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Screening and Characterization of Lactic Acid Bacteria with Broad-Spectrum Antimicrobial Activity from Tibetan Qula, a Yak Milk Cheese

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Abstract

Background

Lactic acid bacteria with natural, effective antibacterial activity, safe and reliable characteristic, gradually become one of the key technologies in food fermentation applications, food preservation and other fields. In this study, 112 presumptive lactic acid bacteria isolated from Tibetan Qula, a fermented yak cheese popular in the Tibetan plateau, were screened for potential probiotic microorganism with antimicrobial activity.

Results

12 lactic acid bacteria were found to have antibacterial activity, and strain QZ50 in particular showed broad-spectrum inhibition against pathogenic bacteria, which retained its antibacterial activity after sequential removal of acids and hydrogen peroxide, indicating the production of a broad-spectrum bacteriocin that could inhibite *Micrococcus luteus* ATCC 28001, *Staphylococcus aureus* ATCC 26003, *Bacillus subtilis* ATCC 63501, *Escherichia coli* ATCC 30105, *Pseudomonas aeruginosa* ATCC 10104, and *Salmonella enterica* ATCC 50094. Strain QZ50 was identified as *Lactobacillus plantarum* based on physicochemical characteristics and 16S rDNA sequencing. And the optimum production conditions were evaluated to obtain the highest yield of plantaricin QZ50. The optimum medium, temperature, initial pH, and inoculum amount for plantaricin QZ50 production were Man, Rogosa, and Sharpe (MRS), 30°C, 6.5, and 3%, respectively. In addition, different C source, N source and stimulating factors in medium show significant effects on plantaricin QZ50 production ($P < 0.05$). The optimum C and N source were respectively glucose and yeast extract, and 2% Tween 80 contributed highest production of plantaricin QZ50. Plantaricin QZ50 exhibited strong heat stability and remained activity at pH 2.0–8.0. In addition, plantaricin QZ50 was inactivated by pepsin, proteinase K, trypsin, papain, and chymotrypsin.

Conclusions

Some strains of *Lactobacillus* isolated from the Qula in the Tibetan plateau have good antibacterial activity which could be considered as potential probiotic. The strain of *Lactobacillus plantarum* QZ50, with a broad-spectrum, stable, safe, and natural antibiotic, has potential applications as a food biopreservative.

Background

Yak, an animal that lives in cold climates and high altitudes, originated in areas more than 3000 m above sea level of the Qinghai-Tibet plateau, the world's highest plateau [1]. Qula, which is a white or yellow dried, grainy cheese hand-made from yak milk, is traditionally consumed by Tibetans. Compared with industrially manufactured cheese, hand-made Qula is rich in lactic acid bacteria.

During production and storage, cheese is susceptible to contamination from spoilage and pathogenic bacteria, which limits the shelf life of the product. Chemical preservatives are commonly used to prevent microbial spoilage; however, studies have reported that these preservatives cause cancer, malformations, and food poisoning [2]. Therefore, it is important to identify broad-spectrum, efficient, stable, and safe biopreservatives.

LAB, the main microorganisms in fermented food, produce bacteriocins, which are antimicrobial peptides or proteins with growth-inhibitory effects against closely related species, including *Listeria monocytogenes*, *Salmonella*, *Escherichia coli*, and *Staphylococcus aureus* [2]. Bacteriocins are considered to be safe because they do not contribute to intestinal dysbiosis or drug resistance. Several bacteriocin-producing LAB have been successfully isolated from fermented foods, including *Lactobacillus plantarum*(*L. plantarum*) UG1 [3], *Lactobacillus acidophilus*(*L. acidophilus*) NX2-6 [4], and *Lactobacillus sakei*(*L. sakei*) LSJ618 [5]. Even though LAB can produce different types of bacteriocins, nisin is the only bacteriocin currently used as a food preservative. Nisin effectively inhibits the growth of *Clostridium botulinum* and the production of its toxin and extends the shelf life of products even at room temperature [6]. Research has shown that 500–1000 IU/g nisin effectively inhibits *C. botulinum* in cheese made from pasteurized milk [2].

However, the yield and antimicrobial spectrum of bacteriocins is low; certain bacteriocins cannot inhibit Gram-negative bacteria, yeast, or mold. Moreover, the yield and antibacterial spectrum of different bacteriocins are dependent on the bacterial strain and habitat [6]. Therefore, it is important to identify LAB that synthesize high levels of broad-spectrum bacteriocins.

In this study, we screened and identified LAB strains producing a novel broad-spectrum bacteriocin. For maximum production and improved applications of bacteriocins, we optimized the bacteriocin production conditions, and the physicochemical characteristics of the resulting bacteriocins were evaluated. Further, this study was the first to date to identify the bacteriocin-producing *L. plantarum* strains found in Qula cheese.

Results

screening of of lactic acid bacteria with antibacterial activity

A total of 112 acid-producing bacteria strains were isolated from traditional hand-made Qula cheese. Among these isolates, 12 strains numbered QZ11, QZ14, QZ21, QZ29, QZ41, QZ42, QZ50, QZ57, QZ58, QZ67, QZ71 and QZ81 had broad inhibitory activity against Gram-positive and Gram-negative bacteria by inhibiting the ETEC K88 and *Salm. enterica* ATCC 13076.

Physiological and Biochemical Characteristics and Antibiotic Susceptibility Tests

The 12 strains of lactic acid bacteria with antibacterial activity from the primary screening were tested for temperature, pH and NaCl tolerance. All strains were grown at temperatures 10°C, 30°C, at pH 4.0, 4.5, 5.0, 8.0 and 9, and at 3.0% NaCl. QZ11, QZ14,QZ21,QZ50 and QZ71 could grow at 5°C, QZ21, QZ50, QZ67, QZ81 could survive at 50°C, while all other strains cannot survive at 5°C, or 50°C.QZ14, QZ21, QZ50, QZ67, QZ71 can tolerate growth in a high salinity environment of 6.5% Nacl.QZ11, QZ14, QZ21, QZ50, QZ57, QZ71 and QZ81 can grow at pH 3.5 and and only two strains QZ50, QZ71 can survive in pH 3.0 environment. Taken together, the seven strains, strains QZ11, QZ14, QZ21, QZ50, QZ67, QZ71 and QZ81 had better environmental tolerance.

Screening of LAB producing broad-spectrum bacteriocin

In order to further screen the lactic acid bacteria with good antibacterial activity, we re-screened the 12 strains of lactic acid bacteria obtained from the initial screening. They were tested for their inhibitory ability against 7

different pathogenic bacteria, as shown in Table 1. The experimental results revealed that the 6 strains inculing QZ11, QZ14, QZ21, QZ50, QZ58, QZ71 and QZ81 had obvious bacterial inhibition and showed significant inhibitory effects on at least four pathogenic bacteria. Only the cell-free supernatant of QZ50 retained its inhibitory activity against all indicator strains after eliminating the organic acid effect (Table 1). Additionally, QZ50 had the strongest inhibitory effect against *Micrococcus luteus ATCC 28001*. Following catalase treatment, the cell-free supernatant of QZ50 still retained its inhibitory activity (data not shown). Following treatment with two kinds of protease, the antimicrobial activity of QZ50 disappeared.

Table 1
Antibacterial activity after eliminating organic acid effects

Strains	G^+				G^-		
	Micrococcus luteus ATCC 28001	*ETEC K88*	*Staphylococs aureus* ATCC 26003	*Bacillus subtilis* ATCC 63501	*Escherichia coli* ATCC 30105	*Pseudomonas aeruginosa* ATCC 10104	*Salmonella enterica* ATCC 50094
QZ11	++	+++	++	-	-	-	+
QZ14	+++	++	++	++	+	-	-
QZ21	++	+	-	++	+	-	-
QZ29	+++	++	++	++	-	-	-
QZ41	+++	+++	++	+	-	-	-
QZ42	+++	++	++	+	-	-	-
QZ50	+++	+++	++	++	++	+	++
QZ57	++	+	++	+	-	-	-
QZ58	+++	++	++	++	-	-	-
QZ67	+++	++	++	+	-	+	-
QZ71	+++	+++	+	++	-	-	++
QZ81	+++	++	++	++	-	-	-

+, Diameter of inhibition zone: 8.00 to 12.00 mm; ++, 12.00 to 16.00 mm; +++, 16.00 to 20.00 mm; ++++, more than 20.00 mm; -, not detected; the diameter of inhibition zone including that of Oxford cup (7.80 mm).

Identification of selected Strains

Physiological and biochemical tests were performed to identify QZ50 (Table 2). Strain QZ50 grew at 10, 15, and 45°C and weakly at 50°C. The strain grew in 3 and 6.5% NaCl and at pH 3–10. Strain QZ50 produced gas from glucose, and utilized ribose, galactose, glucose, fructose, mannose, mannitol, sorbitol, amygdalin, arbutin, esculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, melezitose, raffinose, and gentiobiose, but not glycerol, erythritol, L-arabinose, D-xylose, adonitol, β-methyl-xyloside, sorboseE, rhamnose,

dulcitol, inositol, α-methyl-D glucoside, inulin, starch, glycogen, xylitol, D-turanose, D-tagatose, and L-arabitol. Moreover, it could not completely utilize D-arabitol and gluconate.

Table 2
Physiological and biochemical identification of strain QZ50

Character	QZ50	Substrate	QZ50	Substrate	QZ50
Fermentation type	Homo	Glycerol	-	Sorbitol	+
Growth at temperature (°C):		Erythritol	-	α-Methyl-D Glucoside	-
10	+	D- arabinose	-	Amygdalin	+
15	+	L- arabinose	-	Arbutin	+
45	+	Ribose	+	Esculin	+
50	w	D-xylose		Salicin	+
Growth in NaCl:		Adonitol	-	Cellobiose	+
3.00%	+	β-Methyl –Xyloside	-	Lactose	+
6.50%	+	Galactose	-	Maltose	+
Growth at pH:		Glucose	+	Lactose	+
3	+	Fructose	+	Melibiose	w
3.5	+	MannosE	+	Sucrose	+
4	+	SorbosE	-	Trehalose	+
4.5	+	Rhamnose	-	Raffinose	+
5	+	Dulcitol	-	Gluconate	w
9.5	+	Inositol	-	Inulin	-
10	+	Mannitol	+	Glycogen	-
Note: +, positive; -, negative; w, weakly positive; Homo, homofermentative.					

For the 16S rRNA gene identification of QZ50, a phylogenetic tree was constructed using a neighbor-joining method (Fig. 1). In the *Lactobacillus* cluster, strain QZ50 was grouped with *L. pentosus*, *L. plantarum*, and *L. paraplantarum*, but it could not be identified to a species level based on 16S rRNA gene sequence analysis. The amplification products gained from the recA gene was displayed in Fig. 2. Strain QZ50 and type strain *L. plantarum* subsp. *plantarum* JCM 1149^T all produced 318 bp products. Thus, it was identified as *L. plantarum* subsp. *plantarum*. All the above strains with good antibacterial activity were identified, QZ11, QZ21, QZ71 and QZ81 were identified as *Lactobacillus plantarum*, QZ58 was identified as *Lactococcus lactis*, and QZ14, QZ71 were identified as *Lactobacillus johnsonii.*

Determination and Comparison of the performance of selected lactic acid bacteria.

The antibiotic susceptibility of the 12 selected strains with antibacterial activity was tested and the results were shown in Table 3. All the selected LAB strains were resistant to CN, CIP, CT, VA and P, while most strains exhibited sensitivity to AMP, TE and C. For erythromycin (E), the QZ11, QZ14, QZ21, QZ42,QZ57, QZ58 and QZ81 had sensitivity, while QZ29, QZ41, QZ50 and QZ67 were moderately resistant, and QZ71 was resistant to it. As to rifampicin (RD), QZ29, QZ41 QZ57, QZ58, QZ81 were resistant to it. Strain QZ57 was found to be resistant to most antibiotics except erythromycin and chloramphenicol.

Table 3
Profile of antibiotic susceptibility of selected LAB strains

Strain	AMP	CN	TE	CIP	E	CT	C	VA	P	RD
QZ11	S	R	S	R	S	R	S	R	R	S
QZ14	S	R	R	R	S	R	S	R	R	S
QZ21	S	R	S	R	S	R	R	R	R	S
QZ29	S	R	S	R	M	R	S	R	R	R
QZ41	S	R	S	R	M	R	S	R	R	S
QZ42	R	R	S	R	S	R	S	R	R	S
QZ50	S	R	S	R	M	R	S	R	R	S
QZ57	R	R	R	R	S	R	S	R	R	R
QZ58	R	R	S	R	S	R	S	R	R	R
QZ67	S	R	S	R	M	R	R	R	R	S
QZ71	S	R	S	R	R	R	S	R	R	S
QZ81	S	R	S	R	S	R	S	R	R	R
Note:										
[a]The concentrations of antibiotics are expressed in micrograms per disk (μg/disk), R, resistant; M, moderate resistance; S, susceptible										
[b]Ampicillin (AMP, 10 μg/disk), gentamicin (CN, 10 μg/disk), tetracycline (TE, 30 μg/disk), ciprofloxacin (CIP, 5 μg/disk), erythromycin (E, 15 μg/disk), colistin sulphate (CT, 10 μg/disk), chloramphenicol (C, 30 μg/disk), vancomycin (VA, 30 μg/disk), penicillin (P, 10 μg/disk), rifampicin (RD, 5 μg/disk).										

The surface hydrophobicity and agglutination of the Lactobacillus strains were measured. As shown in Fig. 3, the surface hydrophobicity of the representative strains of LAB ranged from 19–48%, with strains QZ21, QZ58, QZ34 and QZ50 showing high surface hydrophobicity, while the remaining the remaining 2 strains showed lower surface hydrophobicity. As shown in Fig. 4, the self-agglutination of LAB strains varied from 26.3–58.3%, with strains QZ71 and QZ50 showing significantly higher self-agglutination than the remaining 4 strains. The self-agglutination of 2 strains, QZ71 and QZ81, was significantly lower than that of the remaining strains.

The acid production capacity of the six selected strains of LAB is shown in the Fig. 5, there was no significant difference in acid production capacity, and strain QZ50 has the strongest acid production capacity, at 36 hours,

the pH was reduced to 3.98. Combined with the above experimental results and analysis of acid production capacity and surface hydrophobicity and self-aggregation and bacterial inhibition, three strains of bacteria, QZ14 QZ50 and QZ71 were selected for simulated gastrointestinal survival experiments. As shown in the Fig. 6, all three strains of LAB survived after 7h incubation in the simulated gastrointestinal tract. After 3h of artificial gastric juice, the viable counts of the three strains of LAB decreased to different degrees, among which the viable counts of QZ14 and QZ71 were significantly reduced, indicating that these two strains were less tolerant to the artificial gastric juice. After transferring the lactic acid bacteria from the simulated gastric juice to the artificial intestinal fluid, the viable counts of QZ14, QZ71 and QZ50 were significantly reduced, and the viable counts of all three strains of LAB were significantly reduced compared with the initial ones after 4h incubation in the artificial intestinal fluid. Collectively, the strains of LAB were less affected by the artificial gastric fluid, especially ZX50 had no significant decrease in viable bacteria count after incubation in gastric fluid. In contrast, all strains showed a significant decrease of at least 0.89 log10 CFU/mL after incubation in intestinal fluid.

Optimization of media and culture conditions

Some more in-depth studies were performed on Strain QZ50, a broad-spectrum bacteriocin-producing strain with the highest antibacterial activity. The effects of different media, temperature, initial pH values, and inoculum amount on bacteriocin production are shown in Fig. 7. The highest inhibition zone diameter was obtained in MRS broth, at a temperature of 30°C, a pH value of 6.5, and an inoculum amount of 3%. The lowest inhibition zone diameter was obtained with M17 broth, at 25°C, a pH of 4.5, and a 1% inoculum amount. Compared with the conditions of MRS broth, 30°C, pH 6.5, the antimicrobial activity of strain QZ50 under other conditions was significantly different ($P < 0.001$). Compared with the inoculum amount of 3%, other inoculum amount except 2% was significantly different ($P < 0.001$).

Optimization of medium components

The effects of different C and N sources on bacteriocin production are respectively shown in Fig. 8 and Fig. 9. The antimicrobial activity of strain QZ50 due to different C sources was significantly different ($P \leq 0.001$). As shown in Fig. 8, Glucose contributed to the highest inhibition zone diameter, while soluble starch contributed to no bacteriocin production. Lower inhibition zone diameters were obtained in a descending order as: fructose, sucrose, maltose, lactose, and cellobiose.

As shown in Fig. 9, yeast extract contributed to the highest diameter of inhibition, while inorganic N contributed to no bacteriocin production. Except ammonium citrate and ammonium chloride, the antimicrobial activity of strain QZ50 due to different N sources was significantly different (P 0.05). Lower inhibition zone diameters were obtained in a descending order as: tryptone, peptone, casein peptone and beef extract.

The effect of different stimulating factors on bacteriocin production is presented in Fig. 10(A). The diameter of inhibition of QZ50 was the highest with Tween 80 as surfactant. The antimicrobial activity of strain QZ50 due to different surfactants was significantly different ($P \leq 0.01$). As shown in Fig. 10(B), the levels of stimulating factor in the culture medium had significant effect on bacteriocin production($P < 0.01$). The diameter of inhibition of QZ50 was the highest with 2% v/v Tween 80 and the lowest with no Tween 80.

Resistance of bacteriocin against heat, pH, and enzymes

As shown in Table 4, the antibacterial activity of plantaricin QZ50 completely disappeared following treatment with pepsin, protease K, trypsin, papain, and chymotrypsin, while amylase and lipase had no effect. Plantaricin QZ50 retained its activity following incubation at 60°C for 15 and 30 min, but activity slightly decreased at 80, 100, and 121°C. It also retained its activity at pH 2.0–8.0, but complete inactivation was founded at pH 9.0 or 10.0.

Table 4
Effects of different treatment on the activity of plantaricin QZ50

Treatments		**Antimicrobial activity (%)[a]**
Enzymes	α-Amylase	100.0%±1.12
	β-Amylase	100.0%±2.13
	Lipase	100.0%±1.24
	pepsin; protease K; trypsin ; papain ;chymotrypsin	0.0
Heat(°C)	60(15 min)	100.0%±2.17
	60(30 min)	100.0%±1.30
	80(15 min)	87.4%±1.69
	80(30 min)	85.0%±1.78
	100(15 min)	83.6%±0.89
	100(30 min)	81.7%±1.34
	121(15 min)	86.7%±1.52
pH	2.0	100.0%±2.10
	3.0	100.0%±1.27
	4.0	100.0%±0.84
	5.0	100.0%±1.35
	6.0	100.0%±2.10
	7.0	100.0%±1.10
	8.0	100.0%±1.63
	9.0	0.0
	10.0	0.0
Note: [a]The bacteriocin activity of the untreated sample was 1500 AU ml^{-1}.		

Discussion

Since nisin was approved as a food preservative, LAB and other bacteriocin-producing bacteria have become an important research focus. In this study, 112 acid-producing bacteria strains were isolated from traditional Tibetan Qula cheese and screened for broad-spectrum antibacterial activity. In addition to bacteriocin, LAB produce other antibacterial compounds such as organic acids and hydrogen peroxide. Therefore, the isolates were screened for antibacterial activity after eliminating for organic acid and hydrogen peroxide effects. Among the isolates, only strain QZ50 retained its broad-spectrum antibacterial activity. The antimicrobial compound produced by strain QZ50 showed sensitivity towards proteases, indicative of a protein nature. In addition, QZ50 showed stronger inhibition against *Micrococcus luteus* ATCC 28001, *Staphylococs aureus* ATCC 26003, and *Bacillus subtilis* ATCC 63501 than against *Escherichia coli* ATCC 30105, *Pseudomonas aeruginosa* ATCC 10104, and *Salmonella enterica* ATCC 50094. These results, consistent with previous observations [21, 22], confirmed that bacteriocins produced by Gram-positive bacteria generally exhibit stronger antimicrobial activity against Gram-positive bacteria than against Gram-negative bacteria.

Cheese represents a good source of LAB. Certain bacteriocin-producing LAB such as *Enterococcus faecium* MMT21 [23], *Enterococcus faecalis* WHE 96 [24], and *Enterococcus faecium* SD1 [25] have been successively isolated from cheese. However, to the best of our knowledge, this is the first report on a bacteriocin-producing *L. plantarum* strain isolated from Qula cheese.

Culture conditions and medium composition are very important for bacteriocin production. The production of bacteriocins usually require complex media, and several media have been tested for bacteriocin production [26]. In the present study, MRS was a more suitable medium for bacteriocin production than other media, consistent with the findings of Karthikeyan and Santosh (2009) [27] and Hoda et al. (2013) [26]. Additionally, culture temperature and pH can affect bacteriocin production [26]. In this study, the maximum bactericidal activity was obtained at 30°C, in agreement with the research findings of Chen et al. (1995)[28], Moonchai et al. (2005) [29], and Hoda et al. (2013) [26], but different from those of Geisen et al. (1993) [30] and Leroy et al. (2003) [31], who reported that the optimum temperature for bacteriocin production from *Leuconostoc carnosum* LA54A and *Enterococcus faecium* RZS C5 was 25 and 35°C, respectively. Cell growth and metabolite accumulation are dependent on pH [32]. In this study, the production of plantaricin QZ50 was significantly affected by the initial pH value of the culture medium. This is consistent with observations obtained from *Pediococcus acidilactici* BA28 and *Bacillus cereus* XH25 [32, 33]. However, in the study of Embaby et al. (2014) [34], the production of bacteriocin from *Bacillus* sp. YAS 1 was not affected by the initial pH. The optimum pH for bacteriocin production is not consistent across LAB strains. The optimum pH for bacteriocin production is 6 from *Lactobacillus* spp. [26], and < 5 from *L. sakei* CCUG 42687, *L. acidophilus* JCM 1132, and *L. plantarum* LL41 [29, 35, 36]. In this study, the optimum pH for bacteriocin production from *L. plantarum* QZ50 was pH 6.5, which might contribute to the transcription and synthesis of plantaricin QZ50. The composition of the medium has important effects on bacteriocin production. To enhance the production and reduce the cost of the medium, several studies have been performed to optimize the composition [26, 37, 38]. The C source, N source, and percentage of Tween 80 in MRS medium are the most common studied factors [38]. In this study, different C sources contributed to different levels of plantaricin QZ50. This finding was in agreement with earlier reports, which showed that the C source had a significant effect on bacteriocin production [38, 39]. Additionally, glucose contributed to the highest bacteriocin production. Pal et al. (2010) [38], Kamoun et al. (2009) [40] and Amin et al. (2012) [41] have reported that glucose has a significant effect on bacteriocin production from *Bacillus* sp. GU057, *Bacillus thuringiensis* subsp. *kurstaki* strain, and *Weissella paramesenteroides* DFR-8, while Embaby et

al. (2014) reported no significant effects on bacteriocin production from *Bacillus sp.* YAS 1 [34]. In this study, lactose was utilized by *L. plantarum* QZ50 to a lower level than fructose or sucrose. However, lactose favored the production of bacteriocin from *Enterococcus faecium* RZS C5 and *Lactobacillus* sp. MSU3IR [31, 42]. Organic N had a significant effect on bacteriocin production, in agreement with earlier reports [38, 39]. Our findings revealed that yeast extract was the optimum N source, consistent with the reports of Kim et al. (2000) [43], but different from Pal et al. (2010) [38]. In the study of Pal et al. (2010), tryptone was optimum for production of bacteriocin by *Weissella paramesenteroides* DFR-8 [38]. Inorganic N resulted in no bacteriocin production. However, Iyapparaj et al. (2013) reported that inorganic N contributed to increased bacteriocin yield [42]. In this study, plantaricin QZ50 production was the highest with Tween 80. Similar observations were reported with *Lactobacillus* sp. MSU3IR [42] and *L. plantarum* 423 [44]. As a surfactant, Tween 80 reduces the surface tension between bacteriocins and bacteria, thereby improving permeability of cell membranes, which improves the release of bacteriocin from the cell surface [45]. The activity of plantaricin QZ50 varied with different concentrations of Tween 80 in MRS medium, and 2% Tween 80 was the optimum for plantaricin QZ50 production. Hoda et al. (2013) [26] and Iyapparaj et al. (2013) [42] reported that the optimum Tween 80 concentrations ranged from 1 to 1.5%. Based on the results obtained, it is important to conduct optimization experiments for novel bacteriocin-producing isolates.

Next, the partial biochemical characteristics of plantaricin QZ50 were evaluated. In general, bacteriocins are sensitive to proteolytic enzymes. The antibacterial activity of plantaricin QZ50 was lost following treatment with pepsin, proteinase K, trypsin, papain, and chymotrypsin; however, it was not affected by amylase or lipase, which confirmed that plantaricin QZ50 was protein-based. Plantaricin QZ50 retained its antibacterial activity at 60–121°C. Bacteriocin from strain Bacillus sp., BAC YAS 1 from *Bacillus sp.* is stable at 45–80°C [34], Sh10 is stable at 30–121°C [46], and bacteriocin from *Lactobacillus sake* C2 remained activity at 80–121°C [47]. The resistance of plantaricin QZ50 against heat makes it applicable in food processing and preservation. In this study, the antibacterial activity of plantaricin QZ50 fluctuated with increasing temperatures. This result did not agree with past studies, which reported that the activity of bacteriocin declined with increasing temperatures [34, 42, 47, 48]. Plantaricin QZ50 was stable at pH 2–8, similar to bacteriocins from *Bacillus subtilis* 14B and *Lactobacillus brevis* FPTLB3 [48, 49]. In studies by Embaby et al. (2014) [34], bacteriocins from *Bacillus sp.* YAS 1 exhibited a higher pH stability range (3–12) than that of plantaricin QZ50. In the studies by Magnusson and Li et al. (2015) [11] and Schnürer (2001) [50], the activity of bacteriocins from strains ZZU 203 and 204 and *Lactobacillus coryniformis* subsp. coryniformis Si3 disappeared at pH > 6.0. The stability of plantaricin QZ50 at low pH values makes it useful for applications in acidic environments. Differences in physicochemical characteristics among various bacteriocins is mainly caused by differences in amino acid composition. The analysis of the amino acid composition of plantaricin QZ50 is currently underway. In addition, we can control and select the conditions of various bacteriocins based on their physicochemical properties, which will greatly improve their applications.

Conclusions

In this study, some strains of LAB isolated from the Qula in the Tibetan plateau had good antibacterial activity which could be considered as potential probiotic. Especially, *L. plantarum* QZ50 had obvious broad-spectrum antibacterial activity and the inhibition ability was influenced by the change of conditions. The optimum medium, temperature, initial pH, and inoculum amount for plantaricin QZ50 production were Man, Rogosa, and

Sharpe (MRS), 30°C, 6.5, and 3%, respectively. Overall, the strain of *L. plantarum* QZ50, with a broad-spectrum, stable, safe, and natural antibiotic, has potential applications as a food biopreservative.

Methods

Samples and LAB isolation

A total of 31 traditional hand-made Qula cheese samples were collected from herdsmen's families in different areas of Qinghai province, China. The samples were collected in sterilized bags, stored in ice boxes, and immediately sent to the laboratory. Qula samples (10 g) were mixed with 90 ml sterilized water and serially diluted (10 ×) with sterilized water. The 10^{-1} to 10^{-5} dilutions (10 µl) were inoculated into Man, Rogosa, and Sharpe (MRS) agar [1] and incubated at 30°C for 48 h under anaerobic conditions. Following the 48-h incubation, we selected single colonies based on size, shape, and color and purified them on MRS agar. Gram staining and catalase test were performed on the purified colonies for the first identification of isolates. Only Gram-positive and catalase-negative isolates were selected for the tests described below.

Preliminary Screening of of Lactic Acid Bacteria with Antibacterial Activity

The Oxford cup double-layer plate method described by Muhammad et al in 2019 [7] was used for the preliminary screening of all the isolated presumptive lactic acid bacteria from the hand-made Qula cheese samples, screening out the lactic acid bacteria with antimicrobial activity, the indicator strains were *E. coli* K88 and *Salm. enterica* ATCC 13076, which were kept in our laboratory. The materials and methods were as follows: measure 15 mL of NA solid medium poured into a Petri dish and cooled as the lower layer of medium, optical density (OD) at 600 nm adjusted to 1 and inoculated into the NA solid medium cooled to about 50°C at 3% inoculum, mixed well, and poured 5 mL into the lower medium with a pipette. 200µL of overnight culture of *Lactobacillus* cell-free supernatant in the Oxford cup, incubate at 37℃ for 24h, and observe whether there are inhibition circles, and measure the diameter of inhibition circles using vernier calipers.

Physiological and Biochemical Characteristics

All of the strains were initially dentified by physiological and biochemical characteristics, including experiments of Gram reactions, catalase activity and gas production in the presence of glucose as described by Kozaki et al. in 1992 [8]. The bacterial growth at different temperatures (5, 10, 30, 45, and 50°C) and pH values (3.0, 3.5, 4.0, 4.5, 5.0, 8, 9, and 10.0), and tolerance against salt (3.0% and 6.5% NaCl) were measured according to the methods reported by Zhang et al [9].

Screening of LAB strains producing broad-spectrum bacteriocins

The antimicrobial activity of the isolates was examined by the agar diffusion assay method [10] with *Micrococcus luteus* ATCC 28001, *Staphylococcus aureus* ATCC 26003, *Bacillus subtilis* ATCC 63501, *Escherichia coli* ATCC 30105, *Pseudomonas aeruginosa* ATCC 10104, and *Salmonella enterica* ATCC 50094 as indicator strains. Overnight cultures in MRS broth were centrifuged at 10,000 *g* for 20 min. Each indicator strain was suspended in sterile water and standardized to an absorbance of 1 at 600 nm following activation. An

aliquot of cell-free supernatant (300 µl) was placed in wells (7.80 mm in diameter) of MRS agar plates and inoculated with 10% (v/v) of indicator strains. Following incubation at 37°C for 24 h, the diameters of the zone of inhibition were measured.

To eliminate the antimicrobial effect of organic acids, the pH value of the cell-free supernatants and MRS broth (control) was adjusted to 6.0 with NaOH (2 mol/L) and lactic acid (2 mol/L), respectively. To eliminate the antimicrobial effect of hydrogen peroxide, we incubated supernatants (pH 6.0) at 37°C for 2 h with catalase (5 mg/ml) and without catalase (control) [11].

Cell-free supernatants still remaining antimicrobial activity were screened for testing the possible protein nature of antimicrobial compounds. The cell-free supernatants (pH 6.0) were incubated at 37°C for 2 h in the presence of trypsin (3 mg/ml), protease K (3 mg/ml), and no enzyme (control).

Identification of selected strains by 16S rRNA gene sequence analysis

Genomic DNA of the selected strains were extracted using the Genomic DNA Mini Preparation Kit (Beyotime, Hangzhou, China). The amplification of the 16SrRNA gene was performed in a thermocycler using prokaryotic 16S ribosomal DNA universal primers: 27 F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492 R (5'-GGTTACCTTGTTACGACTT-3'). The homologies between the obtained sequences and those in the GenBank database was assessed with BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (NCBI). A bootstrap phylogenetic tree (1000 random resampling) was constructed by the neighbor-joining method using MEGA 5.0 [12].

For further identification of QZ50, a multiplex PCR assay was performed using the recA gene-based primers: paraF (5'-GTCACAGGCATTACGAA AAC-3'), pentF (5'-CAGTGGCGCGGTTGATATC-3'), planF (5'-CCGTTTATGCGGAACACCTA-3'), and pREV (5'-TCGGGATTACCAAACATCAC-3'). The amplification product of the recA gene was compared according to Torriani et al in 2001 [13].

Antibiotic Susceptibility

Antibiotic susceptibility testing was performed by the drug-sensitive tablet method described by Wang X et al [14] to test the susceptibility to 10 common antibiotics. Measure 15 mL of MRS solid medium into a Petri dish for cooling as the lower medium, add the overnight culture of *Lactobacillus* strains. to MRS agar medium at 5% inoculum and mix well, weigh 5 mL and pour it onto the cooled lower plate, and after cooling and solidifying, place the antibiotic susceptibility tablets on the agar plate with sterile forceps and incubate anaerobically at 30°C for 24 h. After that, use Vernier calipers to measure the diameter of the inhibition circle, 3 parallel experiments were performed to take the average value.

Cell Surface Hydrophobicity and Aggregation Assay

The cell surface hydrophobicity and auto-aggregation assay of selected strains were performed according to methods described by Sirichokchatchawan et al in 2018 [15]. Firstly, the preparation of *Lactobacillus* suspension was carried out, and Lactobacillus was cultured overnight in MRS liquid, centrifuged to take the bacteria, washed twice with PBS at pH 6.5, and then the bacteria were resuspended in PBS and the absorbance was adjusted to 0.6 at 600 nm (A0). The hydrophobicity of Lactobacillus cell surface was measured by the method of microbial adherence to hydrocarbons. The absorbance (At) of the aqueous phase at 600 nm was

measured by taking 3 mL of Lactobacillus cell suspension and mixing it well with 1 mL of xylene and leaving it at 37°C for 40 min. The cell surface hydrophobicity (%) was calculated by the following formula: (1-At/A0)×100.

Self-agglutination was measured according to the method of De Souza et al in 2019 [16]. Firstly, 1 mL of lactic acid bacteria suspension was taken and left for 2 h at 37°C, and the absorbance (A1) of its upper liquid layer at 600 nm was measured, then its self-agglutination (%) = 1 - ($A1 \div A0$) × 100, where A0 and A1 represent the absorbance at 0 h and 2 h, respectively. Each experiment was performed in triplicate.

Determination of Acid Production Capacity of representative Strains

The activated lactic acid bacteria were incubated in MRS liquid and the pH value were measured at selected time points (0,4,8,12,36 hours).

Resistance to Simulated Gastrointestinal Tract (GIT) Conditions

The tolerance of lactic acid bacteria under simulated human gastrointestinal was determined as described by Wang W et al [17]. Simulated gastric fluid: 0.35 g of pepsin was diluted in 100 mL 0.2% saline, pH was adjusted to 2.5 with dilute hydrochloric acid, filtered and sterilized. Mock intestinal fluid: 0.1g of trypsin, 1.8g of bovine bile salt, 1.1g of sodium bicarbonate and 0.2g of sodium chloride were added to 100mL of sterile water, pH was adjusted to 8.0 with sodium hydroxide, filtered and sterilized. The overnight culture of lactobacilli was inoculated into the simulated gastric juice at 5% inoculum, vortex shaken for 30s, and incubated at 37℃ for 3h. 100μL of the 3h solution was added into 900μL of simulated intestinal juice, vortex shaken for 30s, and incubated at 37℃ for 4h. The viable counts of lactobacilli after 0h, 3h and 7h were measured by dilution coating method. The viability of the bacteria in the simulated gastrointestinal tract was calculated.

Optimization of plantaricin QZ50 production

Effect of different media. Complete medium (CM) [18], MRS, medium 17 (M17) [19], and all-purpose Tween (APT) [20] were used to assess the optimum medium for bacteriocin production at 30°C for 24 h. Broth medium (pH 7.0) was used as the fermentation medium.

Optimization of culture conditions. The effect of temperature, inoculum amount, and initial pH on bacteriocin production were evaluated. Strain QZ50 was cultured in MRS broth at 30°C for 24 h. MRS broth (5 ml) was inoculated with 1% (v/v) QZ50 culture and incubated at 25, 30, 37, or 42°C for 24 h to determine the optimal temperature. MRS broth was adjusted to pH 4.5, 5.0, 6.0, 6.5, 7.0, 7.5, 8.0, or 9.0 to determine the effect of initial pH on bacteriocin production. Each tube was inoculated with 1% (v/v) QZ50 culture and incubated at 30°C for 24 h. MRS broth was inoculated with 1, 2, 3, 4, or 5% (v/v) QZ50 and incubated at 30°C for 24 h to determine the optimum inoculum amount.

Optimization of medium components. To assess the effect of different C sources on bacteriocin production, glucose, fructose, sucrose, maltose, lactose, cellobiose, or soluble starch were added at 2% (w/v) to MRS medium. To study the effect of different N sources, MRS medium was modified to a basal medium by removing yeast and beef extracts, peptone, and ammonium citrate. The modified medium was supplemented with 2% of different N sources (casein peptone, peptone, beef extract, yeast extract, tryptone, ammonium citrate, or ammonium chloride).

To evaluate the effect of stimulating factor on bacteriocin production, 2% Tween 80, Tween 20, or polyethylene glycol (PEG) 6000 was added to MRS medium. Additionally, Tween 80 at different concentrations (0, 1, 2, 3, 4, 5, and 6%) was used to determine the optimum concentration of Tween 80. In each experiment, antibacterial activity was conducted as previously described.

Resistance of bacteriocin against heat, pH, and enzymes

The resistance of bacteriocin to heat, pH, and enzymes was examined. Cell-free culture supernatants were heated to 60, 80, or 100°C in a water bath and to 120°C in an autoclave for 15 and 30 min. To assess the sensitivity to different pH values, cell-free culture supernatants were adjusted to pH 2, 3, 4, 5, 6, 7, 8, 9, or 10 and incubated at 37°C for 2 h. Cell-free culture supernatants were treated with α-amylase (1 mg/ml), β-amylase (1 mg/ml), lipase (1 mg/ml), pepsin (3 mg/ml), trypsin (3 mg/ml), protease K (3 mg/ml), papain (3 mg/ml), or chymotrypsin (3 mg/ml) at 37°C for 2 h. Prior to the 2-h incubation, the pH of the cell-free supernatants was adjusted to the optimum pH of each enzyme. After all treatments, antibacterial activity was determined as previously described.

Statistical analysis

Each assay was repeated on three independent occasions with triplicate determinations. Statistical analysis was performed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA) with statistical significance determined at $P < 0.01$ or 0.05. Results are expressed as the mean and standard error of the mean of three independent experiments. One-way ANOVA followed by Least significant difference test was used to determine significant differences of the antimicrobial activity due to the different media, culture conditions, medium components.

Abbreviations

LAB
Lactic acid bacteria
ATCC
American Type Culture Collection
L. plantarum
Lactobacillus plantarum
L. acidophilus
Lactobacillus acidophilus
L. sakei
Lactobacillus sakei
C. botulinum
Clostridium botulinum

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article and are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

BZ, JC and ZT designed the study and wrote the manuscript. BZ, JC, LW and HP performed the experiments. BZ, JC and GW conducted the statistical and bioinformatics analysis. GQ, MZ and YL performed the analysis of some of the data. All the authors reviewed and approved the final version of the manuscript.

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Figures

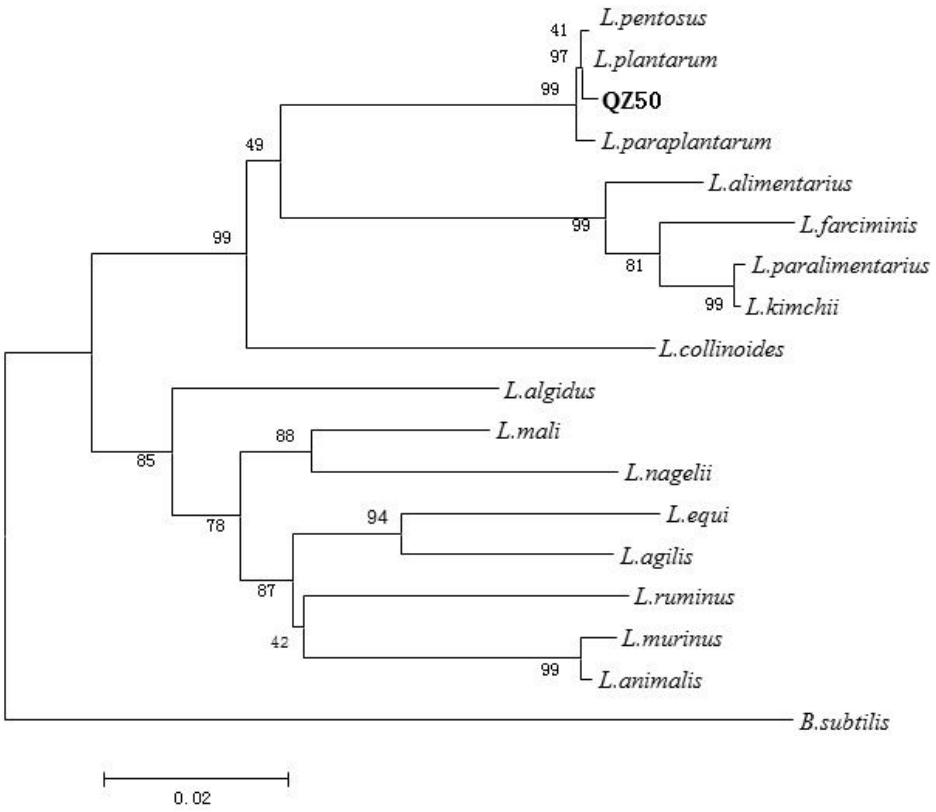

Figure 1

Phylogenetic trees based on the neighbor-joining method using 16S rRNA gene sequences. All sequences were from LAB strains. Numbers at the nodes represent bootstrap values for 100 replicates. B. subtilis was the outlier. Bar 0.02 represents sequence divergence.

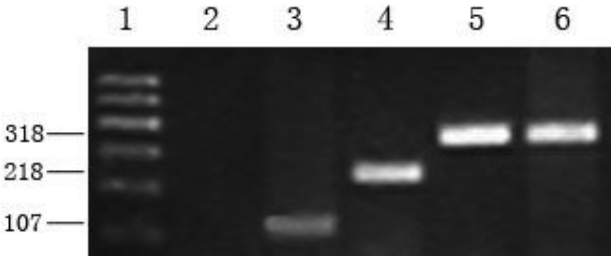

Figure 2

Amplification products obtained from the recA multiplex assay. Lane 1 contained a 600bp PLUS DNA ladder (Tiangen Biotech CO., Ltd., Beijing, China). Lanes 2, 3, 4, and 5, respectively contain the PCR amplification products from L. casei JCM 16167T (negative control), L. paraplantarum JCM 12533T, L. pentosus JCM 1558T, and L. plantarum subsp. plantarum JCM 1149T; Lanes 6 contain the PCR amplification products from strain QZ50.

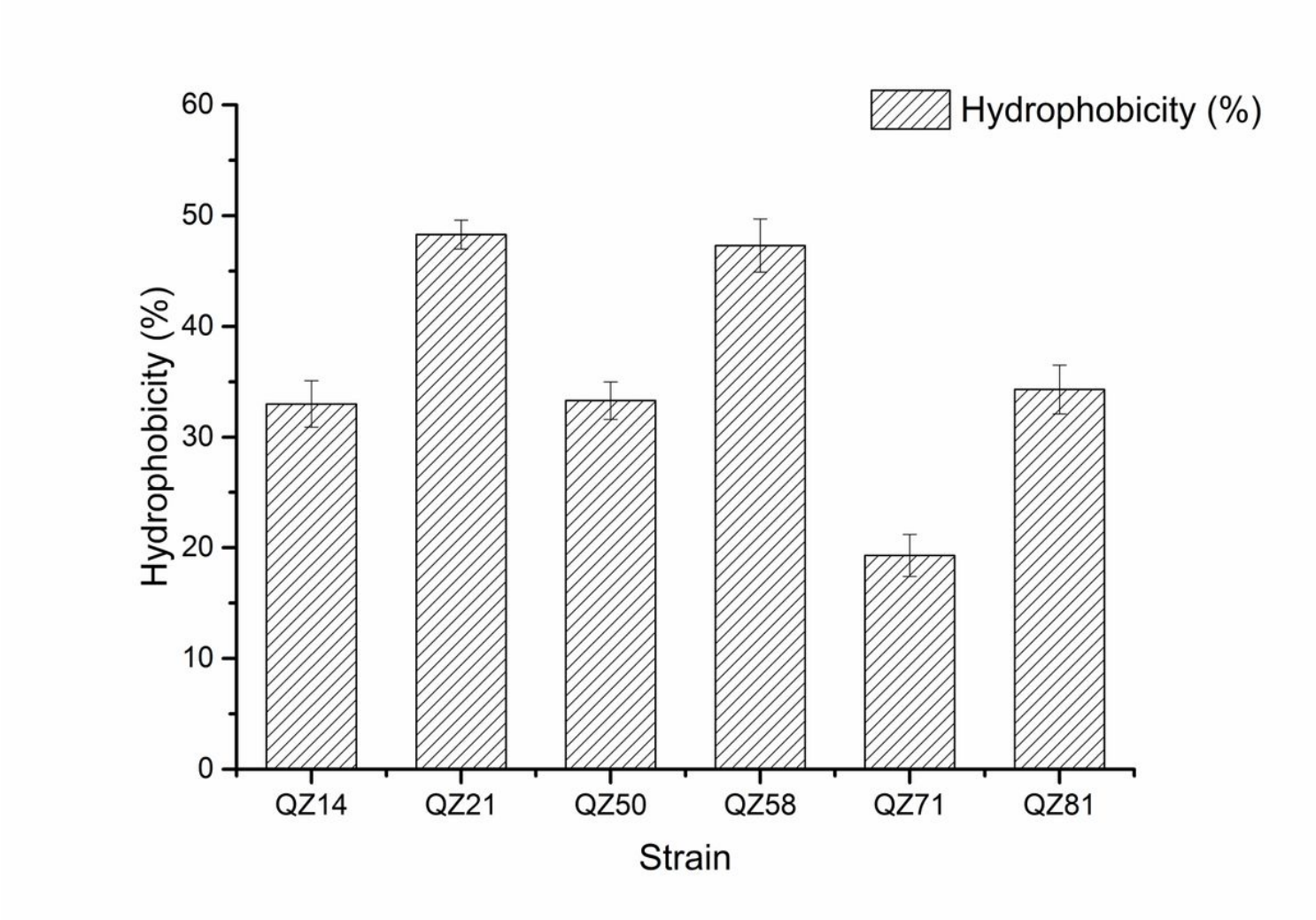

Figure 3

Cell surface hydrophobicity (%) ability of 6 selected lactic acid bacterial strains. Different lowercase letters denote significant difference ($p < 0.05$). The results were expressed as mean ± standard deviation (SD) (n = 3).

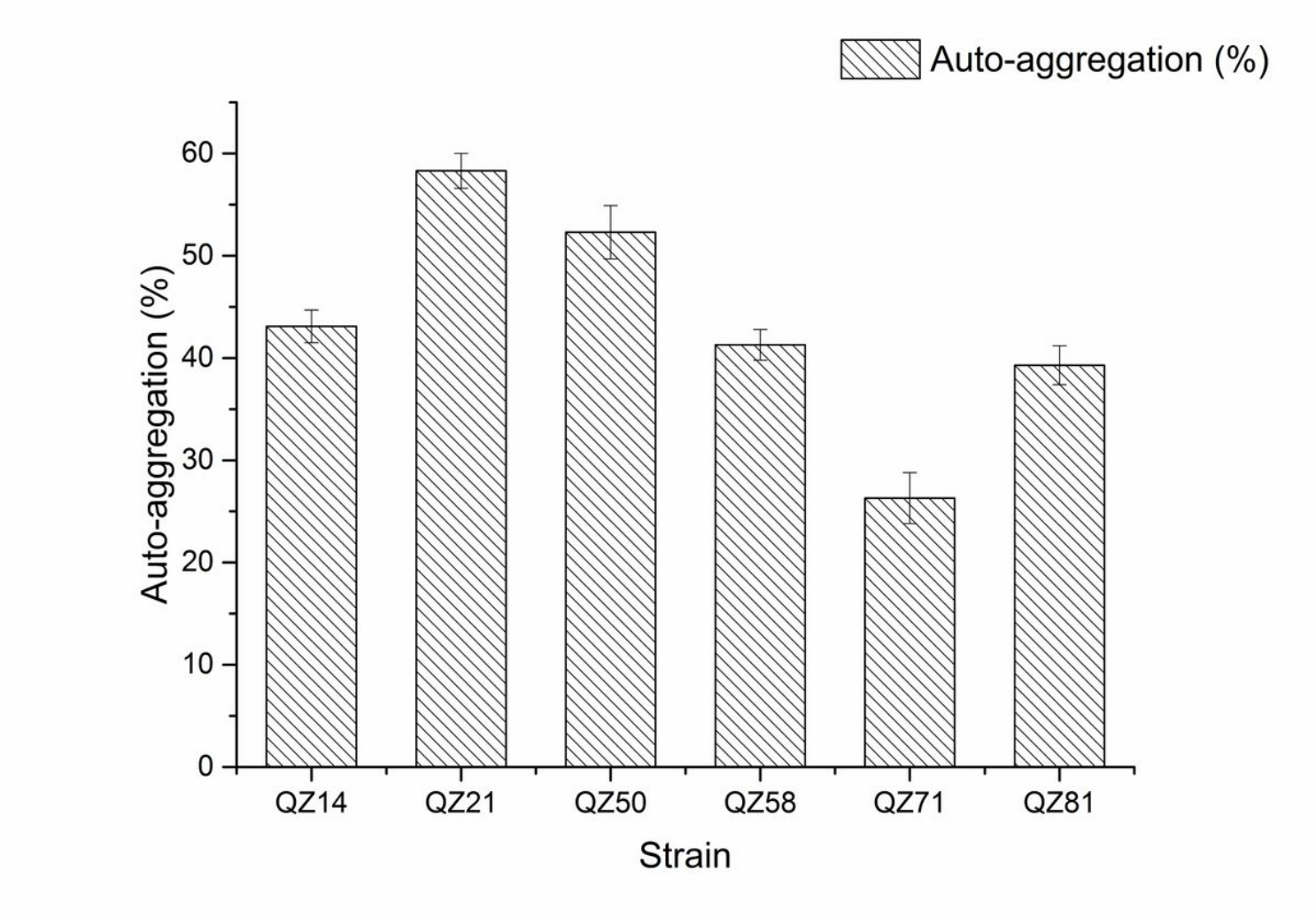

Figure 4

Cell surface auto-aggregation (%) ability of 6 selected lactic acid bacterial strains. Different lowercase letters denote significant difference ($p < 0.05$). The results were expressed as mean ± standard deviation (SD) (n = 3).

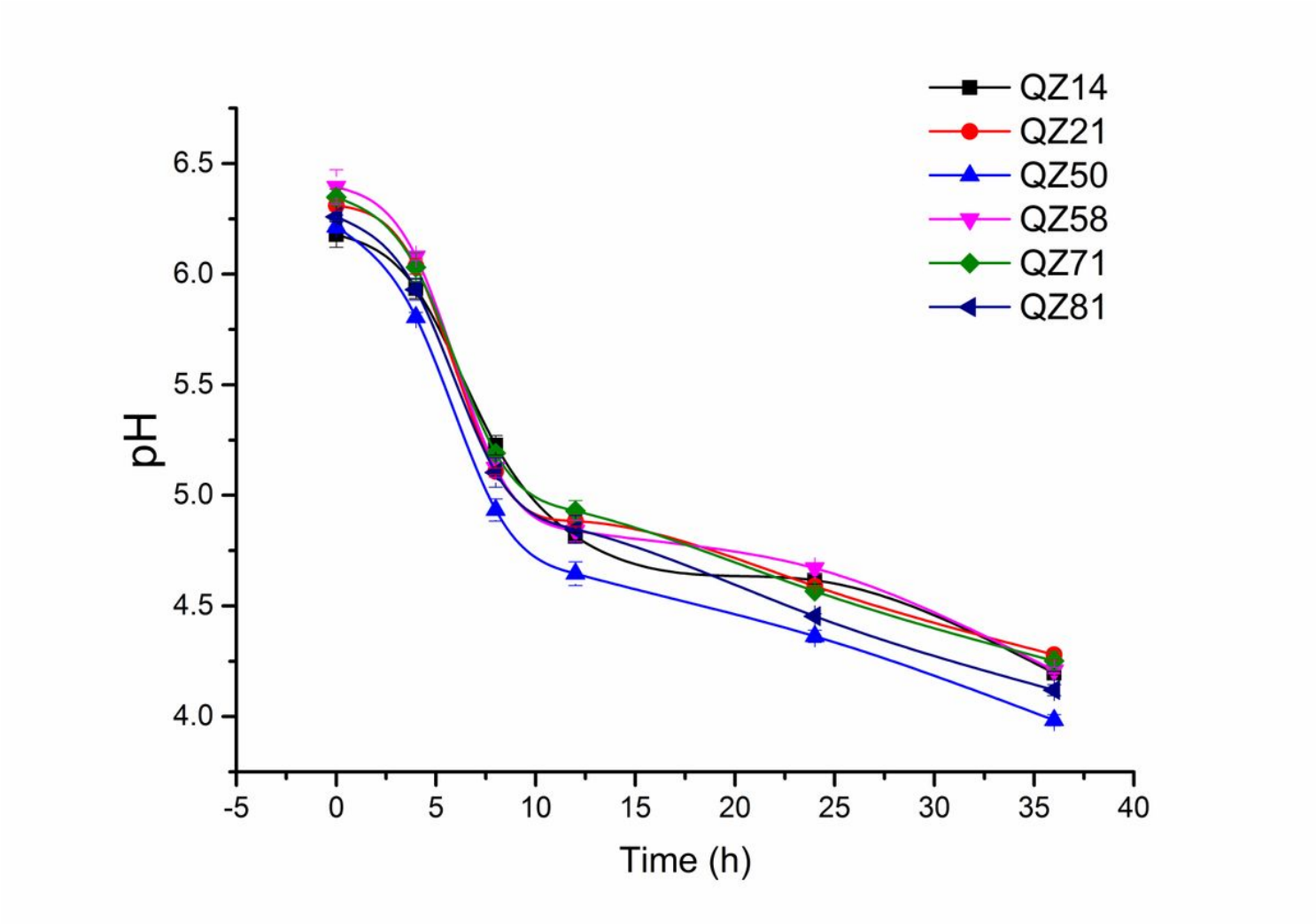

Figure 5

Acid production capacity of 6 selected lactic acid bacterial strains.

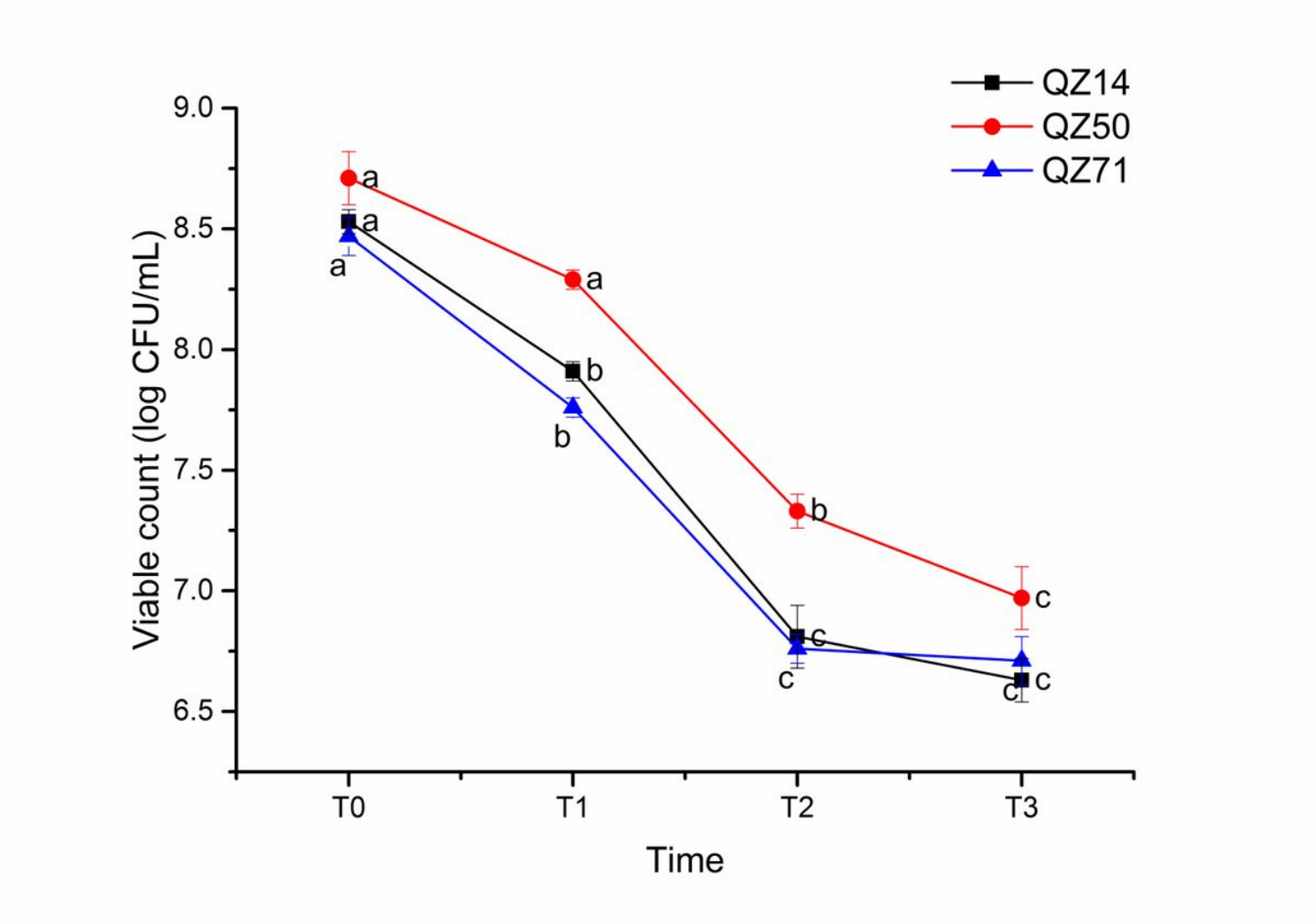

Figure 6

Viable count (log CFU/mL) of LAB after simulated gastrointestinal tract (GIT) conditions. Gastric juice T0 = viability at the beginning of gastric juice, gastric juice T1 = viability after simulation of gastric conditions, intestinal juice T2 = viability at the beginning of gastric juice, intestinal juice T3 = viability after simulation of enteric conditions. Different lowercase letters on the same row denote significant difference ($p < 0.05$) during the assay. The results are expressed as mean ± SD (n =3).

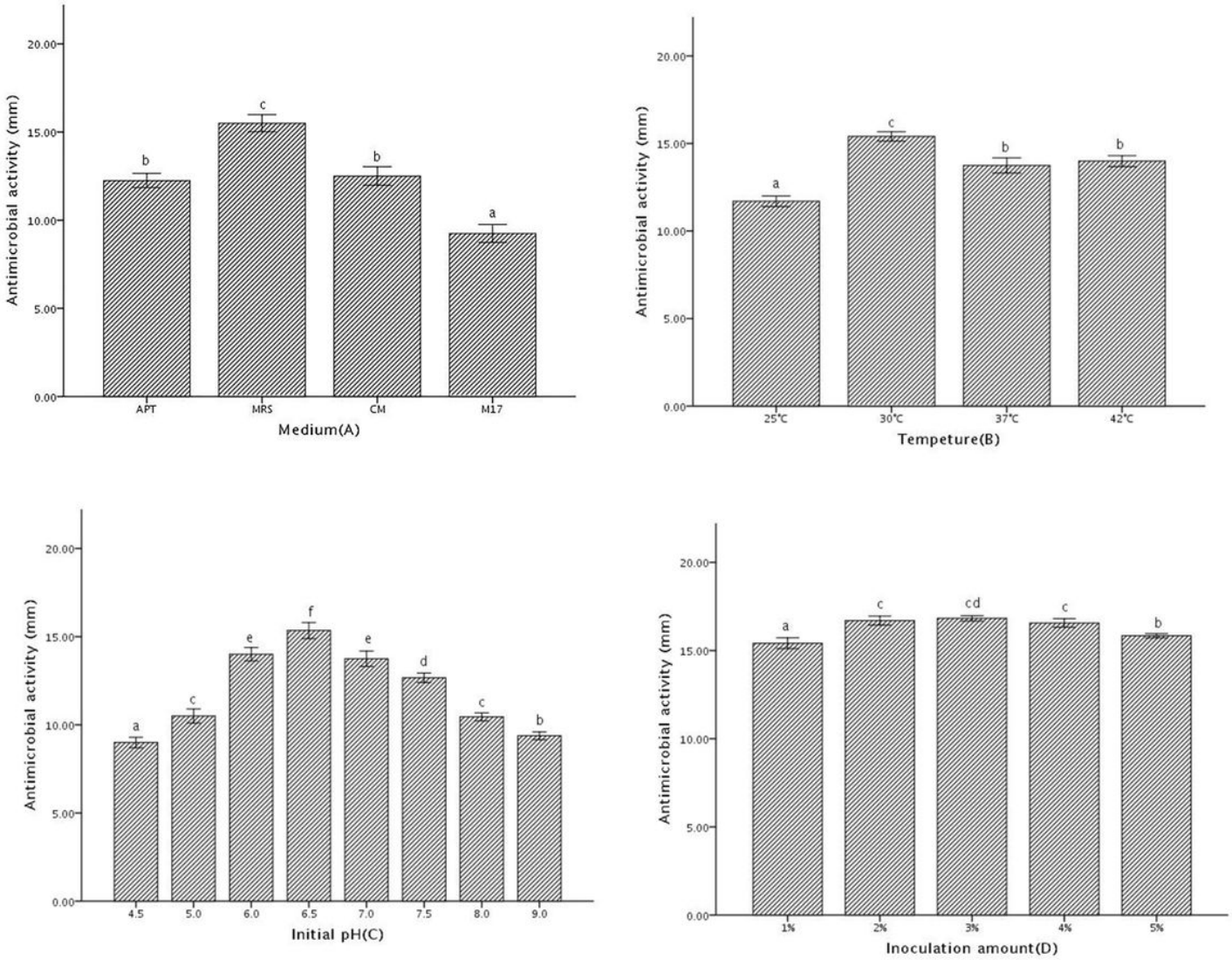

Figure 7

Effect of different media (A), temperature (B), initial pH (C), and inoculum amount (D) on bacteriocin production. Note: a,b,c,d,e,f Superscripts of the same letters indicate no significant inter-group differences, superscripts of different letters indicate significant inter-group differences ($P < 0.05$), and non-continuous letters indicate extremely significant inter-group difference ($P < 0.01$).

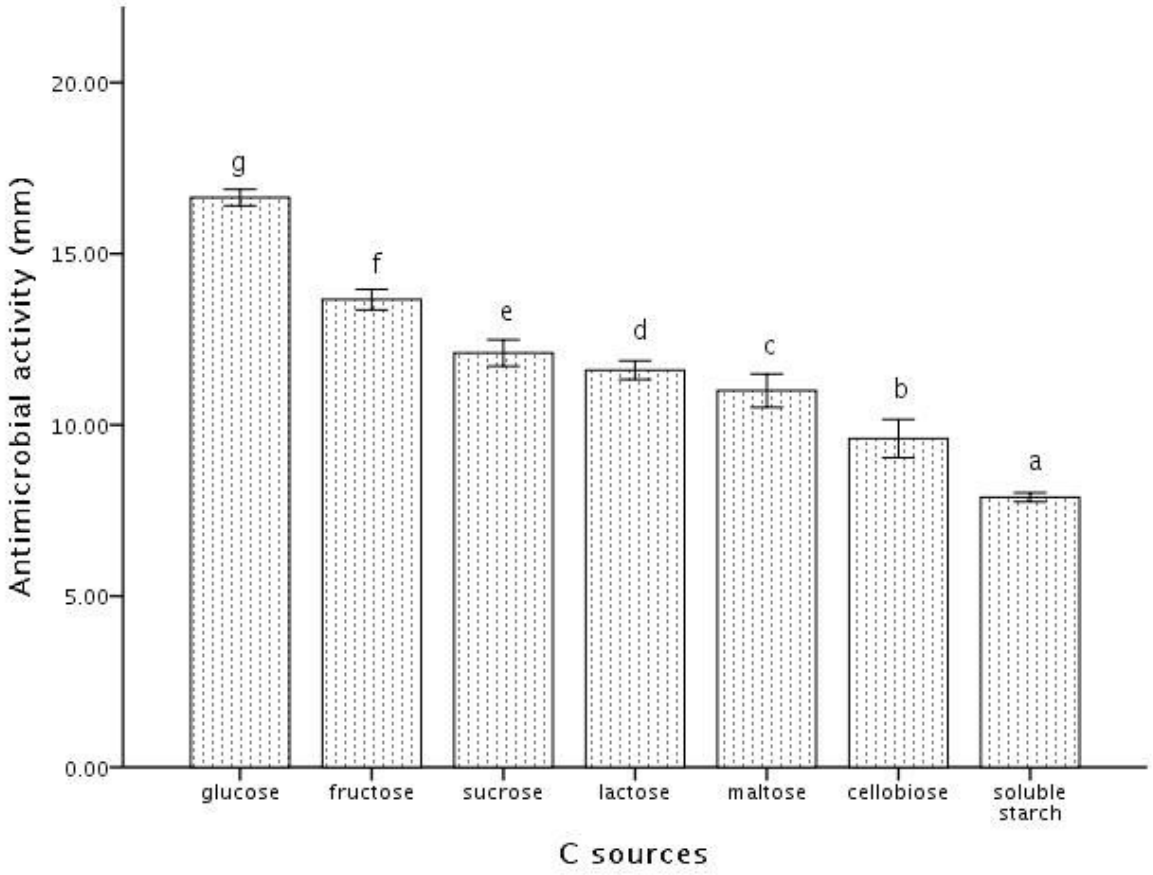

Figure 8

Effect of different C source on bacteriocin production. Note: a,b,c,d,e,f,g Superscripts of the same letters indicate no significant inter-group differences, superscripts of different letters indicate significant inter-group differences ($P < 0.05$), and non-continuous letters indicate extremely significant inter-group difference ($P < 0.01$).

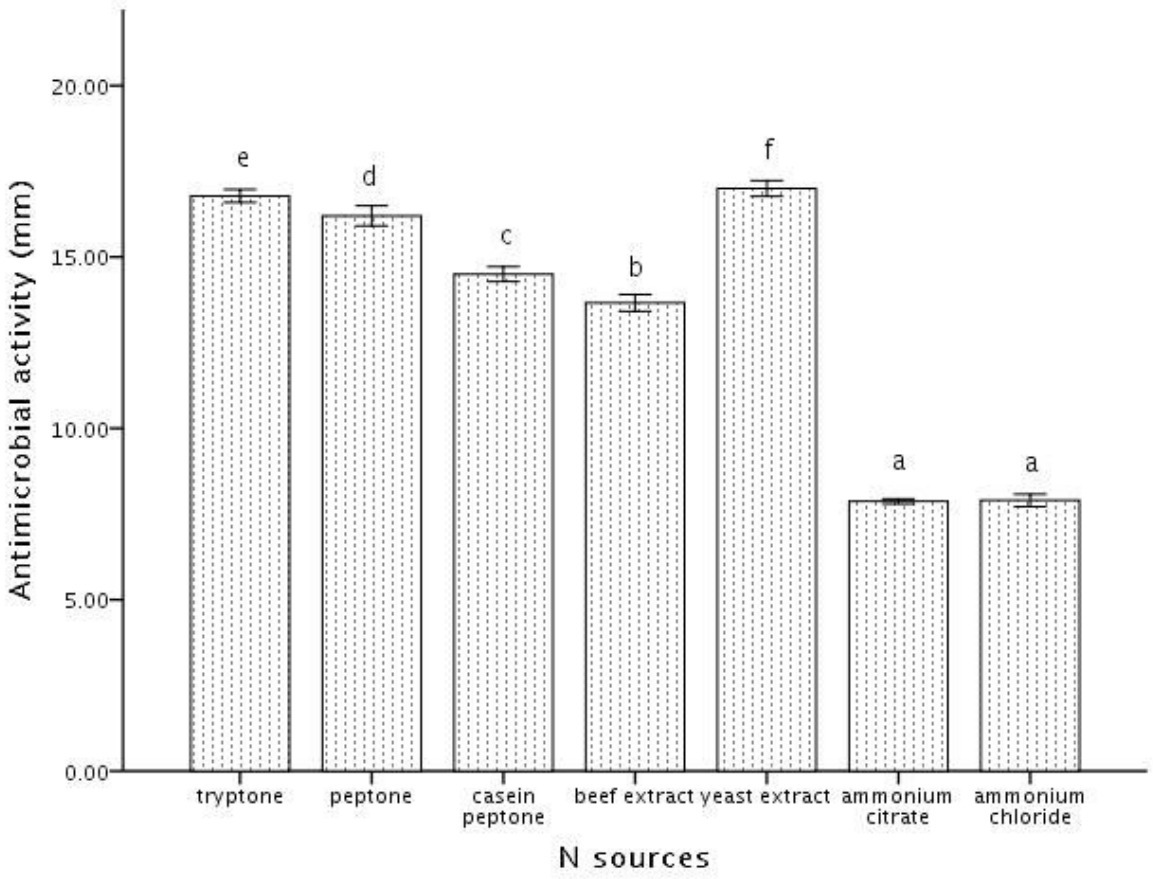

Figure 9

Effect of different N source on bacteriocin production. Note: a,b,c,d,e,f Superscripts of the same letters indicate no significant inter-group differences, superscripts of different letters indicate significant inter-group differences ($P < 0.05$), and non-continuous letters indicate extremely significant inter-group difference ($P < 0.01$).

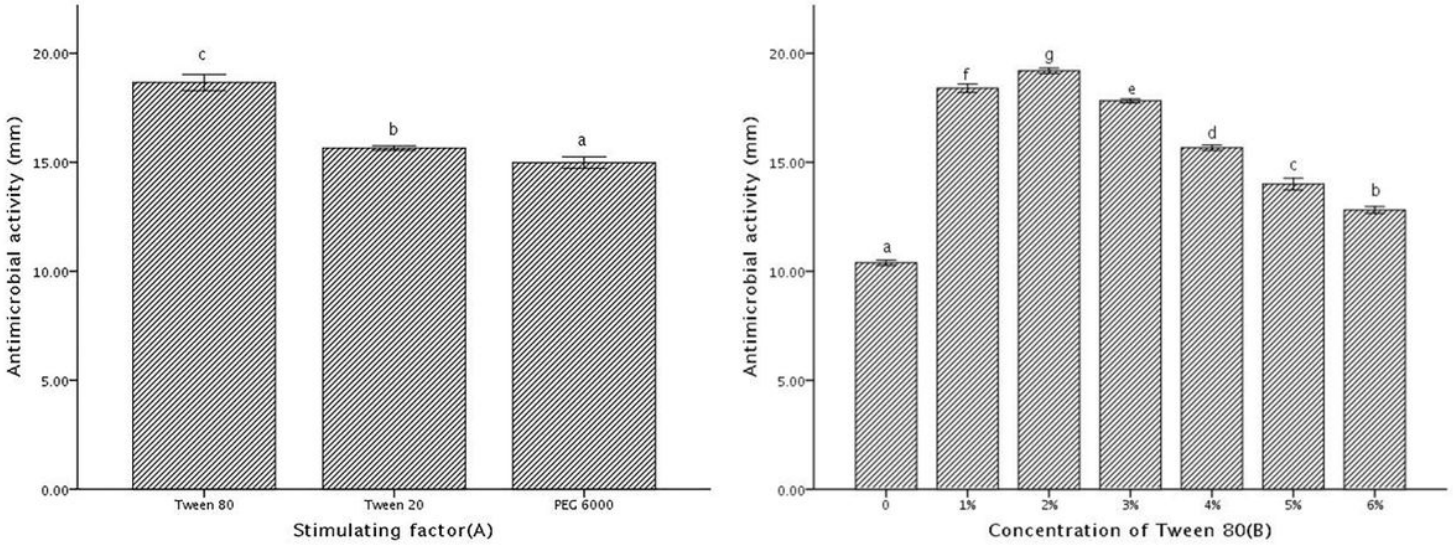

Figure 10

Effect of different stimulating factor (A) and concentration (B) on bacteriocin production. Note: a,b,c,d,e,f,g Superscripts of the same letters indicate no significant inter-group differences, superscripts of different letters indicate significant inter-group differences ($P < 0.05$), and non-continuous letters indicate extremely significant inter-group difference ($P < 0.01$).

Supplementary Files

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