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ORIGINAL ARTICLE

Characterization of a broad spectrum bacteriocin produced by *Lactobacillus plantarum* MXG-68 from Inner Mongolia traditional fermented koumiss

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Abstract

An agar well diffusion assay (AWDA) was used to isolate a high bacteriocin-producing strain with a broad spectrum of antibacterial activity, strain MXG-68, from Inner Mongolia traditional fermented koumiss. *Lactobacillus plantarum* MXG-68 was identified by morphological, biochemical, and physiological characteristics and *16S rDNA* analysis. The production of antibacterial substance followed a growth-interrelated model, starting at the late lag phase of 4 h and arriving at a maximum value in the middle of the stationary phase at 24 h. Antibacterial activity was abolished or decreased in the presence of pepsin, chymotrypsin, trypsin, proteinase, and papain K. The results showed that antibacterial substances produced by *L. plantarum* MXG-68 were proteinaceous and could thus be classified as the bacteriocin, named plantaricin MXG-68. The molar mass of plantaricin MXG-68 was estimated to be 6.5 kDa, and the amino acid sequence of its N-terminal was determined to be VYGPAGIFNT. The mode of plantaricin MXG-68 action was determined to be bactericidal. Bacteriocin in cell-free supernatant (CFS) at pH 7 was stable at different temperatures (60 °C, 80 °C, 100 °C, 121 °C for 30 min; 4 °C and − 20 °C for 30 days), as well as at pH 2.0–10.0. Antibacterial activity maintained stable after treatment with organic solvents, surfactants, and detergents but increased in response to EDTA. Response surface methodology (RSM) revealed the optimum conditions of bacteriocin production in *L. plantarum* MXG-68, and the bacteriocin production in medium optimized by RSM was 26.10% higher than that in the basal MRS medium.

Keywords Bacteriocin · Screening · *Lactobacillus plantarum* · Characterization · Response surface methodology

Introduction

Food safety is essential to human health, and food spoilage can bring about not only huge economic losses but also serious illnesses (Goyal et al. 2018). Microbial pollution caused by pathogens such as *Salmonella Typhimurium*, *Listeria monocytogenes*, *Shigella flexneri*, *Escherichia coli*, and *Staphylococcus aureus* is a primary cause of food spoilage (Gaspar et al. 2018; Lv et al. 2018a). Use of biopreservatives such as lactic acid bacteria (LAB) and their bacteriocins is a promising way to inhibit microbial pollution, lengthen shelf life, and ameliorate food quality (Wayah and Philip 2018; Xi et al. 2018).

Antibacterial peptides known as bacteriocins that are ribosomally synthesized by bacteria can inhibit the growth of similar or closely related bacterial strains under common circumstance (Nishie et al. 2012). Bacteriocins of LAB play a very important role in the food industry as natural preservatives, and most bacteriocins produced by LAB are generally recognized as safe (GRAS) (Kumar et al. 2016; Lan et al. 2012; Merzoug et al. 2016). Nevertheless, nisin is the only FDA (1988) and WHO (1961) certified bacteriocin in food application and the only commercially licensed lactic acid bacteriocin, being approved as a natural food additive by about 50 countries at present (Komora et al. 2017; López et al. 2017; Peres et al. 2012). Oladunjoye et al. (2016)

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showed that 5000 IU/mL nisin could effectively inhibit the growth of *Listeria monocytogenes* in fresh-cut tomato, while Na-kyoung et al. (2015) showed that the shelf life of beef jerky was prolonged and the growth of spoilage bacteria and pathogenic bacteria was inhibited by treatment with 100 IU nisin/g for 3 days and 500 IU nisin/g for 21 days respectively. Nisin also has practical applications as a preservative in foods, such as cheese, cream, pasteurized milk, canned vegetables, and alcoholic beverages (Gharsallaoui et al. 2016). However, the number of certified and commercialized bacteriocins produced by LAB is limited because of their narrow antibacterial spectrum, poor stability, comparatively low yield or unclear action mechanism. Hence, broad-spectrum bacteriocins with good stability secreted by LAB strains from different food sources are required, which is beneficial to both food safety and commercialized application.

Bacteriocin-producing strains of *L. plantarum* have been reported from many food sources, including milk, meat and meat products, cheese, doughs, fermented cucumber, olives, silage, suan-tsai, grapefruit juice, and pineapple (Todorov et al. 2014). Certain bacteriocins produced by *L. plantarum* have been reported to inhibit pathogenic bacteria and spoilage bacteria including Gram-positive bacteria and Gram-negative bacteria, as well as to have good stability. These include bacteriocin LD4, bacteriocin BH-1, plantaricin KL-1Y, plantaricin LpU4, and plantaricin MG (Botthoulath et al. 2018; Kumar et al. 2016; Man et al. 2014; Milioni et al. 2015; Rumjuankiat et al. 2015). The action mode of *L. plantarum* bacteriocins is usually bactericidal, although bacteriostatic effects have been reported for plantaricin C19 and plantaricin TF711 (Milioni et al. 2015). Given the broad antimicrobial spectrum against pathogenic bacteria and the stability characteristics under adverse conditions, the bacteriocin of *L. plantarum* has the potential for application in food safety as well as therapeutics.

Koumiss is a traditionally fermented mare's milk popular among the people of Mongolia and has been used for decades for its health-promoting potential, which includes increasing immunity and treating cardiovascular disease and tuberculosis (Rong et al. 2015). The composition of microorganisms in koumiss is very complex and includes various types of *Lactobacillus*, with the dominant species being *L. plantarum*, *L. helveticus*, and *L. casei* (Sun et al. 2010; Wu et al. 2009). Some studies have screened and characterized *Lactobacillus* strains from koumiss (Pan et al. 2010; Sedláček et al. 2010; Wang et al. 2011). However, only a few studies have characterized *L. plantarum* bacteriocin originating from koumiss, which possesses a broad spectrum of antibacterial activity (Xie et al. 2011).

Therefore, this study was conducted to screen and identify strains originating from Inner Mongolia traditional fermented koumiss for potential isolates with a broad antibacterial spectrum. The second goal was to confirm the characteristics of the antibacterial substances, and to optimize the nutrients required for the production of antibacterial substances using RSM.

Materials and methods

Samples, indicator strains, and cultivation conditions

Twenty koumiss samples were collected from the Zhongqi, Houqi, Zhaqi, Zharuteqi, and Namanqi areas of Tongliao, Inner Mongolia Autonomous Region of China. Media and chemical compounds were purchased from Oxoid and Sigma respectively. All indicator strains were preserved at − 80 °C. The indicator strains were grown as follows: *Lactobacillus plantarum*, *Lactobacillus sakei*, and *Lactobacillus acidophilus* were cultured at 37 °C in De manrogosa-sharpe medium (MRS) (Oxoid, England); *Lactococcus lactis* was cultivated at 37 °C in M17 medium (Oxoid, England); *Clostridium perfringens* and *Clostridium sporogenes* were incubated at 37 °C in reinforced clostridial medium (RCM) (Oxoid, England); *Listeria monocytogenes* was cultured at 37 °C in tryptic soy broth enriched with 0.6% yeast extract (TSA-YE) (Oxoid, England); *Pseudomonas aeruginosa* and *Enterococcus faecalis* were cultivated at 37 °C in Luria-Bertani medium (LB) (Oxoid, England); all other indicator strains including *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*, *Shigella dysenteriae*, *Micrococcus luteus*, *Escherichia coli*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Salmonella Typhimurium*, and *Salmonella enterica Typhimurium* were grown at 37 °C in nutrient broth medium (NB) (Oxoid, England).

Preparation of cell-free supernatant (CFS) and determination of antibacterial activity

Cell-free supernatants were obtained by centrifuging the cultures at 12,000×*g* for 10 min at 4 °C, after which they were adjusted to pH 7.0 using 1 M NaOH and treated with hydrogen peroxidase to eliminate the antibacterial influence of acids and hydrogen peroxide and then filter-sterilized using a filter with a pore size of 0.22 μm.

The antibacterial activity was measured by agar well diffusion assay (AWDA). Briefly, homologous medium agar (0.7%) inoculated with indicator strain (10^7 CFU/mL) was overlaid onto 10 mL of 1.2% agar. Wells with a 6-mm diameter were cut out of the plate and filled with 50 μL of CFS. The plates were held at room temperature for 3 h in a laminar air flow hood, then cultivated at 37 °C for 12 h, after which the inhibition diameters on the plate were measured using a digital

vernier caliper. Inhibition diameters were reported as the mean ± standard deviation (SD) in millimeter ($n = 3$).

Isolation and screening of antibacterial substance-producing strains

A total of 25 mL of koumiss was added to 225 mL sterilized normal saline, after which tenfold serial dilutions were performed. Next, 100 μL of suitable dilutions were plated on the corresponding medium for isolation of LAB. MRS agar medium and M17 agar medium were used to isolate lactobacilli and lactococci strains. The plates were cultured at 37 °C for 48–72 h. Gram-positive strains that were catalase negative were considered potential LAB, and needed to be isolated and purified for further characterization. Additionally, lactobacilli and lactococci strains (about 10^9 CFU/mL) were further cultivated in MRS medium and M17 medium, respectively, at 30 °C for 24 h. CFS samples were used to screen for antibacterial substance-producing strains by the AWDA method.

Identification of antibacterial substance-producing strains

Microscopic observation and Gram staining were applied to observe the individual morphologies of strain MXG-68. Colony morphology of strain MXG-68 was observed after cultivation on MRS agar medium for 48 h at 37 °C. Additionally, biochemical tubes were used to confirm the biochemical and physiological characteristics of the strain. Bergey's Manual of Systematic Bacteriology was then utilized for preliminarily species identification of strain MXG-68.

Molecular identification was conducted by amplification and sequencing of the *16S rDNA* gene of strain MXG-68. The total genomic DNA of strain MXG-68 was obtained using a TIANamp Bacteria DNA Kit (Tiangen, China). The MXG-68-F and R primers (5′-GACG AACGCTGSCGGCGTGCCTAAT-3′ and 5′-GGTG ATCCAAC CGCAGGTTCTCCTA-3′) based on known sequences of *16S rDNA* in *L. plantarum* (GenBank accession numbers KC429782.1, NR_115605.1, NR_113338.1, NR_104573.1, NR_042394.1) were utilized to amplify the *16S rDNA* by PCR using the following program: 94 °C for 5 min followed by 30 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 min, 30 cycles, and then final extension at 72 °C for 10 min, after which the samples were preserved at 4 °C. The PCR products were identified by agarose gel electrophoresis at 100 V and then sequenced. The sequences were subsequently utilized for nucleotide BLAST searches of the NCBI database, and the neighbor-joining method was used to construct a phylogenic tree.

Characterization of antibacterial substances produced by *L. plantarum* MXG-68

Determination of growth kinetics and antibacterial substance production

One percent of *L. plantarum* MXG-68 (about 10^9 CFU/mL) inoculated in MRS medium was cultivated at 30 °C for 32 h, during which time samples were taken at 2 h intervals for enumeration (CFU/mL) by the plate colony counting method. The CFS of cultures was used to measure antibacterial activity by the AWDA method, with *S. Typhimurium* ATCC14028 as the indicator strain.

Determination the nature of antibacterial substance

One percent of *L. plantarum* MXG-68 (about 10^9 CFU/mL) inoculated in MRS medium (pH 6.5) was incubated at 30 °C for 24 h, after which the CFS was gathered for use in future experiments.

To ascertain the nature of the antibacterial substance, CFS was amended with pepsin, trypsin, chymotrypsin, papain, proteinase K, α-amylase, or lipase to a final concentration 1 mg/mL, and was then cultivated at 37 °C for 2 h. A portion of untreated CFS was cultured for 2 h at 37 °C as a control. The inhibition diameters were determined by the AWDA method using *S. typhimurium* ATCC14028 as an indicator strain.

Measurement of molar mass and N-terminal amino acid sequence of antibacterial substance

Tricine-SDS-PAGE was used to test the molar mass of the antibacterial compound. Briefly, CFS was subjected to ammonium sulfate precipitation to 50% saturation, resuspended in sterile water, and dialyzed at 4 °C for 24 h against potassium-sodium phosphate buffer (20 mM, pH 6.0). The partially purified bacteriocins with antibacterial activity were analyzed by Tricine-SDS-PAGE along with protein molar mass marker at 100 V for about 5 h. The gel was then divided into two parts after electrophoresis, one that was dyed with Coomassie Brilliant Blue R250 (CBB R250) and another that was washed with sterile water and overlaid with indicator *S. typhimurium* ATCC14028 (10^7 CFU/mL) in NB semisolid medium to determine the antibacterial activity. The bacteriocin was further purified by ion exchange chromatography, gel filtration chromatography, and reverse-phase chromatography, after which the purified bacteriocin was used to detect the N-terminal amino acid sequence by the Edman degradation method utilizing a 494cLC automated protein sequencer (Applied Biosystems, USA). The N-terminal amino acid sequence that we acquired was compared with sequences available in the NCBI database (https://blast.ncbi. nlm.nih.gov/Blast) to

determine the homology with sequences that were previously reported.

Determination of action mode of antibacterial substance

Cell-free supernatants (20 mL) were mixed with 4-h-old cultures of *S. typhimurium* ATCC14028, and then added to 80 mL of NB medium. Additionally, 20 mL of MRS medium was used in place of the CFS as a control. The samples were then incubated at 37 °C for 14 h, during which time, samples were taken at regular intervals of 1 h for enumeration of viable cells and determination of the cell densities (OD_{600}) of *S. typhimurium* ATCC14028 by the plate colony counting method and turbidimetric assay at 600 nm, respectively.

Influence of different temperatures, pH, organic solvents, surfactants, and detergents on antibacterial activity

To identify the temperature stability of the antibacterial substances, CFS samples were treated at 60 °C, 80 °C, or 100 °C for 30 min in a water bath, autoclaved at 121 °C for 30 min, or refrigerated at 4 °C and − 20 °C for 30 days. Untreated CFS at room temperature was used as a control.

To determine the pH stability of the antibacterial substance, CFS was adjusted to various pH values between 2.0 and 10.0 with 1 M HCl or 1 M NaOH, respectively, after which samples were incubated at 37 °C for 2 h and the pH was readjusted to pH 7.0. To ascertain the influence of organic solvents on antibacterial substance, CFS was supplemented with 1% (*v*/*v*) ethanol, isopropanol, methanol, acetonitrile, acetone, ethyl acetate, or surfactants. The impact of surfactants on the antibacterial substance was measured by adding 1 mg/mL of Tween-80, Tween-20, and Triton X-100 and 0.1 mg/mL, 1.0 mg/mL, and 5.0 mg/mL of EDTA into CFS. CFS supplemented with 1 mg/mL of urea and SDS was used to determine the impact of the detergents on antibacterial substances. All samples were cultivated at 37 °C for 2 h. In addition, untreated CFS incubated at 37 °C for 2 h was used as a control. The inhibition diameters against *S. typhimurium* ATCC14028 were then measured by AWDA.

Optimization of nutrients for the bacteriocin production using response surface methodology (RSM)

The Box-Behnken design (BBD) of RSM was applied to investigate the influence of modified MRS media on bacteriocin production. The design was consisted of 29 runs including five replications at the center points for appraising the purely experimental indeterminacy variance. The modified MRS media were consisted of the basal MRS media and lactose, tryptone, ascorbic acid, and EDTA at different levels (Table 4). Twenty-nine types of modified MRS media and basal MRS medium were used to cultivate *L. plantarum* MXG-68 for 24 h at 30 °C separately. The supernatants were then acquired, and their inhibition diameters (the response) against *S. typhimurium* ATCC14028 were determined by the AWDA method.

Statistical analysis

All experiments were operated in triplicate and the results were reported as the mean ± standard deviation (SD). SPSS 17.0 was used to conduct statistical analysis of the experimental results, while Design Expert 8.0.6.1 was used to identify the nutrients required for bacteriocin production by *L. plantarum* MXG-68. Additionally, Fisher's test for analysis of variance (ANOVA) was used to determine the statistical significance of the model.

Results

Isolation and screening of antibacterial substance-producing strains

Eighty-six lactobacilli and lactococci strains were isolated from Inner Mongolia traditional koumiss samples, of which 41 demonstrated antibacterial activity against *L. monocytogenes* ATCC15313, *B. cereus* ATCC11788, *E. coli* ATCC25922, and *S. typhimurium* ATCC14028. The inhibition diameters of strain MXG-68 were significantly higher than those of the other 40 strains ($P < 0.01$), hence, it was selected for further experiments. Strain MXG-68 demonstrated a broad spectrum of antibacterial activity against both Gram-negative bacteria and Gram-positive bacteria as shown in Table 1.

Identification of strain MXG-68

Strain MXG-68 was identified by morphological, biochemical, physiological, and molecular identification methods. The strain was a rod-shaped and Gram-positive bacillus that produced milky-white, small, dome-shaped colonies with protrusions and opacification. Strain MXG-68 was negative for the gelatin liquefaction, nitrate reduction, H_2S, indole, catalase, ammonia production, starch hydrolysis, V. P, arginine hydrolysis, and rhamnose fermentation tests, while it was positive for gas production from glucose fermentation. Additionally, strain MXG-68 had the ability to ferment many carbohydrates including glucose, sucrose, esculin, fructose, maltose, galactose, lactose, mannitol, raffinose, sorbitol, ribose, pectinose, xylose, melibiose, and cellulose. The growth of strain MXG-68 was not evidently affected by 6.5%

Table 1 The activity of antibacterial substances produced by *L. plantarum* MXG-68 based on selected indicator strains

Species	Medium[a]	Type[b]	strain	Source[c]	Antibacterial activity[d]
Staphylococcus aureus	NB	G^+	ATCC12600	ATCC	++
Staphylococcus aureus	NB	G^+	ATCC25923	ATCC	+
Staphylococcus aureus	NB	G^+	PTCC1112	PTCC	+
Clostridium perfringens	RCM	G^+	ATCC3624	ATCC	++
Clostridium sporogenes	RCM	G^+	PTCC1265	PTCC	+
Listeria monocytogenes	TSA-YE	G^+	NICPBP54002	NICPBP	++
Bacillus subtilis	TSA-YE	G^+	ATCC6051	ATCC	+
Bacillus cereus	NB	G^+	ATCC10987	ATCC	+
Bacillus cereus	NB	G^+	ATCC11788	ATCC	++
Micrococcus luteus	NB	G^+	ATCC9341	ATCC	++
Lactobacillus plantarum	MRS	G^+	CGMCC1.128	CGMCC	-
Lactobacillus plantarum	MRS	G^+	CGMCC1.556	CGMCC	-
Lactobacillus sakei	MRS	G^+	PTCC1712	PTCC	-
Lactobacillus acidophilus	MRS	G^+	ATCC4356	ATCC	+
Lactococcus lactis	M17	G^+	ATCC15577	ATCC	-
Lactococcus lactis	M17	G^+	PTCC1403	ATCC	-
Enterococcus faecalis	LB	G^+	CGMCC1.125	PTCC	++
Escherichia coli	NB	G^-	ATCC25922	PTCC	+++
Escherichia coli	NB	G^-	CGMCC1.1580	CGMCC	++
Escherichia coli	NB	G^-	ATCC35150	ATCC	++
Pseudomonas fluorescens	NB	G^-	ATCC17485	CGMCC	++
Pseudomonas putida	NB	G^-	CGMCC1.645	ATCC	++
Pseudomonas aeruginosa	LB	G^-	ATCC49189	ATCC	+
Salmonella Typhimurium	NB	G^-	ATCC13311	CGMCC	++
Salmonella Typhimurium	NB	G^-	CGMCC1.1552	ATCC	+
Salmonella Typhimurium	NB	G^-	ATCC14028	ATCC	+++
Salmonella enterica Typhimurium	LB	G^-	ATCC14028	CGMCC	+
Salmonella enterica Typhimurium	LB	G^-	ATCC43972	TCC	++

[a] *NB*, nutrient broth medium; *RCM*, reinforced clostridial medium; *TSA-YE*, tryptic soy broth supplemented with 0.6% (*w*/*v*) yeast extract; *MRS*, De man-Rogosa-Sharpe medium; *LB*, Luria-Bertani medium. [b] G^+ Gram-positive bacteria; G^-: Gram-negative bacteria. [c] *ATCC*, American Type Culture Collection; *PTCC*, Persian Type Culture Collection; *NICPBP*, National Institute for the Control of Pharmaceutical and Biological Products (China); *CGMCC*, China General Microbiological Culture Collection Center. [d] -, no inhibition zone; +, 5–10 mm; ++, 10–15 mm; +++, 15–20 mm

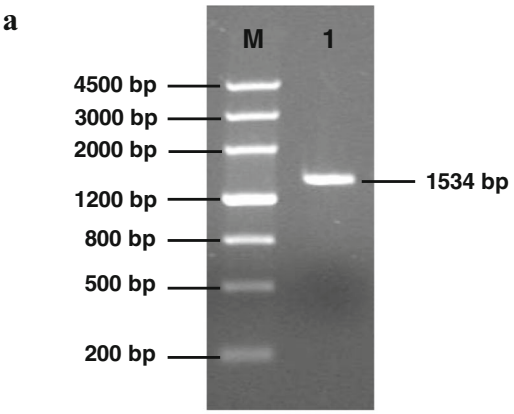
NaCl or 10% NaCl ($P > 0.05$). Based on the comparison of the morphological, biochemical, and physiological features to those of reference strains in Bergey's Manual of Systematic Bacteriology, strain MXG-68 was preliminarily identified as *L. plantarum*.

Molecular identification was conducted by amplification and sequencing of the *16S rDNA* in strain MXG-68. The band produced by PCR method was approximately 1500 bp, and sequencing confirmed that the band was 1534 bp (Fig. 1a). The sequence was submitted to GenBank (NCBI) under accession number KY750314. To further determine if strain MXG-68 belonged to *L. plantarum*, a phylogenetic tree was generated to compare its *16S rDNA* to that of other lactobacilli strains. Upon analysis, *16S rDNA* of strain MXG-68 (KY750314), *L. plantarum* JCM1149 (NR_115605.1), *L. plantarum* NBRC15891 (NR_113338.1), *L. plantarum* CIP103151 (NR_104573.1), and *L. plantarum* NRRL B-14768 (NR_042394.1) were clustered into one group (Fig. 1b). Additionally, the *16S rDNA* of strain MXG-68 exhibited high homology of 99.47–99.93% with other members of this group. These results further demonstrated that strain MXG-68 was *L. plantarum*.

Characterization of antibacterial substance produced by *L. plantarum* MXG-68

Growth kinetics and production of antibacterial substance

As shown in Fig. 2, *L. plantarum* MXG-68 exhibited a presentative sigmoidal growth curve composing of an ephemeral lag phase of 4 h, arriving late log phase at 14 h and afterward



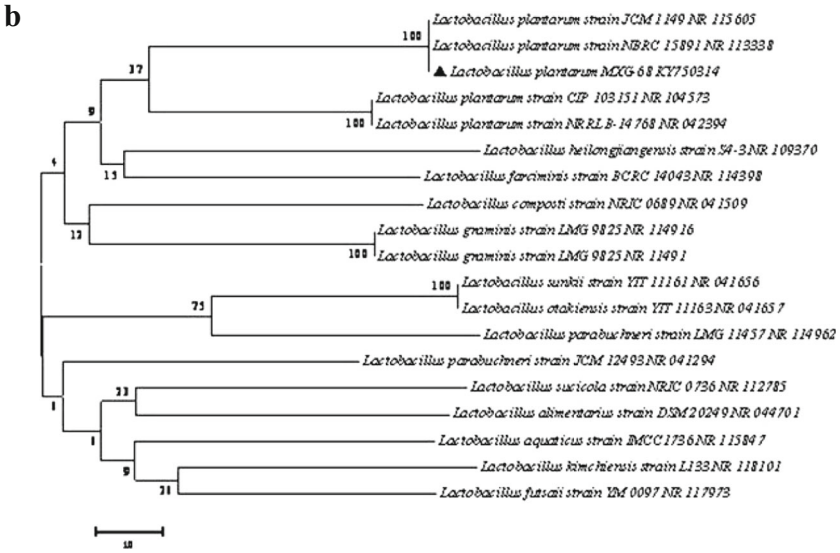


Fig. 1 **a** Agarose gel electrophoresis of PCR amplification products with primers MXG-68-F and MXG-68-R designed to amplify the *16S rDNA* of strain MXG-68. M, marker III. Lane 1: amplicon of *16S rDNA* in strain MXG-68. **b** Phylogenetic trees derived from the *16S rDNA* sequence of *L. plantarum* MXG-68. All sequences were from lactobacilli strains

stationary phase. The production of the antibacterial substance followed a growth-interrelated model, beginning at the late lag phase of 4 h and arriving at the maximum value in the middle of the stationary phase (24 h). Antibacterial activity exhibited a rapid upward trend from 4 to 24 h, as well as a slow downward trend from 24 to 32 h.

Nature of antibacterial substance

Entire inactivation was discovered after treatment of CFS with pepsin and trypsin, and antibacterial activity was obviously reduced after treatment of CFS with chymotrypsin, papain and proteinase K ($P < 0.01$); however, α-

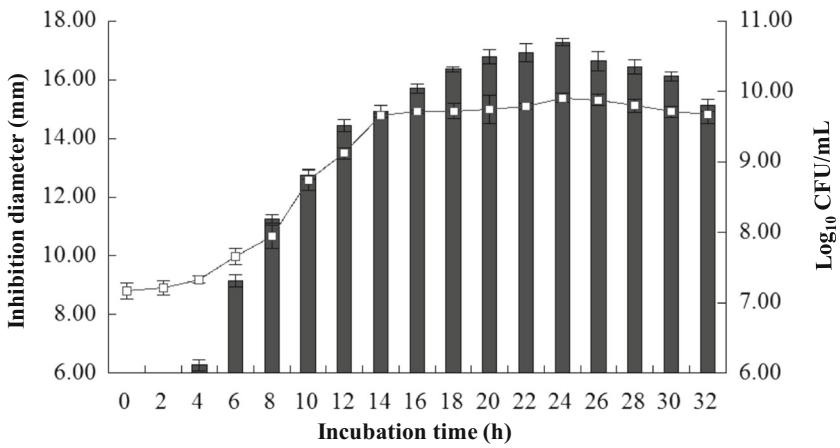


Fig. 2 Growth (turning curve) and antibacterial substance production (column) kinetics of *L. plantarum* MXG-68 at 30 °C for 32 h. Cell numbers are expressed as the mean ± SD of Log_{10} CFU/mL ($n = 3$). Inhibition diameter against *Salmonella typhimurium* ATCC14028 is expressed as the mean ± SD of mm ($n = 3$)

amylase and lipase did not impact its antibacterial activity ($P > 0.05$) (Table 2). These findings indicated that the antibacterial substance was of a proteinaceous nature and could therefore be grouped in the bacteriocin family. The bacteriocin produced by *L. plantarum* MXG-68 was named plantaricin MXG-68.

Molar mass and N-terminal amino acid sequence of bacteriocin

Upon Tricine-SDS-PAGE, the partially purified bacteriocin generated a protein band with a molar mass of about 6.5 kDa in the stained part of the gel, while the other part overlaid with *S. typhimurium* ATCC14028 generated an inhibition zone corresponding to the protein band (Fig. 3).

The N-terminal amino acid sequence of plantaricin MXG-68 was VYGPAGIFNT which showed no apparent homology with other known bacteriocins produced by *L. plantarum* upon a BLAST search of the GenBank database.

Action mode of plantaricin MXG-68

As shown in Fig. 4, the growth of *S. typhimurium* ATCC14028 was restrained in response to the addition of CFS, but there was no entire loss in viable cell numbers of indicator strain *S. typhimurium* ATCC14028. The presence of CFS brought about 99.7% decrease in the viable cell numbers of *S. typhimurium* ATCC14028 in the late logarithmic phase (10 h). Moreover, the OD_{600} of *S. typhimurium* ATCC14028 which included both dead and viable cells, showed no significant changes during co-cultivation with CFS from 4 h to 14 h. These results demonstrated that plantaricin MXG-68 produced by *L. plantarum* MXG-68 exerted bactericidal action rather than bacteriostatic action.

Table 2 Effect of hydrolytic enzymes on antibacterial activity

Serial number	Treatment	Inhibition diameter (mm)
1	Control: untreated CFS	17.26 ± 0.02
2	CFS with 1 mg/mL pepsin	-
3	CFS with 1 mg/mL trypsin	-
4	CFS with 1 mg/mL chymotrypsin	15.41 ± 0.32
5	CFS with 1 mg/mL papain	13.63 ± 0.17
6	CFS with 1 mg/mL proteinase K	13.06 ± 0.45
7	CFS with 1 mg/mL α-amylase	17.20 ± 0.26
8	CFS with 1 mg/mL lipase	17.23 ± 0.18

-, no inhibition zone

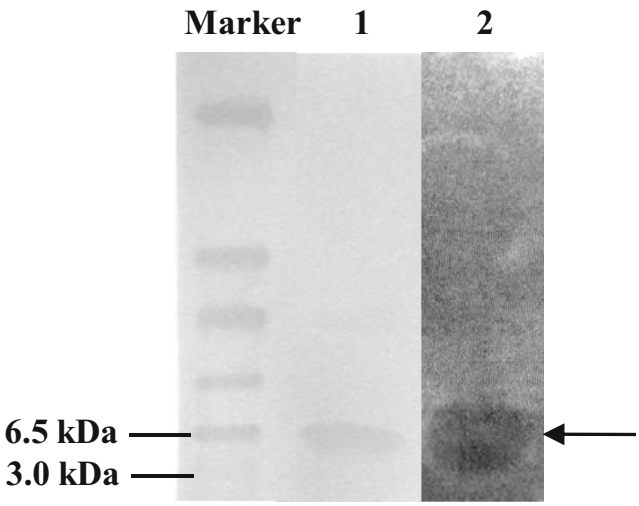


Fig. 3 Tricine-SDS-PAGE analysis and direct determination of antibacterial activity. Marker, protein molar mass marker. Lane 1, partially purified bacteriocins. Lane 2, gel overlaid with the indicator strain, *S. typhimurium* ATCC14028. The arrow demonstrates the inhibition zone

Effects of temperature, pH, organic solvents, surfactants, and detergents on plantaricin MXG-68 stability

As shown in Table 3, the antibacterial activity of plantaricin MXG-68 was not obviously affected by treatment at 60 °C, 80 °C, or 100 °C for 30 min. Moreover, 97.69% of its antibacterial activity remained after sterilization at 121 °C for 30 min. These results demonstrated that plantaricin MXG-68 was highly thermostable. Moreover, plantaricin MXG-68 kept nearly 100% of its antibacterial activity after refrigeration at 4 °C and − 20 °C for 30 days. The activity of plantaricin MXG-68 was stable at pH values between 2.0 and 10.0, with the optimum value occurring at pH 6.0, and activities of 95.95 and 97.45% at pH 2.0 and pH 10.0, respectively.

The antibacterial activity of plantaricin MXG-68 did not obviously decrease after treatment with organic solvents, detergents, or partial surfactants, including Tween-80, Tween-20, and Triton X-100, relative to the control ($P > 0.05$). However, treatment with 0.1 mg/mL, 1 mg/mL, and 5 mg/mL EDTA greatly increased the antibacterial activity of plantaricin MXG-68 relative to the control ($P < 0.01$).

Nutritional optimization for bacteriocin production

The inhibition diameters of 29 runs using the BBD design are shown in Table 4. The final equation generated by the analysis, which is as follows, could be applied to make predictions about the responses for given levels of each factor. $Y = -26.63083 + 7.97133*A + 18.68633*B + 3.50817*C + 13.15000*D - 0.49000*A*B + 0.21500*A*C + 0.020000*A*D - 0.020000*B*C - 0.060000*B*D$

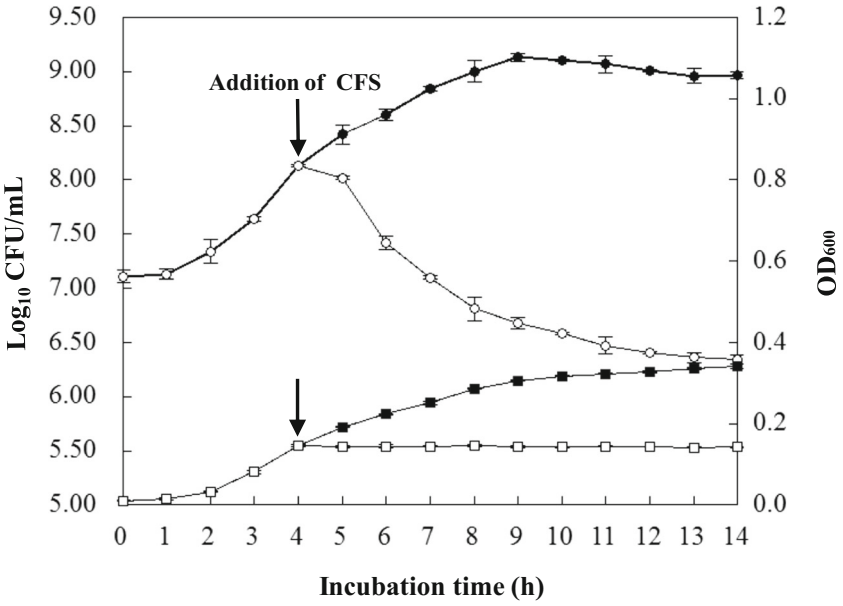


Fig. 4 Influence of antibacterial substances on the growth of *S. typhimurium* ATCC14028. Viable cell numbers (CFU/mL) were observed in the absence (black circle) and presence (white circle) of CFS. Cell densities at 600 nm (OD_{600}) were observed in the absence (black square) and presence (white square) of CFS. Viable cell numbers and cell densities are expressed as the mean ± SD of Log_{10} CFU/mL ($n = 3$) and OD_{600} ($n = 3$)

$-0.010000*C*D-4.74067*A^2-8.86567*B^2-1.08892*C^2-1.31517*D^2$. The results of ANOVA of the model are shown in Table 5. The significance of the model ($P < 0.01$) and the non-significance of lack-of-fit ($P > 0.05$) were obviously advantageous, indicating high predictability of the model. All linear coefficients and quadratic coefficients, as well as a portion of two interaction coefficients (AB and AC), were found to be significant. The R^2 value, which is the proportion of the interpretable variation to total variation, was 0.9977, further supporting that there was an accurate correlation between the predicted and actual values. The adjusted R^2 of 0.9953 demonstrated that the model could coincide with the process of bacteriocin production. Additionally, the coefficient of variation was low (CV% = 0.50), demonstrating that the model was exact and dependable. The Pred R^2 of 0.9876 was reasonably consistent with the adjusted R^2. The high value for the adeq precision (61.543) suggested that the polynomial quadratic model could be applied to navigate the design space.

The normal probability versus residuals was drawn on a graph demonstrating that the data were extremely close to a straight line and located on either side of the line, indicating that the model was reasonably good (Fig. 5). Three-dimensional plots expressing the influence of two variables on the bacteriocin production while other variables were kept at zero indicated the bacteriocin production increased and then decreased as one variable increased when the other variable was fixed (Fig. 6).

The maximum inhibition diameter of 21.79 mm was achieved at a lactose concentration of 0.84%, tryptone concentration of 1.01%, ascorbic acid concentration of 1.66 ppm, and EDTA concentration of 4.98 mg/mL in basal MRS medium. Under these conditions, the bacteriocin production was 26.10% higher than that in the basal MRS medium. Verification experiments conducted in triplicate to check the optimization findings and confirm the precision of the model revealed an inhibition diameter from *L. plantarum* MXG-68 of 21.77 mm. The applicability of the model was effectively confirmed by the consistency between the predicted and verification values.

Discussion

In our study, antibacterial substance-producing strains were separated from Inner Mongolia traditional koumiss, which is known to be a good source of lactic acid bacteria. In previous studies, antibacterial substance-producing strains have been isolated from other foods, such as *E. faecium* and *S. thermophilus* from cheeses and yogurts (Yang et al. 2012), *L. paracasei* ST11BR and *L. pentosus* ST151BR from fermented yak milk (Luo et al. 2011), *L. plantarum* ST13BR and *L. lactis* ST34BR from South African barley beer (Todorov and Dicks 2004), *L. plantarum* JJ18 and *L. plantarum* JJ60 from idli batter (Agaliya and Jeevaratnam 2013), *L. plantarum* from Chinese and Mongolian traditionally fermented foods (Yu et al. 2015), *L. plantarum* LD1 from dosa batter (Gupta and Tiwari 2014), *L. plantarum* ZJ008 from fresh milk (Zhu et al. 2014), *L. plantarum* H5 from Persian sturgeon, *L. plantarum* LD4 from fermented dosa of southern India (Ghanbari et al. 2013), and *L. plantarum* from Chinese traditional low salt fermented whole fish (Zeng et al. 2014). Two categories of media were selected for the separation of LAB, MRS to isolate a general range of LAB from the samples, followed by M17 medium to separate lactococci strains. Therefore, the selection of food sources and media

Table. 3 Effect of temperature, pH, organic solvents, surfactants, and detergents on antibacterial activity

Factors		Inhibition diameter (mm)
Temperature	Control: untreated CFS at room temperature	17.28 ± 0.08
	60 °C for 2 0 min	17.13 ± 0.10
	80 °C for 20 min	16.99 ± 0.17
	100 °C for 20 min	16.90 ± 0.45
	121 °C for 20 min	16.88 ± 0.28
	4 °C for 30 days	17.19 ± 0.17
	− 20 °C for 30 days	17.22 ± 0.13
pH	Control: untreated CFS	17.26 ± 0.02
	pH 2.0	16.58 ± 0.22
	pH 3.0	16.84 ± 0.43
	pH 4.0	17.14 ± 0.16
	pH 5.0	17.30 ± 0.08
	pH 6.0	17.32 ± 0.25
	pH 7.0	17.26 ± 0.12
	pH 8.0	17.21 ± 0.33
	pH 9.0	16.97 ± 0.19
	pH 10.0	16.84 ± 0.26
Organic solvents	Control: untreated CFS	17.26 ± 0.02
	Ethanol (1%, *v/v*)	17.21 ± 0.24
	Methanol (1%, *v/v*)	17.18 ± 0.13
	Isopropanol (1%, *v/v*)	17.21 ± 0.48
	Acetone (1%, *v/v*)	17.15 ± 0.37
	Ethyl acetate (1%, *v/v*)	17.24 ± 0.15
	Acetonitrile (1%, *v/v*)	17.22 ± 0.19
Surfactants	Control: untreated CFS	17.26 ± 0.02
	Tween-80 (1 mg/mL)	17.21 ± 0.54
	Tween-20 (1 mg/mL)	17.16 ± 0.21
	Triton X-100 (1 mg/mL)	17.22 ± 0.33
	EDTA (0.1 mg/mL)	17.51 ± 0.18
	EDTA (1 mg/mL)	17.81 ± 0.27
	EDTA (5 mg/mL)	18.02 ± 0.46
Detergents	Control: untreated CFS	17.26 ± 0.02
	Urea (1 mg/mL)	17.23 ± 0.39
	SDS (1 mg/mL)	17.25 ± 0.20

played an important role in successfully isolating antibacterial substance-producing strains (Yang et al. 2012).

Most bacteriocins produced by different LAB possess a narrow antibacterial spectrum and ineffectively inhibit Gram-negative bacteria. This is because the outer membrane of these LAB blocks the locus for bacteriocin action, for example, plantaricin W from *L. plantarum* LMG 2379, plantaricin C from *L. plantarum* LL441, plantaricin D from *L. plantarum* BFE905, and plantaricin T from *L. plantarum* LPCO10 (Gong et al. 2010). Investigations of bacteriocins with broad antibacterial spectra have been important to human health and the food industry. To date, only a few bacteriocins have been reported to inhibit Gram-negative strains (Perez et al. 2014). As a result of its broad antibacterial spectrum, plantaricin MXG-68 produced by *L. plantarum* MXG-68 strain isolated in the present study may be of interest as a food preservative. Antibacterial substance produced by *L. plantarum* MXG-68 is able to constrain not only Gram-positive strains but also Gram-negative strains. Similar findings have been described for plantaricin from *L. plantarum* TF711, plantaricin MG, *L. plantarum* MF6, and *L. plantarum* MF13 (Agaliya and Jeevaratnam 2013; Gupta and Tiwari 2014; Hu et al. 2013; Man et al. 2012; Yu et al. 2015; Zhu et al. 2014). Our results combined with those of previous studies indicated that the antibacterial spectrum of LAB was strain-specific. Strain MXG-68 was preliminarily identified as *L. plantarum* based on morphological, biochemical, and physiological characteristics and confirmed based on the nucleotide sequence of its *16S rDNA*.

The results of growth kinetics and antibacterial substance production indicated that the secretion of bacteriocin was closely associated with the growth of *L. plantarum* MXG-68. The highest growth and antibacterial substance production occurred in the middle of the stationary phase and at 24 h simultaneously, which is different from that observed for plantaricin BM-1 and bacteriocin LD4 (Kumar et al. 2016). Antibacterial substance from *L. plantarum* MXG-68 has a bactericidal impact on the impressible strain. Similar results have been reported for other bacteriocins from LAB, such as Lactococcin MMT24 from *L. lactis* MMT24 (Ghrairi et al. 2006) and plantaricin 423 from *L. plantarum* 423 (Gong et al. 2010).

Notably, the antibacterial activity against *S. typhimurium* ATCC14028 was obviously reduced or completely lost after treatment with pepsin, trypsin, chymotrypsin, papain, and proteinase K. However, lipase and α-amylase did not influence the antibacterial activity, carbohydrates, and lipids played no role in antibacterial activity (Todorov and Dicks 2004). These results demonstrated that antibacterial substances produced by *L. plantarum* MXG-68 were proteinaceous in nature, and could therefore be divided into various types of bacteriocin. The bacteriocin produced by *L. plantarum* MXG-68 was named plantaricin MXG-68. Different bacteriocins have shown diverse reactions to proteolytic enzymes, for example, bacteriocin JJ18, plantaricin MG, and brevicin 37 (Agaliya and Jeevaratnam 2013; Gong et al. 2010). The molar mass of plantaricin MXG-68 was about 6.5 kDa based on Tricine-SDS-PAGE electrophoresis, which is relatively small for a polypeptide (Kumar et al. 2016).

The N-terminal amino acid sequence of plantaricin MXG-68 was compared to the known sequences of other bacteriocins in the NCBI database based on a BLAST search. The results indicated that the sequence VYGPAGIFNT differed from that of any known bacteriocins (Lv et al. 2018b; Tiwari

Table 4 Experimental data based on BBD design for the bacteriocin production of *L. plantarum* MXG-68

Runs	Variables				Code value				Response inhibition diameter (mm) *Y*
	A: lactose (%)	B: tryptone (%)	C: ascorbic acid (ppm)	D: EDTA (mg/mL)	X_1	X_2	X_3	X_4	
1	0.50	0.50	2.00	5.00	−1	−1	0	0	18.63 ± 0.31
2	1.00	1.00	2.00	5.00	0	0	0	0	21.54 ± 0.18
3	1.00	1.00	1.00	6.00	0	0	−1	1	19.82 ± 0.14
4	1.00	1.50	3.00	5.00	0	1	1	0	17.60 ± 0.28
5	1.00	1.00	1.00	4.00	0	0	−1	−1	20.02 ± 0.15
6	1.00	1.50	2.00	4.00	0	1	0	−1	18.15 ± 0.21
7	1.00	1.00	2.00	5.00	0	0	0	0	21.52 ± 0.30
8	1.00	0.50	1.00	5.00	0	−1	−1	0	18.77 ± 0.12
9	0.50	1.00	3.00	5.00	−1	0	1	0	19.26 ± 0.18
10	1.00	1.00	2.00	5.00	0	0	0	0	21.60 ± 0.21
11	1.00	1.50	1.00	5.00	0	1	−1	0	18.90 ± 0.14
12	1.50	1.00	3.00	5.00	1	0	1	0	18.00 ± 0.26
13	1.00	0.50	3.00	5.00	0	−1	1	0	17.51 ± 0.18
14	1.00	1.00	3.00	4.00	0	0	1	−1	18.50 ± 0.13
15	1.50	1.00	2.00	4.00	1	0	0	−1	18.28 ± 0.30
16	0.50	1.00	1.00	5.00	−1	0	−1	0	20.87 ± 0.22
17	1.00	0.50	2.00	6.00	0	−1	0	1	18.05 ± 0.12
18	1.50	1.50	2.00	5.00	1	1	0	0	17.43 ± 0.28
19	1.00	1.00	3.00	6.00	0	0	1	1	18.26 ± 0.32
20	1.50	1.00	2.00	6.00	1	0	0	1	18.22 ± 0.13
21	1.50	0.50	2.00	5.00	1	−1	0	0	17.44 ± 0.27
22	1.00	1.00	2.00	5.00	0	0	0	0	21.48 ± 0.15
23	1.50	1.00	1.00	5.00	1	0	−1	0	19.18 ± 0.18
24	0.50	1.00	2.00	4.00	−1	0	0	−1	19.80 ± 0.11
25	1.00	0.50	2.00	4.00	0	−1	0	−1	18.06 ± 0.21
26	1.00	1.00	2.00	5.00	0	0	0	0	21.62 ± 0.09
27	0.50	1.50	2.00	5.00	−1	1	0	0	19.11 ± 0.23
28	1.00	1.50	2.00	6.00	0	1	0	1	18.02 ± 0.14
29	0.50	1.00	2.00	6.00	−1	0	0	1	19.70 ± 0.25

et al. 2008). This apparent lack of similarities indicates that plantaricin MXG-68 may be a novel and unique bacteriocin. To obtain more information regarding the N-terminal amino acid sequence of plantaricin MXG-68, additional mass spectrometry techniques should be conducted in the future.

Hot and cold treatments are essential to food processing and storage, hence, it is necessary for bacteriocin to be stable under various temperatures to enable its use as a biological preservative. Similar to bacteriocins secreted by *L. brevis* OG1 and *L. plantarum* F1, plantaricin MXG-68 was thermostable under different temperatures. Unlike paracaseicin A and bacteriocin ST44AM, plantaricin MXG-68 showed obvious heat stability at 121 °C for 30 min, indicating it can be used during food processing (Todorov and Dicks 2009). Additionally, plantaricin MXG-68 was stable at a wide range of pH (2.0–10.0), with an optimum value at 6.0. These findings differ from those of previous reports, in which bacteriocins synthesized by LAB have been found to be generally stable under acidic conditions but deactivated under alkaline and neutral conditions. For example, paracaseicin A and bacteriocin ST31 were found to have high activity under acidic conditions but remarkably decreased activity at pH 6.0 and no activity at pH 7.0–9.0 (Bendjeddou et al. 2012).

Similar to plantaricin LC74, bacteriocin BacTN635 and lactocin RN78, plantaricin MXG-68 was stable after treatment with different organic solvents, indicating its soluble and proteinaceous nature (Perin et al. 2012; Rushdy and Gomaa 2013). Nearly 100% of the antibacterial activity remained after treatment with different organic solvents, detergents, and surfactants, except for EDTA. The chemical stability supported the potential for widespread application of bacteriocin, suggesting that it could maintain its function and structure during different stages of purification. The antibacterial activity of plantaricin MXG-68 was markedly increased after

Table 5 Analysis of variance (ANOVA) of the quadratic model of bacteriocin production generated from the experimental findings

Source	SS	df	MS	*F* value	*P* value	Significant level
Model	53.93	14	3.85	428.47	< 0.0001	Significant
A-lactose	6.48	1	6.48	721.13	< 0.0001	Significant
B-tryptone	0.047	1	0.047	5.21	0.0385	
C-ascorbic acid	5.92	1	5.92	658.77	< 0.0001	Significant
D-EDTA	0.046	1	0.046	5.08	0.0408	Significant
AB	0.060	1	0.060	6.68	0.0216	Significant
AC	0.046	1	0.046	5.14	0.0397	Significant
AD	4.000E-004	1	4.000E-004	0.044	0.8360	
BC	4.000E-004	1	4.000E-004	0.044	0.8360	
BD	3.600E-003	1	3.600E-003	0.4	0.5371	
CD	4.000E-004	1	4.000E-004	0.044	0.8360	
A^2	9.11	1	9.11	1013.51	< 0.0001	Significant
B^2	31.86	1	31.86	3544.62	< 0.0001	Significant
C^2	7.69	1	7.69	855.57	< 0.0001	Significant
D^2	11.22	1	11.22	1248.04	< 0.0001	Significant
Residual	0.13	14	8.990E-003			
Lack of fit	0.11	10	0.011	3.39	0.1253	
Pure error	0.013	4	3.320E-003			
Cor total	54.05	28				
Std. dev.	0.095		R-Squared	0.9977		
Mean	19.15		Adj R-Squared	0.9953		
C. V. %	0.50		Pred R-Squared	0.9876		
PRESS	0.67		Adeq Precision	61.543		

treatment with EDTA, indicating that EDTA chelated divalent cations from the protective external cell membrane of bacteria, making them susceptible to hydrophobic peptides such as bacteriocins.

RSM, which is a highly effective strategy for optimizing microbial metabolite production, is a design method for collecting statistical data, appraising the influences of factors, and determining the optimum conditions to fulfill a desirable purpose (Körbahti et al. 2007). This method has been successfully applied to optimization of the bacteriocin production of lactic acid bacteria, including *L. paracasei* NTU 101, *L. plantarum* NTU 102, *E. faecium* MTCC 5695, *L. brevis* DF01 (Lee et al. 2012), and *L. casei* LA-1 (Kumar et al. 2012). The results revealed that lactose, tryptone, ascorbic acid, and

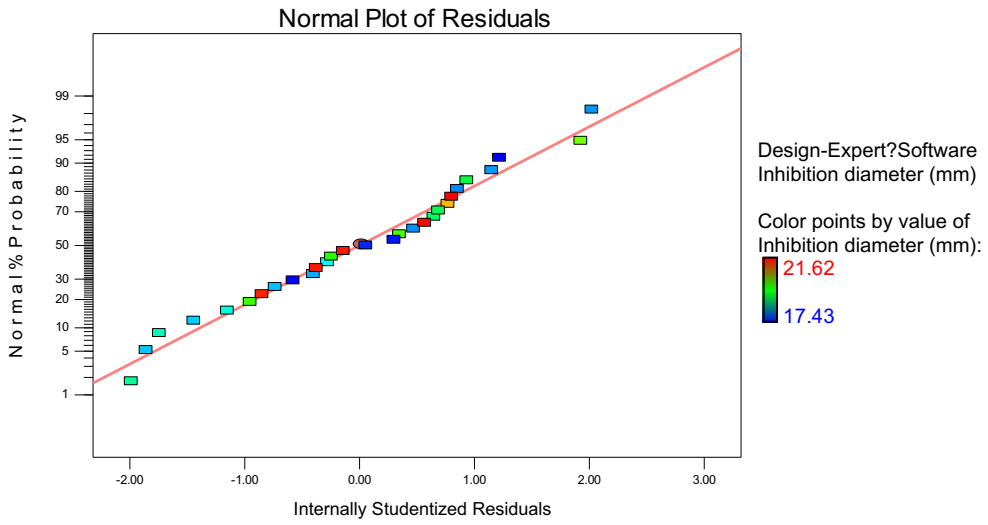


Fig. 5 Plot of expected normal values versus residuals

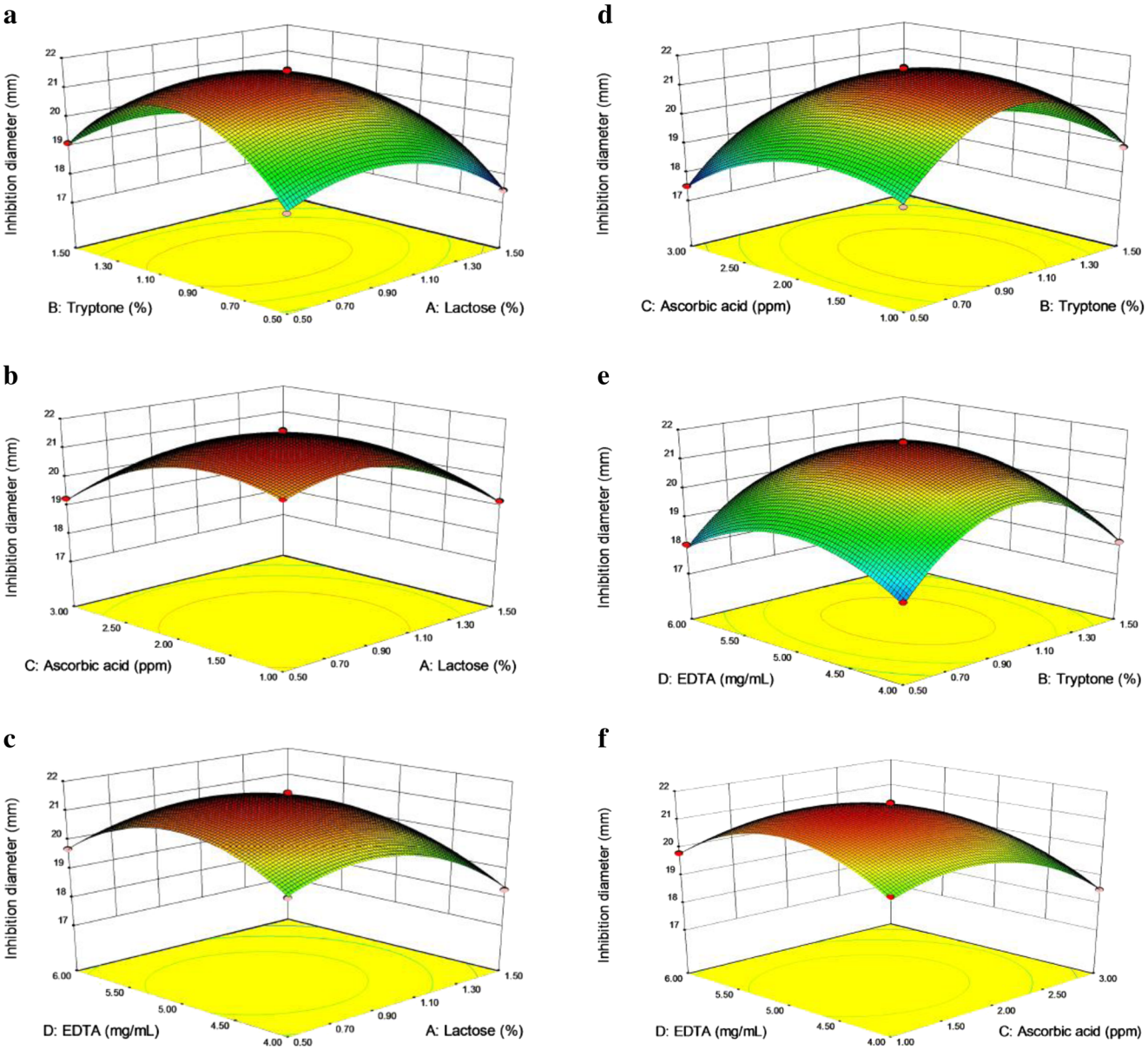


Fig. 6 3D response surface curve of various variables. Influences of lactose and tryptone (**a**), lactose and ascorbic acid (**b**), lactose and EDTA (**c**), tryptone and ascorbic acid (**d**), tryptone and EDTA (**e**), and ascorbic acid and EDTA (**f**) on bacteriocin production by *L. plantarum* MXG-68

EDTA had positive effects on bacteriocin production of *L. plantarum* MXG-68 (data are not shown), thus, RSM was applied to further optimize the amount of these four factors to maximize bacteriocin production. An empirical model was established through RSM to analyze the linkages between variables (lactose, tryptone, ascorbic acid, and EDTA). The goodness of the model can be determined by the corresponding coefficient, including R^2 (0.9977), adjusted R^2 (0.9953), and Pred R^2 (0.9876). A coefficient value closer to 1 indicates a better connection between the predicted and actual values. The signal-to-noise ratio is evaluated by adeq precision, with a ratio greater than 4 considered very good. The ratio of 61.543 indicates a sufficient signal and that the model is suitable for analysis of the design space. The results of ANOVA indicate that the effects of the investigated factors on bacteriocin production occurred in the order lactose > ascorbic acid > tryptone > EDTA. Overall, the results indicated that the model fits the bacteriocin production of *L. plantarum* MXG-68 very well.

In this study, lactose, tryptone, ascorbic acid, and EDTA were found to be beneficial to the bacteriocin production of *L. plantarum* MXG-68. Previous studies have shown that the carbon sources required for the production of bacteriocin by lactic acid bacteria differed obviously, possibly because of different energy types and carbon skeletons of bacteriocins. For instance, the carbon sources for bacteriocin production of *L. plantarum* LB-B1, *Pediococcus acidilactici* C20, *L. plantarum* ST23LD, and *L. pentosus* 31-1 are glucose,

maltose, sorbitol, and lactose, respectively (Halami and Chandrashekar 2005; Todorov and Dicks 2006). Tryptone, as an organic nitrogen source, can significantly promote bacteriocin production, which may be related to the mechanism of bacteriocin production in bacterial growth. Some components of organic nitrogen can induce the initiation and expression of bacteriocin synthetic genes. Bacteriocin itself is a protein, and tryptone contains more unique amino acids that can provide materials for bacteriocin production. Ascorbic acid was found to increase the bacteriocin production by *L. plantarum* MXG-68 in our study, similar to the results observed for *L. plantarum* KC21 in a study conducted by Lim (2010). These findings differ from those of another study (Aasen et al. 2000), in which vitamin supplementation had no effect on sakacin P production by *L. sakei* CCUG 42687. As previously shown, EDTA could enhance the antibacterial activity of bacteriocin secreted by *L. casei* AP8 and plantaricin MG (Castellano et al. 2011).

Author contributions Li-Li Man and Dian-Jun Xiang contributed equally to this article. Li-Li Man and Dian-Jun Xiang performed the experiments and contributed significantly to the data analysis, results discussion and manuscript preparation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics statement No animals or humans were used in this study.

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