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Partial Characterization of Bacteriocin Produced by Halotolerant *Pediococcus acidilactici* Strain QC38 Isolated from Traditional Cotija Cheese

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Abstract

During a screening of lactic acid bacteria producing bacteriocin from Cotija cheese, the strain QC38 was isolated. Based on the 16S rRNA gene nucleotide sequencing (516 pb accession no KJ210322) and phylogenetic analysis, the isolate was identified as *Pediococcus acidilactici*. Neutralized cell-free supernatant was tested for antimicrobial activity against 17 Gram-negative and Gram-positive pathogens. Growth inhibition was achieved against *Listeria monocytogenes* (supplier or indication or source), *Staphylococcus aureus*, *Vibrio vulnificus*, *Vibrio cholerae* O1 Ogawa, *Vibrio cholerae* NO 01 and *Salmonella enterica* subsp. *Enterica* serovar Typhimurium. Bacteriocin-like substance, after heating at 121°C for 15 min it remained stable and its antimicrobial activity was observed at pH ranging from 1.0 to 10.0 but inactivated by α-chymotrypsin and proteinase K. Strain QC38 was able to grow in 1–9% NaCl concentration. The plate overlay assay showed an approximate size of bacteriocin-like substance between 3.4 and 6.5 kDa. *P. acidilactici* QC38 harboured a plasmid that contains a gene for a pediocin (PA-1).

Key words: *Pediococcus acidilactici*, bacteriocin, cheese, cotija, lactic acid bacteria

Introduction

According to the U.S. Food and Drug Administration, some of the most common foodborne pathogenic bacteria are *Campylobacter jejuni*, *Clostridium botulinum*, *Clostridium perfringes*, pathogenic *Escherichia coli*, *Listeria monocytogenes*, *Salmonella enteritidis*, *Samonella* Typhimurium, *Shigella*, *Staphylococcus aureus*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, and *Yersinia enterocolitica*. Foodborne pathogens in the United States, cause losses in several billions of dollars (Hoffmann *et al*., 2012). Since the therapeutic antibiotics are restricted in foods, the utilization of antimicrobial peptides from lactic acid bacteria (LAB) that target food pathogens without toxic or other adverse effects has received important attention.

Bacteriocins produced by LAB are a diverse group of ribosome-synthesized antimicrobial peptides that may be divided into two main groups: class I peptides, which contain post-translational modifications, and class II, or unmodified peptides (Rea *et al*., 2011). Their production is a desirable trait among LAB from the perspective of controlling microbial populations in fermented foods in order to extend product shelf-life and also for safety purposes (Zacharof and Lovitt, 2012).

Cotija cheese is an artisanal raw-milk product named after a village of Michoacán State in Mexico. The texture of this cheese is hard and crumbly with a strong salty flavor. It is made of unpasteurized cow's milk in a very warm climate with 4% salt as a preserve without addition of lactic acid starters, usually 25 kg cylinders with a cream color crust and aged for about 3 months up to a year (Cervantes *et al*., 2008). The contribution of native microbiota may play an important role in its flavor, sensory properties and the quality of the Cotija cheese being produced (Wouters *et al*., 2002). Halotolerant (*Lactobacillus pentosus* and *Weisella thailandensis*) as well as the halophilic LAB (*Lactobacillus acidipiscis*

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and *Tetragenococcus halophilus*) have previously been detected in Cotija cheese (Morales *et al*., 2011) without identification of those producing bacteriocins.

The present work, aimed to identify the halotolerant lactic acid bacteria isolated from Cotija cheese and its antimicrobial activity. The bacteriocin produced by the isolate were also characterized.

Experimental

Materials and Methods

Screening for bacteriocin-producing lactic acid bacteria. Cotija cheese samples were collected from the mountain range of Michoacán, México as 20 g of cheese were added to 180 ml of peptone water. Then, the mixture was serially diluted with peptone water and plated on MRS and M-17 agar (Oxoid, Basingstoke, Hampshire, England) and supplemented with cycloheximide at 1 µg/ml to prevent growth of yeast and molds. The plates were incubated at 28°C for two to three days. The isolates were evaluated by cell morphology and Gram's stain reaction. The isolates were screened for bacteriocin production using the spot on the lawn assay using MRS plates (Moraes *et al*., 2010). Five µl of each isolated strain was spotted on MRS plates and incubated at 28°C for 24 h. After incubation, the plates were overlaid with 10 ml of BHI soft agar and inoculated with 10^5 CFU/ml of every tested pathogenic indicator strain. Then, the inhibition zone around the spots revealed potency of antibacterial activity.

Biochemical identification of isolated bacteriocin-producers. Phenotypic identification was performed using Gram´s staining and an H_2O_2 production test. Fermentation of carbohydrates was done by API 50 CHL system (BioMérieux, Inc,.Hazelwood Missouri, USA).

Identification of the isolate by 16S rDNA sequencing and phylogenetic analysis. Bacteriocin-like substance producers were identified by 16S rDNA sequencing to confirm the results obtained from the biochemical identification. DNA was extracted using the modified method described by Wilson (2001). Briefly, the pellet was suspended in 400 µl of TE buffer (1 mM EDTA pH 8.0, 10 mM Tris-HCl pH 8). Ten mg/ml of lysozyme, and 70 U/ml mutanolysin were added to the tube. The mixture was incubated for 30 min at 37°C, then 30 µl of 10% sodium dodecyl sulphate and 5 µl of proteinase K (20 mg/ml) were added. The tube was mixed and incubated at 37°C for 30 min. The cell debris were removed by precipitation with 100 µl of 5 M NaCl and 80 µl hexadecyltrimethylammonium bromide-NaCl (CTAB-NaCl) solution at 65°C for 10 min. Thereafter, 500 µl of chloroform-isoamyl alcohol (24:1) was added, mixed and centrifuged at 12000 × *g* for 10 min and the aqueous layer was transferred to a new tube containing an equal volume of isopropanol, mixed gently and stored at –20°C overnight. DNA was recovered by centrifugation and washed with 70% ethanol, dried under vacuum, suspended in 30 µl of molecular grade water and stored at –20°C until use. DNA was used to amplify the variable region (V1-V3) of the 16S rRNA gene (about 510 bp) using primer 4F and pD as previously described (Kommedal *et al*., 2011).

After agarose gel electrophoresis, the PCR products were purified by QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

To test the relationship of the 16S rRNA sequence obtained, against a set of most similar *Pediococcus* 16S sequences deposited in GeneBank, a Maximum Likelihood analysis (ML) was performed. The 16S *Pediococcus* sequence (QC38) was uploaded to the GenBank under accession number KJ210322. GenBank accession numbers included in the study were: AB219053, AB680266, AB682548, AB682554, DQ294959, DQ294960, EF059986, EF059987, EU147314, EU147315, FJ538506, FJ844959, FJ844984, FJ915729, GU904688, GQ421474, GQ421479, GQ222392, GQ222393, GQ222394, HQ288527, HQ315858, HQ315859, JN039348, JN836485, JQ806707, JQ806718, JX431046, and KF193970. Before analysis, all sequences were aligned with Clustal X ver. 2.1 (Thompson *et al*., 1997). Regarding the ML method, the algorithm implemented in the program aLRT-PHYML (Guindon and Gascuel, 2003) was used, along with an optimized base frequency and an estimated ratio of transition/transversion. Optimization of tree topology (rather than branch length) was selected. The reliability of each node was estimated *via* the approximate likelihood ratio test (aLRT) (Anisimova and Gascuel, 2006) with the Shimodaira-Hasegawa-like option. The *Lactobacillus plantarum* (strain B-2-7) was used as the outgroup (accession no. GQ183911).

Indicator pathogenic strains. *S. aureus* ATCC 6538, *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*S.* Typhimurium) ATCC14028 (supplier, sources are missing everywhere there, *Brucella abortus* 2308, *Brucella melitensis* 16M, *Brucella suis* 1330, *E. coli*, *Pseudomonas aeruginosa*, *V. vulnificus*, *V. cholerae* O1 Ogawa, *V. cholerae* non-01, *Vibrio alginoliticus*, *V. parahemolyticus*, *L. monocytogenes* ATCC19115, *Listeria innocua*, *Listeria ivanovii* and *Y. enterocolitica* were used as indicator strains for inhibitory effect. All strains, except *V. vulnificus*, *V. cholerae* O1 Ogawa, *V. cholerae* non-01, *V. alginoliticus*, *V. parahemolyticus*, *B. abortus* 2308, *B. melitensis* 16M and *B. suis* 1330 were cultured in Brain heart infusion broth agar (BHI) (BD, Maryland, USA) but *V. vulnificus*, *V. cholerae* O1 Ogawa, *V. cholerae* non-01, *V. alginoliticus* and *V. parahemolyti-*

cus were cultured onto Tryptone soy agar supplemented with 1% NaCl. Finally, *B. abortus* 2308, *B. melitensis* 16M and *B. suis* 1330 were cultured in Tryptone soy agar (BD, Maryland, USA).

Antimicrobial activity assay. Strain QC38 was grown in MRS broth at 37°C for 24 h. The culture was centrifuged at 8000 × *g* for 10 minutes. The cell free supernatant (CFS) was then adjusted to pH 6.5, treated with catalase (1 mg/ml) and filtered through a 0.22 µm filter (Millipore, Carrigtwohill, Co. Cork, Ireland). Then, agar well diffusion assay (AWDA) was used for detection of antimicrobial activity against the investigated indicator pathogenic strain (Ponce *et al*., 2008). Soft BHI agar (1% w/v) containing approximately 10^5 CFU/ml of indicator strains was overlaid onto MRS plates. Wells of 5 mm in diameter were created in the agar indicator pathogen overlaid plates and then filled with 50 µl of the CFS and overnight incubated at 37°C. Then inhibition zones were measured.

Production of bacteriocin. Strain QC38 was grown in MRS broth (pH 6.5) during 18 h. Then, the cells (2% v/v) were transferred to a bottle with MRS broth and incubated at 37°C. Incubated broth samples were aseptically taken from the culture at periodical time intervals in order to measure optical cell density at 600 nm, pH and antimicrobial substance production. The antimicrobial activity of CFS was measured by the AWDA method as described above. The antimicrobial substance concentration Arbitrary Unit per milliliter (AU/ml) was calculated as the inverse of the most concentrated dilution, which induces the inhibition of *L. monocytogenes* (Todorov, 2008).

Effect of pH and enzymes on bacteriocin activity. The effect of pH on bacteriocin activity in the CFS was evaluated by adjusting the pH from 1 to 10 with 1 M HCl or 1 M NaOH. After 2 h of incubation at 30°C, the samples were adjusted to pH 6.5 and the antimicrobial activity was determined (Ponce *et al*., 2008). The CFS was treated with proteinase K, α-chymotrypsine, and pronase E (final concentration 1 mg/ml) for 1 h, then the antimicrobial activity was measured by the AWDA method using *L. monocytogenes* as an indicator.

Effect of temperature and extender storage. The CFS was heated at 37, 50, 70, 80 and 90°C for 1 h, then the samples were cooled at 4°C and the effect of extender storage at 4 and –20°C for 5, 10, 20 days and 1 year was also evaluated. Untreated CFS was used as a positive control in each experiment. Residual activity was determined by AWDA, as described above.

Effect of NaCl concentration on the growth of *P. acidilactici* QC38 and bacteriocin production. *P. acidilactici* QC38 was inoculated (1% v/v) into MRS broth containing different concentrations of NaCl (from 0.5–10% w/v) and incubated at 37°C during 24 h. The optical density at 600 nm wavelength was measured to evaluate bacterial growth in the broth culture. Production of bacteriocin in each concentration of NaCl was evaluated by the AWDA assay using *L. monocytogenes* as the indicator of inhibition (Verluyten *et al*., 2004).

PCR of bacteriocin gene. Plasmid DNA isolation was performed by the modified alkaline lysis method (Anderson and McKay, 1983) and DNA was used as a template for the amplification of bacteriocin gene. PCR was performed using 20 µl reaction mixture consisting of 1X PCR buffer, 1 U of *Taq* DNA polymerase, 3 mM of $MgCl_2$, 0.4 mM of dNTPs and 0.2 µM of each primer (PedF and PedR) (Suwanjinda *et al*., 2007). The PCR conditions were an initial denaturation step at 94°C during 5 min, followed by 30 cycles of denaturation at 94°C during 1 min, annealing (51°C, 40 s) and extension (72°C, 3 min). The products were analyzed on 1.5% agarose gel and staining with ethidium bromide.

Molecular size of bacteriocin. The molecular size of bacteriocin present in CFS was determined by tricine-SDS-PAGE (Schagger and von Jagow, 1987) and a low-molecular weight maker (polypeptide SDS-PAGE, Biorad) was used. After electrophoresis, one half gel was fixed and stained with Coomasie blue. The other half was used to determine the position of bacteriocin in the gel (Powell *et al*., 2007). Briefly, the gel was overlayed with *L. monocytogenes* (10^6 CFU/ml), suspended in BHI soft agar and incubated at 37°C for 24 h.

Results

Isolation of the bacteriocin-producing strains. A total of 178 LAB strains were isolated from Cotija cheese. After preliminary identification the isolate, called QC38 was Gram-positive, catalase and oxidase tests negative, and identified by carbohydrate fermentation profile (API 50CH L system) as *P. acidilactici*.

Phylogenetic analysis of isolate QC38. The resulting ML phylogeny revealed that its 16S sequence has a phylogenetic close relationship with other *Pediococcus* 16S sequences deposited in the GenBank. The phylogeny was resolved showing two main clades with strong nodal support (aLRT > 90, not shown; Fig. 1). One of these clades has approximately three times more sequences than the other one. The smaller clade contains our 16S *Pediococcus* sequence (QC38), was closely related to *P. acidilactici* strains.

Antimicrobial activity and production of bacteriocin. Inhibitory activity was observed against *E. coli*, *L. monocytogenes*, *L. innocua*, *S. aureus*, *S.* Typhimurium, *V. cholerae* NO 01, *V. cholerae* O1 Ogawa and *V. vulnificus* (Table I). As bacteriocin production of *P. acidilactici* QC38 was evaluated, the antimicrobial activity was observed since the exponential growth phase but the activity reached its highest bacteriocin

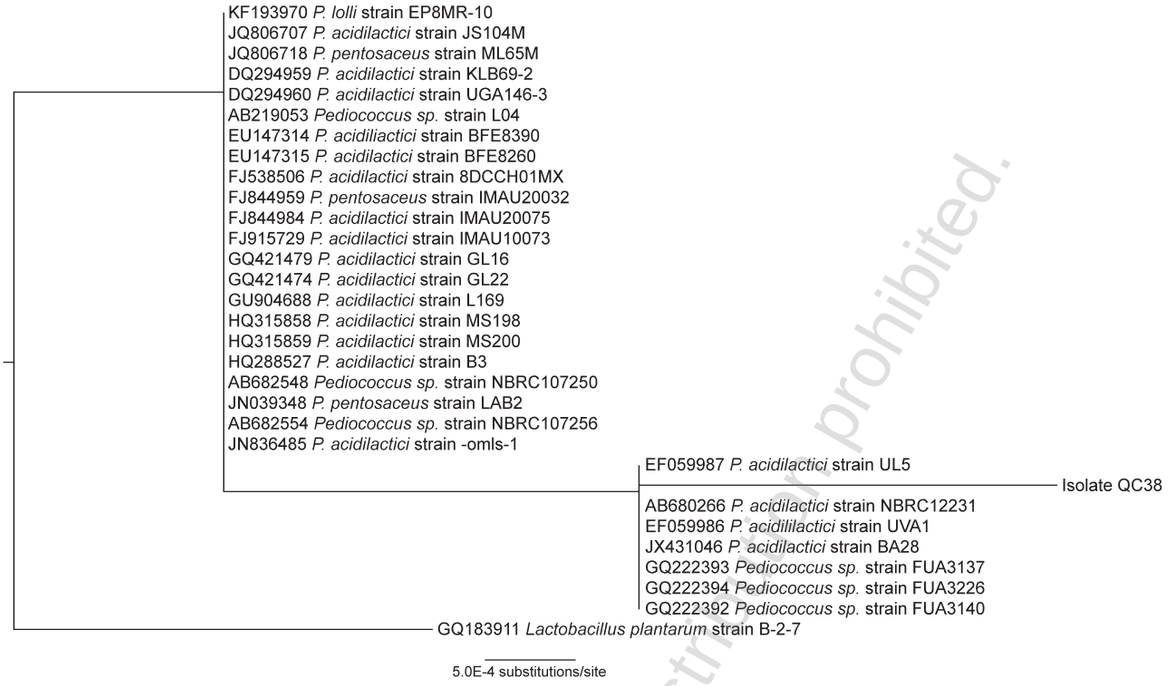

Fig. 1. ML tree reconstruction based on 516 bp from 16S *Pediococcus* species.

Branch support values in the two main clades are > 90 (aLRT). Letters at the beginning of tip names correspond to GenBank accession numbers, followed by *Pediococcus* species and strain. *L. plantarum* strain B-2-7 was used as the outgroup.

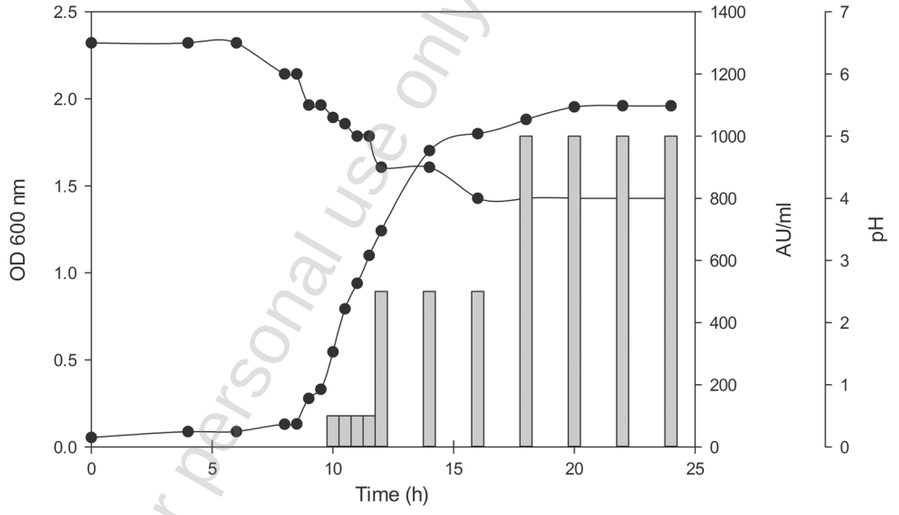

Fig. 2. Bacteriocin production during the growth of *P. acidilactici* QC38 at 37°C.

Changes in optical density measured at 600 nm (●). Antimicrobial activity is presented as AU/ml (bars) against *L. monocytogenes*.

production level in the late exponential phase. The highest antimicrobial activity was maintained during the stationary phase (Fig. 2).

Effect of enzymes, pH and temperature. When CFS of *P. acidilactici* QC38 was treated with different proteolytic enzymes total inactivation of the inhibitory activity was observed with proteinase K and α-chymotrypsin but partially with pronase E. Concerning the pH stability of bacteriocin produced by *P. acidilactici* QC38, the inhibitory activity remained stable after incubation for 2 h at pH values between 1.0 and 10.0 and the CFS was stable at all temperatures tested, even after treated at 121°C, 15 psi pressure for 15 min (Table II).

Growth of *P. acidilactici* QC38 in NaCl and the effect on the bacteriocin production. *P. acidilactici* QC38 was able to grow in 1–9% NaCl. Bacteriocin

Table I
Antimicrobial activity spectrum of *P. acidilactici* QC38.

Indicator microorganism	Activity
Bacillus cereus	–
Brucella abortus 2308	–
Brucella melitensis 16M	–
Brucella suis 1330	–
Escherichia coli	+
Listeria innocua	+++
Listeria ivanovii	–
Listeria monocytogenes	+++
Pseudomonas aeruginosa	–
Salmonella Typhimurium	+
Staphylococcus aureus	++
Vibrio parahemolyticus	–
Vibrio alginoliticus	–
Vibrio cholerae non-01	+
Vibrio cholerae O1 Ogawa	+
Vibrio vulnificus	+
Yersinia enterocolitica	–

(–) No inhibitory activity; (+) 1–9 mm (low inhibitory effect on growth); (++) 9.1–19 mm (medium effect); and (+++) > 19.1 mm (high effect).
*Antimicrobial activity was measured by AWDA.

production was unaffected by 1–3% NaCl, however, higher concentrations of NaCl (4–9%) reduced bacteriocin production.

PCR bacteriocin gene and molecular size. After purification of a plasmid from *P. acidilactici* QC38 it was used as a template to amplify a fragment of the gene encoding for pediocin PA-1 300 bp that indicates the presence of this encoding gene in *P. acidilactici* QC38 (Fig. 3). The molecular size of bacteriocin produced by *P. acidilactici* QC38 was between 3.4–6.5 kDa, as determined by tricine-SDS-PAGE (Fig. 4).

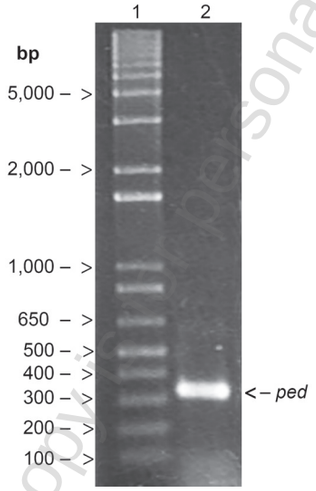

Fig. 3. DNA fragments obtained after PCR with plasmid DNA from *P. acidilactici* QC38 using Pediocin PA1-specific primers.
(Lane 1) 1 kb Plus ladder, and Lane 2) 300 bp amplicon obtained using plasmid DNA.

Table II
Effects of proteolytic enzymes, temperature, and pH on the activity of the bacteriocin produced by *P. acidilactici* QC38.

Treatment	Residual bacteriocin activity (%)
Proteinase K[b]	0
Pronase E[b]	69
α-chymotrypsin[b]	0
50°C[c]	98
70°C[c]	98
80°C[c]	84
90°C[c]	89
121°C, 15 psi for 15 min[d]	84
4°C, 5 days[e]	100
4°C, 10 days[e]	100
4°C, 20 days[e]	98
4°C, 1 year[e]	51
–20°C, 5 days[e]	100
–20°C, 10 days[e]	100
–20°C, 20 days[e]	98
–20°C, 1 year[e]	89
pH[f]	
1	96
2	98
3	100
4	100
5	100
6	100
7	100
8	96
9	84
10	82
Control[g]	100

[b] Bacteriocin treated with proteolytic enzymes at 37°C for 1 h.
[c] Bacteriocin incubated at different temperatures for 1 h each.
[d] Bacteriocin autoclaved at 121°C, 15 psi for 15 min.
[e] Bacteriocin storage at various temperatures and time intervals.
[f] Bacteriocin treated at different pH values at 37°C for 2 h each.
[g] Untreated bacteriocin. Antimicrobial activity was measured by AWDA.

Discussion

The food processing industry has recently focused on applying new strategies for food conservation in order to guarantee their quality and safety. Application of bacteriocin can be an useful alternative, since these peptides do not alter the food flavor, aroma and texture.

Bacteriocins are commonly produced by LAB and have been isolated from fermented foods such as dairy

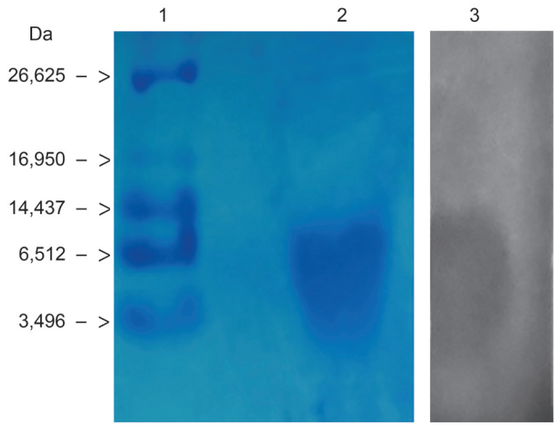

Fig. 4. Tricine-SDS-PAGE of bacteriocin produced by *P. acidilactici* QC38.
Lane 1) Molecular weight marker; Lane 2) Bacteriocin produced by *P. acidilactici* QC38 stained with Coomassie blue; Lane 3) Zone of growth inhibition.

products. It has been reported that LAB isolated from dairy products are good candidates for evaluating antimicrobial substance production, as they are not pathogenic bacteria and most of them have been used as probiotic strains (De Vuyst and Leroy, 2007).

In Mexico, different kinds of cheeses are manufactured without starter cultures such Cotija cheese. In this work different LAB were isolated from Cotija cheese, but one of them was able to inhibit pathogenic Gram-positive and Gram-negative bacteria. It was identified as *P. acidilactici* belonging to LAB which is involved in different fermentation processes. The ability of LAB to inhibit the growth of pathogenic and undesirable bacteria in food is well known due to the production of organic acids, hydrogen peroxide or bacteriocins (De Vuyst and Leroy, 2007). The proteinaceous nature of bacteriocin produced by *P. acidilactici* QC38 was confirmed by using proteolytic enzymes. Anti-Listerial activity has been reported for other *P. acidilactici* strains isolated from dairy and fermented products, these bacteriocins have a molecular weight of about 4.6–15 kDa (Gurira and Buys, 2005; Abbasiliasi *et al.*, 2012; Zhang *et al.*, 2012). Bacteriocins produced by strains of *P. acidilactici* varied in their antimicrobial activity whereas those isolated from a sausage inhibited *L. monocytogenes* but could not inhibit *S. aureus* or *Salmonella* Typhimurium (Albano *et al.*, 2007). Nevertheless, *P. acidilactici* QC38, was not only able to inhibit the growth of *L. monocytogenes* but also *E. coli*, *L. innocua*, *S. aureus*, *S.* Typhimurium, *V. cholerae* NO 01, *V. cholerae* O1 Ogawa, and *V. vulnificus*. The inhibitory activity against Gram-positive and Gram-negative is less prevalent in the bacteriocin reports. Since Cotija cheese is made of raw unpasteurized milk, *Brucella* spp. was evaluated as an indicator, however the three tested strains were not inhibited in the study. The antimicrobial activity of bacteriocin of *P. acidilactici* QC38 showed similar results of those produced by different LABs previously reported (Cladera-Olivera *et al.*, 2004; Todorov and Dicks, 2006). PCR plasmid investigation of *P. acidilactici* QC38 detected the presence of the gene encoding the pediocin PA-1 in the purified plasmid which is a bactericidal peptide produced *via* plasmid-linked operon (Halami and Chandrashekar, 2005; Manjulata and Halami, 2011). The bacteriocin produced by *P. acidilactici* QC38 showed a size between 3.4–6.5 kDa similar to that one reported for pediocin, however bacteriocin produces by QC38 showed antimicrobial activity against broad range of foodborne pathogenic bacteria not reported previously.

Conclusions

Due to strict regulations in the food industry, there is an increasing demand in controlling food-borne pathogenic bacteria such as *L. monocytogenes*, *S. aureus*, *S.* Typhimurim *etc*. To our knowledge, this is the first study on a bacteriocinogenic strain of *P. acidilactici* isolated from Cotija cheese. The bacteriocin reported in this work was able to inhibit some virulent Gram-positive and Gram-negative food-borne pathogens and it was resistant to a wide range of pH storage and NaCl rather than being a heat stable bacteriocin. All these properties nominates this peptide as a potential preservative for food products.

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Conflict of interest

The authors declare that they have no conflict of interest.

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