 (**B**), and *L. monocytogenes* ATCC 221633 (**C**) in the pork spoilage model. Different asterisks represent significant difference (*$P < 0.05$, **$P < 0.01$)

first 5 days but increased dramatically in the next 5 days despite the addition of rLactoliserin BU. Specifically, rLactoliserin BU decreased the production of TVB-N during pork-meat storage compared with the control, and the TVB-N concentration of samples treated with 20, 40, and 60 μg/mL rLactoliserin BU was 10.9, 10.8, and 10.3 on the 4th day. However, the TVB-N value of the control was 17.15, which exceeded the minimum concentration of TVB-N as illustrated by National Standard GB 2707–2016. Thus, the shelf life of pork-meat samples can be extended to 4, 5, and 6 days when individually treated with 20, 40, and 60 μg/mL rLactoliserin BU during pork-meat storage, respectively.

As shown in Fig. 7C, the TBARS of all samples increased during the storage period, whereas that of the rLactoliserin BU treatment groups constantly increased more slowly than did the control. The TBARS of the control was 0.68 mg/100 g at day 10, which was higher than that of the rLactoliserin BU treatment groups (mean = 0.58 mg/100 g), and the TBARS of meat samples treated with various concentrations of rLactoliserin BU showed no significant differences during storage. This result was in agreement with the antioxidant activity of rLactoliserin BU, which revealed that rLactoliserin BU can inhibit the lipid oxidation of pork meat and thus had the potential to be a promising preservative for meat storage.

Sensory analysis

Sensory analysis is a simple and intuitive method of evaluating the color, texture, odor, and overall acceptability of food quality [32]. In the current study, the sensory results are shown in Table 2. The sensory scores of all pork-meat samples decreased with prolonged storage time, and the sensory characteristics gradually deteriorated. Nevertheless, the scores of all groups on day 1 did not differ from that on day 0. Meanwhile, the score of the control significantly decreased in the next 2 days, and the score on day 4 was 4.0. which was unacceptable as the evaluation criteria stated. Conversely, the rLactoliserin BU treatment groups showed acceptable scores of 7, 8, and 8.5 on day 6 with 20, 40, and 60 μg/mL rLactoliserin BU addition. These results were in accordance with those of bacteriological and physicochemical investigations, indicating that 40 or 60 μg/mL rLactolisterin BU was suitable for pork storage.

Microbiological-challenge tests

Pork-meat samples treated with various concentrations of rLactolisterin BU were artificially contaminated with 5×10^5 CFU/g *S. aureus* ATCC 25923, *E. coli* H7:O157 ATCC 35150, and *L. monocytogenes* ATCC 221633 and stored at 4 ℃ to determine the potential of rLactoliserin BU as a preservative in a pork-spoilage model. As shown in Fig. 8A–C, after incubation for 24 h at 37 ℃ since inoculation, the growth of *E. coli* H7:O157 ATCC 35150, *S. aureus* ATCC 25923, and *L. monocytogenes* ATCC 221633 obviously decreased to 1.90, 2.62, and 3.08 Log 10 CFU/g by 60 μg/mL rLactolisterin BU in a dose-dependent manner. Owing to the abundant nutrient contents of pork and its products, they are easily contaminated by microorganisms including *E. coli*, *L. monocytogenes*, and *S. aureus*, thereby reducing the shelf life of pork [33]. Thus, new technologies such as bio-preservation, are receiving research interest. AMPs such as nisin [7], bacaucin-1 [34], and sakacin P [35] have been evaluated in pork, seafood, or other meat products, showing excellent effect in food-spoilage models. Results showed the potent antibacterial effect of rLactoliserin BU in a pork-spoilage model, in agreement with reported AMPs used in food storage and suggesting that rLactoliserin BU could be a promising preservative.

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Authors' contributions

Data curation, BD, JW, CLS and YJL; Funding acquisition, BD, JW, CLS and YJL; Investigation, GWZ and CLY; Methodology, BD, JW, TW and YJL; Project administration, BD; Writing—original draft, BD; Writing—review & editing, BD and CLS. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Declarations

Competing interests

The authors declare no conflict of interest.

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