Phenotypic, genetic and technological characterisation of *Lactococcus garvieae* strains isolated from a raw milk cheese

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Abstract

A series of *Lactococcus garvieae* strains isolated as the majority population of a Spanish traditional, starter-free cheese made from raw milk were phenotypically and genotypically characterised in order to address their biochemical potential, safety requirements, and technological properties. As expected, all *L. garvieae* cheese strains fermented lactose but grew slowly in UHT-treated milk. Enzymatic activities of *L. garvieae* were similar to those of *Lactococcus lactis,* although higher esterase and lipase activities were recorded for *L. garvieae* strains. Profiles of the volatile compounds produced from milk by *L. garvieae* and *L. lactis* strains were also comparable. *L. garvieae* strains did not produce haemolysin, gelatinase and the biogenic amines tyramine and histamine. Five *L. garvieae* stains showed tetracycline resistance encoded by a *tet*(M) gene. The use of *L. garvieae* strains as starter or adjunct cultures might be recommended for certain cheese types, provided that the safety of the strains has been demonstrated.

1. Introduction

The sensorial properties of cheeses made from raw milk depend on a number of key factors, such as type of milk, animal breed and nutrition and technology of manufacture. Traditional cheeses made from pasteurised milk used to lack the intense flavour of cheeses made from raw milk (Albenzio et al., 2001). Besides inactivation of native milk enzymes, pasteurisation reduces to a great extent the complex cheese microbiota which develops in raw milk cheeses (Corroler et al., 1998; Wouters et al., 2002). Microbial types which are not restored by the addition of commercial starter cultures, may play a pivotal role in the development of typical sensory profiles (Smit et al., 2005).

Although *Lactococcus garvieae* has been identified as the etiological agent of the lactococcosis in fish, it has been reported recently as a majority component of the autochthonous microbial populations of certain artisanal cheeses (Fortina et al., 2003; Foschino et al., 2006) and fermented milk products (El-Baradei et al., 2008) manufactured from raw milk. *L. garvieae* strains of dairy origin have been shown to be free of virulence determinants, such as haemolysins and gelatinase (Fortina et al., 2007), suggesting that *L. garvieae* dairy strains are unrelated to the pathogenic ones (Foschino et al., 2008). This agrees well with the lack of association between consumption of raw milk cheese and human diseases. In addition, it is believed that the activity of *L. garvieae* strains in dairy products may contribute to their final sensory characteristics. This has prompted some authors to propose the use of characterised strains as starter or adjunct cultures, provided that the safety of the strains has been determined unequivocally (Fortina et al., 2007). However, scarce data are available on the biotechnological properties of *L. garvieae* strains from dairy origin, strengthening the need for a complete phenotypic and genetic characterization of more strains.

During a recent microbial typing of Casín, a Spanish traditional, starter-free cheese made from raw milk, high populations of *L. garvieae* strains were observed at day three by both culturing and culture-independent analyses (Alegría et al., 2009). As this species might be associated with the authenticity and originality of the cheese and could be valuable for the designing of new starter and/or adjunct cultures for other cheese types, this work was aimed to characterise a series of representative *L. garvieae* strains isolated throughout Casín manufacturing and ripening for their relevant physiological, genetic, and technological properties. In addition, a primary evaluation of their safety was also assessed.

2. Material and methods

2.1. Bacterial strains, media and culture conditions

Forty-seven *L. garvieae* isolates from Casín cheese were used in this study. The strains *L. garvieae* CECT 4531^T, *L. lactis* subsp. *lactis* LMG 6890^T and *L. lactis* subsp. *cremoris* NCIMB 700608 from the Spanish Type Culture Collection (CECT, Valencia, Spain), Belgian Co-ordinated Collections of Micro-organisms (BCCM^{TM}, University of Ghent, Ghent, Belgium), and National Collection of Industrial and Marine Bacteria (NCIMB Ltd., Aberdeen, UK), respectively, were used as controls in the different assays. *Listeria monocytogenes* Scott A (laboratory collection), *Staphylococcus aureus* CECT 4520^T and *Enterococcus faecalis* LMG 8222 were used as controls in the safety assays. Unless otherwise stated, isolates and control strains were cultured in liquid and agarised M17 medium (Scharlau, Barcelona, Spain) at 30ºC for 48 h.

2.2. Genotypic typing

2.2.1. Isolation of total DNA.

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

2.2.2. Amplified ribosomal DNA restriction analysis (ARDRA)

Total DNA from isolates was used as a template to amplify a segment of the 16S rRNA gene by the polymerase chain reaction (PCR) technique using the universal prokaryotic primers S-D-Bact-0008-a-S-20 (27F) (5'-AGAGTTTGATCCTGGCTCAG-3') and S-*-Univ-1492R-b-A-21 (1492R) (5'-GGTTACCTTGTTACGACTT-3'). Amplicons were purified through GenElute^{TM} PCR Clean-Up columns (Sigma-Aldrich), digested with

*Hae*III and *Hha*I restriction enzymes (Invitrogen Ltd., Pasley, UK) and electrophoresed in agarose gels. Gels were stained with ethidium bromide (0.5 μg mL^{-1}) and photographed under UV light.

2.2.3. Typing analysis.

Isolates were grouped by combined analysis of repetitive element (REP) typing using primer BoxA2R (5'-ACGTGGTTTGAAGAGATTTTCG-3') as reported by Koeuth et al. (1995), and random amplification of polymorphic DNA-PCR (RAPD) typing with primers OPA-18 (5'-AGGTGACCGT-3') and M13 (5'-GAGGGTGGCGGTTCT-3'), as reported by Mättö et al. (2004) and Rossetti and Giraffa (2005), respectively.

2.3. Phenotypic characterization

Carbohydrate fermentation profiles of isolates and control strains were determined using the commercial PhenePlate™ system (Bactus, Stockholm, Sweden) as recommended by the supplier.

Enzyme activities were measured by the commercial, semi-quantitative API-ZYM system (bioMérieux, Montalieu-Vercieu, France) following the manufacturer's recommendations. Sixty-five μL of a cellular suspension corresponding to McFarland standard 5 (spectrophotometric equivalent of 3×10^9 cfu mL^{-1}) were inoculated into each well of the API-ZYM strips, incubated for 4 h at 30ºC and developed as recommended.

2.4. Safety assessment

2.4.1. Production of gelatinase and haemolysins

Production of gelatinase was tested on Todd-Hewitt agar containing 30 g of gelatin/litre, as described by Coque et al. (1995). The production of haemolysins was analysed by a plate assay in Columbia agar plates containing 5% defibrinated human blood (Merck, Darmstadt, Germany). The presence of haemolysis is indicated by the formation of clear zones surrounding the colonies.

2.4.2. Production of biogenic amines

Production of the biogenic amines tyramine and histamine was analysed by an agar plate assay, as reported by Bover-Cid and Holzapfel (1999). Amino acid precursors, tyroxine and histidine, respectively, were added at 1% to the growth medium.

2.4.3. Antibiotic resistance

The minimum inhibitory concentration (MIC) was determined by microdilution in VetMIC™ plates for lactic acid bacteria (National Veterinary Institute of Sweden, Uppsala, Sweden), containing two-fold serial dilutions of 14 antibiotics. Colonies grown in LSM (Klare et al., 2005) agar plates were suspended in 2 mL of sterile saline (Oxoid, Basingstoke, Hampshire, UK) to obtain a density corresponding to McFarland standard 1 (spectrophotometric equivalent 3×10^8 cfu mL^{-1}). The suspension was further diluted 1:1000 with LSM (final cell concentration 3×10^5 cfu mL^{-1}). One hundred microlitres of this inoculum were added to each well of the VetMIC™ plate, and the plate was incubated at 28ºC for 48 h. The MICs were defined as the lowest antibiotic concentration at which no visual growth was observed.

The presence of tetracycline resistance genes was checked by PCR using the universal primers for genes encoding ribosomal protection proteins DI (5'-

135 GAYACICCIGGICAYRTIGAYTT-3') and DII (5'-G
136 CCCARWAIGGRTTIGGIGGIACYTC-3') (Clermont et al., 1997), and following the
137 reported PCR conditions. Amplicons were purified and sequenced by cycle extension in an
138 ABI 370 DNA sequencer (Applied Biosystems, Foster City, CA, USA). Sequences were
139 finally compared with others in databases.
140
141 *2.5. Technological characterisation*
142 2.5.1. Growth and acidification of milk
143 Acid production was determined in UHT-treated milk (CAPSA, Siero, Spain). A 1%
144 inoculum from an overnight culture was used to inoculate UHT milk, which was then
145 incubated at 22ºC; samples were scored for clotting at 15, 18 and 24 h. The pH was
146 measured at 24 h with a Crison pH-meter (Crison Instruments S.A., Barcelona, Spain).
147 Appearance of the coagulum by visual inspection was also recorded.
148 2.5.2. Production of volatile compounds
149 Analysis of volatile compounds was performed after growth of the strains at 30ºC in
150 UHT-treated milk (CAPSA) for 48 h. Cultures were supplied with 100 μL of internal
151 standard (cyclohexanone, 0.36 mg mL^{-1}) and stored at −80°C until analysis. The separation
152 and quantification of volatile compounds was carried out by head space/gas
153 chromatography/mass spectrometry analysis in an Agilent equipment composed of a G
154 1888 HS, a 6890 GC and a 5975B MSD (Agilent Technologies, Wilmington, DE, USA),
155 and equipped with a capillary column HP-Innovax 60 m × 0.25 μm (Agilent). Sample
156 preparation and gas chromatographic separation were performed as recently described by
157 Salazar et al. (2008). Peaks were quantified as the relative total ionic count abundance with

158 respect to the internal standard. Concentration (mM) of some volatile compounds
159 (acetaldehyde, diacetyl, 2-propanone, acetic acid, 2-butanone and ethanol) was calculated
160 using linear regression equations ($R^2 \geq 0.99$) from the corresponding curves of standards
161 obtained using five representative concentrations.
162
163 **3. Results and discussion**
164 *3.1. Identification and typing of L. garvieae strains*
165 *L. garvieae* isolates had been identified previously by amplifying a 16S rDNA region,
166 sequencing and comparing the sequences against those in databases (Alegría et al., 2009).
167 As ribosomal DNA had been amplified from cell extracts obtained directly from the
168 counting plates, isolates were subcultured twice in M17 plates and isolated colonies were
169 utilised to inoculate M17 broth. Total DNA was purified from overnight cultures in this
170 medium. The identity of the isolates was verified by partial ARDRA, for which ribosomal
171 DNA was amplified with primers 27F and 1492R and digested with the restriction enzymes
172 *Hae*III and *Hha*I. As judged by a previous *in silico* analysis, the latter enzyme generated
173 recognisably different restriction patterns for *L. garvieae* species and its relative *L. lactis*.
174 All isolates utilized in this study displayed ARDRA patterns for these two enzymes
175 identical to those of *L. garvieae* CECT 4531^T, as shown in Fig. 1 for three isolates.
176 Once the identity of the isolates was assured, they were all subjected to REP typing
177 with primer BoxA2R, and to RAPD analysis with primers OPA-18 and M13. *L. garvieae*
178 isolates had been obtained from a single cheese batch, which anticipated the isolation of
179 replicates. Surprisingly, the REP profiles with primer BoxA2R were found to be identical
180 (as shown for 15 isolates in Fig. 2A), suggesting isolates could also be the same strain.
181 However, four RAPD patterns were obtained with primer M13; although the profiles were

highly similar and shared over 80% identity, they differed in a few faint bands (data not shown). Production of strain-specific bands in bacterial species with primer BoxA2R has been repeatedly reported (Koeuth et al., 1995; Malathum et al., 1998; Esteves et al., 2006). Furthermore, homogeneous RAPD groups of *L. garvieae* isolates from different cheeses obtained with primer M13 has been reported recently (Foschino et al., 2008).

In the search for a more discriminating RAPD analysis, the OPA-18 primer was identified, which yielded three clear-cut distinct profiles (Fig. 2B). Thirty six isolates where shown to belong to genotype (a), nine to genotype (b) and the remaining two to genotype (c) (Fig. 2B). These results demonstrated the unambiguous presence of at least three different genetic strains. Parallel genetic variability and relatedness has recently been reported for *L. garvieae* strains isolated from traditional Italian cheeses (Foschino et al., 2008). Furthermore, the *L. lactis* population of Casín (Alegría et al., 2009) and other traditional cheeses made from raw milk has been reported repeatedly to consist of a similar number of strains (Corroler et al., 1998; Mannu et al., 2000; Delgado and Mayo, 2004).

3.2. Phenotypic analysis

Based on the genotypic similarity, a set of eight representative strains of the different RAPD profiles [5 of genotype (a), 2 of genotype (b), and 1 of genotype (c) in Fig. 2B] was selected for further analysis. For comparison, three control strains were also subjected to all assays, namely *L. garvieae* CECT 4531^{T}, *L. lactis* subps. *lactis* LMG 6890^{T} and *L. lactis* subsp. *cremoris* NCIMB 700608. The carbohydrate fermentation profiles were assayed by the PhenePlate^{TM} system, by which the fermentation of 23 different substrates was analysed (Table 1). The profiles of the different *L. garvieae* strains showed small, if any, differences. All strains assimilated galactose, maltose, cellobiose, trehalose, lactose, mannose, inosine,

206 mannitol, amygdalin, tagatose and salicin, but showed variability in the utilisation of
207 palatinose and sorbitol (weak reaction by one strain each), arbutin (two positive strains and
208 four weak utilisers) and gluconate (utilised weakly for all but one strains). Profiles of *L.*
209 *garvieae* cheese strains were more similar to that of *L. garvieae* type strain compared with
210 the fermentation profiles of *L. lactis* strains. However, *L. garvieae* CECT 4531^T was
211 recorded as lactose negative. The number of carbohydrates utilised by the *L. lactis* strains
212 analysed was shorter; in particular, only four substrates from the PhenePlates were utilised
213 by *L. lactis* subsp. *cremoris* NCIMB 700608.
214 As measured by the semi-quantitative API-ZYM method, the enzymatic profiles
215 displayed by the *L. garvieae* strains were rather weak (Table 2). All strains presented
216 moderate esterase (C4), esterase-lipase (C8) and leucine arylamidase activities, and low
217 levels of acid phosphatase and naphtol-AS-BI-phosphohydrolase. Though positive strains
218 showed low activity, variability among strains was found for alkaline phosphatase, valine
219 arylamidase, trypsin, α-chymotrypsin, β-galactosidase, β-glucuronidase, and α-
220 glucosidase. Similar reduced enzymatic profiles were observed for control strains. In spite
221 of this similarity, *L. lactis* subsp. *lactis* LMG 6890^T showed high α- and β-glucosidase
222 activities. The latter activity was absent in the assayed *L. garvieae* strains.
223 Scarce data are available on the phenotypic and biochemical properties of *L. garvieae*
224 strains from dairy sources. In spite of a high genetic similarity among strains, a certain
225 degree of variability was observed for carbohydrate utilisation and some enzymatic
226 activities. In contrast with fish pathogens, *L. garvieae* strains from dairy origin are all
227 lactose fermenters (Teixeira et al., 1996; Fortina et al., 2007). This agrees well with the
228 results obtained in this work, in which the only lactose negative strain was *L. garvieae*

229 CECT 4531^{T}, which is a fish isolate. However, Fortina et al. (2007) further described a
230 large variation in the utilisation of many other carbohydrates, such as melibiose, L-
231 arabinose, tagatose and N-acethyl-glucosamine. These authors assayed a large number of
232 strains from milk, curd and cheeses of the Toma Piemontese (cows' milk) and Caprino
233 Lombardo (goats' milk) varieties, which may explain the larger variations. Moreover, the
234 use of a different microdilution system (API 50 CHL) may further account for some of the
235 observed differences. Minor discrepancies were also observed among the enzymatic
236 activities displayed by strains from Casín and those isolated from Italian cheeses (Fortina et
237 al., 2007). Interestingly, esterase and esterase-lipase activities of *L. garvieae* strains seem to
238 be stronger than those displayed by the two *L. lactis* control strains analysed in this work
239 and those of wild isolates from traditional cheeses (unpublished results). Casín cheese is
240 characterised by an intense lipolysis, which may partially be due to a weekly manual
241 kneading step during ripening (Alegría et al., 2009), but activity of bacterial esterases and
242 lipases could also contribute to the degradation of fats.
243
244 *3.3. Safety assays*
245 All 47 wild isolates were assayed for the presence of haemolysin and gelatinase
246 activity, as well as for the production of biogenic amines. Haemolysis (of either β- or α-
247 type) and gelatinase activities were never detected. In addition, none of the strains was
248 shown to produce either tyramine or histamine on the plate assays. These results agree well
249 with those reported by Fortina et al. (2007), except for the presence in their isolates of 11
250 strains from Caprino Lombardo cheese producing tyramine. Thought *L. garvieae* is a well
251 recognized fish pathogen (Vendrell et al., 2006), and has been implicated in subclinical

252 cases of mastitis in cows (Teixeira et al., 1996), true factors of pathogenesis in this species
253 have never been reported, except for the presence of capsule, which is associated with
254 pathogenicity in fish (Vendrell et al., 2006). Initial microscopic analysis of cheese strains
255 indicates that they all are non-capsulated (data not shown). In addition, genome sequences
256 of *L. garvieae* strains are not yet available, hampering the search for virulence determinants
257 that should be absent in strains intended to be used in food systems.
258 The antibiotic resistance/susceptibility profiles of the strains were analysed by
259 microdilution. The MIC values obtained for the 14 antibiotics assayed with the VetMIC™
260 system are summarised in Table 3. On one hand, the MICs were low and similar for
261 erythromycin, chloramphenicol, ampicillin, penicillin G, vancomycin, linezolid and
262 ciprofloxacin. On the other hand, the MICs of aminoglycosides were rather high (8–16 μg
263 mL^{-1} for gentamicin and 32–256 μg mL^{-1} for kanamycin, streptomycin and neomycin) in all
264 but the *L. lactis* subsp. *cremoris* control strain (which proved to be very susceptible to all
265 antibiotics). High MIC values in the *L. garvieae* strains were also scored for clindamycin (≥
266 16 μg mL^{-1}), trimethoprim (64- ≥ 64μg mL^{-1}) and rifampicin (34- ≥ 64μg mL^{-1}). However,
267 equal distribution of these MICs between strains suggested the presence of inherent
268 resistance mechanisms to all these antibiotics. In fact, the differential resistance of *L.*
269 *garvieae* strains to clindamycin as compared with *L. lactis* has been proposed as a
270 phenotypic test for distinguishing between the two species (Elliot and Facklam, 1996;
271 Zlotkin et al., 1998). However, the recent spread of the macrolides, lincosamides, and
272 streptogramin B resistance (MLS_B phenotype) encoded by *erm*(B) among *L. lactis* makes
273 this test inappropriate (Walther et al., 2008).

The MICs to tetracycline were shown to behave differently, as values varied from 0.5–2 μg mL^{-1} in all control strains and three wild *L. garvieae* strains, to ≥ 64 μg mL^{-1} in five wild strains [all of which belonged to OPA-18 genotype (a); Fig. 2B]. Positive amplification was obtained in all resistant strains with universal primers for tetracycline resistance genes encoding ribosomal protection proteins (Fig. 3). Sequencing of the amplicons and analysis of the sequences showed that a *tet*(M) gene, identical to each other and to many others in databases, was responsible for the high tetracycline resistance. Genes *tet*(M) and *tet*(S) encoding ribosomal protection proteins already have been identified in both dairy and fish isolates (Kim et al., 2004; Fortina et al., 2007; Maki et al., 2008; Walther et al., 2008). Tetracycline resistance in *L. garvieae* has been associated to transferable plasmids (Maki et al., 2009) or transposons of the Tn*1545*-Tn*916* family (Fortina et al., 2007). Association of the *tet*(M) gene identified in this work with transferable elements has yet to be determined. However, resistant strains should not be used as cheese cultures in order to avoid spread of antibiotic resistance genes via the food chain.

3.4. Technological characterization

In contrast to *L. lactis* LMG 6890^T and NCIMB 700608, none of the *L. garvieae* strains coagulated UHT-treated milk after 24 h of incubation at 22ºC. Indeed, *L. garvieae* strains lowered the pH of the milk from 6.6–6.7 to only 5.50–5.35 compared with a final pH of 4.52 and 4.22 for LMG 6890^T and NCIMB 700608, respectively. Despite this, at least seven volatile compounds could be detected by GC/MS after growth of the strains in milk at 30ºC for 48 h (Table 4). Furthermore, volatile profiles of *L. lactis* and *L. garvieae* strains were shown to be similar in both qualitative and quantitative terms. Though differences

297 among strains were found, ethanol was the major volatile compound of the profiles and was
298 produced by all strains. Small amounts of acetaldehyde and 2-propanone were also
299 produced by all strains. Marginal levels of acetoin and diacetyl were detected in samples
300 fermented by all eight and three *L. garvieae* strains, respectively. The repeatability of this
301 analysis was shown to be high, as the coefficient of variation for the different volatile
302 compounds and strains varied from 1 to 8%.
303 *L. garvieae* cheese isolates from this work and others (Fortina et al., 2007) have shown
304 to present a slow rate of acidification, but still comparable to wild lactococcal isolates
305 (Delgado et al., 2002; Wouters et al., 2002; Topisirovic et al., 2006). *L. garvieae* dairy
306 strains have been found to present other desirable technological traits, such as assimilation
307 of lactose and low proteolytic activity (Fortina et al., 2007), which makes their use as
308 starter or adjunct cultures feasible.
309
310 **4. Conclusions**
311 Phenotypic and genetic studies of *L. garvieae* strains are still necessary in order to
312 understand the functional and ecological significance of this species in dairy products. In
313 this work, a series of 47 *L. garvieae* isolates from the manufacturing and ripening stages of
314 a traditional cheese made from raw milk and a set of *L. garvieae* and *L. lactis* control
315 strains were subjected to several assays to assess their phenotypic and genotypic diversity,
316 as well as address their safety and technological properties. These analyses allowed an
317 initial characterization of the *L. garvieae* cheese strains and their comparison with *L. lactis*.
318 Similar functional properties were observed for strains of the two species, which included
319 qualitative and some quantitative volatile profiles from milk. In addition, no particular
320 undesirable traits were encountered in the *L. garvieae* strains analysed in this work, except

for the presence of *tet*(M) in five the isolates. The suitability of the wild *L. garvieae* strains free of tetracycline resistance, as starters for cheese making is currently being addressed either alone or in combination with *L. lactis*. The small biochemical differences shown by *L. garvieae* strains as compared to those of *L. lactis* could be amplified along cheese ripening giving rise to distinctive or unique sensory properties.

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6. References

Albenzio, M., Corbo, M. R., Rehman, U. S., Fox, P. F., de Angelis, M., Corsetti, A., Sevi, A., Gobbetti, M., 2001. Microbiological and biochemical characteristics of Canestrato Pugliese cheese made from raw milk, pasteurized milk or by heating the curd in hot whey. *International Journal of Food Microbiology, 67,* 35-48.

Alegría, A., Álvarez-Martín, P., Sacristán, N., Fernández, E., Delgado, S., Mayo, B., 2009. Diversity and evolution of microbial populations during manufacture and ripening of Casín, a traditional Spanish, starter-free cheese made from cow's milk. *International Journal of Food Microbiology,* (In Press).

344 Bover-Cid, S., Holzapfel, W. H., 1999. Improved screening procedure for biogenic amine
345 production by lactic acid bacteria. *International Journal of Food Microbiology, 53,* 33-41.
346 Clermont, D., Chesneau, O., de Cespedes, G., Horaud, T., 1997. New tetracycline
347 resistance determinants coding for ribosomal protection in streptococci and nucleotide
348 sequence of *tet*(T) isolated from *Streptococcus pyogenes* A498. *Antimicrobial Agents and*
349 *Chemotherapy, 41,* 112-116.
350 Coque, T. M., Patterson, J. E., Steckelberg, J. M., Murray, B. E., 1995. Incidence of
351 hemolysin, gelatinase, and aggregation substance among enterococci isolated from patients
352 with endocarditis and other infections and from the feces of hospitalized and community-
353 based persons. *Journal of Infectious Diseases, 171,* 1223-1229.
354 Corroler, D., Mangin, I., Desmasures, N., Guéguen, M., 1998. An ecological study of
355 lactococci isolated from raw milk in the Camembert cheese registered designation of origin
356 area. *Applied and Environmental Microbiology, 64,* 4729-4735.
357 Esteves, A., Aymerich, T., Garriga, M., Patarata, L., Fontes, M. C., Martins, C., 2006.
358 Tracing *Salmonella* in Alheira processing plants. *Journal of Applied Microbiology, 103,* 1-
359 10.
360 Delgado, S., Delgado, T., Mayo, B., 2002. Technological performance in milk of several
361 lactococci and enterococci strains of dairy origin. *Journal of Food Protection, 65,* 1590-
362 1596.
363 Delgado, S., Mayo, B., 2004. Phenotypic and genetic diversity of *Lactococcus lactis* and
364 *Enterococcus* spp. strains isolated from Northern Spain starter-free farmhouse cheeses.
365 *International Journal of Food Microbiology, 90,* 309-319.
366 El-Baradei, G., Delacroix-Buchet, A., Ogier, J. C., 2008. Bacterial diversity of traditional
367 Zabady fermented milk. *International Journal of Food Microbiology, 121,* 295-301.

368 Elliot, J. A., Facklam, R. R., 1996. Antimicrobial susceptibilities of *Lactococcus lactis* and
369 *Lactococcus garvieae* and a proposed method to discriminate between them. *Journal of*
370 *Clinical Microbiology, 34,* 1296-1298.
371 Flórez, A. B., Ammor, M. S., Mayo, B., 2008. Identification of *tet*(M) in two *Lactococcus*
372 *lactis* strains isolated from a Spanish traditional starter-free cheese made of raw milk and
373 conjugative transfer of tetracycline resistance to lactococci and enterococci. *International*
374 *Journal of Food Microbiology, 121, 189-194.*
375 Fortina, M. G., Ricci, G., Acquati, A., Zeppa, G., Gandini, A., Manachini, P. L., 2003.
376 Genetic characterization of some lactic acid bacteria occurring in an artisanal protected
377 denomination origin (PDO) Italian cheese, the Toma Piedemontese. *Food Microbiology*,
378 *20,* 397-404.
379 Fortina, M. G., Ricci, G., Foschino, R., Picozzi, C., Dolci, P., Zeppa, G., Cocolin, L.,
380 Manachini, P.L., 2007. Phenotypic typing, technological properties and safety aspects of
381 *Lactococcus garvieae* strains from dairy environments. *Journal of Applied Microbiology,*
382 *103,* 445-453.
383 Foschino, R., Picozzi, C., Borghi, M., Cerliani, M. C., Cresci, E., 2006. Investigation on the
384 microflora of Caprino Lombardo cheese from raw goat milk. *Italian Journal of Food*
385 *Science, 18,* 33-49.
386 Foschino, R., Nucera, D., Volponi, G., Picozzi, C., Ortoffi, M., Bottero, M. T., 2008.
387 Comparison of *Lactococcus garvieae* strains isolated in northern Italy from dairy products
388 and fishes through molecular typing. *Journal of Applied Microbiology, 105,* 652-662.
389 Kim, S. R., Nonaka, L., Suzuki, S., 2004. Occurrence of tetracycline resistance genes
390 *tet*(M) and *tet*(S) in bacteria from marine aquaculture sites. *FEMS Microbiology Letters,*
391 *237,* 147-156.

Klare, I., Konstabel, C., Muller-Bertling, S., Reissbrodt, R., Huys, G., Vancanneyt, M., Swings, J., Goossens, H., Witte, W., 2005. Evaluation of new broth media for microdilution antibiotic susceptibility testing of lactobacilli, pediococci, lactococci, and bifidobacteria. *Applied and Environmental Microbiology, 71,* 8982-8986.

Koeuth, T; Versalovic, J., Lupski, J. R., 1995. Differential subsequence conservation of interspersed repetitive *Streptococcus pneumoniae* BOX elements in diverse bacteria. *Genome Research, 5,* 408-418.

Maki, T., Hirono, I., Kondo, H., Aoki, T., 2008. Drug resistance mechanism of the fish-pathogenic bacterium *Lactococcus garvieae*. *Journal of Fish Diseases, 31*, 461-468.

Maki, T., Santos, M. D., Kondo, H., Hirono, I., Aoki, T., 2009. A transferable 20-kilobase multiple drug resistance-conferring R plasmid (pKL0018) from a fish pathogen (*Lactococcus garvieae*) is highly homologous to a conjugative multiple drug resistance-conferring enterococcal plasmid. *Applied and Environmental Microbiology, 75,* 3370-3372.

Malathum, K., Singh, K. V., Weinstock, G. M., Murray, B. E. 1998. Repetitive sequence-based PCR versus pulsed-field gel electrophoresis for typing of *Enterococcus faecalis* at the subspecies level. *Journal of Clinical Microbiology, 36,* 211-215.

Mannu, L., Paba, A., Pes, M., Scintu, M. F., 2000. Genotypic and phenotypic heterogeneity among lactococci isolated from traditional Pecorino Sardo cheese. *Journal of Applied Microbiology, 89,* 191-197.

Mättö, J., Marinen, E., Suihko, M. L., Alander, M., Palva, A., Saarela, M., 2004. Genetic heterogeneity and functional properties of intestinal bifidobacteria. *Journal of Applied Microbiology, 97,* 459-470.

414 Rossetti, L., Giraffa, G., 2005. Rapid identification of dairy lactic acid bacteria by M13-
415 generated, RAPD-PCR fingerprint databases. *Journal of Microbiological Methods, 63,* 135-
416 144.
417 Salazar, N., Gueimonde, M., Hernández-Barranco, A. M., Ruas-Madiedo, P., de los Reyes-
418 Gavilán, C. G., 2008. Exopolysaccharides produced by intestinal *Bifidobacterium* strains
419 act as fermentable substrates for human intestinal bacteria. *Applied and Environmental*
420 *Microbiology, 74,* 4737-4745.
421 Smit, G., B. Smit, A., Engels, W. J., 2005. Flavour formation by lactic acid bacteria and
422 biochemical flavour profiling of cheese products. *FEMS Microbiological Reviews, 29,* 591-
423 610.
424 Teixeira, M. L., Merquior, V. N. C., Vianni, M. C. E., Carvaho, M. g. S., Fracalanzza, S. E.
425 L., Steigerwalt, A. g., Brenner, D. J., Facklam, R. R., 1996. Phenotypic and genotypic
426 characterization of atypical *Lactococcus garvieae* strains isolated from water buffalos with
427 subclinical mastitis and confirmation of *L. garvieae* as a senior subjective synonym of
428 *Enterococcus seriolicida*. *International Journal of Systematic Bacteriology, 46,* 664-668.
429 Topisirovic, L., Kojic, M., Fira, D., Golic, N., Strahinic, I., Lozo, J., 2006. Potential of
430 lactic acid bacteria isolated from specific natural niches in food production and
431 preservation. *International Journal of Food Microbiology, 112,* 230-235.
432 Vendrell, D., Balcázar, J. L., Ruiz-Zarzuela, I., de Blas, I., Gironés, O., Múzquiz, J. L.,
433 2006. *Lactococcus garvieae* in fish: a review. *Compendium of Immunology and*
434 *Microbiology of Infectious Diseases, 29,* 177-198.
435 Walther, C., Rossano, A., Thomann, A., Perreten, V., 2008. Antibiotic resistance in
436 *Lactococcus* species from bovine milk: presence of a mutated multidrug transporter *mdt*(A)
437 gene in susceptible *Lactococcus garvieae* strains. *Veterinary Microbiology, 131,* 348-357.

438 Wouters, J. T. M., Ayad, E. H. E., Hugenholtz, J., Smit, G., 2002. Microbes from raw milk
439 for fermented dairy products. *International Dairy Journal, 12,* 91-109.
440 Zlotkin, A., Eldar, A., Ghittino, C., Bercovier, H., 1998. Identification of *Lactococcus*
441 *garvieae* by PCR. *Journal of Clinical Microbiology, 36,* 983-985.

Table 1.- Carbohydrate fermentation profiles of *L. garvieae* strains assayed by the PhenePlate system (Bactus).

Sugar	Strain										
	T1-1	T1-41	T2-4	T2-17	T3-18	T3-22	Q1-21	Q2-41	CECT 4531Ta	LMG 6890Ta	NCIMB 700608[a]
Arabinose	-	-	-	-	-	-	-	-	-	-	-
Xylose	-	-	-	-	-	-	-	-	-	+	-
Galactose	+	+	+	+	+	+	+	+	+	+	+
Maltose	+	w[b]	w	w	w	+	+	+	+	+	w
Cellobiose	+	+	+	+	+	+	+	+	+	+	-
Trehalose	+	+	+	+	+	+	+	+	+	+	-
Palatinose	-	-	-	-	w	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-	-	-	-	-
Lactose	+	+	+	+	+	+	+	+	-	+	+
Melibiose	-	-	-	-	-	-	-	-	-	-	-
Mannose	+	+	+	+	+	+	+	+	+	+	+
Melezitose	-	-	-	-	-	-	-	-	-	-	-
Inosine	+	+	+	+	+	+	+	+	+	-	-
Mannitol	+	+	+	+	+	+	+	+	+	-	-
Arbutin	-	w	+	w	+	-	w	w	-	-	-
Sorbitol	-	-	-	w	-	-	-	-	-	-	-
Galactonolactone	-	-	-	-	-	-	-	-	-	-	-
Sorbose	-	-	-	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-	-	-
Tagatose	+	+	+	+	+	+	+	+	-	-	-
Amydalin	+	+	+	+	+	+	+	+	+	-	-
Gluconate	w	w	w	w	w	-	w	w	w	-	-
Salicin	+	+	+	+	+	+	+	+	+	+	-

[a]CECT 4531^{T} is the *L. garvieae* type strain, while LMG 6890^{T} and NCIMB 700608 are *L. lactis* subsp. *lactis* (type strain) and *L. lactis* subsp. *cremoris*, respectively.
[b]w, weak reaction.

Table 2.- Enzymatic activities of *L. garvieae* strains measured with the API ZYM system (bioMérieux).

Enzymatic activity[a]	**Strain**										
	T1-1	T1-41	T2-4	T2-17	T3-18	T3-22	Q1-21	Q2-41	CECT 4531^{Tb}	LMG 6890^{Tb}	NCIMB 700608[b]
Alcaline phosphatase	0	0	2.5	0	0	0	0	0	0	2.5	0
Esterase (C4)	5	10	10	5	5	2.5	5	5	2.5	0	2.5
Esterase-Lipase (C8)	10	20	10	10	10	5	10	10	5	5	5
Lipase (C14)	0	0	0	0	0	0	0	0	0	0	0
Leucine arylamidase	30	20	20	20	20	10	10	10	5	30	5
Valine arylamidasa	0	0	2.5	0	2.5	0	0	0	0	2.5	2.5
Cysteine arilamidase	0	0	0	0	0	5	0	0		5	2.5
Trypsin	0	0	0	0	2.5	0	0	0	0	0	0
α-Chymotrypsin	0	0	0	0	2.5	0	0	0	0	0	0
Acid phosphatase	2.5	2.5	2.5	2.5	2.5	5	2.5	2.5	2.5	20	5
Naphtol-AS-BI-phosphohydrolase	10	2.5	2.5	2.5	5	5	2.5	2.5	2.5	5	5
β-Galactosidase	0	0	2.5	0	0	2.5	0	0	0	2.5	0
β-Glucuronidase	0	0	0	0	0	2.5	0	0	0	0	0
α-Glucosidase	2.5	0	2.5	2.5	2.5	0	0	2.5	2.5	∞ 40	0
β-Glucosidase	0	0	0	0	0	0	0	0	0	∞ 40	0

[a]Activity was recorded as the approximate nanomoles of hydrolysed substrate. Strains were all negative for α-galactosidase, N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase.
[b]CECT 4531^{T} is the *L. garvieae* type strain, while LMG 6890^{T} and NCIMB 700608 are *L. lactis* subsp. *lactis* (type strain) and *L. lactis* subsp. *cremoris*, respectively.

Table 3.- Minimum Inhibitory Concentration (MIC) in μg mL^{-1} of fourteen antibiotics to *L. garvieae* strains by broth microdilution.

Antibiotic	Strain										
	T1-1	T1-41	T2-4	T2-17	T3-18	T3-22	Q1-21	Q2-41	CECT 4531Ta	LMG 6890Ta	NCIMB 700608[a]
Gentamicin	8	8	16	8	16	8	16	8	8	16	0.5
Kanamycin	64	64	64	64	256	128	64	32	32	32	2
Streptomycin	64	64	64	64	128	64	64	64	64	64	2
Neomycin	128	64	128	64	256	128	64	32	32	32	1
Tetracycline	≥ 64	≥ 64	1	0.5	≥ 64	1	≥ 64	≥ 64	1	2	0.5
Erythromycin	0.12	0.12	0.25	0.25	0.5	0.25	0.25	0.12	1	0.5	0.25
Clindamycin	≥ 16	≥ 16	≥ 16	≥ 16	≥ 16	≥ 16	≥ 16	≥ 16	≥ 16	1	0.12
Chloramphenicol	4	8	4	8	8	4	4	4	8	8	8
Ampicillin	1	2	1	2	1	1	1	1	2	0.5	0.25
Penicillin G	1	1	0.5	0.5	1	0.5	0.5	1	1	0.5	0.25
Vancomycin	2	1	1	2	2	1	1	1	4	0.5	0.5
Virginiamycin	≥ 8	≥ 8	8	8	8	8	≥ 8	8	8	8	1
Linezolid	2	4	2	2	4	4	2	2	2	4	2
Trimetoprim	≥ 64	≥ 64	64	64	≥ 64	64	64	64	64	≥ 64	64
Ciprofloxacin	8	8	4	8	8	4	4	4	4	8	8
Rifampicin	≥ 64	≥ 64	64	64	≥ 64	≥ 64	64	32	32	≥ 64	≥ 64

[a]CECT 4531^{T} is the *L. garvieae* type strain, while LMG 6890^{T} and NCIMB 700608 are *L. lactis* subsp. *lactis* (type strain) and *L. lactis* subsp. *cremoris*, respectively.

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

Volatile compound	**Strain**										
	T1-1	T1-41	T2-4	T2-17	T3-18	T3-22	Q1-21	Q2-41	CECT 4531Ta	LMG 6890Ta	NCIMB 700608[a]
Methanethiol	0	0	0	0	0	0	0	0	0	0	0
Acetaldehyde	1.31 (5.02)[b]	0.68 (2.56)	1.69 (5.79)	0.95 (3.55)	1.01 (3.68)	0.51 (2.25)	0.74 (2.46)	0.93 (1.41)	0.52 (1.54)	1.04 (3.81)	0.51 (0.64)
2-Methyl-propanal	0	0	0	0	0	0	0	0	0	0	0
2-Propanone	0.44 (1.46)	0.20 (0.78)	0.80 (1.09)	0.28 (0.87)	0.39 (0.90)	0.50 (1.10)	0.25 (0.53)	0.51 (0.85)	0.33 (1.30)	0.34 (1.34)	0.30 (1.39)
3-Methyl-butanal	0	0	0	0	0	0	0	0	0	0	0
Ethanol	35.84 (352.9)	14.60 (151.9)	6.09 (66.8)	10.64 (204.9)	3.27 (27.0)	28.36 (129.6)	8.60 (86.36)	16.67 (173.0)	12.16 (123.5)	12.89 (138.9)	7.42 (77.5)
Diacetyl	0	0.11 (0.36)	0	0.10 (0.15)	0.18 (0.90)	0	0	0	0	0	0
2-Methyl-1-propanol	0	0	0	0	0	0	0	0.20	0	0.26	0
2+3-Methyl-1-butanol	0	0	0	0	0	0	0	0.76	0	1.06	0
Acetoine	0.61	0.32	1.13	0.43	0.96	0.23	0.34	0.31	0	0	0
Acetic acid	0	0	0	0	0	0	0	0	0	0	0

[a]CECT 4531^{T} is the *L. garvieae* type strain, while LMG 6890^{T} and NCIMB 700608 are *L. lactis* subsp. *lactis* (type strain) and *L. lactis* subsp. *cremoris*, respectively.
[b]In parenthesis, concentration of the compounds in the fermented milk in $\mu g\ mL^{-1}$.

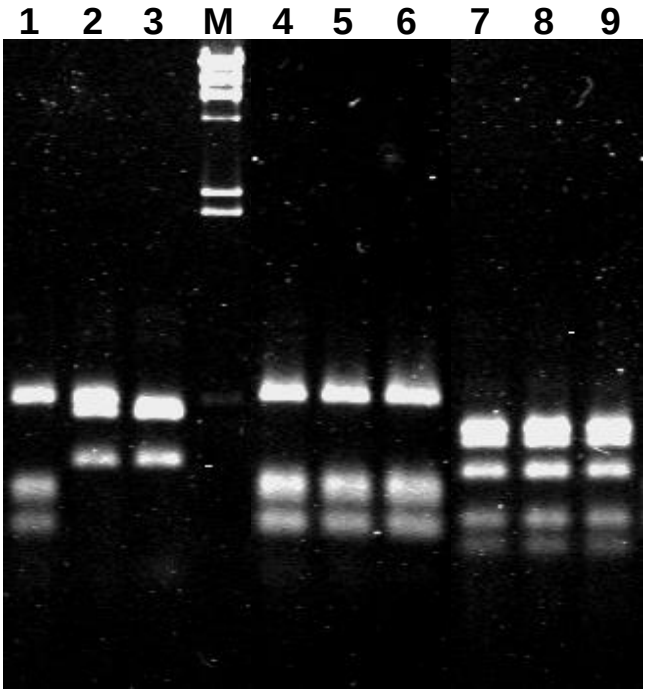

Figure 1

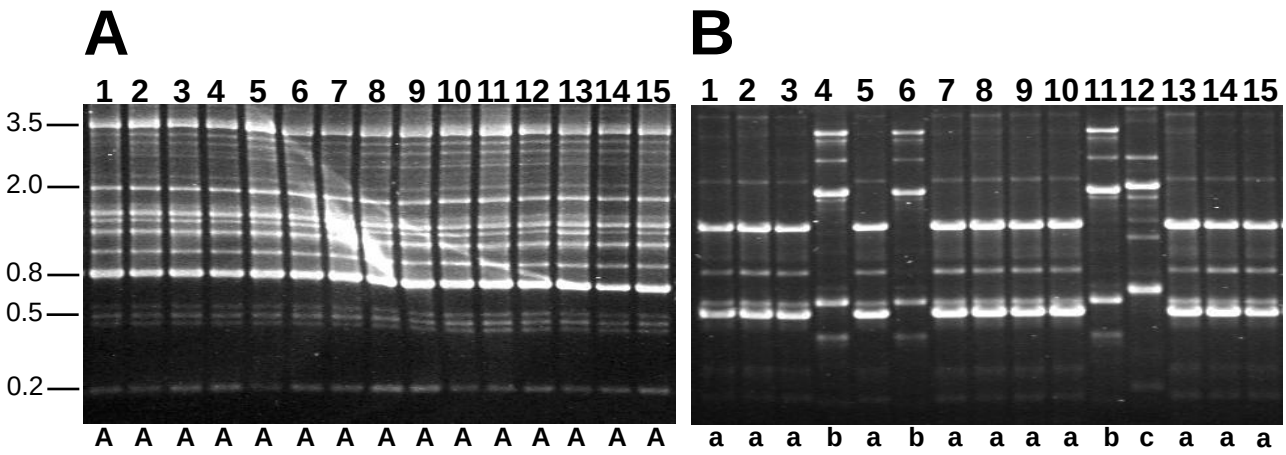
A
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
3.5
2.0
0.8
0.5
0.2
A A A A A A A A A A A A A A A
a a a b a b a a a a b c a a a

Figure 2

Figure

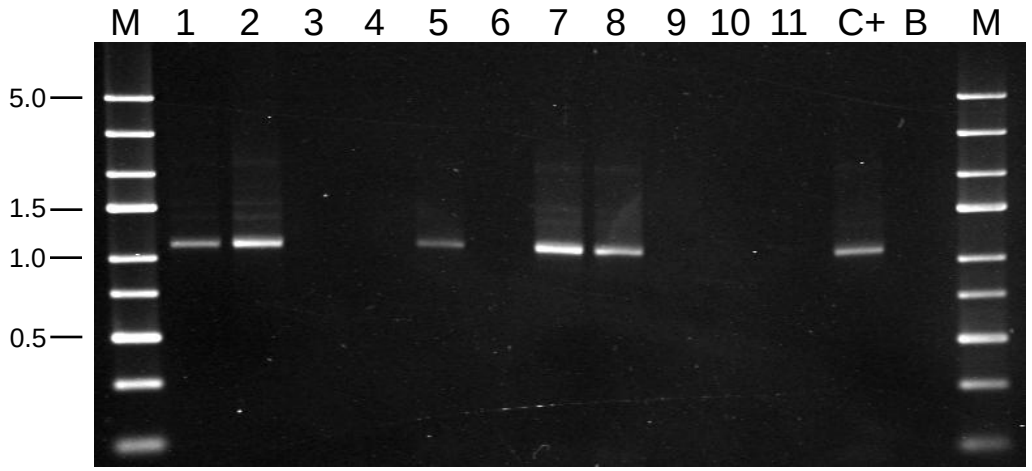

Figure 3

Figure Legends

Figure 1.- Partial ARDRA profiles of *Lactococcus garvieae* strains. Amplicons were obtained with primers S-D-Bact-0008-a-S-20 (27F) and S-*-Univ-1492R-b-A-21 (1492R) and digested with restriction enzymes *Hae*III and *Hha*I. Order: Line 1-3, amplicons from *L. garvieae* CECT 4531^T, *L. lactis* subsp. *lactis* LMG 6890^T, and *L. lactis* subsp. *cremoris* NCIMB 700608, respectively, digested with *Hha*I. Lines 4-9, amplicons from three wild *L. garvieae* strains digested with *Hha*I (4-6) and *Hae*III (7-9). M, molecular weight marker: Lambda DNA digested with *Hin*dIII.

Figure 2.- Typing of fifteen *L. garvieae* isolates from Casín cheese with primers BoxA2R (A) and OPA-18 (B). The different profiles identified with the two primers are indicated by a letter code. On the left, the molecular weight (in kbp) of some amplicons is indicated.

Figure 3.- Amplification of tetracycline resistance genes with the universal primers DI and DII (Clermont et al., 1997) for ribosomal protection genes from *L. garvieae* strains. Order: Lines 1-11, order of the strains as in the tables (T1-1, T1-41, T2-4, T2-17, T3-18, T3-22, Q1-21, Q2-41, CECT 4531^T, LMG 6890^T, and NCIMB 700608); C+, positive amplification control (DNA from *Lactococcus lactis* AA29, which carries a functional *tet*(M) gene; Flórez et al., 2008); B, blank to which DNA was not added; M, Gene Ruler Express^{TM} DNA ladder (Fermentas Gmbh., Germany); the molecular weight (kbp) of some bands is indicated on the left.