

# Identification and Characterization of a Bacteriocin-Like Substance, Produced by *Leuconostoc mesenteroides*, as a Bio-Preservative Against *Listeria monocytogenes*

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**Abstract:** By conducting a systemic screening from Chinese traditional Tofu, a bacteriocin producer with broad antimicrobial spectrum was found. The producer was identified by 16S rDNA sequencing, and it was identified as *Leuconostoc mesenteroides*. The producer possessed typical bacteriocin traits, such as be heat-stable and proteinase-labile. Its inhibition spectrum contained many distantly related genera of Firmicutes, comprising most lactic acid bacteria (LAB) as well as problematic species of *Bacillus*, *Listeria* and *Staphylococcus*. In addition, the bacteriocin producer showed significantly inhibition effects on controlling *Listeria monocytogenes* in milk. Taken together, the results indicate that this is a novel and potent bacteriocin from *Leu. mesenteroides* and that its very broad inhibition spectrum can be use in food preservation as well as in infection treatments caused by Gram-positive pathogens.

**Keywords:** Bacteriocins, Lactic Acid Bacteria, Inhibition Spectrum, Applications, *Listeria monocytogenes*

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## 1. Introduction

Cow milk is considered as an important source for healthy and balanced diet, it offers essential nutritive requirements for human consumption. However, because of its nutritional properties, various bacteria can gain access to the raw or unpasteurized milk, pasteurized milk and milk products, causing food-borne illness associated with ingestion of those possible contamination sources [1-3].

*Listeria monocytogenes* is one of the most important and common etiological agents in the milk [1], [4]. Adverse health effects due to consumption milk contaminated with *L. monocytogenes* have been reported in decades [5-7]. In addition, several opportunistic bacteria have been detected in the milk seems to vary prevalence of illnesses associated with milk and dairy products [3], [8]. Interests on discovering alternative methods to maintain the quality of milk thus have

been increasing.

Traditionally, vaccine and antibiotics are given to the cows to cure the pathogenic inflammation and bovine mastitis [9-10]. However, as the emergence of bacterial antibiotic resistance, potential harmful bacteria residue in the milk could transmit virulent factors to customers. Therefore, novel treatment or alternative sources of antimicrobial agents were expected to satisfy with food safety demands and preservation requirements. Bacteriocins produced by Lactic Acid Bacteria (LAB) were regarded as the promising sources of antimicrobial, providing the potential application as bio-preservatives in food.

Bacteriocins are the antimicrobial peptides or proteins produced by different group of bacteria [11]. Their inhibition spectrum is towards closely related species with nano-molar concentration, while several bacteriocins produced by LAB have broad inhibition activity, including food spoilage and pathogenic microorganisms. Besides that, bacteriocins

produced by LAB with desirable properties, like safe compounds, pH and heat tolerant provide prospective benefits for food preservation, importantly, bacteriocins have not exhibited lethal to humans, and thus discovery of new bacteriocins produced by LAB have been taken more attentions [12-13].

Screening for the bacteriocin producers, as the first step for discovery of novel bacteriocin, has been utilized from several products, and the methods have been utilized recently [13-15]. Bacteriocin-producers, isolated and analyzed from dairy product, and their characteristics have been partly or fully understood as well [16-17]. However, only little bacteriocin has been successfully applied. Therefore, discovering the putative bacteriocin-producing strains, as well as applying the bacteriocin producer on food as bio-preservative, is of important practical meaning for industrial purposes.

The objectives of this present study were: 1) to isolate, identify and characterize the bacteriocin-producing strains by LAB from Chinese traditional fermented products; 2) to determine the potential inhibition spectrum of bacteriocin-like substances; 3) to analyze the properties of bacteriocin producer(s) compared with known bacteriocin(s); 4) to apply bacteriocin on the milk as bio-preservatives against pathogens.

## 2. Materials and Methods

### 2.1. Sample Collection, LAB Isolation and Culture Conditions

The fermented Chinese Tofu was purchased from supermarket in Shanghai. The samples were obtained in 10 g and stored in sterile test tubes. Samples subsequently have been delivered at 2-4°C within 4h into the lab. The samples were analyzed on the De Man, Rogosa and Sharpe (MRS) supplemented with cysteine-HCl (0.5 g/L) and M17 agar plates with 0.4% of glucose in anaerobic incubation. Based on the morphological structure of all the colonies, gram staining, catalase reaction and acid production have been performed to obtain LAB. Two to three colonies of LAB were picked and inoculated in proper agar. The bacteria in transported media were streaked on appropriate agar plates to recover the isolates. All isolates were sub-cultured in the broth, and the stocks with 13% glycerol were maintained at -80°C.

### 2.2. Detection of Antimicrobial Activity

The agar diffusion bioassay described by Kanatani and Oshimura [18] was used to screen for bacteriocin producing by LAB among isolates. Semi solid Brain Heart Infusion soft agar (BHI broth plus 0.8% bacteriological agar) was used to detect the activity of bacteriocin production by LAB described previously [18]. *Lactococcus lactis* IL1403, *Lactobacillus sakei* LFM2313, *Lactobacillus plantarum* LFM2003, *Listeria innocua* LFM2710 and *Staphylococcus aureus* LFM3242 were used as indicator bacteria in first round.

The bacteriocin producers whose activity was against *L.*

*lactis* were continuously tested with another four different indicators (*L. lactis* LFM2122, *Enterococcus faecalis* LFM3088, *L. lactis* LFM2081, *L. lactis* LFM2130) for the second round. To confirm the production of inhibitory compound of bacteriocin, samples have been treated by proteinase K (20 µg/mL) was measured. Heat-treated at 100°C for 15 min of cell-free supernatants was assayed to analyze the heat tolerance of bacteriocin.

### 2.3. DNA Isolation, PCR Reaction and Sequencing Determination

To identify the species of bacteriocin producers, 16s rDNA gene sequencing was used. Total genomic DNA was isolated by using of fast prep (Bio101/Savant) and DNA mini kit (Omega Bio-tek Inc., GA). Amplification of 16s rDNA was carried out for 30 cycles in a 50-µL mixture solution including 5 µL 10× thermoPol reaction buffer, 1 µL dNTPs, 1 µL template DNA and 0.25 µL Taq DNA polymerase as described by BioLabs®. Primers 5F (5'-GGTTACCTTGTTACGACTT-3') and 11R (5'-TAACACATGCAAGTCGAACG-3') were used in the study [15]. PCR products were purified by using NucleoSpin Extract II (Macherey-Nagel, Düren, Germany). The PCR running program at 50-µL mixture solution was briefly described as follows: after incubation at 95°C for 30 seconds and for 15 seconds at 95°C, template DNA was performed for 1 min at the annealing temperature of 54°C, followed by 68°C for 90 seconds; the reaction was accomplished at 68°C for 5 min and maintained at 4°C. Sequencing reaction was carried out by using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, CA).

### 2.4. API Test and Hemolytic Analysis

The API test was performed by using bioMerieux®sa, France. The results were checked after 24 h and confirmed after 48 h. Hemolytic reaction was carried out by appearance of clear zones (β-hemolysis), greenish zones (α-hemolysis) and absence of zones (γ-hemolysis) on sheep blood agar (Oxoid).

### 2.5. Bacteriocin Preparation and Assays

Bacteriocin was concentrated from supernatant of over-night culture with 45% ammonium sulfate. The activity of bacteriocin was tested by using microtiter plates against *L. lactis* as an indicator. Antimicrobial unit (AU) was defined as the amount of bacteriocin that showed 50% inhibition activity of indicators within 200 µL of culture.

### 2.6. Inhibition Activity Against *L. Monocytogenes* in the Milk

To assess the effects of bacteriocin against potential pathogenic bacteria in the milk, crude bacteriocin with different concentration (0, 500 and 2000 AU/mL) was applied on the milk. Overall 100µL of culture with 107 CFU/mL of bacteria, *L. monocytogenes* (DH7001 and DH7002) isolated from milk, was inoculated in to the sterilized milk and growth overnight at 37°C. Total viable count (TVC), pH and sensory

evaluation were analyzed after 24 h by using Total Viable Count Agar (Oxoid) and PALCAM agar as a selective media used for *L. monocytogenes* (Oxoid).

### 2.7. Sensory and Physical Evaluation of Milk Applied with Bacteriocin

Sensory evaluation of milk was performed with eight experienced and trained panelists according to Hayaloglu et al. [19]. The milk was evaluated for appearance, color, odor, consistency and overall acceptability using a score from 0 point (bad) to 5 points (good). Sterilized milk, stored at the same condition after 24 h was analyzed as a control.

## 3. Results

### 3.1. Bacterial Isolation and Screening

LAB belong to the large group of microorganisms united by formation of lactic acid appears to have broad spectrum. Based on the Gram staining, low pH, catalase reaction and growth in two different media, GM17 and MRS broth, a total of 64 LAB were obtained in this study. To screen the antimicrobial activity of the whole isolates, five indicators were analyzed (Table 1). The results illustrated that, against *L. lactis* 38 from total 64 (59.4%) isolates were shown activity for the first round. For the second round, only four isolates showed activity against all the indicators. All those bacteriocin producers had sensitively responses to proteinase K, inhibitory activities of culture supernatants of bacteriocin producers which still maintained the activity after heat treatment.

### 3.2. Determination of Bacteriocin Producers by PCR Reaction

A total of four bacteriocin producers of LAB were identified to species by 16S rDNA sequencing analysis. According to the results performed in the BLAST at NCBI, all the samples were verified to be *Leu. mesenteroides* with identity range up to 1100 to 1300 nucleotides and identity ration more than 99%.

**Table 1.** The portion of isolates producing antimicrobial activity against the five indicators.

Indicators	Isolates with antimicrobial activity <sup>a</sup>
<i>L. lactis</i> IL 1403	38 (59.4 %)
<i>L. sakei</i> LFM 2313	29 (45.3 %)
<i>L. innocua</i> LFM 2710	46 (71.9 %)
<i>S. aureus</i> LFM 3242	40 (62.5 %)
<i>L. plantarum</i> LFM 2003	27 (42.2 %)
All five <sup>b</sup>	4 (6.3%)

a Total isolates screened for antimicrobial activity were 64

b Number of isolates with antimicrobial activity against all the five indicators

### 3.3. API Test and Hemolytic Analysis

Fermentation characteristics of LAB isolated from different sources present distinctively properties [20]. The

bacteriocin producer of *Leu. mesenteroides* has the capacity to metabolize the basic sugar, indicating that the strain is likely to be isolated from the fermented products. Meanwhile, the measurement for hemolysis about *Leu. mesenteroides* was conducted on the blood agar, and all those isolates showed no hemolytic activity.

### 3.4. Inhibitory Spectrum of Bacteriocin

Bacteriocins produced by *L. lactis* strains revealed broad inhibitory activities against gram positive bacteria [11]. In our study, bacteriocin produced by *Leu. mesenteroides* displayed broad spectrum against Gram-positive bacteria stains, including all the closely related genera of Firmicutes, comprising most lactic acid bacteria (LAB) as well as problematic species of *Bacillus*, *Listeria* and *Staphylococcus* (Table 2).

### 3.5. Application of Bacteriocin Against *Listeria Monocytogenes* on the Milk

In order to test the potential application of bacteriocin, produced by *Leu. mesenteroides*, as biopreservatives on the milk, 50x crude bacteriocin with 500 and 2000 AU/mL were applied on the sterilized milk inoculated with 10<sup>7</sup> CUF/mL *L. monocytogenes* (KS 7001, KS 7002). Additional, pH and sensory analysis were performed as well. Results showed that no colonies were detected on both BHI and selective agar plates when the milk was treated with two doses (500 and 2000 AU/mL) of bacteriocin. The significant differences suggest the bacteriocin could inhibit the growth of *L. monocytogenes* in the milk.

The pH of sterilized milk originally was about 6.626, it changed to 6.632 after 24h at 37°C. There are no significant differences between the sterilized milk treated with/without bacteriocin and *L. monocytogenes*. To test the satisfaction and acceptability of the sterilized milk with bacteriocin, sensory evaluation was carried out with 8 experienced panelists. Overall acceptability of the sterilized milk with bacteriocin showed no differences compared with sterilized milk without bacteriocin within groups. Low concentration of bacteriocin was recommended to apply on the milk against *L. monocytogenes*, suggesting that the quality of milk would not be affected from bacteriocin produced by *Leu. mesenteroides*.

**Table 2.** Inhibition spectrum of *Leu. mesenteroides* compared with nisin against different indicators.

Indicators	<i>Leu. mesenteroides</i>	nisin
<i>L. monocytogenes</i>	+	+
<i>L. innocua</i>	+	+
<i>Strep. Thermophylu</i>	+	+
<i>S. aureus</i>	+	+
<i>E. faecalis</i>	+	+
<i>E. faecium</i>	+	+
<i>B. cereus</i>	+	+
<i>E. coli</i>	-	-

## 4. Discussion

Over the last decades, a variety of novel bacteriocins produced by LAB have been described, and some of

bacteriocin producers have been isolated from different sources, especially in milk and dairy products and applied on food as novel biopreservatives [21-22]. In this study, we contributed to the screening and identification of bacteriocin-producing LAB from Chinese traditional Tofu, with the purposes of discovering novel bacteriocins against *L. lactis*, and then narrowed down the bacteriocin producer to *Leu. mesenteroides* for further investigation. Prosperities and inhibition spectrum of the bacteriocin were characterized. Eventually, application of bacteriocin producer on milk against *L. monocytogenes* was analyzed.

Completely 38 isolates out of 64 isolates had inhibitory effect on *L. lactis*. Moreover, another four different indicators were determined within those 38 isolates, four bacteriocin producers have been confirmed to be sensitive to all indicators with response to proteinase K. *Leu. mesenteroides* eventually has been identified by analysis on the 16S rDNA sequence. This bacteriocin producer showed broad inhibition spectrum as nisin, which indicates this producer has the potential against pathogens in the food.

To date, bacteriocin has been effectively studied, as strategies for food bio-preservation, to control the pathogenic bacteria in food [23-24]. However, only nisin and pediocin PA-1 were commercially applied [25]. Moreover, continuous emergence of nisin-resistance bugs lead to the worries and explorations for discovery the novel treatment for food bio-preservation [25]. Therefore, screening for new bacteriocins with a broad inhibition spectrum, such as bacteriocins produced by *Leu. mesenteroides*, that solve the instability of nisin, may play beneficial roles in the control of undesirable pathogens in food. In this study, we tested the inhibition activities of bacteriocin, produced by *Leu. mesenteroides* against pathogens isolated from dairy products. Those results implied that the bacteriocin products might have the potential against pathogens in milk. Pujato *et al.* [26] recently reported a bacteriocin produced by *Leu. citreum* MB1 as biopreservatives against *L. monocytogenes* in milk, claiming bacteriocin producer with low or equal concentration ( $10^4$  CFU/mL) could be able to delay its growth at refrigerated temperature, while bacteriocin producer with higher concentration ( $10^6$  CFU/mL) could totally inhibit *L. monocytogenes*. Similar results were also obtained against *L. monocytogenes* by applying 1000 AU/mL crude bacteriocin in milk [27]. However, their bacteriocin showed weaker activity against *L. monocytogenes* in milk.

Sensory aspects of milk affect the acceptance by consumers [27]. However, there is no literature introducing the sensory evaluation on milk with bacteriocin treated. In this study, milk treated with low concentrated bacteriocin received higher scores overall, whereas there was no significant differences among samples ( $P < 0.05$ ), and the acidity of the samples varied little ( $P < 0.05$ ) as well. Those promising patterns and characteristics suggest bacteriocin with low concentration might be applied on the milk with appearance satisfaction for consumers.

## 5. Conclusion

Bacteriocins/bacteriocin producers have gained much

interest in recent years due to their great potential in antimicrobial applications in food safety and in medicine. Particularly, screening for the bacteriocin producers with broad inhibition spectra is of practical applications, because there is an urgent need for a new source of antimicrobial to fight antibiotic resistant pathogens causing serious problems in infection treatments worldwide. In this work we describe a new bacteriocin producer from *Leu. mesenteroides* isolated from Chinese traditional Tofu, this bacterium showed a potent broad inhibition spectrum against many gram-positive pathogens including the problematic species of *Enterococcus*, *Listeria* and *Staphylococcus*. In addition, it showed great potentials on inhibiting the *L. monocytogenes* in milk.

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