Plantaricin MG active against Gram-negative bacteria produced by *Lactobacillus plantarum* KLDS1.0391 isolated from "Jiaoke", a traditional fermented cream from China

H.S. Gong, X.C. Meng *, H. Wang

Key Laboratory of Dairy Science (Ministry of Education), Northeast Agricultural University, Harbin, People's Republic of China
Food Science and Technology College, Northeast Agricultural University, Harbin, People's Republic of China

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ABSTRACT

A bacteriocin named plantaricin MG produced by *Lactobacillus plantarum* KLDS1.0391 which was isolated from "Jiaoke", a traditional, naturally fermented cream from Inner Mongolia in China is reported in this article. Plantaricin MG was purified by ammonium sulfate precipitation followed by sequential gel filtration chromatography and reverse-phase chromatography. Mass spectrometry analysis showed the mass of plantaricin MG to be approximately 2180 Da. The bacteriocin showed a broad inhibitory activity against Gram-positive and Gram-negative bacteria including *Listeria monocytogenes, Staphylococcus aureus, Salmonella typhimurium* and *Escherichia coli*. The bacteriocin was extremely heat-stable (30 min at 121 °C) and remained active after incubation at pH 2.0–10.0. It was found to be sensitive to proteolytic enzymes (pepsin, trypsin, papain, α-chymotrypsin, proteinase K, Neutrase and Alcalase). The mode of action of plantaricin MG was identified as bactericidal.

1. Introduction

"Jiaoke" is a traditional, naturally fermented cream which is hand-made in Inner Mongolia of China. The fat content of "Jiaoke" is more than 45%. "Jiaoke" is made from unpasteurised bovine milk and is allowed to ferment spontaneously in a crock for 1–2 days at ambient temperature. The microbial flora responsible for the fermentation is derived from the air, raw milk and the containers. After initial fermentation without stirring, the cream floats then the cream layer is removed and poured into a cotton bag. The cotton bag is hanged and other milk components are exuded from the pores. Furthermore, the cream in the bag is further fermented spontaneously at ambient temperature. After 2 days fermentation, "Jiaoke" is successfully made. The lactic acid bacteria (LAB) that are naturally present in the air, raw milk and the containers are responsible for the fermentation. No additional cultures are inoculated into the cream during the fermentation. "Jiaoke" is a good source of lactic acid bacteria. Main LAB strains isolated from "Jiaoke" are *Lactobacillus* spp., *Lactococcus* spp., and to a lesser extent of *Leuconostoc* spp. and *Enterococcus* spp. It is sure that lactic acid and low pH mainly contribute to the storability of "Jiaoke", but "Jiaoke" has longer shelf-life than other products with lactic acid and the same pH, so we supposed that some antibacterial substances e.g. bacteriocins are responsible for its storability.

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by one bacterium that are active against similar or closely related bacteria, and as with host defence peptides, cell signalling mechanisms can also be involved (Bowdish, Davidson, & Hancock, 2005; Cotter, Hill, & Ross, 2005). Although bacteriocins are produced by many Gram-positive and Gram-negative species, those produced by LAB are of particular interest due to their potential application in the food industry as natural preservatives (Paul, Morgan, & Hill, 2002), because many of these bacteria have generally regarded as safe (GRAS) status. Furthermore, as the majority of bacteriocin-producing LAB are natural food isolates, they are ideally suited to food applications (Deegan, Cotter, Hill, & Ross, 2006).

According to the definition of Klaenhammer (Klaenhammer, 1988), bacteriocins produced by LAB are active against closely related bacteria. The structure and composition of the outer membrane of Gram-negative bacteria does not allow access of bacteriocins to the cytoplasmic membrane. However, a few exceptions have been described: e.g. thermophilin 81 produced by *Streptococcus thermophilus* (Ivanova et al., 1998); plantaricin 35d produced by *Lactobacillus plantarum* (Messi, Bondi, Sabia, Battini, & Manicardi, 2001); bacteriocin AS-48 and Enterocin AS-48RJ produced by *Enterococcus faecalis* (Abriouel, Valdivia, Gálvez, & Maqueda, 2001; Abriouel et al., 2005); bacteriocin ST34BR produced by *Lactococcus lactis* subsp. *lactis* (Todorov & Dicks, 2004); bacteriocins ST26MS and ST28MS produced by *Lb. plantarum* (Todorov & Dicks,

* Corresponding author. Present address: Key Laboratory of Dairy Science (Ministry of Education), Northeast Agricultural University, Harbin, People's Republic of China. Tel.: +86 451 55191813; fax: +86 451 55190340.
E-mail address: xchmeng@hotmail.com (X.C. Meng).

2005); a bacteriocin produced by *Enterococcus mundtii* ST15 (De Kwaadsteniet, Todorov, Knoetze, & Dicks, 2005) and bacteriocin AMA-K produced by *Lb. plantarum* AMA-K (Todorov, Nyati, Meincken, & Dicks, 2007). Overall, these bacteriocins have the activities against Gram-negative bacteria.

The objective of this study was to isolate the lactobacilli strains in "Jiaoke" and screen the bacteriocin-producing strain. The second objective was to purify the bacteriocin and examine the partial characteristics of the bacteriocin.

2. Materials and methods

2.1. Lactobacilli isolation and culture conditions

Lactobacilli strains were isolated from 38 samples of "Jiaoke". The samples were serially diluted in sterile 0.9% (w/v) sodium chloride solution, plated onto De man, Rogosa and Sharpe (MRS) agar (Oxoid, England), and incubated at 37 °C for 48 h. Gram-positive, catalase-negative bacilli were identified as presumptive lactobacilli. All presumptive lactobacilli strains were screened for bacteriocins production.

All indicator strains used in this research were stored at −80 °C in 40% (v/v) glycerol. Lactobacilli strains were grown at 37 °C in MRS broth. Lactococcal strains were propagated at 37 °C in M17 broth (Oxoid) supplemented with 0.5% (w/v) lactose. *Listeria monocytogenes* was grown at 37 °C in tryptic soy broth (Oxoid) supplemented with 0.6% (w/v) yeast extract (Oxoid). *Clostridium perfringens* was grown at 37 °C in anaerobic beef liver broth (Aoboxing, Beijing, China). Other indicator strains were incubated at 37 °C in beef extract–peptone media (Aoboxing).

2.2. Screening for antibacterial activity

All lactobacilli strains were grown in MRS broth for 28 h at 30 °C. Cultures were centrifuged at 12,000g for 10 min at 4 °C and supernatants were collected, adjusted to pH 6.5 with 3 M NaOH, and filtered through a 0.22 μm filter (Millipore Corporation, Bedford, MA, USA) to prepare cell-free supernatants (CFSs).

CFSs of all lactobacilli strains were screened for antibacterial activity by agar well diffusion assay (AWDA) as described by Schillinger and Lücke (1989), with some modifications. Ten milliliters of 1.2% (w/v) agar (Sanland-chem International Inc., Xiamen, China) was poured into a sterile plate and dried. Molten soft beef extract–peptone agar (0.7%, w/v) was first seeded with the indicator strain (36 μl of overnight culture per 6 ml of agar, i.e. approximately 10^7 cells). The inoculated soft agar was rapidly dispensed onto an agar plate. After solidification, the soft agar was dried for 30 min under a laminar flow hood. Six millimeters diameter wells were made in these agar plates, and 50 μl of the bacteriocin solution was placed into each well. Plates were held at room temperature for 3 h with their wells unsealed to allow the bacteriocin completely diffused, and these plates were incubated for 24 h at 37 °C. Bacteriocin activity was expressed as the diameter of zone of inhibition (including the well) which was measured by a vernier caliper. In some experiments, antibacterial activity was expressed as arbitrary units (AU). To obtain the titer (AU/ml), serial dilutions of bacteriocin were prepared and dispensed in wells. The titer (AU/ml) was defined as the reciprocal of the highest dilution which gave a definite zone was multiplied by the conversion factor (20 when 50 μl of the bacteriocin solution was used) (Ryan, Rea, Hill, & Ross, 1996). The definite zone was defined as clear zone with the width of 2 mm around the well (Anastasiadou, Papagianni, Filiousis, Ambrosiadis, & Koidis, 2008). Strain with the largest zone of inhibition was selected for the following studies.

2.3. Identification of strain

The sugar fermentation pattern of strain that produced bacteriocin was tested with the API 50 CHL system (bioMérieux, Marcy l'Etoile, France). The obtained sugar fermentation pattern was analyzed by running the APILAB Plus software (bioMérieux). Genotypic identification was confirmed by 16S rDNA sequence analysis. The identity was confirmed by PCR using genus-specific primers (Scarpellini, Diego, Silvia, & Laura, 2002), then the PCR product was sequenced on an automatic sequencer (ABI PRISM 3730; Applied Biosystems SA). Further identification was conducted by PCR-generated DNA banding patterns obtained with species-specific primers for *Lb. plantarum*, *Lactobacillus pentosus* and *Lactobacillus paraplantarum* as described by Torriani, Felis, and Dellaglio (2001).

2.4. Spectrum of antibacterial activity

Five hundreds milliliters CFS was precipitated by 70% saturated ammonium sulfate (Guangfu, Tianjin, China). After overnight stirring at 4 °C the resulting precipitate was collected by centrifugation at 12,000g for 15 min and dissolved in 10 ml of 20 mM sodium acetate buffer (pH 6.5), then the samples precipitated by ammonium sulfate were obtained. The antibacterial activities of the samples were tested against Gram-positive and Gram-negative bacteria. The indicator strains were inoculated in the appropriate soft agar media and the antibacterial activities were determined by AWDA previously described. All experiments were conducted in triplicate.

2.5. Growth and bacteriocin production kinetics

Twenty milliliters of the overnight strain culture was inoculated into 2 L of MRS medium and incubated at 30 °C. At 4-h intervals, 1 ml samples were removed and the viable cell count was determined by enumeration on beef extract–peptone plate agar. Meanwhile 10 ml culture was removed to prepare CFS (pH 6.5). The antibacterial activities of the CFSs were determined by AWDA previously described. *Salmonella typhimurium* ATCC14028 was used as the indicator strain.

2.6. Purification of plantaricin MG

Purification steps were performed at room temperature using ÄKTA purifier 100 and ÄKTA explorer 10 (GE Healthcare). All columns used were purchased from GE Healthcare (Uppsala, Sweden).

Plantaricin MG was purified from 1 L CFS by a three-step procedure. Proteins, including the bacteriocin, were precipitated by 70% saturated ammonium sulfate. After overnight stirring at 4 °C the resulting precipitates were collected by centrifugation at 12,000g for 15 min and dissolved in 20 ml of 0.05 M sodium acetate and 0.1 M sodium chloride (pH 5.0) (buffer A). This solution was load on a gel filtration column (Hiload 26/60 superdex 75 prep grade column) in ÄKTA purifier 100. The column was washed with 480 ml buffer A at a flow rate of 3 ml/min. All peak fractions were collected and tested antibacterial activities by AWDA previously described by using *S. typhimurium* ATCC14028 as the indicator strain. The active fraction obtained from gel filtration was applied to a SOURCE 5RPC ST 4.6/150 column in ÄKTA explorer 10. The active fraction was eluted with a gradient of water–acetonitrile at a flow rate of 1 ml/min, 0–5 ml, 10% (v/v) acetonitrile; 5–44 ml, 10–50% (v/v) acetonitrile; and 44–48 ml, 50% (v/v) acetonitrile. All peak fractions were collected and the antibacterial activities were determined as described above. The purity of the bacteriocin was tested by SinoChrom ODS-BP 4.6/250 column in a high-performance liquid chromatography (HPLC) system (Waters 2695, USA). Mobile phase A was water/0.1% trifluoroacetic acid (TFA) and

mobile phase B was 90% acetonitril/0.1% TFA. The bacteriocin was eluted with 60% mobile phase A and 40% mobile phase B at a flow rate of 1 ml/min. The protein concentration of each fraction was determined by an Enhanced BCA Protein Assay Kit (Beyotime, Jiangsu, China).

2.7. Mass spectrometry

The molecular mass of purified plantaricin MG was analyzed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MS) by a mass spectrometer (UltraFlex 2, Bruker, Germany). A model VSL-337 ND S-nitrogen laser (337 nm) was used. The ion acceleration voltage and laser intensity were set to 20 kV and 35%, respectively. Five microliters of suspension of purified bacteriocin sample was carefully mixed with an equal volume of α-cyano-hydroxicinnamic acid (CHC). One microliter of the prepared solution was spotted onto a Perspective 10×10 MALDI plate and left to dry at room temperature for 10 min before analysis.

2.8. Effect of enzymes, surfactants, pH and temperature on antibacterial activity

The active fraction obtained from gel filtration chromatography was adjusted to 1 mg protein/ml as partial purified bacteriocin (pH 6.5), and then was used to examine antibacterial properties. Aliquots of partial purified bacteriocin were co-incubated with the following enzymes at 37 °C for 2 h, pepsin (800 U/ml, 0.05 M citric acid buffer, pH 2.0, Sigma, USA), papain (600 U/ml, 0.05 M citric acid buffer, pH 2.0, Sigma), trypsin (250 U/ml, 0.05 M sodium phosphate buffer, pH 7.0, Sigma), proteinase K (38 U/ml, pH 7.5, 0.05 M sodium phosphate buffer, Amresco, USA), α-chymotrypsin (100 U/ml, 0.05 M sodium phosphate buffer, pH 7.5, Sigma), Neutrase (250 U/ml, pH 7.0, 0.05 M sodium phosphate buffer, Novozymes, Denmark), Alcalase (250 U/ml, 0.05 M sodium phosphate buffer, pH 8.0, Novozymes) and catalase (2000 U/ml, 0.05 M sodium phosphate buffer, pH 7.0, Sigma). After incubation, all samples were adjusted to pH 6.5 with sterile 3 M NaOH or 3 M HCl and tested for antibacterial activity by AWDA previously described by using *S. typhimurium* ATCC14028 as the indicator strain. Partial purified bacteriocin in buffers without enzyme and buffers alone were used as controls. All experiments were performed in triplicate.

In a separate experiment, the effect of surfactants on antibacterial activity was tested by adding 1% (w/v) sodium dodecyl sulfate (SDS), Tween 20, Tween 80, Triton X-100 and urea, respectively, to partial purified bacteriocin (1 mg protein/ml). Ethylenediaminetetraacetic acid (EDTA) was added to partial purified bacteriocin to yield final concentrations of 0.1 mmol/L, 2.0 mmol/L and 5.0 mmol/L, respectively (Todorov et al., 2007). Untreated partial purified bacteriocin and surfactants at each of these concentrations were used as controls. All samples were incubated at 37 °C for 5 h and then tested for antimicrobial activity by AWDA previously described by using *S. typhimurium* ATCC14028 as the indicator strain.

The effect of pH on antibacterial activity was tested by adjusting partial purified bacteriocin (1 mg protein/ml) to values ranging from 2.0 to 10.0 (at increments of one pH unit) with sterile 3 M NaOH or 3 M HCl. After 72 h of incubation at 37 °C, the samples were re-adjusted to pH 6.5 with sterile 3 M NaOH or 3 M HCl and tested for antibacterial activities by AWDA previously described. All experiments were conducted in triplicate.

The effect of temperature on antibacterial activity was tested by heating partial purified bacteriocin (1 mg protein/ml) at 60 °C/30 min, 80 °C/30 min, 100 °C/30 min and 121 °C/30 min, respectively, then the samples were cooled by cold water immediately, and residual antibacterial activities were tested by AWDA previously described by using *S. typhimurium* ATCC14028 as the indicator strain. In experiments of 121 °C/30 min, heating and cooling time were about 15 min, respectively. All experiments were conducted in triplicate.

2.9. Mode of action of plantaricin MG

To determine the mode of action of plantaricin MG, 50 mg partial purified bacteriocin (the active fraction obtained from gel filtration chromatography and freeze-dried) was added to 4 h-old cultures of *S. typhimurium* ATCC14028 in 100 ml beef extract–peptone medium (Final concentration was 0.5 mg protein/ml). The cultures of *S. typhimurium* ATCC14028 were used as control. Changes in cell density were recorded at 600 nm and the numbers of viable cells (cfu) were determined by plating the samples on extract–peptone agar (1.8%, w/v).

2.10. The efficacy of plantaricin MG against spore form of bacteria

Bacillus cereus and *Bacillus subtilis* were inoculated into beef extract–peptone agar and *C. perfringens* were inoculated into anaerobic beef liver agar (Aoboxing). Both agar were supplemented with 10 mg/L of $MnSO_4 \cdot H_2O$ and incubated at 30 °C for 6 days. The spores were collected with peptone buffered saline (PBS) and the cultures were heated at 85 °C for 15 min in a water bath then rapidly cooled to room temperature with cold water. The spore suspensions were centrifuged (7000g, 20 min, 20 °C) and resuspended in PBS. The spore counts were determined by plating on plate count agar (PCA) at 30 °C for 72 h (Katina, Sauri, Alakomi, & Mattila-Sandholm, 2002; Menteş, Ercan, & Akçelik, 2007). One milligram partial purified bacteriocin (the active fraction obtained from gel filtration chromatography and freeze-dried) was added to 1 ml spore suspension (about 2×10^2 cfu/ml), After 48 h of incubation at 30 °C, the viable cells (cfu) of spore suspensions were determined by plating the samples on extract–peptone agar or anaerobic beef liver agar (1.8%, w/v) at 30 °C for 72 h. The spore suspensions without bacteriocin were used as control group.

2.11. Data analysis

Results are presented as mean value ± standard deviation (SD). The Microsoft Excel 2003 and SAS 9.1.3 statistical program were used for data analysis.

3. Results

### 3.1. Isolation of lactobacilli strains and screening for bacteriocins production

Sixty-seven lactobacilli strains were isolated from 38 samples of "Jiaoke". These isolates belonged to *Lb. plantarum*, *Lb. pentosus*, *Lactobacillus casei*, *Lactobacillus acidophilus*, *Lactobacillus brevis* and *Lactobacillus buchneri*. Fifty-six strains showed antibacterial activity and four strains showed activity against *S. typhimurium* ATCC14028 (data are not shown). From a total of four strains screened for bacteriocin production against *S. typhimurium* ATCC14028, strain KLDS1.0391 with the largest zone of inhibition was selected for the following studies.

3.2. Identification of strain

KLDS1.0391 is a rod-shaped bacillus. Based on sugar fermentation reactions using the API 50 CHL system, its sugar fermentation pattern was 99.9% related to *Lb. plantarum* ATCC14917 (data are not shown). Approximately 1.5 kb fragment of the 16S rDNA was amplified (Fig. 1), the gene sequence of strain KLDS1.0391 showed
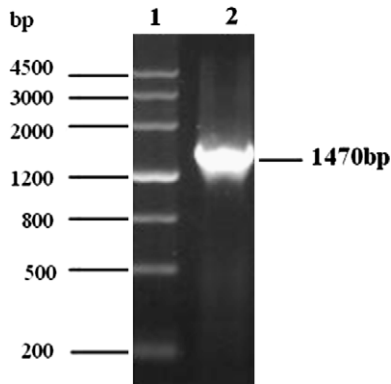
Fig. 1. Agarose gel electrophoresis showing the amplification of the 1470 bp fragment of 16S rDNA for KLDS1.0391. Lane 1, Marker; Lane 2, PCR amplification products.
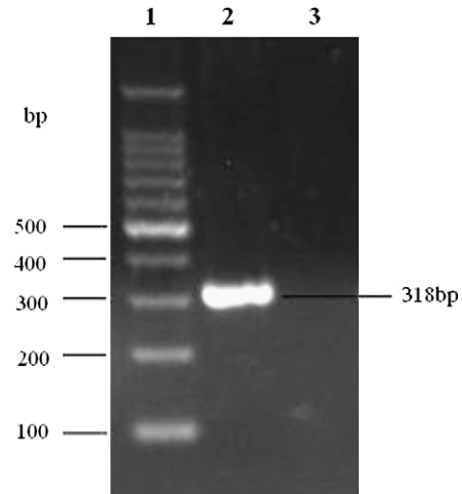
Fig. 2. Amplification products obtained from the *recA* multiplex assay. Lane 1, 100 bp ladder marker. Lane 2, amplification products from KLDS1.0391. Lane 3, amplification products from *Lb. casei* KLDS1.0319 (negative control).

99.8% identity to the 16S rDNA of *Lb. plantarum* ATCC14917 (data are not shown). Amplification of genomic DNA produced a 318 bp fragment of the *recA* gene (Fig. 2) and the DNA sequence amplified revealed 100% homology to the partial *recA* gene sequences of *Lb. plantarum* ATCC14917 (data are not shown). Considering these results, we concluded that strain KLDS1.0391 belonged to *Lb. plantarum*.

3.3. Antibacterial activity spectrum

The antibacterial activity spectrum of samples precipitated by 70% ammonium sulfate for *Lb. plantarum* KLDS1.0391 was assayed by AWDA against a wide range of microorganisms (Table 1). The

antibacterial activity of plantaricin MG was not only evident against Gram-positive bacteria but also against Gram-negative bacteria. Plantaricin MG can inhibit several food spoilage bacteria and food-borne pathogens, Gram-positive bacteria including *L. monocytogenes*, *Staphylococcus aureus Micrococcus luteus*, *C. perfringens*, *B. cereus* and *B. subtilis*; Gram-negative bacteria including enteropathogenic *Escherichia coli*, enterotoxigenic *E. coli*, enteroinvasive *E. coli*, *Pseudomonas fluorescens*, *Pseudomonas putida* and *S. typhimurium*. Plantaricin MG showed high activity against

Table 1
Antibacterial activity spectrum of plantaricin MG produced by *Lb. plantarum* KLDS1.0391.

Species	Strain	Source[a]	Diameter (mm) of zone of inhibition[b]
Streptococcus thermophilus	KLDS3.0203	KLDS-DICC	13.58 ± 0.30
Lc. lactis subsp. *lactis*	KLDS4.0303	KLDS-DICC	13.69 ± 0.01
Lc. lactis subsp. *cremoris*	KLDS4.0316	KLDS-DICC	14.50 ± 0.02
Enterococcus faecalis	KLDS6.0316	KLDS-DICC	16.00 ± 0.18
Enterococcus faecium	KLDS6.0332	KLDS-DICC	14.90 ± 0.32
Lb. delbrueckii subsp. *bulgaricus*	KLDS1.9201	KLDS-DICC	13.34 ± 0.02
Lb. delbrueckii subsp. *delbrueckii*	KLDS1.0324	KLDS-DICC	15.38 ± 0.08
Lactobacillus acidophilus	KLDS1.0380	KLDS-DICC	–
Lactobacillus casei	KLDS1.0319	KLDS-DICC	15.17 ± 0.09
Lactobacillus paracasei	KLDS1.0201	KLDS-DICC	15.36 ± 0.06
Lactobacillus casei subsp. *casei*	KLDS1.0407	KLDS-DICC	12.52 ± 0.28
Lactobacillus plantarum	KLDS1.0314	KLDS-DICC	–
Lactobacillus acetotolerans	KLDS1.0340	KLDS-DICC	12.18 ± 0.14
Lactobacillus brevis	KLDS1.0408	KLDS-DICC	14.50 ± 0.10
Lactobacillus buchneri	KLDS1.0405	KLDS-DICC	14.05 ± 0.05
Lactobacillus rhamnosus	KLDS1.0385	KLDS-DICC	15.21 ± 0.19
Lb. coryniformis subsp. *coryniformis*	KLDS1.0321	KLDS-DICC	12.11 ± 0.09
Lactobacillus alimentarius	KLDS1.0442	KLDS-DICC	13.06 ± 0.02
Staphylococcus aureus	ATCC25923	ATCC	18.24 ± 0.41
Micrococcus luteus	ATCC 9341	ATCC	20.55 ± 1.06
Bacillus subtilis	ATCC6051	ATCC	15.56 ± 0.93
Listeria monocytogenes	NICPBP54002	NICPBP	15.75 ± 0.35
Bacillus cereus	ATCC10987	ATCC	16.32 ± 0.21
Clostridium perfringens	CVCC44	CVCC	15.78 ± 0.45
Escherichia coli	ATCC25923	ATCC	17.50 ± 0.03
Enteropathogenic *E. coli*	CMCC44706	CMCC	16.23 ± 0.60
Enterotoxigenic *E. coli*	CMCC44247	CMCC	16.51 ± 0.60
Enteroinvasive *E. coli*	CMCC44350	CMCC	16.18 ± 0.41
Pseudomonas fluorescens	ATCC17485	ATCC	13.37 ± 0.37
Pseudomonas putida	ATCC13525	ATCC	16.27 ± 0.17
Salmonella typhimurium	ATCC14028	ATCC	16.13 ± 0.27

[a] KLDS-DICC, Key Laboratory of Dairy Science-Dairy Industrial Culture Collection (China); ATCC, American Type Culture Collection; NICPBP, National Institute for the Control of Pharmaceutical and Biological Products (China); CVCC, China Veterinary Culture Collection; CMCC, China Medical Culture Collection.

[b] Wells (6 mm in diameter) were filled with 50 μl samples precipitated by 70% ammonium sulfate; mean counts of tri-trials (mean ± SD); "–"no inhibitory zone was observed.

M. luteus, E. coli, P. fluorescens, P. putida and *S. typhimurium*, but showed low activity against most *Lactobacillus*. spp.

### 3.4. Kinetics of growth and bacteriocin production

Lb. plantarum KLDS1.0391 started to produce bacteriocin at 12 h during the logarithmic growth phase in MRS broth at 30 °C (Fig. 3). Maximum bacteriocin production was reached after 28 h of incubation (the stationary phase). The maximum cell numbers were also reached at 28 h of incubation. Afterward, the antibacterial activity slowly decreased.

3.5. Purification and mass spectrometry

Plantaricin MG produced by *Lb. plantarum* KLDS1.0391 was purified from CFS to homogeneity. By ammonium sulfate precipitation, Hiload 26/60 superdex 75 prep grade column gel filtration chromatography, and SOURCE 5RPC ST 4.6/150 column reverse-
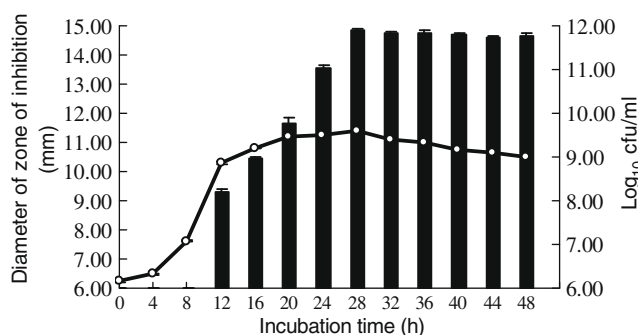
Fig. 3. Plantaricin MG production during growth of *Lb. plantarum* KLDS1.0391 in MRS broth at 30 °C. Legends indicate that the bars are the diameters of zone of inhibition and the line represents $\log_{10}$ cfu/ml. The growth of *Lb. plantarum* KLDS1.0391 was evaluated as viable cell count by enumeration on plate agar, and the activity of plantaricin MG against *Salmonella typhimurium* ATCC14028 was measured by diameter (mm) of zone of inhibition. The experiment was conducted in triplicate, the error bars were standard deviation (SD).
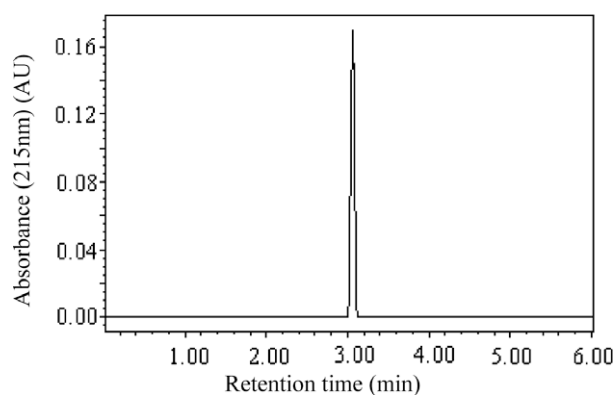
**Fig. 4.** Purity of plantaricin MG identified by HPLC.

phase chromatography, overall, a 25,225-fold purification and 12% yield were obtained, as summarized in Table 2. The purity of plantaricin MG was tested by HPLC. Only one peak appeared at 3 min (Fig. 4). Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) MS analysis of purified bacteriocin revealed that its molecular masses is approximately 2180 Da (Fig. 5).

3.6. Effect of enzymes, surfactants, pH and temperature on antibacterial activity

Completely inactivation activity was observed after treatment of the partial purified bacteriocin with pepsin, trypsin. Partial inactivation of antimicrobial activity was observed after treatment of the partial purified bacteriocin with papain, proteinase K, α-chymotrypsin, Neutrase and Alcalase (Table 3), confirming its proteinaceous nature. Treatment of partial purified bacteriocin with catalase did not result in any changes of antibacterial activity (Table 3), indicating that the inhibition activity was not caused by hydrogen peroxide. Partial purified bacteriocin is not sensitive to treatment with 1% (w/v) SDS, Tween 20, Tween 80, Triton X-100 and urea. When 0.1 mmol/L, 2.0 mmol/L and 5.0 mmol/L EDTA were added to the partial purified bacteriocin, respectively, the antibacterial activity of the mixture is stronger than EDTA or bacteriocin. Partial purified bacteriocin remained stable after 72 h of incubation at pH values between 2.0 and 10.0 (Table 3). No decrease in activity was observed after treatment at 121 °C for 30 min (Table 3). The results suggest that partial purified bacteriocin is strongly heat resistant.

3.7. Mode of action of plantaricin MG

The addition of partial purified bacteriocin (0.5 mg protein/ml) to cells of *S. typhimurium* ATCC14028 in their mid-logarithmic growth phase (4 h-old) resulted in 96.8% decrease in the number
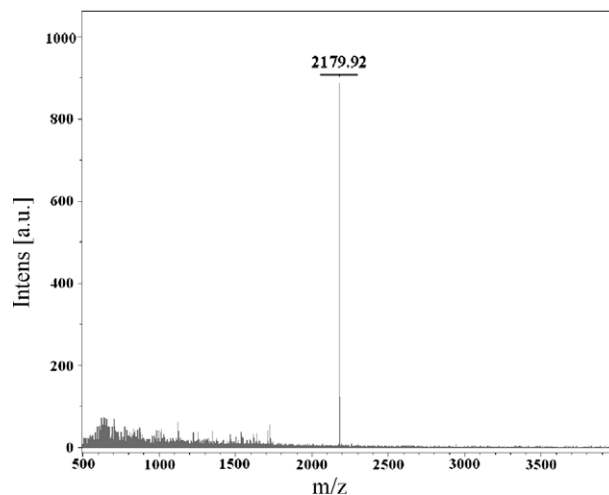
Fig. 5. MALDI-TOF MS-determined mass spectrum of plantaricin MG (mass, 2179.92 Da).

Table 2
Purification of plantaricin MG produced by *Lb. plantarum* KLDS1.0391.

Step	Volume (ml)	Total activity (AU)[a]	Total protein (mg)	Specific activity (AU/mg)	Purification (fold)	Yield (%)
Cell-free supernatant	1000	9600	26,048	0.37	1	100
Ammonium sulfate precipitation	28.6	9552	1711	5.35	14	95
Gel filtration	28.2	4512	101	44.64	120	47
Reverse-phase chromatography	1.4	1120	0.12	9333.33	25,225	12

[a] Antibacterial activity (in arbitrary units [AU]) was assayed by agar well diffusion assay using *Salmonella typhimurium* ATCC14028 as an indicator strain.

Table 3
Effect of enzymes, surfactants, pH and temperature on antibacterial activity of plantaricin MG.

Factors		Diameter (mm) of zone of inhibition[a]
Enzymes	Without enzyme	13.87 ± 0.22
	Pepsin (800 U/ml)	–
	Trypsin (250 U/ml)	–
	Papain (600 U/ml)	10.15 ± 0.27
	Proteinase K (38 U/ml)	10.21 ± 0.20
	α-Chymotrypsin (100 U/ml)	10.51 ± 0.51
	Neutrase (250 U/ml)	10.78 ± 0.42
	Alcalase (250 U/ml)	10.42 ± 0.35
	Catalase (2000 U/ml)	13.92 ± 0.18
Surfactants	SDS (1 mg/ml)	13.85 ± 0.17
	Tween 20 (1 mg/ml)	13.71 ± 0.09
	Tween 80 (1 mg/ml)	13.80 ± 0.11
	Triton X-100 (1 mg/ml)	13.81 ± 0.19
	Urea (1 mg/ml)	13.82 ± 0.09
	EDTA (0.1 mmol/L) without plantaricin MG	10.71 ± 0.41
	EDTA (0.1 mmol/L) with plantaricin MG	14.16 ± 0.48
	EDTA (2 mmol/L) without plantaricin MG	13.66 ± 0.56
	EDTA (2 mmol/L) with plantaricin MG	16.88 ± 0.47
	EDTA (5 mmol/L) without plantaricin MG	14.89 ± 0.42
	EDTA (5 mmol/L) with plantaricin MG	18.61 ± 0.52
pH	pH 2.0	13.02 ± 0.31
	pH 3.0	13.28 ± 0.45
	pH 4.0	13.57 ± 0.32
	pH 5.0	13.76 ± 0.34
	pH 6.0	13.78 ± 0.26
	pH 7.0	13.70 ± 0.15
	pH 8.0	13.63 ± 0.21
	pH 9.0	13.34 ± 0.18
	pH 10.0	13.26 ± 0.27
Temperature	25 °C for 30 min	13.88 ± 0.24
	60 °C for 30 min	13.89 ± 0.32
	80 °C for 30 min	13.89 ± 0.20
	100 °C for 30 min	13.87 ± 0.33
	121 °C for 30 min	13.88 ± 0.28

[a] Wells (6 mm in diameter) were filled with 50 μl partial purified bacteriocin; Mean counts of tri-trials (mean ± SD). "–"no inhibitory zone was observed.
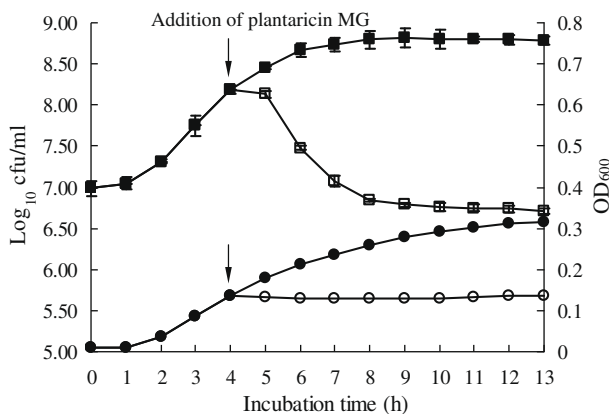
Fig. 6. The effect of plantaricin MG on the growth of *Salmonella typhimurium* ATCC14028. Optical density at 600 nm was observed in the absence (●) and presence (○) of plantaricin MG, respectively; viable cell counts (cfu/ml) were observed in the absence (■) and presence (□) of plantaricin MG, respectively. The experiment was conducted in triplicate, the error bars were standard deviation (SD).

of viable cells (1.6×10^8–5.1×10^6 cfu/ml) over the following 9 h (Fig. 6). In the same experiment, the optical density readings of *S. typhimurium* ATCC14028 remained constant through co-incubation. These results indicate that plantaricin MG acts bactericidally rather than bacteriolytically on sensitive cells.

3.8. The efficacy of plantaricin MG against spore form of bacteria

The addition of partial purified bacteriocin (1 mg protein/ml) to spore suspensions of *B. cereus*, *B. subtilis* and *C. perfringens* (about 2×10^2 cfu/ml), after 48 h of incubation at 30 °C, the spore number of *B. cereus*, *B. subtilis* and *C. perfringens* were reduced to 7×10^1 cfu/ml, 4×10^1 cfu/ml and 6×10^1 cfu/ml, respectively. However, the spore number of *B. cereus*, *B. subtilis* and *C. perfringens* of control groups remain about 2×10^2 cfu/ml. These results indicate that plantaricin MG can inhibit spore form of *B. cereus*, *B. subtilis* and *C. perfringens*.

4. Discussion

We select "Jiaoke" as raw materials for screening bacteriocin-producing lactobacilli strains because it is a good source of lactic acid bacteria strains. In this study, a new *Lb. plantarum* KLDS1.0391 which produced a bacteriocin has been isolated from "Jiaoke". The bacteriocin produced by *Lb. plantarum* KLDS1.0391 termed plantaricin MG has been purified and partially characterized. To our knowledge, this is the first report on the isolation and the partial characterization of bacteriocins produced by *Lb. plantarum* from "Jiaoke".

Plantaricin MG is capable of inhibiting a wide range of Gram-positive bacteria mainly against *L. monocytogenes*, *S. aureus*, *M. luteus*, *B. subtilis* *C. perfringens*, *B. cereus* and some LAB. The activity of plantaricin MG observed against Gram-negative bacteria (*Escherichia*, *Pseudomonas* and *Salmonella*) is an unusual phenomenon. Stevens, Sheldon, Klapes, and Klaenhammer (1991) theorized that bacteriocins of lactic acid bacteria are inefficient to inhibit Gram-negative bacteria because the outer membrane hinders the site for bacteriocin action. Most bacteriocins produced by *Lb. plantarum* merely inhibit Gram-positive bacteria and even the spectrum of activity are rather narrow, e.g. plantaricin S and T produced by *Lb. plantarum* LPCO10 (Jiménez-Díaz, Rios-Sánchez, Desmazeaud, & Ruiz-Barba, 1993); plantaricin C produced by *Lb. plantarum* LL441 (González, Arca, Mayo, & Suárez, 1994); plantaricin D produced by *Lb. plantarum* BFE905 (Franz, Du Toit, Olasupo, Schillinger, & Holzapfel, 1998); plantaricin W produced by *Lb. plantarum* LMG 2379 (Holo, Jeknic, Daeschel, Stevanovic, & Nes, 2001). However, a few bacteriocins produced by *Lb. plantarum* have been reported to be active against Gram-negative bacteria. Bacteriocins ST26MS (2.8 kDa) and ST28MS (5.5 kDa) produced by *Lb. plantarum* ST26MS and ST28MS, respectively, can inhibit *Acinetobacter*, *Escherichia* and *Pseudomonas* (Todorov & Dicks, 2005). Bacteriocin AMA-K (2.9 kDa) produced by *Lb. plantarum* AMA-K can inhibit *E. coli* (Todorov et al., 2007). Plantaricin MG is different from above bacteriocins produced by *Lb. plantarum* in that it has different molecular masses and it can not only inhibit *E. coli*, *P. fluorescens*, *P. putida*, but also inhibit *S. typhimurium*.

Maximal activity of plantaricin MG was observed at stationary growth phase at 30 °C. Maximal activity of a bacteriocin produced by *E. faecium* MMT21 (Ghrairi, Frere, Berjeaud, & Manai, 2008), bacteriocin ST8KF produced by *Lb. plantarum* ST8KF (Powell, Witthuhn, Todorov, & Dicks, 2007) and thoeniicin 447 produced by *Propionibacterium thoenii* 447 (Van der Merwe, Bauer, Britz, & Dicks, 2004) have been also recorded during stationary growth phase. The slow decrease of the antibacterial activity in the later stationary growth phase might be due to the partial digestion of the antibacterial compound by proteolytic enzymes released from the cells.

Treatment of plantaricin MG with proteolytic enzymes (pepsin, trypsin, papain, α-chymotrypsin, proteinase K, Neutrase and Alcalase) resulted in loss of activity, confirming its proteinaceous nature. Some other plantaricins have similar but not absolutely same

characteristics. Plantaricin C activity was completely lost upon treatment with 1 mg/ml pronase, trypsin, and α-chymotrypsin but was not affected by other proteases, such as pepsin and proteinase K, or by α-amylase or lipase (González et al., 1994); plantaricin D was inactivated by α-chymotrypsin, trypsin, pepsin and proteinase K, but not by papain (Franz et al., 1998). No change in activity was observed when partial purified bacteriocin treated with catalase, indicating that hydrogen peroxide was not responsible for inhibition. Plantaricin MG is not sensitive to treatment with 1% (w/v) SDS, Tween 20, Tween 80, Triton X-100 and urea. Similar results were recorded for bacteriocin AMA-K produced by *Lb. plantarum* AMA-K (Todorov et al., 2007). However, Bacteriocins ST26MS and ST28MS produced by *Lb. plantarum* ST26MS and ST28MS are sensitive to treatment with 1% (w/v) Tween 20, Tween 80, Triton X-114 and Triton X-100. But 1% (w/v) SDS and urea had no effect on antimicrobial activity (Todorov & Dicks, 2005); plantaricin C19 produced by *Lb. plantarum* C19 lost its activity after treatment with SDS or Triton X-100 (Atrih, Rekhif, Moir, Lebrihi, & Lefebvre, 2001); bacteriocin ST8KF produced by *Lb. plantarum* ST8KF lost its activity after treatment with Triton X-100 (Powell et al., 2007). EDTA enhanced partial purified bacteriocin antimicrobial activity against *S. typhimurium* ATCC14028. EDTA has antimicrobial activity and is known to potentiate the antimicrobial activities of bacteriocin and antibiotics, especially against Gram-negative microorganisms. Gram-negative bacteria are shielded from partially hydrophobic molecules such as bacteriocin by the outer membrane lipopolysaccharides (LPS). EDTA permeabilizes the outer membrane by releasing the LPS. This allows bacteriocin to reach the cytoplasmic membrane, and explains why combinations of bacteriocin with EDTA inhibited Gram-negative strains. Bacteriocins produced by LAB are generally highly stable under acidic conditions, but many of them, including nisin, are easily inactivated under neutral and alkaline conditions (Delves-Broughton, Blackburn, Evans, & Hugenholtz, 1996). This has been an obstacle in the expansion of their application. Plantaricin MG was found to be stable after incubation for 72 h at pH values between 2.0 and 10.0, i.e. it remained stable under neutral and alkaline pH conditions. Plantaricin MG was also thermostable, and thermostability would be a very useful characteristic if it was to be used as a food preservative, because many food-processing procedures involve the heating step. In conclusion, plantaricin MG was stable over a wide range of pH and after heat treatment, similarly to other plantaricins, e.g. plantaricin 35d (Messi et al., 2001), plantaricin TF711 (Hernández, Cardell, & Zárate, 2005), bacteriocin ST8KF (Powell et al., 2007), bacteriocin AMA-K (Todorov et al., 2007), and bacteriocin J23 (Rojo-Bezares et al., 2007). On the other hand, there are also other heat susceptible plantaricins which are active in a narrow range of pH, this is the case of plantaricin F (Fricourt, Barefoot, Testin, & Hayasaka, 1994), plantaricin LC74 (Rekhif, Atrih, & Lefebvre, 1994), and plantaricin UG1 (Enan, el-Essawy, Uyttendaele, & Debevere, 1996).

Plantaricin MG has a bactericidal effect on sensitive strain with no concomitant cell lysis, since 96.8% decrease in the number of viable cells (1.6×10^8–5.1×10^6 cfu/ml) of *S. typhimurium* ATCC14028 in the presence of plantaricin MG was detected without OD600 nm decrease. A similar mode of action has been observed in other bacteriocins from LAB, e.g. plantaricin 423 produced by *Lb. plantarum* 423 (van Reenen, Dicks, & Chikindas, 1998) and Lactococcin MMT24 produced by *Lactococcus lactis* MMT24 (Ghrairi, Frère, Berjeaud, & Manai, 2005). Plantaricin 423 has a weakly bactericidal effect on *Oenococcus oeni* 19Cl, since a slow decrease in the number of viable cells (2.5×10^6–1×10^6) over a period of 24 h was detected without OD600 nm decrease. Lactococcin MMT24 has a bactericidal effect on *Lc. cremoris* ATCC11603 with no concomitant cell lysis, since a decrease in the number of viable cells (3.5×10^7–6.2×10^4 cfu/ml) in the presence of Lactococcin MMT24 (160 AU/ml) was detected without OD600 nm decrease.

Although *B. cereus*, *B. subtilis* and *C. perfringens* spores occur naturally in soil and atmosphere, they occasionally contaminate raw meats and milk. Because of their relatively high resistance to heat, low water activity (a_w), and high salt, they survive the curing and pasteurization treatments given to minimally processed meat products and dairy products. Plantaricin MG can inhibit not only viable cells but also spores form of these bacteria. These results indicated that plantaricin MG has potential value in minimally thermally processed foods. Similar results were recorded for *Lb. plantarum* VTT E-78076, *Pediococcus pentosaceus* VTT E-90390, *Lb. plantarum* LMO25 and *Lb. alimentarius* LMO7. These bacteria were shown inhibition against the growth of rope-forming *Bacillus* strains (*B. subtilis* and *B. licheniformis*) in wheat bread (Katina et al., 2002; Menteş et al., 2007).

Due to the properties of plataricin MG, i.e. broad antibacterial activity spectrum, heat stability and stability over a wide range of pH, plantaricin MG can effectively be used as biopreservative in a wide number of foods.

Acknowledgment

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