Identification, typing and functional characterization of *Leuconostoc* spp. strains from traditional, starter-free cheeses

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Abstract

Selected *Leuconostoc* strains are required as aroma producers in dairy starters. In this work, 42 *Leuconostoc* isolates from different raw milk traditional cheeses made without the addition of commercial starters were identified by molecular methods. These isolates were assigned to the species *Leuconostoc citreum* (24), *Leuconostoc mesenteroides* (13) and *Leuconostoc lactis* (5). Typing the isolates by repetitive extragenic profiling (Rep-PCR) identified 22 strains, out of which 14 were selected for further characterization based on their behaviour in milk. The biochemical, technological and food safety variables recorded were examined to select appropriate candidates for use as adjunct cultures. In agreement with the wide genetic diversity recorded by Rep-PCR, wide phenotypic biodiversity was seen among the species and strains in terms of enzyme and carbohydrate fermentation profile, acidification rate, the production of volatile compounds in milk, and growth under different pH, salt and temperature conditions. No production of biogenic amines was recorded, even when suitable amino acid precursors were present in the growth medium. All 14 strains proved to be susceptible or intrinsically resistant to a set of 16 antibiotics, except for one *Leuc. citreum* strain that showed resistance to ciprofloxacin. The remaining 13 strains could be confidently used as components of adjunct cultures in the dairy industry.

1. Introduction

Leuconostoc species are heterofermentative lactic acid bacteria (LAB) used as components of mesophilic cultures to produce aroma during milk fermentation. Presently, the genus *Leuconostoc* includes 13 species, *Leuc. carnosum, Leuc. citreum, Leuc. fallax, Leuc. gasicomitatum, Leuc. gelidum, Leuc. holzapfelii, Leuc. inhae, Leuc.*

kimchii, *Leuc. lactis*, *Leuc. mesenteroides* (with four subspecies, *cremoris*, *dextranicum*, *mesenteroides* and *suionicum*), *Leuc. miyukkimchii*, *Leuc. palmae*, and *Leuc. pseudomesenteroides* (http://www.bacterio.cict.fr/alintro.html; www.dsmz.de). Of these, *Leuc. mesenteroides* subsp. *cremoris* and *Leuc. lactis* are included as components of some mesophilic commercial starters (Hemme and Focaud-Scheunemann, 2004).

Leuconostocs lack a functional extracellular caseinolytic proteinase (Liu et al., 2010), which is essential for reaching high cell densities in milk. They are therefore usually employed as adjunct cultures in combination with fast-acid-producing lactococci. *Leuconostoc* species metabolize the citrate of milk to produce diacetyl (Hemme and Focaud-Scheunemann, 2004), a key odour compound in butter, buttermilk and certain cheese varieties (Smit et al., 2005). They also participate in the formation of other aroma and flavour compounds, such as lactic, acetic acid, and ethanol (Hemme and Focaud-Scheunemann, 2004). These and other metabolic end products contribute not only to the flavour profile of fermented products but, via their antimicrobial action, to their preservation (Hemme and Focaud-Scheunemann, 2004). As many other LAB species (de Vuyst and Leroy, 2007), some dairy *Leuconostoc* strains produce pediocin-like bacteriocins (Chang and Chang, 2010; Sawa et al., 2010), which may further contribute to food safety while reducing food spoilage. As obligate heterofermenters, *Leuconostoc* spp. produce CO_2 (Hemme and Focaud-Scheunemann, 2004), which favours the opening of the curd so important in the manufacture of blue cheese.

Though their numbers may vary widely, *Leuconostoc* species have been reported to be present in many traditional dairy products made from raw milk with or without starters (Joansen and Kibenich, 1992; Server-Busson et al., 1999; Cibik et al., 2000; Sánchez et al., 2005; Nieto-Arribas et al., 2010). Together with the mesophilic lactobacilli, *Leuconostoc* species contribute to the non-starter lactic acid bacteria

(NSLAB) populations of dairy environments. As NSLAB members, they are deemed to play a pivotal role in maintaining flavour and typicity of traditional dairy products (Nieto-Arribas et al., 2010; Sánchez et al., 2005). These products therefore provide a reservoir of phenotypic and genetic biodiversity, from which new strains with novel properties might be selected for improving adjunct cultures.

In this work, *Leuconostoc* isolates from traditional cheeses made from raw milk without commercial starters were identified by molecular methods and genotyped. Representative strains of these species were subjected to complete biochemical, genetic and technological characterization in order to select appropriate candidates for use as adjunct cultures in the dairy industry.

2. Materials and Methods

2.1. Bacterial strains and growth conditions

Leuconostoc isolates (n=42) were obtained from cheesemilk (3), curd (5) and ripen cheeses (34) of different batches of five Spanish traditional varieties; i.e., Cabrales (18), Casín (12), Genestoso (6), Peñamellera (3) and Porrúa (3). Isolates were recovered from the counting plates of *Leuconostoc* in Mayeux, Sandine and Elliker agar (MSE; Biokar Diagnostics, Beauvais, France), purified in de Man, Rogosa and Sharpe agar (MRS) (Merck, Darmstad, Germany) and kept frozen in MRS broth with 15% glycerol at -80ºC. Unless otherwise stated, cultures were grown aerobically and statically in MRS broth at 30ºC for 18-24 h.

2.2. Isolation and purification of total DNA

Total genomic DNA from the isolates was purified using the GenElute Bacterial Genomic DNA kit (Sigma-Aldrich, St. Louis, MO, USA), following the manufacturer's

recommendations. In short, 1.5 mL from an overnight culture was centrifuged, washed in sterile saline (0.9%) and suspended in a lysozyme solution (30 µg mL^{-1}) supplemented with mutanolysin (100 units mL^{-1}). Cell were incubated at 37ºC for 30 min and treated with proteinase K (20 µg mL^{-1}) at 55ºC for 15 min. Lysates were obtained by adding a lysis solution containing SDS, and the DNA was purified through the commercial columns of the kit and eluted using molecular biology-grade water (Sigma-Aldrich). DNA was then stored until analysis at -20ºC.

2.3. Identification by ARDRA and 16S rDNA sequencing

Total DNA was used as a template to amplify a segment of the 16S rRNA gene by PCR using the universal prokaryotic primers S-D-Bact0008-a-S-20 (27F) (5'-AGAGTTTGATCCTGGCTCAG-3') and S-*-Univ1492R-b-A-21 (1492R) (5'-GGTTACCTTGTTACGACTT-3'). The PCR conditions were as follows, one cycle at 95°C for 5 min, 35 cycles at 94°C for 30 s, 50°C for 45 s and 72°C for 2 min, and a final extension step at 72 °C for 10 min. Amplicons were purified through GenElute PCR Clean-Up columns (Sigma–Aldrich), digested with HaeIII and HhaI restriction enzymes (Invitrogen, Pasley, UK), and electrophoresed in 1.5% agarose gels. The gels were then stained with ethidium bromide (0.5 mg mL^{-1}) and visualized under UV light.

Isolates were grouped according to their ARDRA profile. Representative amplicons of each group were then sequenced using the 27F primer, employing an ABI 373 DNA sequencer (Applied Biosystems, Foster City, Ca., USA). The sequences obtained (larger than 800 bp on average) were compared with those in the GenBank database using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/), and with those in the Ribosomal Database Project database (http://rdp.cme.msu.edu/index.jsp). Isolates were allocated to a given species based on percentages of sequence identity (Stackebrandt et

al., 2002), but also by manual inspection of sequences at key variable positions as compared to those of *Leuconostoc* spp. type strains.

2.4. Strain typing by Rep-PCR

The intraspecies diversity of the isolates was assessed by repetitive extragenic profiling (Rep-PCR) using primer BoxA2R (5'-ACGTGGTTTGAAGAGATTTTCG-3'), as reported by Koeuth et al. (1995). Reproducibility studies of the Rep-PCR technique (independent amplifications with the same DNA and different reactions with independently-purified DNA) showed a percentage of similarity among the patterns of over 85%. Clustering was performed using the unweighted pair group method using arithmetic averages (UPGMA), with the GeneTools software (Syngene, San Diego, CA, USA). The similarity of the patterns was expressed by the Sørensen-Dice coefficient.

2.5. Acidification of milk

Single colonies of the strains cultivated on MRS agar plates at 30ºC for 48 h were grown in UHT milk (CAPSA, Siero, Spain). Overnight milk cultures were used to inoculate 5 mL of UHT milk at 1% (v/v), which were then incubated at 30ºC for up to 5 days. Culture growth was examined at 24 h, 48 h and 5 days by counting of dilutions on MRS agar plates, pH measurement and visual inspection of the clotting. Assays were performed in triplicate. In order to check production of glucans and/or dextrans influencing clotting texture, strains were also grown in UHT milk supplemented with 5% (w/v) sucrose (Merck) and 0.005% bromocresol purple as a pH indicator. Fermented milk was gently stirred and the viscosity was measured at 5ºC by the Posthumus funnel methods as reported elsewhere (Ruas-Madiedo et al., 2002).

2.6. Metabolic activities

2.6.1. Carbohydrate fermentation

The carbohydrate fermentation profiles of the isolates were determined using the commercial API50 CHL system (bioMérieux, Montalieu-Vercieu, France), following the supplier's recommendations.

2.6.2. Enzyme activities

Enzyme activities were measured using the commercial, semiquantitative API-ZYM system (bioMérieux), following the manufacturer's recommendations.

2.7. Technological characterisation

2.7.1. Proteolytic activity

The proteolytic activity of the strains was investigated by the *o*-phthaldialdehyde (OPA) method (Church et al., 1983). Briefly, after incubation of the strains in milk at 30ºC for 48 h, proteins were precipitated by the addition of 2 mL of 0.75 N trichloroacetic acid (TCA) and 0.2 mL of water to 1 mL milk samples. Mixtures were vortexed for 2 min and then filtered using Whatman nº2 filter paper (Whatman, Maidstone, UK). The OPA reagent (Sigma-Aldrich) was added to the filtrates and the absorbance measured at 340 nm using a Benchmark PlusMicroplate Spectrophotometer (BioRad, Hercules, CA, USA). Results were expressed as mM glycine L^{-1} using an appropriate calibration curve (concentration range 0.1-10 mM). Assays were done in triplicate, which allows subjecting the results to statistical analysis.

2.7.2. Production of volatile compounds

Volatile compound analysis was performed after growth of the strains at 30ºC in UHT milk for 24 and 48 h and 5 days. The separation and quantification of the volatile compounds was performed by headspace/gas chromatography/mass spectrometry

(HS/GC/MS), using an Agilent apparatus (Agilent Technologies, Wilmington, DE, USA) equipped with a capillary column DB-WAXetr 60 m x 0.25 mm x 0.25µm (Agilent). Sample preparation and gas chromatographic separation were performed as previously described (Salazar et al., 2008). Compounds were quantified as the normalized value of their chromatogram peak areas using cyclohexanone (3.6 µg per mL^{-1}) as an internal standard, which was given a value of 100.

2.7.3. Production of antimicrobial substances

Antimicrobial activity was first examined by an agar spot test, followed by a well-diffusion assay using neutralized, filter-sterilized supernatants. *Lactococcus lactis* subsp. *cremoris* MG 1363, *Lactobacillus sakei* CECT 906^T, *Listeria innocua* 86/26 and *Staphylococcus aureus* CECT 86^T were used as indicators. *Leuconostoc* spp. and indicator strains were grown as reported elsewhere (Alegría et al., 2010).

2.7.4. Resistance to NaCl and low pH, and growth at different temperatures

Resistance to NaCl was assessed by inoculation of the strains (at 1% v/v) into a series of 100 µL MRS broths containing concentrations of NaCl ranging from 4 to 7% (w/v) with 0.5% intervals. To test the resistance of the strains to acid, they were inoculated (at 1% v/v) into 100 µL MRS broth adjusted with acetic acid to pH values between 3.5 and 6.5 with 0.5 unit intervals. As a control for both tests, a culture in standard MRS (pH 6.8, no extra NaCl) was used. Finally, strains were inoculated (1% v/v) into MRS broth and incubated at 10, 25, 32, and 45ºC to determine their growth at different temperatures. In all cases, optical density (OD) was measured at 595 nm.

Results after 24 h of incubation were expressed as the OD_{595} difference between standard MRS and the corresponding NaCl, pH or temperature condition. All assays were performed in triplicate and the results subjected to statistical analysis.

2.8. Safety of *Leuconostoc* strains

2.8.1. Antibiotic resistance

The minimum inhibitory concentrations (MIC) of several antibiotics of biological and clinical significance were determined by a microdilution method as reported by Flórez et al. (2007), using plates containing serial two-fold dilutions of 16 antibiotics (VetMIC^{TM}, National Veterinary Institute of Sweden, Uppsala, Sweden). Briefly, individual colonies on Mueller-Hinton agar plates (Oxoid, Basingstoke, Hampshire, UK) were suspended in 2 mL of sterile saline solution (Oxoid) until a density corresponding to McFarland standard 1 (around $3x10^8$ cfu mL^{-1}). This suspension was then diluted 1:1000 in the same medium to a final concentration $3x10^5$ cfu mL^{-1}. An aliquot of 100 µL of this inoculum was then added to each well of the VetMIC^{TM} plates, which were then incubated at 30ºC for 48 h. MICs were defined as the lowest antibiotic concentration at which no visual growth was observed.

2.8.2. Production of biogenic amines

The production of the toxic amines tyramine and histamine from their respective precursor amino acids (tyrosine and histidine) was investigated using the plate assay described by Bover-Cid and Holzapfel (1999).

3. Results and Discussion

3.1. Identification and typing of *Leuconostoc*

Leuconostoc spp. levels in the analyzed cheeses ranged from undetectable levels in some cheesemilk samples up to $4.8x10^6$ cfu mL^{-1} in a two month-old Cabrales cheese. Levels varied widely between the cheese types (from 2.1x102 to $4.8x10^6$ cfu mL^{-1}) and, as a general trend, counts increased moderately as ripening progressed. After purification, the 42 *Leuconostoc* spp. of this study were subjected to a molecular

identification scheme consisting in amplification of a major part of their 16S rRNA gene, digestion of the amplicons with two restriction enzymes (ARDRA), sequencing and sequence comparison. The restriction enzyme HaeIII yielded two ARDRA profiles, profile 1 (24 isolates) and profile 2 (18 isolates) (Fig. 1A, lanes 1 and 2, respectively). All 24 isolates of profile 1 with HaeIII gave the same HhaI profile (Fig. 1B; lane a). In contrast the 18 isolates of profile 2 with HaeIII gave two different profiles with HhaI - profile (b) (13 isolates) and profile (c) (5 isolates) (Fig. 1B; lanes b and c, respectively). After sequencing and comparison of 14 out of the 42 amplicons, the profiles were assigned to the species *Leuc. citreum* (profiles 1 and a; 24 isolates), *Leuc. mensenteroides* (profiles 2 and b; 13 isolates), and *Leuc. lactis* (profiles 2 and c; 5 isolates). The intraspecies genetic diversity of the isolates belonging to the different species was assessed by Rep-PCR. Sixteen fingerprinting profiles were returned by the 24 *Leuc. citreum* isolates, nine for the 13 *Leuc. mesenteroides* isolates, and three for the five *Leuc. lactis* isolates (Fig. 2). Using the cut-off obtained in the reproducibility study (85%), 15 strains were contemplated among the 24 *Leuc. citreum* isolates, five among the 13 *Leuc. mesenteroides*, and two among the 5 *Leuc. lactis* (Fig. 2). All 22 strains were cultured in UHT milk, and eight of which showed no activity (neither grew nor acidified) were discarded from further analysis.

The phenotypic and metabolic activity of the remaining 14 strains was analyzed using the API 50 CHL and API-ZYM systems. Tables 1 and 2 show the results obtained. In agreement with the high genetic diversity detected by Rep-PCR, high phenotypic diversity was encountered. Most strains showed single patterns in both phenotypic assays. Moreover, combining carbohydrate utilization and enzyme profiles, strains could all be individually distinguished by their phenotype. All the *Leuconostoc* strains fermented D-glucose, D-fructose, N-acetyl-glucosamine, D-lactose, D-maltose,

247 D-mannose and D-sucrose. However, diversity in the utilization of another 17
248 carbohydrates was detected (Table 1). Twelve of the 19 enzymatic activities tested were
249 detected among the analyzed strains. Of these, some activities (such as those of leucine
250 arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase,
251 β-galactosidase and α-glucosidase) were shown by all or most strains. In contrast, other
252 activities (such as those of esterase lipase C8, cystine arylamidase, α-chymotrypsin and
253 α-galactosidase) were only rarely detected (Table 2).
254
255 **3.2. Technological characterization of *Leuconostoc* strains**
256 Table 3 shows the behaviour of the 14 selected strains in UHT milk. Within the time
257 points analyzed, all strains attained a similar maximum cell density after 24 h of
258 incubation, declining thereafter the number of live cells. Acidification and milk clotting
259 progressed slowly. By day 5, pH 5.0 or lower was reached with most *Leuc. citreum*
260 strains, and a stable coagulum had been produced. However, the final pH of the milk
261 fermented by the *Leuc. mesenteroides* and *Leuc. lactis* strains was higher, and the
262 viscosity of the coagulum much lower. In some cases, small- and medium-sized gas
263 bubbles were clearly observed within the coagulum, indicating the active production of
264 CO_2. Gas production by *Leuc. citreum* 7A7b was so strong that the curd broke, allowing
265 for some whey drainage (Table 3). In sucrose-supplemented milk, the *Leuc. citreum*
266 strains generally attained higher numbers than those of *Leuc. mesenteroides* and *Leuc.*
267 *lactis*. All but two strains (*Leuc. mesenteroides* 3AC2 and 3AC16) developed well and
268 turned the pH indicator yellow. However, an increase in viscosity, as determined by the
269 Posthumus funnel method, was not observed, suggesting glucans and dextrans are not
270 produced under the culture conditions of this study.

271 Table 4 shows the production of volatile compounds in milk by the strains. At least
272 eight volatile compounds were detected by GC/MS. Though differences among strains
273 were found, ethanol was the major volatile compound in the profiles of all strains.
274 Acetic acid was also produced by all strains, except for *Leuc. lactis* 4AB2. Small
275 amounts of 2-propanone and ethyl-acetate were produced by most strains. Compounds
276 such as 2-butanone, diacetyl, 2-heptanone and butanoic were detected rarely.
277 The pH and NaCl content of the medium, and the incubation temperature, had great
278 influence on the final cell density attained by the *Leuconostoc* cultures. Fig. 3 shows the
279 results of growth under different conditions. For reasons of clarity, only key results are
280 shown in the figure panels. Fig. 3A shows the strain to strain variation in growth at the
281 different temperatures assayed. Similar ODs were obtained for all strains at 10, 25 and
282 32ºC. However, at 45ºC none of the strains grew, except for the two *Leuc. lactis* strains
283 (4AB2 and 7G3a), which showed reduced but still appreciable growth at this
284 temperature. The growth of all strains was inversely related to the NaCl content of the
285 growth medium (Fig. 3B). *Leuc. citreum* strains 4AC15, 7A7b and 7G2c reached higher
286 cell densities than all the other strains at all salt concentrations up to 6.5%. The strain
287 *Leuc. lactis* 7G3a also grew well up to an NaCl concentration of 5.5%. Compared to
288 growth in unadjusted MRS (pH 6.8), all strains showed 20% reductions in OD at pH
289 5.5, and more than 80% at pH 5. None of the strains grew at pH 4.5 or lower (Fig. 3C).
290 Small differences between strains were observed at all pHs, although at pH 5 *Leuc.*
291 *mesenteroides* 3AC2 and 3AC16 doubled the optical density reached by all others.
292 The OPA assay detected significant differences in proteolytic activity between the
293 isolates (Table 5). *Leuc. lactis* 7G3a was associated with the strongest release of amino
294 acids from milk proteins, followed by *Leuc. mesenteroides* 3AC16, to equivalents of

295 8.09±0.01 and 13.66±0.02 mM L^{-1} of glycine, respectively. In contrast, one strain each
296 of these two species showed no detectable proteolytic activity in the OPA test.
297 Most strains produced antimicrobial substances against one or more indicators in the
298 agar spot test. However, in the well-diffusion assay, only *Leuc. citreum* 4AC4 showed a
299 consistent and specific inhibitory effect against the *L. innocua* indicator.
300

301 **3.3. Safety of *Leuconostoc* strains**

302 Where defined, MICs were compared to the microbiological breakpoints stated for
303 the purpose of distinguishing acquired resistant from susceptible or inherent
304 (intrinsically) resistant bacteria by the Panel on Additives and Products or Substances
305 used in Animal Feed (FEEDAP) (EFSA, 2012). For most of the antibiotics, the MIC
306 values were lower than the FEEDAP cut-offs. The MICs of kanamycin and
307 chloramphenicol for some strains were one dilution higher than their corresponding cut-
308 offs. The MIC values of all the antibiotics for the different strains suggested that all but
309 one were either susceptible or intrinsically resistant. The exception was *Leuc. citreum*
310 7A7b, for which the MIC of ciprofloxacin was 64 μg mL^{-1}; a value eight-fold higher
311 than that associated with most other strains.
312 None of the strains produced tyramine or histamine on plates containing their
313 precursor amino acids tyrosine or histidine. Production of these and other biogenic
314 amines has been further excluded by ultra-high pressure liquid chromatography (UPLC)
315 analysis (Ladero et al., unpublished).
316

317 **4. Discussion**

318 Traditional cheeses are a good source of new dairy LAB strains, including
319 *Leuconostoc* spp. (Server-Bussson et al., 1999; Cibik et al., 2000; Sánchez et al., 2005;

Nieto-Arribas et al., 2010; Cardamone et al., 2011). In this work, 42 *Leuconostoc* isolates were identified and typed by molecular methods. Rep-PCR identified 22 different strains, 14 of which were selected for further study based on their acidification and growth behaviour in milk. As a whole, strains of *Leuc. citreum* reached higher cell densities and clotted milk faster than strains from the other two species. The use of growth and acidification of milk as a selective criterion might further allow the use of single strains or strains mixtures of *Leuconostoc* spp. as the sole starter in milk-based fermentations. Large diversity in carbohydrate fermentation (by the API50 CH system) was observed among species and strains. Not surprisingly, the molecular and phenotypic identification did not match, as the latter method has been repeatedly reported to be not reliable for LAB (Cibik et al., 2000; Delgado and Mayo, 2004). Nevertheless, the genetic diversity revealed by Rep-PCR correlated well with the biochemical diversity seen in the different phenotypic assays. Similar levels of phenotypic and genetic diversity has been reported elsewhere for *Leuconostoc* spp. isolated from traditional, starter-free, raw-milk cheeses (Cibik et al., 2000; Sánchez et al., 2005; Nieto-Arribas et al., 2010).

High variation in technological characteristics among *Leuconostoc* spp. strains has also been reported frequently (Sánchez et al., 2005; Nieto-Arribas et al., 2010; Cardamome et al., 2011). However, the use of distinct methodologies, assays and/or culture conditions impedes a proper comparison of the results from different works. Qualitative and quantitative variations between the strains in terms of their volatile compound profiles in milk were recorded. Surprisingly, diacetyl was only identified as a minority component in the profiles of two strains, *Leuc. mesenteroides* 2G5b and *Leuc. lactis* 4AB2. In *Lactococcus lactis* it is well known that the transport of citrate only occurs after acidification of the medium during cell growth (García-Quintans et al.,

345 1998). Similar pH-mediated induction of the components required for citrate utilization
346 in *Leuconostoc* may hamper the formation of diacetyl at the high pH recorded for all
347 cultures at 48 h (Table 3). Furthermore, as *Leuconostoc* does not grow well in milk
348 when on its own, diacetyl production, and perhaps that of other volatile compounds,
349 might be enhanced when strains are cultivated with lactococci. Such production might
350 reflect greater growth or the complementation of metabolic routes (Ayad et al., 2001).
351 Proteolysis is the most critical process for the formation of volatile compounds
352 during the ripening of dairy products (Smit el al., 2005). Differences in the proteolytic
353 ability of the strains, as judged by the OPA test, were noted. Though proteolysis of
354 casein is essential for full growth in milk, the activity recorded did not correlate with the
355 acidification and clotting of milk (Table 3). Proteolytic levels higher than those reported
356 in the literature (Nieto-Arribas et al., 2010) were scored for two strains. Since no
357 extracellular proteinases have ever been described for *Leuconostoc* species (Liu et al.,
358 2010), differential lysis during growth in milk and the release of intracellular proteolytic
359 enzymes might explain such differences; though this result did not correlate with a
360 concomitant decrease of viability.
361 The antimicrobial activity of *Leuconostoc* in solid media has been attributed to
362 metabolic end products such as lactic and acetic acid and diacetyl (Hemme and
363 Foucaud-Sheunemann, 2004), as well as colony-associated compounds such as fatty
364 acids and H_20_2 (Hemme and Foucaud-Sheunemann, 2004; de Vuyst and Leroy, 2007).
365 Consequently, the inhibitory effects detected by the agar spot test are not always
366 confirmed in liquid (Alegría et al., 2010; Dal Bello et al., 2010). In the present work, a
367 single strain (*Leuc. citreum* 4AC4) showed inhibitory activity when neutralized
368 supernatants were used. The production of bacteriocin-like substances by different
369 strains has frequently been reported (de Vuyst and Leroy, 2007; Sawa et al., 2010). The

production of a bacteriocin by 4AC4 is suspected, but the nature of the inhibitory compound was not further examined. The inclusion of bacteriocin-producing strains in starter and adjunct cultures could, under certain conditions, help to improve safety and quality of fermented dairy products.

Antibiotic resistance in a bacterial species can be inherent (referred to as intrinsic or natural resistance) or acquired (atypical resistance) via mutation or more frequently by DNA acquisition through horizontal transmission (EFSA, 2012). The use of starter and adjunct cultures carrying acquired antibiotic resistances should be avoided to prevent the spread of antibiotic resistance determinants through the food chain. The MICs of antibiotics for all assayed strains were lower than the microbiological breakpoints defined by the European Food Safety Authority for acquired resistances (EFSA, 2012), or just a dilution higher. Increased MICs might be the result of methodological limitations, such as a lack of standardized methods for use with *Leuconostoc* species, the absence of appropriate control strains of this genus, or the use of different antibiotic-testing media (Katla et al., 2001; Flórez et al., 2005). Analysis of the MIC distributions for all antibiotics, and comparison with previous results (Katla et al., 2001; Flórez et al., 2005), strongly suggests that the strains of this study are susceptible or intrinsically resistant to all the antibiotics assayed. The exception is *Leuc. citreum* 7A7b, which showed an atypical enhanced resistance to ciprofloxacin. The mechanism of resistance to this antibiotic in this strain deserves further investigation.

The production of biogenic amines by *Leuconostoc* species from wines is well known (Lonvaud-Funel and Joyeux, 1994; Moreno-Arribas et al., 2003), but this activity has never been seen in cheese isolates (Bober-Cid and Holzapfel, 1999; Sánchez et al., 2005; Nieto-Arribas et al., 2010). the formation of biogenic amines by

Leuconostoc spp. may have been selected against through adaptation to the milk environment, as would seem to be the case for *Lact. lactis* (Ladero et al., 2011).

Leuconostoc spp. have been described as moderately susceptible to bacteriophage attack (Dessart and Steenson, 1995). This may result from their low growth rates compared to those of lactococci. *Leuconostoc* species usually reach their maximum numbers during cheese ripening, when the matrix is in a semi-solid state, which might hinder the spread of phages. In the present work, phage resistance -a desirable characteristic for starter and adjunct cultures- was not examined due to the lack of a suitable collection of phages infecting species of this genus.

In conclusion, several *Leuconostoc* strains from traditional cheeses were fully characterized. The genetic and phenotypic diversity detected among the strains of the different species, including their different proteolytic activities, should allow for the rational selection of cultures that would meet different technological demands. Those characterized here might complement currently available strains for the industrial manufacture of dairy products. They could also be used in the design of specific starter and adjunct cultures based on native strains, which might contribute towards the maintenance of typicity in traditional cheeses. Of particular interest might be strains 1C6a, 4AC4 and 7G2a. These strains showed good behaviour in milk and belonged to *Leuc. citreum*, a species absent from the portfolio of most starter manufacturers. The testing of individual strains and mixtures as adjunct cultures, and their evaluation in cheese trials together with starter strains of *Lactococcus lactis* is currently in progress.

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Table 1.- Fermentation of carbohydrates by *Leuconostoc* strains isolated from traditional, starter-free cheeses made of raw milk.

Species/Strain	Fermentation of carbohydrate[a]																
	LARA	RIB	DXYL	GAL	MDG	ARB	ESC	SAL	CEL	MEL	TRE	RAF	GEN	DTUR	GNT	2KGN	5KGN
Leuc. citreum																	
1A3b	+	-	-	+	+	-	-	-	-	-	-	-	-	+	+	-	-
1C6a	+	-	-	+	+	-	-	+	-	-	+	-	-	+	+	-	-
2A5b	+	-	-	+	+	-	-	-	-	-	+	-	-	+	+	-	-
2G3b	-	-	-	+	+	+	+	+	-	-	+	-	-	+	+	-	-
4AC4	+	-	+	+	+	+	+	+	-	+	+	-	-	+	+	+	-
4AC15	+	-	+	+	+	+	+	+	-	+	+	-	-	+	+	+	-
7A7b	-	-	-	+	+	+	+	+	-	-	+	-	-	+	+	-	-
7G2a	-	-	+	+	+	-	-	-	-	+	+	+	-	+	+	+	-
Leuc. mesenteroides																	
1G2d	-	-	-	+	+	-	-	-	-	-	+	-	-	+	-	-	-
2G5b	-	-	-	-	-	+	+	+	-	-	+	-	-	+	+	-	-
3AC2	-	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+
3AC16	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
Leuc. lactis																	
4AB2	-	-	-	+	-	-	-	-	-	+	-	+	-	-	-	-	-
7G3a	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-

[a]Key of carbohydrates: LARA, L-arabinose; RIB, D-ribose; DXYL, D-xylose; GAL, D-galactose; MDG, α-methyl-D-glucoside; ARB, arbutin; ESC, Esculin; SAL, salicin; CEL, D-cellobiose; MEL, D-melibiose; TRE, D-trehalose; RAF, D-raffinose; GEN, gentiobiose; DTUR, D-turanose; GNT, gluconate; 2KGN, 2-keto-gluconate; and 5KGN, 5-keto-gluconate. All strains fermented D-glucose, D-fructose, D-mannose, D-maltose, N-acetyl-glucosamine, D-lactose, and D-sucrose, while proved to be negative for glycerol, erythritol, D-arabinose, L-xylose, adonitol, β-methyl-D-xyloside, L-sorbose, L-rhamnose, dulcitol, inositol, D-manitol, D-sorbitol, α-methyl-D-mannoside, amygdalin, inulin, melezitose, starch, glycogen, xylitol, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, and L-arabitol.

Table 2.- Diversity of enzymatic activities of *Leuconostoc* strains from traditional, starter-free cheeses made of raw milk.

Species/strain	Enzymatic activity[a]											
	Alkaline phosphatase	Esterase C4	Esterase Lipase C8	Leucine arylamidase	Valine arylamidase	Cystine arylamidase	α-chymotrypsin	Acid phosphatase	Naphthol-AS-BI-phosphohydrolase	α-galactosidase	β-galactosidase	α-glucosidase
Leuc. citreum												
1A3b	5	5	0	5	5	0	0	10	5	0	40	10
1C6a	5	0	0	5	5	0	0	10	5	0	40	10
2A5b	5	0	0	5	5	0	0	10	5	0	40	10
2G3b	5	5	0	5	5	0	0	10	5	0	40	10
4AC4	0	10	5	5	5	0	0	5	5	5	40	10
4AC15	0	10	5	5	5	0	0	5	5	0	40	20
7A7b	5	0	0	5	5	5	0	20	5	0	40	20
7G2a	0	5	0	5	5	0	0	5	5	0	40	10
Leuc. mesenteroides												
1G2d	5	0	0	10	0	5	0	5	5	0	40	10
2G5b	5	5	0	5	5	0	0	5	5	0	40	10
3AC2	0	20	0	5	5	0	0	5	5	0	40	5
3AC16	0	20	0	5	5	0	0	5	5	0	40	5
Leuc. lactis												
4AB2	5	0	0	10	5	0	10	5	0	5	0	0
7G3a	5	0	0	10	5	0	0	10	5	0	20	0

[a]Units of activity are expressed as nanomoles of substrate hydrolyzed under the assay conditions.
Lipase C14, trypsin, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase activities were never recorded.

Table 3.- Behaviour of *Leuconostoc* strains from traditional, starter-free cheeses in UHT-milk.

Species/strain	Incubation in milk for[a]								
	24 h			48 h			5 days		
	pH[b]	Log_{10} cfu mL^{-1}	Clotting of milk	pH	Log_{10} cfu mL^{-1}	Clotting of milk	pH	Log_{10} cfu mL^{-1}	Clotting of milk
Leuc. citreum									
1A3b	6.07	8.81	+/-[c]	5.45	8.46	+/-	5.00	8.02	+++ (gb)[d]
1C6a	6.03	8.35	+/-	5.50	8.59	+	5.02	7.81	+++
2A5b	6.21	8.30	+/-	5.55	8.35	++	5.11	7.78	+++ (gb)
2G3b	6.28	8.19	-	5.93	8.05	+	5.21	8.61	+
4AC4	6.05	8.38	+/-	5.53	7.32	+	4.80	7.16	+++
4AC15	6.06	8.54	+/-	5.50	8.26	++	5.01	6.30	+++ (gb)
7A7b	6.32	8.52	-	6.08	7.79	+	5.70	6.89	+++ (w)[e]
7G2a	6.03	8.61	+/-	5.58	8.25	+	4.97	6.83	+++
Leuc. mesenteroides									
1G2d	6.27	8.21	-	5.75	8.48	+/-	5.66	6.42	+/- (gb)
2G5b	5.99	8.74	-	5.58	8.12	+	5.62	6.48	++
3AC2	6.22	8.30	-	5.80	7.99	+/-	5.39	7.89	+/-
3AC16	6.28	8.23	-	5.81	8.03	+/-	5.39	7.71	+ (gb)
Leuc. lactis									
4AB2	6.24	8.38	-	5.78	8.26	+/-	5.27	7.11	+
7G3a	6.25	8.24	-	6.00	7.80	+/-	5.72	7.96	+/-

[a]Inoculated with 10^6 cfu from overnight cultures in UHT milk and incubated at 30ºC.
[b]pH of uninoculated milk 6.66
[c]+/-, incomplete clot at the bottom of the tube.
[d](gb), visible gas bubbles in the curd.
[e](w), broken curd and whey drainage.

Table 4.- Relative abundance of volatile compounds detected by Head Space, Gas Chromatography, Mass Spectrometry (HS/GS/MS) after growth of the strains at 30ºC for 48 h in UHT-treated milk.

Species/Strain	Volatile compound[a]							
	2-propanone	Ethyl-acetate	2-butanone	Ethanol	Diacetyl	2-heptanone	Acetic acid	Butanoic acid
Leuc. citreum								
1A3b	4.67	-	-	15,576.69	-	-	383.87	-
1C6a	3.31	-	-	14,498.04	-	-	382.99	-
2A5b	5.26	8.44	-	16,817.62	-	-	341.06	-
2G3b	4.49	5.33	-	12,527.85	-	-	261.55	-
4AC4	3.16	8.47	-	18,265.82	-	-	394.64	-
4AC15	4.56	8.75	-	20,265.67	-	-	492.00	8.45
7A7b	7.56	-	-	8,914.78	-	-	19.77	-
7G2a	4.30	6.26	1.08	16,991.22	-	-	407.07	2.67
Leuc. mesenteroides								
1G2d	2.49	-	-	11,349.17	-	-	227.62	-
2G5b	0.28	2.47	1.41	8,208.76	3.12	4.07	117.07	-
3AC2	-	4.75	1.33	10,798.79	-	-	20.04	-
3AC16	-	4.72	-	12,711.59	-	-	30.80	-
Leuc. lactis								
4AB2	0.75	3.67	1.09	7,833.15	2.17	3.65	-	3.48
7G3a	2.65	-	-	6,688.42	-	4.41	92.89	4.76

[a]Results were calculated as the difference between values obtained for the sample and that of a non-inoculated UHT-milk sample incubated under the same conditions.
-, not detected.

Table 5.- Proteolytic activity of *Leuconostoc* strains isolated from traditional, starter-free cheeses as determined by the OPA assay.

Species/strain	Proteolytic activity[a]	
	Average[b]	SD[c]
Leuc. citreum		
1A3b	0.87	0.02
1C6a	0.93	0.02
2A5b	0.46	0.00
2G3b	1.26	0.02
4AC4	1.05	0.46
4AC15	1.13	0.01
7A7b	0.00	0.15
7G2a	0.61	0.01
Leuc. mesenteroides		
1G2d	0.18	0.01
2G5b	1.40	0.01
3AC2	0.00	0.00
3AC16	8.09	0.01
Leuc. lactis		
4AB2	0.00	0.00
7G3a	13.66	0.02

[a]Determined as the mmol of glycine released after incubation in milk under the conditions of the assay (30ºC for 48 h), using a glycine calibration curve.
[b]Average results of three independent experiments.
[c]SD, standard deviation.

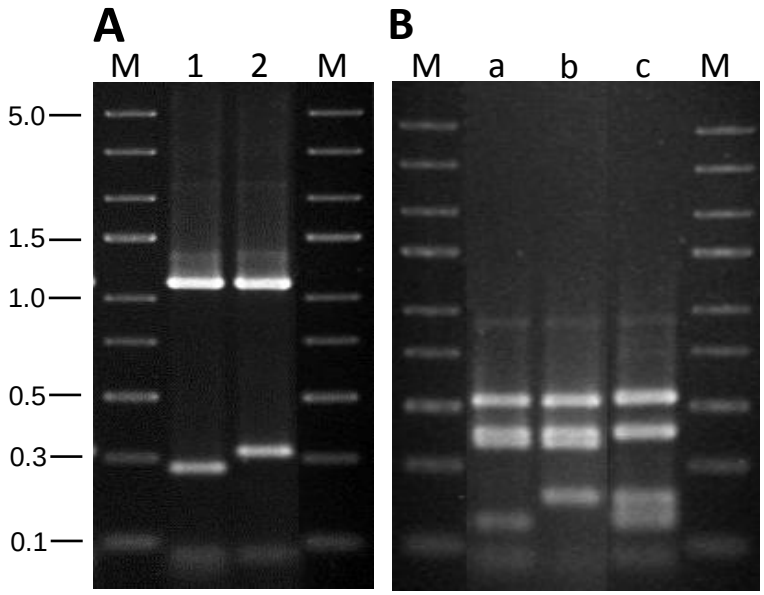
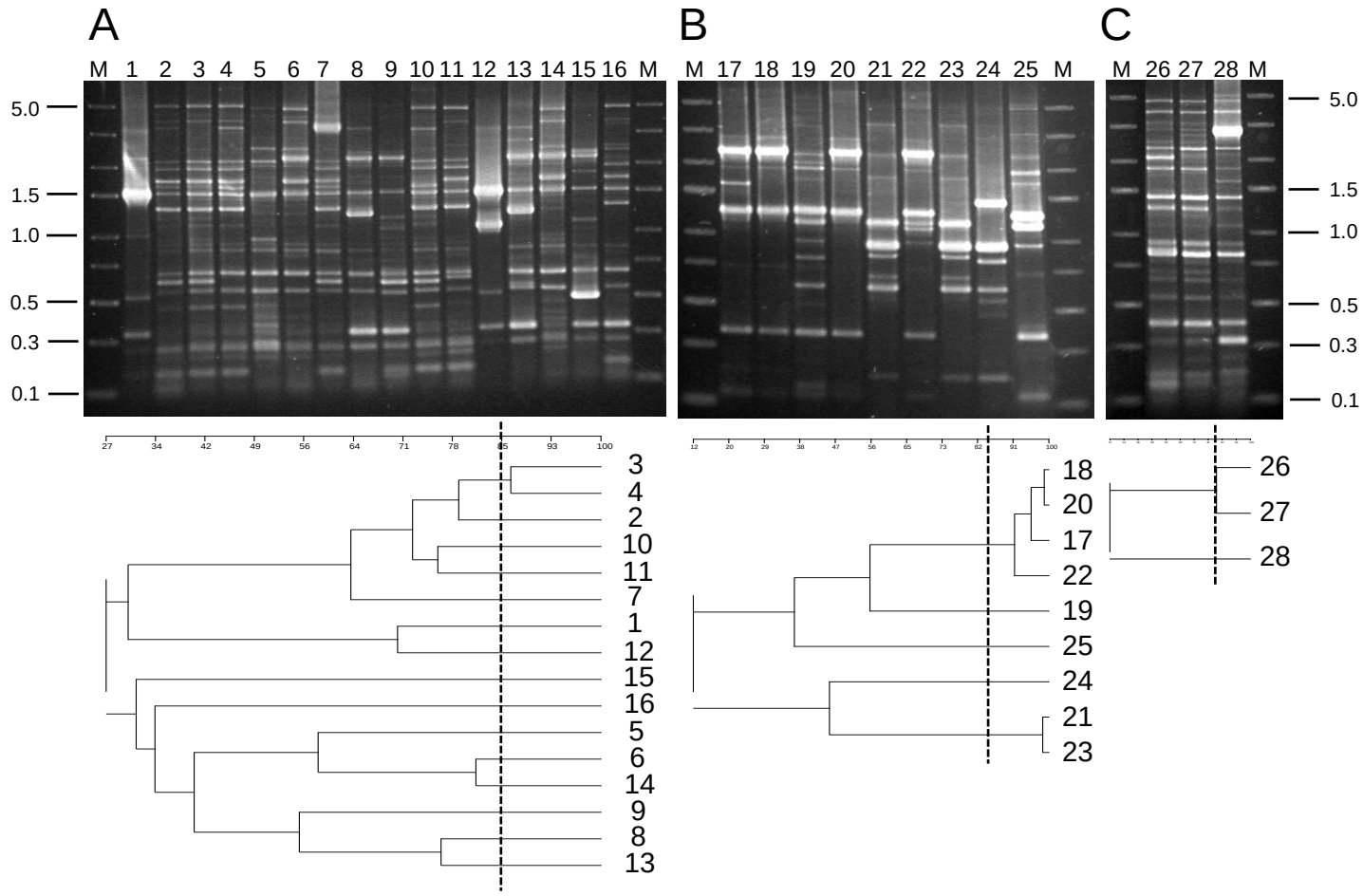


Figure 1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0.1
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3
4
2
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8
13
B
M 17 18 19 20 21 22 23 24 25 M
12 20 29 38 47 56 65 73 82 91 100
18
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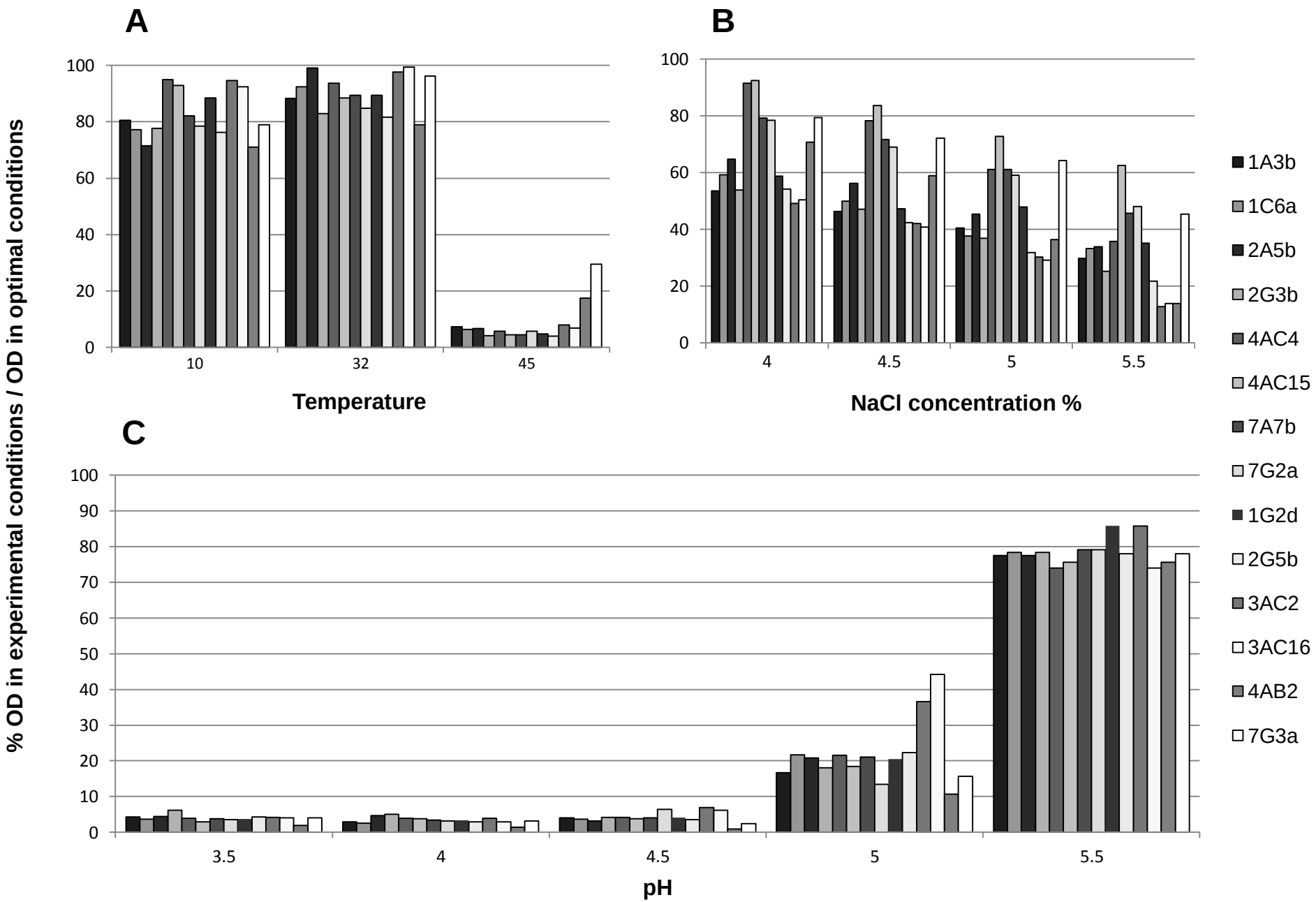
28

Figure 2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60
40
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4
4.5
5
5.5
NaCl concentration %
C
100
90
80
70
60
50
40
30
20
10
0
3.5
4
4.5
5
5.5
pH
% OD in experimental conditions / OD in optimal conditions
1A3b
1C6a
2A5b
2G3b
4AC4
4AC15
7A7b
7G2a
1G2d
2G5b
3AC2
3AC16
4AB2
7G3a

Figure 3

Figure Legends

Figure 1.- ARDRA profiles obtained after amplification of the 16S rRNA genes of the *Leuconostoc* strains with the universal primers 27F and 1492R and digestion of the amplicons with the restriction ennzymes HaeIII (Panel A) and HhaI (Panel B). M, Gene Ruler Express^{TM} DNA ladder (Fermentas, Vilnius, Lithuania); the molecular weight (kbp) of some bands is indicated on the left.

Figure 2.- Rep-PCR profiles obtained with primer BOXA2R corresponding to 16 isolates of *Leuc. citreum* (Panel A), 9 isolates of *Leuc. mesenteroides* (Panel B), and 3 isolates of *Leuc. lactis* (Panel C). M, Gene Ruler Express^{TM} DNA ladder (Fermentas). The dendrograms of similarity of the different profiles are shown below the panels. The broken line indicates the position of 85% of similarity which, as suggested by reproducibility studies, separated different strains.

Figure 3.- Effect of temperature (A), NaCl concentration (B), and acid (pH) (C) on the growth of *Leuconostoc* strains. Results are expressed as the percentage of OD_{595} in modified MRS with respect to OD_{595} in standard MRS after 24 h of incubation. Results are the average of three independent cultures with a standard deviation always lower than 5%.