View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Digital.CSIC

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

3 Silvia Portilla-Vázquez[1], Ana Rodríguez[2], Mario Ramírez-Lepe[1], Patricia G.
4 Mendoza-García[1]* and Beatriz Martínez[2]*

5 *[1]Food Research and Development Unit (UNIDA) Technological Institute of Veracruz, Calz.*
6 *Miguel Ángel de Quevedo 2779, Formando Hogar, 91897 Veracruz, México.*

7 *[2] Dairy Research Institute IPLA-CSIC, Paseo Río Linares, s/n, 33300 Villaviciosa,*
8 *Asturias, Spain.*

9

10 **Correspondence to both authors:**

11 Patricia G. Mendoza-García

12 Food Research and Development Unit (UNIDA) Technological Institute of Veracruz, Calz.

13 Miguel Ángel de Quevedo 2779, Formando Hogar, 91897 Veracruz, México.

14 Phone +52 229-9345701; e-mail: pmendoza@itver.edu.mx

15 Beatriz Martínez

16 IPLA-CSIC. Paseo Río Linares, s/n, 33300 Villaviciosa, Asturias, Spain.

17 Phone +34 985 89 21 31; e-mail: bmfl@ipla.csic.es

18

19

20 **Abstract**

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

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

44 **Introduction**

45

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

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

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

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

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

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

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

92 species and the bacteriocins they produce, and assess their feasibility as adjunct cultures for
93 the manufacture of dairy products. The isolates were screened for antimicrobial activity
94 against pathogens such as *Enterococcus* and also *Listeria monocytogenes* that has been
95 reported as the causative agent of infectious outbreaks associated with the consumption of
96 raw milk fresh cheeses (Moraes et al., 2012; Renye et al. 2008).
97
98 **Material and Methods**
99
100 **Microorganisms and culture conditions**
101 Bacteriocinogenic LAB strains were grown in Man-Rogosa-Sharpe (MRS) broth (BD
102 Difco, Detroit, MI, USA) at 37°C for 18-24 h without agitation. *Lactococcus lactis*
103 IPLA947, used as a starter strain (Cárcoba et al. 2000), was grown in M17 (Biokar) at 30
104 ºC. *Enterococcus faecalis* NRRL-B537, *Listeria innocua* AST 062 and *Listeria*
105 *monocytogenes* ATCC19115 were used as indicators and grown in MRS and Luria-Bertani
106 (LB) (BD Difco, Detroit, MI, USA), respectively, at 37ºC. Clinical isolates from University
107 of Veracruz *Proteus mirabilis, Enterobacter cloacae, Salmonella paratyphi, Klebsiella*
108 *pneumoniae, Pseudomonas aeruginosa and Staphylococcus aureus* were grown in LB at 37
109 ºC. All strains were maintained as frozen stocks at –80 °C in their corresponding broth plus
110 20% (v/v) glycerol.
111
112 **Direct antagonism test**
113 Samples of 15 cheeses elaborated in local farms from Municipio of La Joya, Veracruz
114 (México) were aseptically taken and maintained at 4°C until laboratory processing. Five
115 grams of cheese were homogenized with 10 mL of phosphate buffer (0.1M, pH 7.0). Then,

116 1 mL of homogenized sample was added to 10 mL of MRS broth and incubated for 24 h at
117 37ºC. From each sample, ten-fold serial dilutions were made in 0.85% NaCl solution and
118 100 µl were spread on MRS agar plates which were incubated at 37ºC for 24h. The
119 experiments were carried out by triplicate. Plates with isolated colonies were double-
120 layered with indicator strains. *E. faecalis* NRRL-B537 and *L. innocua* AST 062 were
121 previously cultured for 2 h at 37°C in 10 mL of MRS and LB media, respectively, and used
122 to inoculate the same media supplemented with 1.2% agar that was poured onto the plates.
123 Plates were incubated at 37 °C during 18-20 h until the appearance of inhibition halos was
124 observed. Positive colonies were re-isolated on MRS agar in order to obtain pure strains.
125 All isolated colonies were examined for catalase activity, examined under optical
126 microscopy to determine cell morphology, and tested for Gram-staining reaction (Torres-
127 Llanez et al. 2006).
128
129 **Antimicrobial activity and proteolytic enzyme treatment**
130 Isolates were incubated in 10 mL MRS broth at 37 °C for 18-20 h. The cultures were
131 centrifuged at 12,000 xg for 15 min, and cell-free supernatants were neutralized to pH 6.0
132 with 1M NaOH and subsequently filtered through a 0.22 µm polyethersulfone membrane
133 (Millipore, Darmstadt, Germany). Supernatants were stored at -20 °C until further use. The
134 antimicrobial activity was determined by the agar well diffusion assay as previously
135 described (Schillinger and Lucke 1989). Inactivation by proteinase K (1 mg/mL; Sigma-
136 Aldrich, St. Louis, Mo. USA) was evaluated after incubation for 30, 90 and 120 min at 37
137 °C. After treatment, the residual antimicrobial activity was determined by the agar well
138 diffusion assay using a control sample without addition of enzyme.
139

140 **DNA extraction and molecular identification**

141 Colonies were homogenized in 20 µL of a lysis buffer (0. 25% SDS-50 mM NaOH) and
142 heated at 95°C for 5 min. Subsequently, volume was adjusted with deionized water to 200
143 µL. Samples were centrifuged at 13, 000 xg for 5 min at 4 ºC and the supernatant was kept
144 at -20 °C (Salgado-Ruiz et al. 2015). For PCR reactions, PureTaq Ready-To-Go PCR
145 Beads (GE Healthcare, Munich, Germany) kit was used. For molecular identification of the
146 cultures, primers 27FYM (5' AGAGTTTGATYMTGGCTCAG 3') and 1492R (5'
147 GGTTACCTTGTTACGACTT 3') that partially amplify the 16S rDNA gene were used
148 (Alegría et al. 2013). Reactions were made in a thermocycler (Bio-Rad, Hercules) with the
149 following amplification protocol: 5 min at 95°C; 30 cycles of 30 s at 94°C, 45 s at 50°C,
150 and 2 min at 72°C; 10 min at 72°C. PCR products were analysed by electrophoresis in 0.8
151 % agarose gels and visualized under UV light with a G:Box Syngene™ 126
152 transilluminator (Syngene, Cambridge, UK). The PCR products were sequenced by
153 Macrogen (Korea).

154

155 **Genetic fingerprinting by RAPD-PCR**

156 DNA was extracted from colonies as described above. Primers OPL5 (5'-ACGCAGGCAC-
157 3'), RAPD5 (5'-AACGCGCAAC-3'), P1 (5'-CCGCAGCCAA-3') were used according to
158 Gutiérrez et al. (2011). RAPD-PCR reactions were performed with the following cycling
159 program: four cycles at 94 °C for 45 s, 30 °C for 120 s and 72 °C for 60 s; 26 cycles at 94
160 °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
161 PCR products were analysed by electrophoresis in 0.8% agarose gels. For the construction
162 of the dendrogram, the Pearson product moment correlation coefficient and the unweighted
163 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

188 24 h of incubation at 30 ºC. Sugars and organic acids were analysed by HPLC with an
189 ICSep ICE-ION-300 ion-exchange column, essentially as described by Rehaiem et al.
190 (2012), following an isocratic gradient with sulfuric acid 3mM as a mobile phase at 0.7
191 mL/min flow and a temperature of 65 °C. Organic acids and sugars were detected by a
192 photodiode array PDA 996 and a 410 differential refractometer detector, respectively.
193 Volatile compound analysis was performed after co-culturing in 10 ml of UHT milk in 20
194 ml glass tubes sealed with rubber and metallic cups. Strains were inoculated at 1% (v/v)
195 and incubated for 18 h at 30 ºC and two independent experiments were carried out for each
196 co-culture. Volatile compounds were determined by headspace/gas chromatography/mass
197 spectrometry (HS/GC/MS), using an Agilent apparatus (Agilent Technologies, Wilmington,
198 DE, USA) equipped with a capillary column HP-Innowax 60 m×0.25 mm×0.25 μm
199 (Agilent) (Salazar et al. 2009). Propyl acetate (1μg/ml) was used as an internal standard to
200 determine relative abundances.
201

202 **Statistical analyses**

203 Statistics was performed using the SPSS 15.0 software (SPSS, Chicago, IL, USA). Data
204 related to residual lactose, and production of organic acids and volatile compounds by the
205 acidifying strain *L. lactis* IPLA947 alone and in co-cultures with the bacteriocinogenic
206 strains *P. acidilactici* SP-01, and *L. plantarum* SP-68 and SP-651 at different incubation
207 times (8, 18 and 24 h) were subjected to Oneway ANOVA using as factor the incubation
208 time with four categories (the four strains indicated above). The Student-Newman-Keuls
209 test ($p<0.05$) was applied for means comparison.
210
211

212 **Results and Discussion**

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

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

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

236

237 **Molecular identification of LAB isolates**

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

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

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

260 because these organisms are capable to grow under the highly selective conditions of the
261 cheese environment (Sánchez et al. 2006; Lavilla-Lerma et al. 2013). *Pediococcus*
262 *acidilactici* strains were detected as well. This genus is not often isolated from dairy
263 products (Papagianni and Anastasiadou 2009). However, there is great interest in the
264 application of *P. acidilactici* strains in fermented dairy products as protective secondary
265 cultures, particularly in the manufacture of Italian-style cheeses and also in low pH dairy
266 foods such as yogurt (Somkuti and Steinberg 2010).
267

268 **Genetic fingerprinting by RAPD-PCR**

269 Randomly amplified polymorphic DNA (RAPD-PCR) is a useful method to study the
270 genetic diversity of natural microorganisms present in different kind of cheeses, to
271 monitoring the survival of strains added in the manufacturing process and/or maturation as
272 well as detecting identical isolates within a strain collection (Delgado and Mayo et al. 2004;
273 Sánchez et al. 2006). Some authors pointed out that if this technique is to be used alone,
274 one primer is insufficient for a good discrimination (Gutiérrez et al. 2011; Sánchez et al.
275 2006).

276 In our study, using the RAPD-PCR profiles generated by 3 primers, three major
277 clusters I- III were identified, which could be further discriminated into seven sub–clusters
278 Ia, Ib, IIc, IId, IIIe, IIIf, IIIg with a percentage of similarity of 50 % (Fig. 1). The cluster I,
279 included two isolates of *P. acidilactici* and one of *L. fermentum* grouped into subgroups Ia,
280 and Ib, respectively. The cluster II was the least homogeneous, grouping two strains of *P.*
281 *acidilactici* and *E. faecium* (IIc), and two *L. plantarum* and one strain of *L. fermentum* (IId).
282 The cluster III encompassed most of the *E. faecium* isolates (IIIf), two strains of *L.*
283 *plantarum* (IIIe), 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).
290

291 **Detection of bacteriocin genes**

292 The presence of bacteriocin enconding 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-Bezares 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

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

310 The diversity of bacteriocin genes present in the rest of strains was low. All *E.*
311 *faecium* isolates amplified the gene encoding enterocin P (*entP*) while none of the other
312 enterocin genes (*entA, entB*, *entL50A* and *entAS-48*) were detected (data not shown). The
313 production of more than one enterocin is common among *E. faecium* strains (Alvarado et
314 al. 2005). However, that was not the case of our isolates and their antimicrobial activity is
315 likely due to production of enterocin P only. Enterocin P belongs to the pediocin-like class
316 IIa bacteriocins with strong anti-listeria effect and together with enterocins A and B are
317 prevalent among enterococci (Cintas et al. 1997; Hadji-Sfaxi et al. 2011; Renye et al.
318 2009). Within *P. acidilactici*, the pediocin PA-1 gene was detected in three (SP-01, SP-02,
319 and SP-61) out of the four strains and correlated with the anti-listeria activity shown by
320 these isolates. As previously described, production of pediocin PA-1 is a common
321 phenotype found in *P. acidilactici* strains (Albano et al. 2007; Millette et al. 2008;
322 Papagianni and Anastasiadou 2009).

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

331 synthesis, particularly in the case of those bacteriocins subjected to quorum sensing and
332 may be strain specific (Diep et al. 2009; Maldonado et al. 2003).
333

334 **Induction of bacteriocin NC8 activity**

335 Production of several bacteriocins is regulated by quorum-sensing mediated by peptide
336 pheromones or autoinducer peptides (Diep et al. 2009; Rojo-Bezares et al. 2007). Other
337 factors such as the presence of competing microorganisms have been reported to regulate
338 production of bacteriocins by some LAB. Coordinating bacteriocin production within the
339 population has a greater effect on inhibition of competitors, because a high concentration of
340 bacteriocin is suddenly produced at a certain time, preventing the selection of bacteriocin
341 resistant variants (Diep et al. 2001; Diep et al. 2009; Maldonado et al. 2004; Rojo-Bezares
342 et al. 2007). Given the fact that inducing conditions may be strain specific (Diep et al.
343 2009; Maldonado et al. 2003), co-cultures of *L. plantarum* SP-68 with *L. lactis* IPLA947
344 were carried out to confirm the production of the inducible bacteriocin NC8 by this newly
345 isolate of dairy origin. As shown in Fig. 2, antimicrobial activity against *L. pentosus* 128/2
346 was only detected in cell-free supernatants from the co-cultures while no activity was
347 present when the strains were grown independently. These results are in agreement with
348 those reported previously by Maldonado et al. (2004) and demonstrate the inducible
349 synthesis of the bacteriocin NC8 by *L. plantarum* SP-68.
350

351 **Metabolic profiling of co-cultures in milk**

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

355 flavours precluding their use as bioprotective cultures (Perin et al. 2015; Rilla et al. 2003).
356 Thereby, pH decrease, sugar consumption, production of organic acids and volatile
357 compounds was analysed in co-cultures of the starter strains *L. lactis* IPLA 947 and
358 selected bacteriocin producers including *P. acidilactici* SP-01, *L. plantarum* SP-68 and *L.*
359 *plantarum* SP-651. Acidification was not hindered by the presence of the bacteriocinogenic
360 strains and all the co-cultures reached an average pH of 4.92 (±0.01), 4.28 (±0.01) and 4.25
361 (±0.02) after 8, 18 and 24 h of incubation, respectively (data not shown). Accordingly,
362 lactose consumption as well as the production of lactic acid, considered as the main
363 metabolic product of milk fermentation, followed the same trend regardless of the
364 bacteriocinogenic adjunct culture and no significant differences were noted at the end of the
365 incubation ($p>0.05$) (Fig. 3a,b).

366 More variability was observed with other organic acids such as acetic, formic and
367 succinic acids (Fig. 3). Acetic and formic acids are the result of alternative routes in
368 homofermentative LAB that redirect pyruvate depending on environmental conditions
369 (Mayo et al., 2010). This heterolactic or mixed fermentation appears to be favoured by the
370 adjunct cultures. Production of acetic acid was clearly increased in all co-cultures ($p<0.05$)
371 and reached up to three-fold higher levels in the presence of *P. acidilactici* SP-01 compared
372 to *L. lactis* IPLA947 alone (Fig. 3). Both *L. plantarum* strains also contributed with higher
373 levels of acetic acid ($p<0.05$) that increased from 8 h to 24 h (Fig. 3). The concentration of
374 formic acid were also higher ($p<0.05$) in co-cultures with *P. acidilactici* SP-01 during
375 fermentation but levelled off at the end of the fermentation (Fig. 3). Very interesting, co-
376 culturing with the bacteriocinogenic strains caused a significant increase of succinic acid
377 throughout fermentation ($p<0.05$). Production of succinic acid by LAB has not been studied
378 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.

403 **Acknowledgments**

404 This work was supported by Tecnológico Nacional de México (5486.14.15-P), the Mexican
405 National Council for Science and Technology (Consejo Nacional de Ciencia y Tecnología -
406 CONACYT) and partially by the Ministry of Economy and Competitiveness of Spain
407 (MINECO) through grant BIO2013-46266-R. Funding by GRUPIN14-139 Plan de Ciencia,
408 Tecnología e Innovación 2013-2017 (Principado de Asturias, Spain), supported by FEDER
409 EU funds, is also acknowledged. S. Portilla-Vázquez held a CONACYT fellowship as well
410 as an i-COOP mobility grant COOPA20015 funded by Consejo Superior de Investigaciones
411 Científicas (CSIC), Spain. We thank the cheesemakers from La Joya, (Veracruz, México)
412 for their donation of traditionally-made "Queso fresco".
413

414 **The authors declare that the research was conducted in the absence of any**
415 **commercial or financial relationships that could be construed as a potential conflict of**
416 **interest.**
417

418 **References**

419

420 Albano, H., Todorov, S. D., van Reenen C, Hogg, T., Dicks, L. M. T., Teixeira, P. (2007).
421 Characterization of two bacteriocins produced by *Pediococcus acidilactici* isolated
422 from "Alheira", a fermented sausage traditionally produced in Portugal. *Int. J. Food*
423 *Microbiol*. 116(2):239–47.

424 Alegría, A., Delgado, S., Flórez, A. B., Mayo, B. (2013). Identification, typing, and
425 functional characterization of *Leuconostoc* spp. strains from traditional, starter-free
426 cheeses. *Dairy Sci. Technol*. 93 (6): 657-673.

427 Alvarado, C., García-Almendárez, B. E., Martin, S. E., Regalado, C. (2005). Anti-*Listeria*
428 *monocytogenes* bacteriocin-like inhibitory substances from *Enterococcus faecium*
429 UQ31 isolated from artisan Mexican-style cheese. *Curr. Microbiol*. 51(2):110–115.
430 Cárcoba, R., Delgado, T., Rodríguez, A. (2000). Comparative performance of a mixed
431 strain starter in cow's milk, ewe's milk and mixtures of these milks. *Eur. Food Res.*
432 *Technol*. 211(2):141-146.
433 Caro, I., Mateo, J., Sandoval, M. H., Soto, S., García-Armesto, M. R., Castro, J. M. (2013).
434 Characterization of Oaxaca raw milk cheese microbiota with particular interest in
435 *Lactobacillus* strains. *J. Dairy Sci.* 96(6):3461–3470.
436 Caro, I., Soto, S., Fuentes, L., Gutiérrez-Méndez, N., García-Islas, B., Monroy-Gayosso, K.
437 E., Mateo, J. (2014). Compositional, Functional and Sensory Characteristics of
438 Selected Mexican Cheeses. *Food Nutr. Sci.* 5:366–375.
439 Carr, F.J., Chill, D., Maida, N. (2002). The lactic acid bacteria: a literature survey. *Crit.*
440 *Rev. Microbiol*. 28: 281-370.
441 Cintas, L.M., Casaus, P., Håvarstein, L.S., Hernández, P.E., Nes, I.F. (1997). Biochemical
442 and genetic characterization of enterocin P, a novel sec- dependent bacteriocin from
443 *Enterococcus faecium* P13 with a broad antimicrobial spectrum. *Appl. Environ.*
444 *Microbiol.* 63(11):4321-4330.
445 Delgado, S and Mayo, B. (2004). Phenotypic and genetic diversity of *Lactococcus lactis*
446 and *Enterococcus* spp. strains isolated from Northern Spain starter-free farmhouse
447 cheeses. *Int. J. Food Microbiol.* 90(3):309–319.
448 Diep, D. B., Johnsborg, O., Risøen, P. A., Nes, I. F. (2001). Evidence for dual functionality
449 of the operon plnABCD in the regulation of bacteriocin production in *Lactobacillus*
450 *plantarum. Mol. Microbiol.* 41(3):633–644.

451 Diep, D. B., Straume, D., Kjos, M., Torres, C., Nes, I. F. (2009). An overview of the
452 mosaic bacteriocin pln loci from *Lactobacillus plantarum*. *Peptides*. 30(8):1562–
453 1574.
454 Dobson, A., Cotter, P. D., Ross, R.P., Hill, C. (2012). Bacteriocin production: a probiotic
455 trait? *Appl. Environ. Microbiol.* 78(1):1–6.
456 Dudley, E. G., and Steele, J. L. (2005). Succinate production and citrate catabolism by
457 Cheddar cheese nonstarter lactobacilli. *J. Appl. Microbiol.* 98:14–23.
458 Eckburg, P.B., Bik, E.M., Bernstein, C.N., Purdom, E., Dethlefsen, L., Sargent, M.,
459 Gill,S.R., Nelson, K.E. and Relman, D.A. (2005). Diversity of the human intestinal
460 microbial flora. *Science*. 308: 1635–1638.
461 Favaro L., Barretto Penna, A.L., Todorov, S.D. (2015) Bacteriocinogenic LAB from
462 cheeses – Application in biopreservation? *Trends Food Sci. Tech.* 41(1):37–48.
463 García, P., Rodríguez, L., Rodríguez, A., Martínez, B. (2010). Food biopreservation:
464 promising strategies using bacteriocins, bacteriophages and endolysins. *Trends Food*
465 *Sci. Tech*. 21(8):373–382.
466 Gatti, M., Lindner, J.D.D., De Lorentiis, A., Bottari, B., Santarelli, M., Bernini, V.,
467 Neviani, E. (2008). Dynamics of whole and lysed bacterial cells during Parmigiano-
468 Reggiano cheese production and ripening. *App. Environ. Microbiol.* 74 (19): 6161-
469 6167.
470 Gutiérrez, D., Martín-Platero, A. M., Rodríguez, A., Martínez-Bueno, M., García, P.,
471 Martínez, B. (2011). Typing of bacteriophages by randomly amplified polymorphic
472 DNA (RAPD)-PCR to assess genetic diversity. *FEMS Microbiol. Lett.* 322(1):90–97.

473 Hadji-Sfaxi, I., El-Ghaish, S., Ahmadova, A., Batdorj, B., Le Blay-Laliberté, G., Barbier,
474 G., Chobert, J. M. (2011). Antimicrobial activity and safety of use of *Enterococcus*
475 *faecium* PC4.1 isolated from Mongol yogurt. *Food Control*. 22(12):2020–2027.
476 Lavilla-Lerma, L., Pérez-Pulido, R., Martínez-Bueno, M., Maqueda, M., Valdivia, E.
477 (2013). Characterization of functional, safety, and gut survival related characteristics
478 of *Lactobacillus* strains isolated from farmhouse goat's milk cheeses. *Int. J. Food*
479 *Microbiol.* 163(2-3):136–145.
480 Maldonado, A., Ruiz-Barba, J. L., Jiménez-Díaz, R. (2003). Purification and Genetic
481 Characterization of Plantaricin NC8, a Novel Coculture-Inducible Two-Peptide
482 Bacteriocin from *Lactobacillus plantarum* NC8. *Appl. Environ. Microbiol.*
483 69(1):383–389.
484 Maldonado, A., Ruiz-Barba, J. L., Jiménez-Díaz, R. (2004). Production of plantaricin NC8
485 by *Lactobacillus plantarum* NC8 is induced in the presence of different types of
486 gram-positive bacteria. *Arch. Microbiol.* 181(1):8–16.
487 Martínez, M. I., Rodríguez, E., Medina, M., Hernández, P. E., and Rodríguez, J. M. (1998).
488 Detection of specific bacteriocin-producing lactic acid bacteria by colony
489 hybridization. *J. Appl. Microbiol.* 84(6):1099–1103.
490 Mayo, B., Aleksandrzak-Piekarczyk, T., Fernández, M., Kowalczyk, M., Álvarez-Martín,
491 P., and Bardowski, J. (2010). "Updates in the Metabolism of Lactic Acid Bacteria," in
492 *Biotechnology of Lactic Acid Bacteria: Novel Applications*, 3-33.
493 Millette, M., Dupont, C., Shareck, F., Ruiz, M. T., Archambault, D., Lacroix, M. (2008).
494 Purification and identification of the pediocin produced by *Pediococcus acidilactici*
495 MM33, a new human intestinal strain. *J. Appl. Microbiol.* 104(1):269-275.

496 Moll, G. N., Van Den Akker, E., Hauge, H. H., Nissen-Meyer, J., Nes, I. F., Konings, W.
497 N., Driessen, A. J. M. (1999). Complementary and overlapping selectivity of the two-
498 peptide bacteriocins plantaricin EF and JK. *J. Bacteriol.* 181(16):4848-4852.
499 Moraes, P. M., Perin, L. M., Todorov, S. D., Silva, A., Franco, B. D. G. M., Nero, L. A.
500 (2012). Bacteriocinogenic and virulence potential of *Enterococcus* isolates obtained
501 from raw milk and cheese. *J. Appl. Microbiol.* 113(2):318–328.
502 Ortolani, M. B. T., Moraes, P. M., Perin, L. M., Viçosa, G. N., Carvalho, K. G., Silva
503 Júnior, A., Nero, L. A. (2010). Molecular identification of naturally occurring
504 bacteriocinogenic and bacteriocinogenic-like lactic acid bacteria in raw milk and soft
505 cheese. *J. Dairy Sci.* 93(7):2880–2886.
506 Papagianni, M., and Anastasiadou, S. (2009). Pediocins: The bacteriocins of Pediococci.
507 Sources, production, properties and applications. *Microb. Cell Fact.* 8:3.
508 Perin, L. M., Dal Bello, B., Belviso, S., Zeppa, G., Carvalho, A. F. D., Cocolin, L., and
509 Nero, L. A. (2015). Microbiota of Minas cheese as influenced by the nisin producer
510 *Lactococcus lactis* subsp. *lactis* GLc05. *Int. J. Food Microbiol.* 214**:**159-167.
511 Ramasamy, K., Rahman, N. Z. A., Chin, S. C., Alitheen, N. J., Abdullah, N., Wan, H. Y.
512 (2012). Probiotic potential of lactic acid bacteria from fermented Malaysian food or
513 milk products. *Int. J. Food Sci. Tech.* 47:2175–2183.
514 Rehaiem, A., Martínez, B., Manai, M., Rodríguez, A. (2012). Technological performance
515 of the enterocin A producer *Enterococcus faecium* MMRA as a protective adjunct
516 culture to enhance hygienic and sensory attributes of traditional fermented milk
517 'Rayeb'. *Food Bioprocess Tech.* 5(6):2140-2150.

518 Renye, J. A., Somkuti, G. A., Vallejo-Cordoba, B., Van Hekken, D. L., González-Cordoba,
519 A. F. (2008). Characterization of the microflora isolated from queso fresco made
520 from raw and pasteurized milk. *J. Food Safety*. 28(1):59–75.
521 Renye, J. A., Somkuti, G. A., Paul, M., Van Hekken, D. L. (2009). Characterization of
522 antilisterial bacteriocins produced by *Enterococcus faecium* and *Enterococcus durans*
523 isolates from Hispanic-style cheeses. *J. Ind. Microbiol. Biot.* 36(2):261–268.
524 Rilla N., Martínez B., Delgado T., Rodríguez A. (2003). Inhibition of *Clostridium*
525 *tyrobutyricum* in Vidiago cheese by *Lactococcus lactis* ssp *lactis* IPLA 729, a nisin Z
526 producer. *Int. J. Food Microbiol.* 85(1-2):23-33.
527 Rojo-Bezares, B., Sáenz, Y., Navarro, L., Zarazaga, M., Ruiz-Larrea, F., Torres, C. (2007).
528 Coculture-inducible bacteriocin activity of *Lactobacillus plantarum* strain J23
529 isolated from grape must. *Food Microbiol.* 24(5):482–91.
530 Salazar, N., Ruas-Madiedo, P., Kolida, S., Collins, M., Rastall, R., Gibson, G., de los
531 Reyes-Gavilán, C. G. (2009). Exopolysaccharides produced by *Bifidobacterium*
532 *longum* IPLA E44 and *Bifidobacterium animalis* subsp. *lactis* IPLA R1 modify the
533 composition and metabolic activity of human faecal microbiota in pH-controlled
534 batch cultures. *Int. J. Food* Microbiol. 135(3):260-267.
535 Salgado-Ruiz, T.B., Rodríguez, A., Gutiérrez, D., Martínez, B., García, P., Espinoza-
536 Ortega, A., Martínez-Campos, A.R., Lagunas-Bernabé, S., Vicente, F., Arriaga-
537 Jordán, C.M. (2015). Molecular characterization and antimicrobial susceptibility of
538 *Staphylococcus aureus* from small-scale dairy systems in the highlands of Central
539 México. *Dairy Sci. Technol.* 95 (2): 181-196.

540 Saltijeral, J. A., Alvarez, V. B., García, B. (1999). Presence of listeria in Mexican Cheeses.
541 *J. Food Safety*. 19(4):241–247.
542 Sánchez, I., Seseña, S., Poveda, J. M., Cabezas, L., Palop, L. (2006). Genetic diversity,
543 dynamics, and activity of *Lactobacillus* community involved in traditional processing
544 of artisanal Manchego cheese. *Int. J. Food Microbiol.* 107(3):265–273.
545 Saxer, S., Schwenninger, S. M., Lacroix, C. (2013). Characterization of the microflora of
546 industrial Mexican cheeses produced without added chemical preservatives. *LWT -*
547 *Food Sci. Tech.* 53(1):314–320.
548 Schillinger, U., and Lucke, F. K. (1989). Antibacterial Activity of *Lactobacillus sake*
549 Isolated from meat. *Appl. Environ. Microbiol.* 55(8):1901–1906.
550 Siezen, R. J., Tzeneva, V. A., Castioni, A., Wels, M., Phan, H. T. K., Rademaker, J. L. W.,
551 Starrenburg, M. J. C., Kleerebezem, M., and van Hylckama Vlieg, J. E. T. (2010).
552 Phenotypic and genomic diversity of *Lactobacillus plantarum* strains isolated from
553 various environmental niches. *Environ. Microbiol.*12(3):758-773.
554 Somkuti, G. A., and Steinberg, D. H. (2010). Pediocin production in milk by *Pediococcus*
555 *acidilactici* in co-culture with *Streptococcus thermophilus* and *Lactobacillus*
556 *delbrueckii* subsp. *bulgaricus*. *J. Ind. Microbiol. Biot.* 37(1):65–69.
557 Torres-Llanez, M. J., Vallejo-Cordoba, B., Díaz-Cinco, M. E., Mazorra-Manzano, M. A.,
558 González-Córdova, A. F. (2006). Characterization of the natural microflora of
559 artisanal Mexican Fresco cheese. *Food Control*. 17:683–690.

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

| | PCR primers | Sequence 5'-3' | Annealing °C | Size (bp) | Reference |
|---|---|---|---|---|---|
| ***plnA*** | plnA-F | GTA CAG TAC TAA TGG GAG | 50 | 450 | (Lavilla-Lerma et al. 2013) |
| | plnA-R | CTT ACG CCA ATC TAT ACG | | | |
| ***plnEF*** | plnEF-F | GGC ATA GTT AAA ATT CCC CCC | 50 | 428 | |
| | plnEF-R | CAG GTT GCC GCA AAA AAA G | | | |
| ***plnJ*** | plnJ-F | TAA CGA CGG ATT GCT CTG | 50 | 475 | |
| | plnJ-R | AAT CAA GGA ATT ATC ACA TTA GTC | | | |
| ***plnK*** | plnK-F | CTG TAA GCA TTG CTA ACC AAT C | 50 | 246 | |
| | plnK-R | ACT GCT GAC GCT GAA AAG | | | |
| ***plnN*** | 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 | | | |

560

**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 | *E. faecalis* NRRL-B537[a] | *L. innocua* AST0 62[a] | *L. monocytogenes* ATCC19115[a] | Treatment Proteinase K[b] |
|---|---|---|---|---|---|
| *Lactobacillus plantarum* | SP-24 | - | - | +/- | - |
| | SP-38 | +/- | - | +/- | ND[a] |
| | SP-48 | - | - | +/- | ND |
| | SP-50 | - | +/- | +/- | - |
| | SP-651 | - | - | +/- | - |
| | SP-68 | - | +/- | +/- | - |
| *Lactobacillus fermentum* | SP-13 | + | - | - | ND |
| | SP-23 | + | - | +/- | ND |
| *Enterococcus faecium* | 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 | - | +/- | +/- | - |
| *Pediococcus acidilactici* | 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

561
562

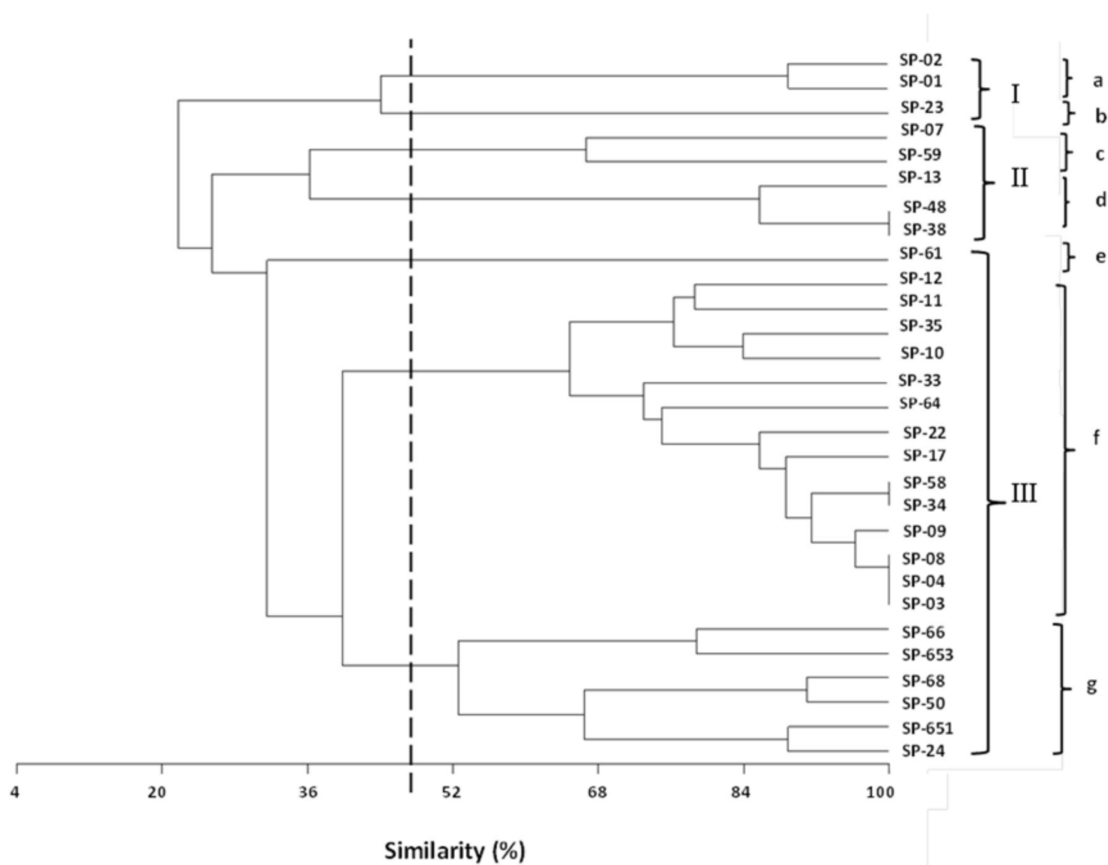
**Table 3.** Presence of bacteriocin genes determined by PCR in bacteriocinogenic lactobacilli isolated from "Queso Fresco" cheese.

| Species | Isolate | Bacteriocin gene | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | *plnA* | *plnEF* | *plnJ* | *plnK* | *plnS* | *plnN* | *pln*NC8 |
| *Lactobacillus plantarum* | SP-24 | + | + | + | + | - | + | - |
| | SP-38 | - | - | - | - | - | - | - |
| | SP-48 | - | - | - | - | - | - | - |
| | SP-50 | - | + | + | + | - | - | + |
| | SP-651 | + | + | + | + | - | + | - |
| | SP-68 | - | + | + | + | - | - | + |
| *Lactobacillus fermentum* | SP-13 | - | - | - | - | - | - | - |
| | SP-23 | - | - | - | - | - | - | - |

563
564
565

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

570

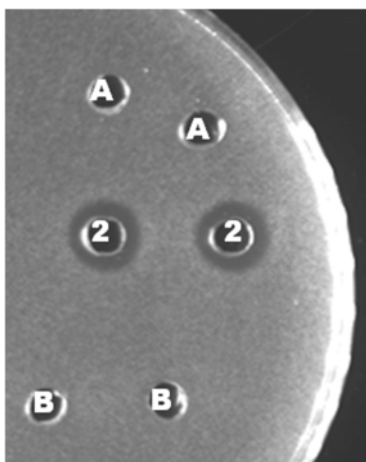



571

572

573

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

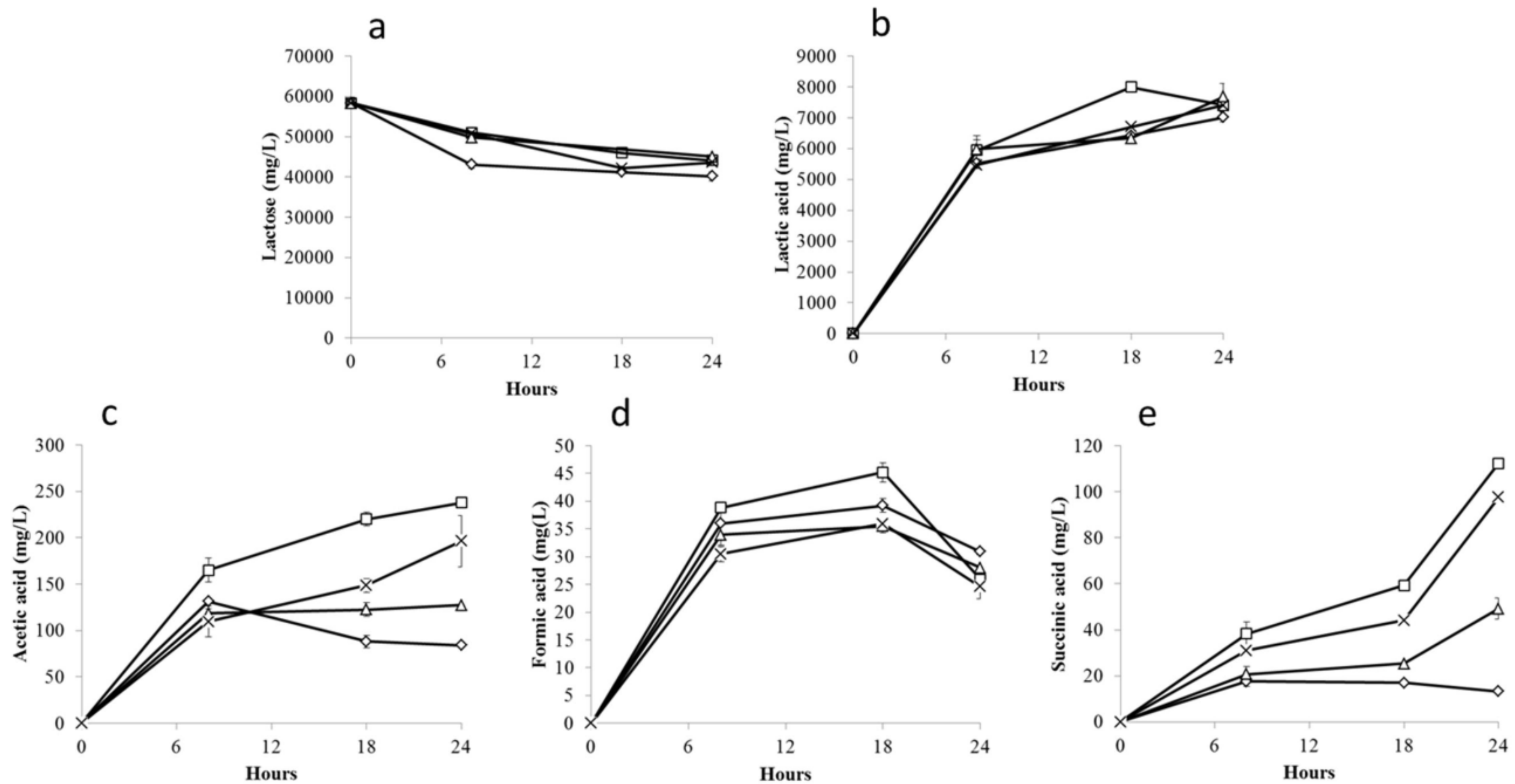



579
580
581
582

583 **Figure 3.** Residual lactose (a) and production of lactic (b), acetic (c), formic (d) and succinic (e) acids during milk fermentation of *L.*

584 *lactis* IPLA947 alone (◊) and co-cultured with the bacteriocinogenic strains *P. acidilactici* SP-01 (□), *L. plantarum* SP-68 (Δ) and *L.*

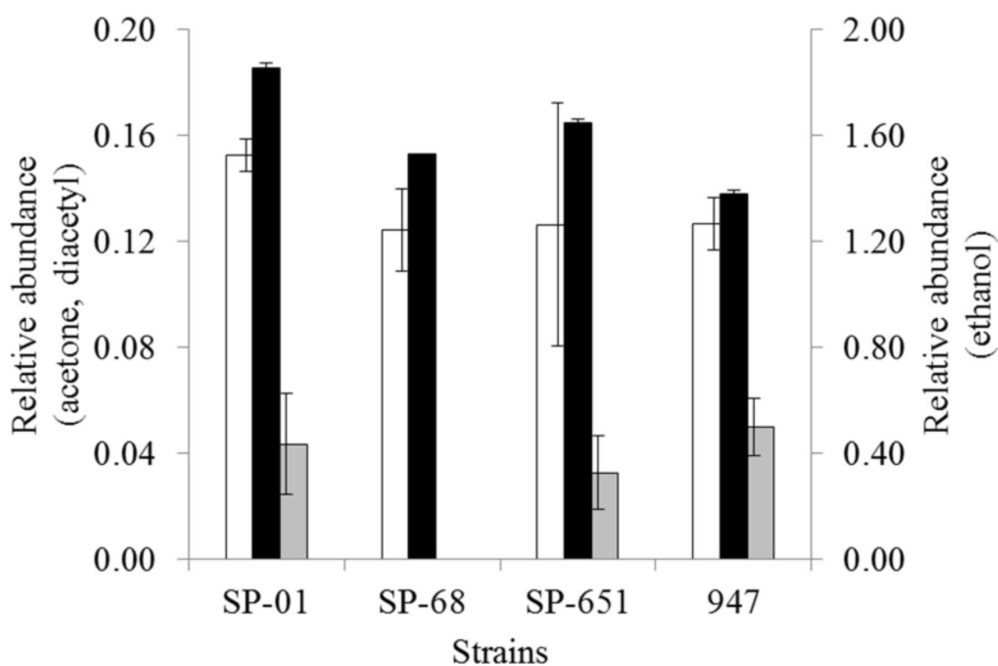
585 *plantarum* SP-651 (x). Data reported are means and standard deviation (vertical bars) of two independent experiments.




586

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

592




593