Biodiversity of bacteriocin-producing lactic acid bacteria from Mexican regional cheeses and their contribution to milk fermentation

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Abstract

The aim of this work was to examine the biodiversity of bacteriocin-producing lactic acid bacteria from homemade cheeses produced in Veracruz (México) and assess their contribution as adjunct cultures in dairy products. Ninety-three presumptive bacteriocinogenic strains were detected by direct antagonism assays and twenty-nine out of them were active against *Enterococcus faecalis* NRRL-B537, *Listeria innocua* 062 AST, or *Listeria monocytogenes* ATCC19115 by the well diffusion test using cell-free supernatants, adjusted to pH 6.0 to exclude inhibition by organic acids. Positive isolates were identified by partial sequencing of the 16s rDNA as *Pediococcus acidilactici* (4 isolates), *Enterococcus faecium* (17 isolates), *Lactobacillus plantarum* (6 isolates) and *Lactobacillus fermentum* (2 isolates). RAPD-PCR discriminated 7 groups with a 50% similarity and revealed the presence of the same isolates. The coding genes for the synthesis of plantaricin EF, plantaricin JK, plantaricin N, plantaricin NC8 and the inducing peptide plantaricin A were detected by PCR in *L. plantarum*. Similarly, enterocin P and pediocin PA-1 genes were amplified from *Enterococcus* and *Pediococcus* genomic DNA, respectively. Overall, co-culturing of bacteriocin producing *Lactobacillus* and *Pediococcus* strains with the dairy starter *Lactococcus lactis* IPLA947 did not interfere with milk acidification. Lactose consumption, acidification rate and production of lactic acid were unchanged. Nonetheless, higher levels of acetic acid, ethanol and succinic acid were detected depending on the strain. Our results demonstrate the diversity of bacteriocinogenic species in homemade Mexican cheeses which may be used as adjunct cultures to enhancing safety of this well-appreciated cheese while providing a richer range of metabolites.

Keywords: milk fermentation, adjunct cultures, bacteriocin, Mexican cheese, raw milk cheese, lactic acid bacteria

Introduction

Lactic acid bacteria (LAB) play an important role in the food industry, because they contribute significantly to the flavour, texture and, in many cases, to the nutritional value of the food products (Carr et al. 2002). LAB are found on rich nutrient habitats which are characterized by the presence of soluble carbohydrates, degraded protein products and vitamins, as well as, low oxygen tensions and include, for example, milk, dairy products, fermented meat, fish, fruits and vegetables. LAB are also part of the gastrointestinal and urogenital tract native microbiota (Dobson et al. 2012; Eckburg et al. 2007)

LAB have the ability to inhibit the development of a large number of pathogenic and food spoilage microorganisms. The primary antimicrobial effect of LAB is due to the competition for nutrients and the synthesis of organic acids (mainly lactic acid and acetic acid), that lower the pH of the environment. However, among the antimicrobial substances produced by LAB, antimicrobial peptides known as bacteriocins, are regarded as the most interesting for food biopreservation (Favaro et al. 2015). Due to their protein nature, they are degraded by proteolytic enzymes in the gastrointestinal tract while they remain active in the food substrate in which they are located. In addition they are not considered toxic or immunogenic (Favaro et al. 2015; García et al. 2010).

The increasing number of consumers who demand for less processed and traditional food products has fostered research on bacteriocins to inhibit pathogenic microorganisms. Bacteriocin-producing LAB can be used as starters or adjuvant cultures in food fermentations (Rehaim et al. 2012; Rilla et al. 2003). Fermented dairy products, especially raw milk artisanal cheeses, have been exploited as a source of bacteriocinogenic LAB. In this context, there are several reports on the isolation of indigenous LAB from different

types of cheeses made through a traditional way that highlight the relevance of the microbial diversity found in these niches (Lavilla-Lerma et al. 2013; Moraes et al. 2012; Ortolani et al. 2010).

In Mexico, as in other countries of Latin America, cheese quality is closely associated with the production region and its traditions (Saxer et al. 2013). Fresh Cheese is the most popular variety consumed in these countries and is also the most popular Hispanic-style cheese found in the United States (Torres-Llanez et al. 2006). A great variety of typical Mexican cheeses can be found such as Panela, Fresh, Oaxaca, Mexican-Manchego style, Manchego-Botanero, Tenate and backpack cheese (Caro et al. 2014; Saxer et al. 2013). These are cheeses made from cow's milk and they are often marketed and consumed fresh or short-ripened. Several aspects of Mexican cheeses dealing with food safety and characterization of native microbiota have been recently reported (Alvarado et al. 2005; Caro et al. 2013).

“Queso fresco” cheese (fresh cheese) is a high moisture cheese characterized by a crisp texture, a slightly salty flavour and a low melting capacity. Artisanal, small-scale production is traditionally made by farmers using raw milk without adding a specific starter culture (Caro et al. 2014; Renye et al. 2008; Torres-Llanez et al. 2006). Most commonly, coagulation is driven by the indigenous LAB present in milk and the action of rennet (Saxer et al. 2013; Torres-Llanez et al. 2006). The high moisture content of the cheese (c.a. 50%) and a nearly neutral pH (6.3 to 6.5) are ideal for supporting the growth of a large number of bacterial species that contribute to their sensory quality but also compromise their hygienic quality (Alvarado et al. 2005; Renye et al. 2008; Saltijeral et al. 1999).

The aim of this work was to isolate bacteriocin-producing LAB from “Queso fresco” cheese made from raw cow's milk, characterize their diversity in terms of bacterial

species and the bacteriocins they produce, and assess their feasibility as adjunct cultures for the manufacture of dairy products. The isolates were screened for antimicrobial activity against pathogens such as *Enterococcus* and also *Listeria monocytogenes* that has been reported as the causative agent of infectious outbreaks associated with the consumption of raw milk fresh cheeses (Moraes et al., 2012; Renye et al. 2008).

Material and Methods

Microorganisms and culture conditions

Bacteriocinogenic LAB strains were grown in Man-Rogosa-Sharpe (MRS) broth (BD Difco, Detroit, MI, USA) at 37°C for 18-24 h without agitation. *Lactococcus lactis* IPLA947, used as a starter strain (Cárcoba et al. 2000), was grown in M17 (Biokar) at 30 °C. *Enterococcus faecalis* NRRL-B537, *Listeria innocua* AST 062 and *Listeria monocytogenes* ATCC19115 were used as indicators and grown in MRS and Luria-Bertani (LB) (BD Difco, Detroit, MI, USA), respectively, at 37°C. Clinical isolates from University of Veracruz *Proteus mirabilis*, *Enterobacter cloacae*, *Salmonella paratyphi*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were grown in LB at 37 °C. All strains were maintained as frozen stocks at –80 °C in their corresponding broth plus 20% (v/v) glycerol.

Direct antagonism test

Samples of 15 cheeses elaborated in local farms from Municipio of La Joya, Veracruz (México) were aseptically taken and maintained at 4°C until laboratory processing. Five grams of cheese were homogenized with 10 mL of phosphate buffer (0.1M, pH 7.0). Then,

1 mL of homogenized sample was added to 10 mL of MRS broth and incubated for 24 h at 37°C. From each sample, ten-fold serial dilutions were made in 0.85% NaCl solution and 100 µl were spread on MRS agar plates which were incubated at 37°C for 24h. The experiments were carried out by triplicate. Plates with isolated colonies were double-layered with indicator strains. *E. faecalis* NRRL-B537 and *L. innocua* AST 062 were previously cultured for 2 h at 37°C in 10 mL of MRS and LB media, respectively, and used to inoculate the same media supplemented with 1.2% agar that was poured onto the plates. Plates were incubated at 37 °C during 18-20 h until the appearance of inhibition halos was observed. Positive colonies were re-isolated on MRS agar in order to obtain pure strains. All isolated colonies were examined for catalase activity, examined under optical microscopy to determine cell morphology, and tested for Gram-staining reaction (Torres-Llanez et al. 2006).

Antimicrobial activity and proteolytic enzyme treatment

Isolates were incubated in 10 mL MRS broth at 37 °C for 18-20 h. The cultures were centrifuged at 12,000 xg for 15 min, and cell-free supernatants were neutralized to pH 6.0 with 1M NaOH and subsequently filtered through a 0.22 µm polyethersulfone membrane (Millipore, Darmstadt, Germany). Supernatants were stored at -20 °C until further use. The antimicrobial activity was determined by the agar well diffusion assay as previously described (Schillinger and Lucke 1989). Inactivation by proteinase K (1 mg/mL; Sigma-Aldrich, St. Louis, Mo. USA) was evaluated after incubation for 30, 90 and 120 min at 37 °C. After treatment, the residual antimicrobial activity was determined by the agar well diffusion assay using a control sample without addition of enzyme.

DNA extraction and molecular identification

Colonies were homogenized in 20 µL of a lysis buffer (0.25% SDS-50 mM NaOH) and heated at 95°C for 5 min. Subsequently, volume was adjusted with deionized water to 200 µL. Samples were centrifuged at 13,000 xg for 5 min at 4 °C and the supernatant was kept at -20 °C (Salgado-Ruiz et al. 2015). For PCR reactions, PureTaq Ready-To-Go PCR Beads (GE Healthcare, Munich, Germany) kit was used. For molecular identification of the cultures, primers 27FYM (5' AGAGTTTGATYMTGGCTCAG 3') and 1492R (5' GGTTACCTTGTTACGACTT 3') that partially amplify the 16S rDNA gene were used (Alegría et al. 2013). Reactions were made in a thermocycler (Bio-Rad, Hercules) with the following amplification protocol: 5 min at 95°C; 30 cycles of 30 s at 94°C, 45 s at 50°C, and 2 min at 72°C; 10 min at 72°C. PCR products were analysed by electrophoresis in 0.8 % agarose gels and visualized under UV light with a G:Box Syngene™ transilluminator (Syngene, Cambridge, UK). The PCR products were sequenced by Macrogen (Korea).

Genetic fingerprinting by RAPD-PCR

DNA was extracted from colonies as described above. Primers OPL5 (5'-ACGCAGGCAC-3'), RAPD5 (5'-AACGCGCAAC-3'), P1 (5'-CCGCAGCCAA-3') were used according to Gutiérrez et al. (2011). RAPD-PCR reactions were performed with the following cycling program: four cycles at 94 °C for 45 s, 30 °C for 120 s and 72 °C for 60 s; 26 cycles at 94 °C for 5 s, 36 °C for 30 s and 72 °C for 30 s and a final step of 10 min at 75 °C. Finally, the PCR products were analysed by electrophoresis in 0.8% agarose gels. For the construction of the dendrogram, the Pearson product moment correlation coefficient and the unweighted pair group method with arithmetic averages (UPGMA) were used (Gutiérrez et al. 2011).

164

165 **Detection of bacteriocin genes**

166 Determination of genes involved in the biosynthesis of bacteriocins were carried out by
167 PCR using the conditions and specific primers as previously reported by Lavilla-Lerma et
168 al. (2013), Hadji-Sfaxi et al. (2011) and Martínez et al. (1998) (Table 1). PCR generated
169 fragments were analysed by electrophoresis in 2% agarose gels and visualized as described
170 before.

171

172 **Induction of plantaricin NC8**

173 Induction of the production of the bacteriocin NC8 in *L. plantarum* SP-68 was studied as
174 previously described (Maldonado et al. 2004). Briefly, MRS broth was inoculated with 1%
175 of an overnight culture of *L. plantarum* SP-68 plus 0.5% of an overnight culture of *L. lactis*
176 IPLA947 strain and incubated at 30 °C during 6, 8, 18 and 24 h. Antimicrobial activity of
177 the supernatants was assayed by the agar well diffusion test using *L. plantarum* 128/2 as the
178 indicator strain. Individual cultures of *L. lactis* IPLA947 and *L. plantarum* SP-68 were used
179 as controls to confirm that the inhibitory activity was due to the induction of NC8.

180

181 **Analysis of sugars, organic acids and volatile compounds in milk co-cultures**

182 Bacteriocinogenic strains *P. acidilactici* SP-01, *L. plantarum* SP-68 and *L. plantarum* SP-
183 651, and the starter strain *L. lactis* IPLA947 were initially pre-adapted in milk by two
184 successive passages in 10 ml of commercially available UHT semi-skimmed milk (Central
185 Lechera Asturiana, Spain). Carbohydrates and organic acids were followed in two
186 independent co-cultures in a final volume of 50 ml of milk inoculated at 1% (v/v) with *L.*
187 *lactis* IPLA947 and the bacteriocin producer. Samples (1 ml) were withdrawn at 8, 18 and

24 h of incubation at 30 °C. Sugars and organic acids were analysed by HPLC with an ICSep ICE-ION-300 ion-exchange column, essentially as described by Rehaïem et al. (2012), following an isocratic gradient with sulfuric acid 3mM as a mobile phase at 0.7 mL/min flow and a temperature of 65 °C. Organic acids and sugars were detected by a photodiode array PDA 996 and a 410 differential refractometer detector, respectively. Volatile compound analysis was performed after co-culturing in 10 ml of UHT milk in 20 ml glass tubes sealed with rubber and metallic cups. Strains were inoculated at 1% (v/v) and incubated for 18 h at 30 °C and two independent experiments were carried out for each co-culture. Volatile compounds were determined by headspace/gas chromatography/mass spectrometry (HS/GC/MS), using an Agilent apparatus (Agilent Technologies, Wilmington, DE, USA) equipped with a capillary column HP-Innowax 60 m×0.25 mm×0.25 µm (Agilent) (Salazar et al. 2009). Propyl acetate (1µg/ml) was used as an internal standard to determine relative abundances.

Statistical analyses

Statistics was performed using the SPSS 15.0 software (SPSS, Chicago, IL, USA). Data related to residual lactose, and production of organic acids and volatile compounds by the acidifying strain *L. lactis* IPLA947 alone and in co-cultures with the bacteriocinogenic strains *P. acidilactici* SP-01, and *L. plantarum* SP-68 and SP-651 at different incubation times (8, 18 and 24 h) were subjected to Oneway ANOVA using as factor the incubation time with four categories (the four strains indicated above). The Student-Newman-Keuls test ($p<0.05$) was applied for means comparison.

Results and Discussion

Isolation of antibacterial LAB from "Queso fresco" (fresh cheese)

Ninety-three colonies grown on MRS plates and isolated from cheese samples obtained from the Municipio of La Joya, Veracruz (México), revealed the presence of inhibition halos against *L. monocytogenes* ATCC19115, *L. innocua* AST 062 or *E. faecalis* NRRL-B537 by the double-layer agar assay. The presumptive LAB strains were Gram-positive microorganisms, catalase negative and with bacillary or coccoid morphology. Subsequently, the cell-free supernatants from these strains were neutralized, sterilized by filtration, and tested by the well-diffusion assay. Twenty-nine strains were found to produce an inhibition zone against at least one of the three indicator strains (Table 2). None of the isolates were active against the genera *Proteus*, *Enterobacter*, *Salmonella*, *Klebsiella*, *Pseudomonas* and *Staphylococcus* (data not shown). Interestingly several isolates demonstrated a strong antilisterial activity (Table 2). Previous studies have reported the antagonistic activity of autochthonous isolates from dairy products against *L. monocytogenes* (Hadj-Sfaxi et al. 2011; Ortolani et al. 2010) which is of relevance as this pathogen is often involved in outbreaks associated with consumption of fresh cheese made from raw milk (Renye et al. 2009; Saltijeral et al. 1999). The activity of some of the isolates was rather weak (shown as +/- in Table 2) and it was not always detected. However, these strains were further characterized given the fact that several bacteriocins are inducible and may require specific environmental conditions to trigger synthesis (Diep et al. 2001; Maldonado et al. 2003; Rojo-Bezarez et al. 2007).

The proteinaceous nature of the antimicrobial activity, typical for bacteriocins, was confirmed in 21 out of the 29 active supernatants by their susceptibility to proteinase K treatment (Table 2).

Molecular identification of LAB isolates

All 29 isolates were subjected to molecular identification. The partial 16S rDNA gene sequences of 29 isolates showed 99% homology with NCBI database retrieved sequences and were identified as follows: 6 strains (21%) as *Lactobacillus plantarum* (GenBank AB601179 and AB713901); 2 strains (7%) as *Lactobacillus fermentum* (GenBank HM058427 and HM058429); 4 strains (14%) as *Pediococcus acidilactici* (Gen Bank JN592051 and JQ801716), and 17 (57%) as *Enterococcus faecium* (GenBank gHQ293030 and EF204317) (Table 2). Bacteriocin production by all these species have been often reported (Albano et al. 2007; Caro et al. 2013; Ortolani et al. 2010; Renye et al. 2009; Torres-Llenez et al. 2006).

As reported by Renye et al. (2009) and Moraes et al. (2012) who studied the microbial composition of fresh cheeses, the most prevalent species in our samples was *E. faecium*. The presence of this species in raw milk and raw milk cheese is very common (Moraes et al. 2012; Alvarado et al. 2005) and *E. faecium* has been considered essential for the development of the sensory qualities associated to Mexican Queso Fresco cheeses (Hadji-Sfaxi et al. 2011). However, its deliberate use in food is still controversial because of the increasing association of *Enterococcus* sp. with nosocomial infections and the presence of multiple antibiotic-resistant genes, putatively transmissible by conjugation to nonpathogenic microorganisms (Delgado and Mayo 2004; Hadji-Sfaxi et al. 2011). Therefore, enterococcal strains often undergo exhaustive safety assessments prior to their use in food.

Lactobacillus species were also isolated although the cheeses used in this study were not ripened. *Lactobacillus* has been reported to become predominant through ripening

because these organisms are capable to grow under the highly selective conditions of the cheese environment (Sánchez et al. 2006; Lavilla-Lerma et al. 2013). *Pediococcus acidilactici* strains were detected as well. This genus is not often isolated from dairy products (Papagianni and Anastasiadou 2009). However, there is great interest in the application of *P. acidilactici* strains in fermented dairy products as protective secondary cultures, particularly in the manufacture of Italian-style cheeses and also in low pH dairy foods such as yogurt (Somkuti and Steinberg 2010).

Genetic fingerprinting by RAPD-PCR

Randomly amplified polymorphic DNA (RAPD-PCR) is a useful method to study the genetic diversity of natural microorganisms present in different kind of cheeses, to monitoring the survival of strains added in the manufacturing process and/or maturation as well as detecting identical isolates within a strain collection (Delgado and Mayo et al. 2004; Sánchez et al. 2006). Some authors pointed out that if this technique is to be used alone, one primer is insufficient for a good discrimination (Gutiérrez et al. 2011; Sánchez et al. 2006).

In our study, using the RAPD-PCR profiles generated by 3 primers, three major clusters I- III were identified, which could be further discriminated into seven sub-clusters Ia, Ib, IIc, IId, IIle, IIIf, IIlg with a percentage of similarity of 50 % (Fig. 1). The cluster I, included two isolates of *P. acidilactici* and one of *L. fermentum* grouped into subgroups Ia, and Ib, respectively. The cluster II was the least homogeneous, grouping two strains of *P. acidilactici* and *E. faecium* (IIc), and two *L. plantarum* and one strain of *L. fermentum* (IId). The cluster III encompassed most of the *E. faecium* isolates (IIIf), two strains of *L. plantarum* (IIle), and one strain of *P. acidilactici* (IIIg). Overall, *E. faecium* isolates were

284 closely related contrasting to the higher diversity found within the species *L. plantarum* and
285 *P. acidilactici*. The RAPD-PCR also contributed to detect isolates with more than 80 % of
286 similarity which suggests that are different isolates from the same strain (Gatti et al. 2008).
287 This is exemplified by the pairs of *L. plantarum* SP-651/SP-24 and SP-50/SP-68 with
288 similar inhibitory activities (Table 2) and the same bacteriocin genes, as described below
289 (see Table 3).

291 **Detection of bacteriocin genes**

292 The presence of bacteriocin encoding genes within the bacteriocinogenic isolates was
293 assessed by PCR. Within *L. plantarum*, two strains, SP-24 and SP-651, were positive for
294 *plnA* (inducing peptide), *plnEF*, *plnJK* (encoding two-peptide bacteriocins), and *plnN*
295 (putative bacteriocin with an N-terminal double glycine leader consensus) (Table 3). This
296 result resembles the organization described in *L. plantarum* C11 and WCFS1 strains (Diep
297 et al. 2009). On the other hand, two of our strains, SP-50 and SP-68, did not amplify *plnA*
298 and *plnN* but were positive for the plantaricin NC8 which has been described previously in
299 *L. plantarum* NC8, isolated from grass silage (Maldonado et al. 2003; 2004). Remarkably,
300 NC8 producing strains are rarely isolated from dairy products (Diep et al. 2009; Maldonado
301 et al. 2003; Rojo-Bezarez et al. 2007). All these bacteriocin producing isolates also carried
302 the *plnEF* and *plnJK* genes demonstrating further the mosaicism and broad presence of
303 several plantaricin genes in *L. plantarum*, regardless the source of isolation (Diep et al.
304 2009; Lavilla-Lerma et al. 2013; Maldonado et al. 2004). The production of several
305 bacteriocins by the same strain plays an important role in niche competition as each
306 bacteriocin may differ in their mode of action, thus widening the spectrum of inhibited
307 bacteria (Diep et al. 2009). In fact, it has been shown that plantaricins EF and JK have a

slightly different mode of action on sensitive cells, the first producing pores with selectivity for cations and the second one with selectivity for anions (Moll et al. 1999).

The diversity of bacteriocin genes present in the rest of strains was low. All *E. faecium* isolates amplified the gene encoding enterocin P (*entP*) while none of the other enterocin genes (*entA*, *entB*, *entL50A* and *entAS-48*) were detected (data not shown). The production of more than one enterocin is common among *E. faecium* strains (Alvarado et al. 2005). However, that was not the case of our isolates and their antimicrobial activity is likely due to production of enterocin P only. Enterocin P belongs to the pediocin-like class IIa bacteriocins with strong anti-listeria effect and together with enterocins A and B are prevalent among enterococci (Cintas et al. 1997; Hadji-Sfaki et al. 2011; Renye et al. 2009). Within *P. acidilactici*, the pediocin PA-1 gene was detected in three (SP-01, SP-02, and SP-61) out of the four strains and correlated with the anti-listeria activity shown by these isolates. As previously described, production of pediocin PA-1 is a common phenotype found in *P. acidilactici* strains (Albano et al. 2007; Millette et al. 2008; Papagianni and Anastasiadou 2009).

It is worth noticing that some isolates (SP-38, SP-48, SP-13, SP-23, SP-59, SP-653 and SP-66) did not amplify any bacteriocin gene. These isolates were characterized by weak activity in their supernatants which was not always detected. Although the presence of other bacteriocin genes cannot be discarded, it is possible that non-producing mutants were selected during sub-culturing. Bacteriocin genes are often located on mobilizable genetic elements (transposons and plasmids) which may be lost when growing under laboratory conditions (García et al. 2010; Hadji-Sfaki et al. 2011). Furthermore, small variations in culturing conditions (media composition, temperature) may reduce bacteriocin

synthesis, particularly in the case of those bacteriocins subjected to quorum sensing and may be strain specific (Diep et al. 2009; Maldonado et al. 2003).

Induction of bacteriocin NC8 activity

Production of several bacteriocins is regulated by quorum-sensing mediated by peptide pheromones or autoinducer peptides (Diep et al. 2009; Rojo-Bezarez et al. 2007). Other factors such as the presence of competing microorganisms have been reported to regulate production of bacteriocins by some LAB. Coordinating bacteriocin production within the population has a greater effect on inhibition of competitors, because a high concentration of bacteriocin is suddenly produced at a certain time, preventing the selection of bacteriocin resistant variants (Diep et al. 2001; Diep et al. 2009; Maldonado et al. 2004; Rojo-Bezarez et al. 2007). Given the fact that inducing conditions may be strain specific (Diep et al. 2009; Maldonado et al. 2003), co-cultures of *L. plantarum* SP-68 with *L. lactis* IPLA947 were carried out to confirm the production of the inducible bacteriocin NC8 by this newly isolate of dairy origin. As shown in Fig. 2, antimicrobial activity against *L. pentosus* 128/2 was only detected in cell-free supernatants from the co-cultures while no activity was present when the strains were grown independently. These results are in agreement with those reported previously by Maldonado et al. (2004) and demonstrate the inducible synthesis of the bacteriocin NC8 by *L. plantarum* SP-68.

Metabolic profiling of co-cultures in milk

Adjunct dairy cultures are often used to enhance the organoleptic properties of dairy products. However, the addition of bacteriocin producing bacteria to milk may interfere with the rate of acidification driven by the starter or contribute to the formation of off-

flavours precluding their use as bioprotective cultures (Perin et al. 2015; Rilla et al. 2003). Thereby, pH decrease, sugar consumption, production of organic acids and volatile compounds was analysed in co-cultures of the starter strains *L. lactis* IPLA 947 and selected bacteriocin producers including *P. acidilactici* SP-01, *L. plantarum* SP-68 and *L. plantarum* SP-651. Acidification was not hindered by the presence of the bacteriocinogenic strains and all the co-cultures reached an average pH of 4.92 (± 0.01), 4.28 (± 0.01) and 4.25 (± 0.02) after 8, 18 and 24 h of incubation, respectively (data not shown). Accordingly, lactose consumption as well as the production of lactic acid, considered as the main metabolic product of milk fermentation, followed the same trend regardless of the bacteriocinogenic adjunct culture and no significant differences were noted at the end of the incubation ($p > 0.05$) (Fig. 3a,b).

More variability was observed with other organic acids such as acetic, formic and succinic acids (Fig. 3). Acetic and formic acids are the result of alternative routes in homofermentative LAB that redirect pyruvate depending on environmental conditions (Mayo et al., 2010). This heterolactic or mixed fermentation appears to be favoured by the adjunct cultures. Production of acetic acid was clearly increased in all co-cultures ($p < 0.05$) and reached up to three-fold higher levels in the presence of *P. acidilactici* SP-01 compared to *L. lactis* IPLA947 alone (Fig. 3). Both *L. plantarum* strains also contributed with higher levels of acetic acid ($p < 0.05$) that increased from 8 h to 24 h (Fig. 3). The concentration of formic acid were also higher ($p < 0.05$) in co-cultures with *P. acidilactici* SP-01 during fermentation but levelled off at the end of the fermentation (Fig. 3). Very interesting, co-culturing with the bacteriocinogenic strains caused a significant increase of succinic acid throughout fermentation ($p < 0.05$). Production of succinic acid by LAB has not been studied deeply but it has been detected in cultures of both *L. plantarum* and *P. acidilactici* and has

been associated to the development of the sensory attributes of fermented foods including cheese (Dudley and Steele 2005; Ramasamy et al. 2012).

The profile of major volatile compounds was also slightly different depending on the bacteriocin producing strain (Fig. 4). Co-cultures with *P. acidilactici* SP-01 and *L. plantarum* SP-651 showed an increased relative abundance of ethanol ($p<0.05$) compared to the single fermentation carried out by *L. lactis* IPLA947, which is consistent with the mixed acid fermentation and the concomitant higher levels of acetic and formic acid (Fig. 3). On the contrary, acetone levels were not significantly different to that of *L. lactis* IPLA947 milk cultures ($p>0.05$). Diacetyl production was reduced in co-cultures with *L. plantarum* SP-68 but not with *L. plantarum* SP-651, reflecting the high biodiversity and the plasticity of this species (Siezen et al. 2010) which was also evident by the profile of organic acids (e.g. acetic and succinic acids) as described above. In general, these results reinforce the importance of analysing the contribution of each adjunct culture to the organoleptic properties of the fermented product in a strain-specific basis.

Conclusions

Mexican craft cheeses are a good source of genetically diverse bacteriocin producing LAB. Their presence in co-cultures with dairy starters does not interfere with milk acidification and, depending on the strain, they may provide a richer range of metabolites likely to impact positively the organoleptic properties of cheese, while possibly contributing to enhance safety.

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Table 1. Primer pairs and PCR conditions used for the detection of bacteriocin genes.

	PCR primers	Sequence 5'-3'	Annealing °C	Size (bp)	Reference
<i>plnA</i>	plnA-F	GTA CAG TAC TAA TGG GAG	50	450	(Lavilla-Lerma et al. 2013)
	plnA-R	CTT ACG CCA ATC TAT ACG			
<i>plnEF</i>	plnEF-F	GGC ATA GTT AAA ATT CCC CCC	50	428	
	plnEF-R	CAG GTT GCC GCA AAA AAA G			
<i>plnJ</i>	plnJ-F	TAA CGA CGG ATT GCT CTG	50	475	
	plnJ-R	AAT CAA GGA ATT ATC ACA TTA GTC			
<i>plnK</i>	plnK-F	CTG TAA GCA TTG CTA ACC AAT C	50	246	
	plnK-R	ACT GCT GAC GCT GAA AAG			
<i>plnN</i>	plnN-F	ATT GCC GGG TTA GGT ATC G	50	146	
	plnN-R	CCT AAA CCA TGC CAT GCA C			
Plantaricin NC8	NC8-F	GGT CTG CGT ATA AGC ATC GC	55	207	
	NC8-R	AAA TTG AAC ATA TGG GTG CTT TAA ATT CC			
Plantaricin S	plnS-F	GCC TTA CCA GCG TAA TGC CC	55	320	
	plnS-R	CTG GTG ATG CAA TCG TTA GTT T			
Enterocin A	AF	GGTACCACTCATAGTGGAAA	58	138	(Hadji-Sfaxi et al. 2011)
	AR	CCCTGGAATTGCTCCACCTAA			
Enterocin B	BF	CAAAATGTAAAAGAATTAAGTACG	56	201	
	BR	AGAGTATACATTTGCTAACCC			
Enterocin P	PF	GCTACGCGTTCATATGGTAATGGT	60	132	
	PR	ATGTCCCATACCTGCCAAACCAGAAG C			
Enterocin L50A	Ent1F	NNNNCCATGGGAGCAATCGCAAAA	56	135	
	Ent1R	NNNNAAGCTTAATGTTTTTTAATCCAT CAAT			
Enterocin AS-48	ASF	GAGGAGTATCATGGTTAAAGA	50	339	
	ASR	ATATTGTTAAATTACCAA			
Pediocin PA-1	Ped-F	AAA ATA TCT AAC TAA TAC TTG	44	723	(Martínez et al. 1998)
	Ped-R	TAA AAA GAT ATT TGA CCA AAA			

Table 2. Antimicrobial activity of cell-free supernatants of lactic acid bacteria isolated from “Queso Fresco” cheese and activity after treatment with proteinase K.

Species	Isolate	<i>E. faecalis</i> NRRL-B537 ^a	<i>L. innocua</i> AST0 62 ^a	<i>L. monocytogenes</i> ATCC19115 ^a	Treatment Proteinase K ^b
<i>Lactobacillus plantarum</i>	SP-24	-	-	+/-	-
	SP-38	+/-	-	+/-	ND ^a
	SP-48	-	-	+/-	ND
	SP-50	-	+/-	+/-	-
	SP-651	-	-	+/-	-
	SP-68	-	+/-	+/-	-
<i>Lactobacillus fermentum</i>	SP-13	+	-	-	ND
	SP-23	+	-	+/-	ND
<i>Enterococcus faecium</i>	SP-03	++	++	+++	-
	SP-04	++	++	+++	-
	SP-08	+	+++	+++	-
	SP-09	++	+	+	-
	SP-10	++	++	+++	-
	SP-11	++	++	+++	-
	SP-12	++	++	+++	-
	SP-17	++	+++	+++	-
	SP-22	++	++	+++	-
	SP-33	+	++	+++	-
	SP-34	++	++	++	-
	SP-35	+	++	+++	-
	SP-58	+	++	++	-
	SP-59	-	+/-	+/-	ND
	SP-64	++	++	++	-
	SP-653	-	+/-	+/-	-
	SP-66	-	+/-	+/-	-
<i>Pediococcus acidilactici</i>	SP-01	+	++	++	-
	SP-02	+/-	+	+	ND
	SP-07	+/-	+	+	ND
	SP-61	+	+	+	ND

^a (+++) Inhibitory zone >13mm, (++) Inhibitory zone 10-13mm, (+) Inhibitory zone 9mm, (+/-) weak inhibition, (-) No inhibition zone, ND: not determined

^b Antimicrobial activity after proteinase treatment was determined against *L. monocytogenes* ATCC19115

Table 3. Presence of bacteriocin genes determined by PCR in bacteriocinogenic lactobacilli isolated from “Queso Fresco” cheese.

Species	Isolate	Bacteriocin gene						
		<i>plnA</i>	<i>plnEF</i>	<i>plnJ</i>	<i>plnK</i>	<i>plnS</i>	<i>plnN</i>	<i>plnNC8</i>
<i>Lactobacillus plantarum</i>	SP-24	+	+	+	+	-	+	-
	SP-38	-	-	-	-	-	-	-
	SP-48	-	-	-	-	-	-	-
	SP-50	-	+	+	+	-	-	+
	SP-651	+	+	+	+	-	+	-
	SP-68	-	+	+	+	-	-	+
<i>Lactobacillus fermentum</i>	SP-13	-	-	-	-	-	-	-
	SP-23	-	-	-	-	-	-	-

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Figure 1. Dendrogram obtained by cluster analysis of RAPD-PCR profiles of lactic acid bacteria (SP) isolated from “Queso fresco” cheese, using the UPGMA method and the Pearson product moment correlation coefficient. Dashed line represents threshold similarity value of 50% for discriminating clusters.

Figure 2. Induction of the antimicrobial activity in *Lactobacillus plantarum* SP-68 by co-culturing with *Lactococcus lactis* IPLA947. Supernatants from individual cultures SP-68 (A) and *L. lactis* IPLA947 (B) and co-cultures after 8 h of incubation at 30 °C (2) were assayed in duplicate by the well diffusion test using *Lactobacillus pentosus* 128/2 as indicator.

Figure 3. Residual lactose (a) and production of lactic (b), acetic (c), formic (d) and succinic (e) acids during milk fermentation of *L. lactis* IPLA947 alone ($\diamond$) and co-cultured with the bacteriocinogenic strains *P. acidilactici* SP-01 ($\square$), *L. plantarum* SP-68 (Δ) and *L. plantarum* SP-651 (x). Data reported are means and standard deviation (vertical bars) of two independent experiments.

Figure 4. Relative abundance of ethanol (black), acetone (white) and diacetyl (grey) in milk cultures incubated at 30 °C for 18 h of *L. lactis* IPLA 947 alone (947) or in co-culture with the bacteriocin producers *P. acidilactici* SP-1, *L. plantarum* SP-68 and *L. plantarum* SP-651. Data reported are means and standard deviation (vertical bars) of two independent experiments.