Comparative Phenotypic and Molecular Genetic Profiling of Wild *Lactococcus lactis* subsp. *lactis* Strains of the *lactis* and *cremoris* Genotypes Isolated from Starter-Free Cheeses Made of Raw Milk

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SHORT TITLE: Wild strains of *Lactococcus lactis* of *lactis* and *cremoris* genotypes

KEY WORDS: *Lactococcus lactis* subsp. *lactis, Lactococcus lactis* subsp. *cremoris*, lactic acid bacteria, starters, dairy lactococci

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ABSTRACT

Twenty *Lactococcus lactis* strains with a *lactis* phenotype isolated from five traditional cheeses made of raw milk with no added starters, belonging to the *lactis* and *cremoris* genotypes (ten strains each), were subjected to a series of phenotypic and genetic typing methods, with the aims of determining their phylogenetic relationships and suitability as starters. Pulsed field gel electrophoresis (PFGE) analysis of intact genomes digested with SalI and SmaI proved that strains were all different except for three isolates of the *cremoris* genotype, which showed identical PFGE profiles. Multilocus sequence typing (MLST) analysis using internal sequences of seven loci (namely *atpA, rpoA, pheS, pepN, bcaT, pepX* and 16S rDNA) revealed considerable inter-genotype nucleotide polymorphism, although deduced amino acid changes were scarce. Analysis of the MLST data for the present strains and others from other dairy and non-dairy sources showed that all of them clustered into the *cremoris* or *lactis* genotype groups, by using both independent and combined gene sequences. These two groups of strains also showed distinctive carbohydrate fermentation and enzyme activity profiles; the strains in the *cremoris* group showing broader profiles. However, the resistance/susceptibility profiles to 16 antibiotics were very similar; showing no atypical resistances, except for tetracycline resistance in three identical *cremoris* isolates. The number and concentration of volatile compounds produced in milk by the strains belonging to these two groups were clearly different, with the *cremoris* strains producing higher concentrations of more branched-chain, derived compounds. Together, the present results support the idea that the *lactis* and *cremoris* genotypes of phenotypic *Lactococcus lactis* subsp. *lactis* actually represent true subspecies. Some strains of this study of the two subspecies appear to be good starter candidates.

INTRODUCTION

Lactococcus lactis is a lactic acid bacterium commonly dominant in milk and fermented dairy products. Not surprisingly, carefully selected strains of *L. lactis* are majority components of starter cultures for dairy fermentations (38). Worldwide, over 100 million metric tons of milk are transformed annually into dairy products using *L. lactis* starters, reflecting the latters' industrial and thus economic importance (38). The growth of *L. lactis* in milk is associated with the rapid production of lactic acid, which provides flavor, assists in curd formation, prevents the growth of pathogenic and spoilage bacteria, and creates optimal biochemical conditions for ripening. Via their proteolytic and amino acid conversion pathways, lactococci further contribute to the final texture (moisture, softness) and flavor of dairy products (47). These functions determine the sensory quality, safety and shelf-life of fermented dairy products. *L. lactis* includes three subspecies (*cremoris, lactis* and *hordniae*) plus a diacetyl-forming biovariety (*L. lactis* subsp. *lactis* biovar *diacetylactis*). The lactose-negative *L. lactis* subsp. *hordniae* (45) has never been found in dairy products. *L. lactis* subsp. *lactis* is distinguished from *L. lactis* subsp. *cremoris* according to five phenotypic criteria: the ability to grow at 40ºC, in 4% NaCl, and at pH 9.2, the ability to ferment maltose, and the capacity to deaminate arginine (25, 45), for all of which the subsp. *cremoris* strains are reported negative. In addition, *L. lactis* subsp. *lactis* biovar *diacetylactis* is distinguished by its ability to assimilate citrate, which is converted into diacetyl, a potent odorous compound.

Current dairy lactococcal starters are thought to be derived from a small number of well-adapted, genetically-related lineages showing similar genetic profiles and phenotypic properties (24, 39, 55). Therefore, there is a great demand for new strains to solve technological problems such as insufficient acid production, frequent culture failure

71 resulting from the attack of bacteriophages, and the development of undesirable flavors (28,
72 33, 37, 54). In addition, interest is spurred by the continuing search for strains harboring
73 unique flavor-forming activities (3) or that produce novel, broad-range antimicrobial agents
74 (6). Strains with these properties might be of use in traditional fermentations, but also to
75 allow new processes to be developed.
76 For some cheese types, *L. lactis* subsp. *cremoris* strains are the preferred starter since
77 their growth response in milk is better and because of the typical aroma profiles associated
78 with them (42, 54). Although found in dairy environments, the natural niche of this
79 subspecies remains elusive, and claims of having isolated novel *L. lactis* subsp. *cremoris*
80 strains from milk and naturally fermented products are regarded as controversial (30, 33,
81 42, 54). Furthermore, distinction between *L. lactis* subsp. *lactis* and *L. lactis* subsp.
82 *cremoris* is difficult since it is based on a set of phenotypic characteristics that may show
83 strain to strain variation. Further, some strains of *L. lactis* showing a subsp. *lactis*
84 phenotype according to the classical distinction criteria have long been known to show a *L.*
85 *lactis* subsp. *cremoris* genotype (27). Recently, phenotypic *L. lactis* subsp. *cremoris*
86 showing a *lactis* genotype have also been reported (30, 52). Therefore, the *L. lactis* species
87 has an unusual structure with two phenotypically distinct groups defining the subspecies
88 *lactis* and *cremoris*, which may belong to two distinct genotype groups (30, 36, 40, 52, 54).
89 This makes the accurate identification of new isolates very difficult, yet this is a crucial first
90 step in the development of new cultures. In addition, the phenotypic and genetic
91 relationships between the subspecies of *L. lactis*, and even within its subspecies, remain
92 unclear.
93 As the use of molecular genetic techniques became universal, strains with a *L. lactis*
94 subsp. *cremoris* genotype have been isolated from many sources, including vegetables and

plants (28, 37) and milk and dairy products (11, 15, 18, 34, 43, 54). A few strains have also been isolated from Spanish traditional, starter-free cheeses made from raw milk (14, 21, 35).

This work reports a comparative phenotypic, genotypic and technological characterization of 20 strains with a *L. lactis* subsp. *lactis* phenotype, ten each belonging to the *lactis* and *cremoris* genotypes. To compare their properties and assess their functionality, the strains were subjected to genetic fingerprinting, carbohydrate fermentation tests, enzyme activity profiling and antibiotic resistance-susceptibility assays. Growth and production of volatile compounds in milk was also examined. These studies allowed the molecular genetic and phenotypic profiles of the strains belonging to the two genotypes to be compared, to make comparisons with results in the literature, and to propose a new classification for the members of this species.

MATERIAL AND METHODS

Strains, media and culture conditions. The bacteria studied were *L. lactis* subsp. *lactis* strains, ten belonging to the *lactis* and ten belonging to the *cremoris* genotypes. They were all isolated during the manufacturing and ripening stages of five traditional, Spanish cheeses made from raw milk without the deliberate addition of commercial starter cultures, which implies that the isolates were all wild strains. The origin of the different strains is as follows: Cabrales (*L. lactis* subsp. *lactis* L39, 1AA59, 3AA15, 2BA36 and 4AA10 of the *lactis* genotype; and *L. lactis* subsp. *lactis* 1AA23, 3AA9, 3AA11, 3AA23, and 4AA28 of the *cremoris* genotype); Peñamellera (*L. lactis* subsp. *lactis* 1A38 and 2A83 of the *lactis* genotype; and *L. lactis* subsp. *lactis* 2A5, 2A22, and 2A27 of the *cremoris* genotype), Genestoso (*L. lactis* subsp. *lactis* GE-1 of the *lactis* genotype; and *L. lactis* subsp. *lactis*

GE2-14 of the *cremoris* genotype), and Casín (*L. lactis* subsp. *lactis* CAS3 and Q1-6 of the *lactis* genotype; and *L. lactis* subsp. *lactis* LC44 of the *cremoris* genotype). These strains were previously identified by the sequencing of the 16S rRNA gene and comparison of the sequences against those in the GenBank and Ribosomal Database Project II databases (1).

L. lactis subsp. *lactis* CECT 185^T (=ATCC19435^T) (genotype and phenotype *lactis*), *L. lactis* subsp. *cremoris* CECT 967^T (=NCDO 607^T) (genotype and phenotype *cremoris*), *L. lactis* subsp. *lactis* MG 1363 (phenotype *lactis*, genotype *cremoris*), and *L. lactis* subsp. *lactis* IL 1403 (ex-phenotype *lactis* biovar *diacetylactis*, genotype *lactis*) strains were used as controls throughout this study. Unless otherwise stated, strains were grown statically in M17 broth at 30ºC for 18-24 h.

Molecular identification of the strains. The identification of the isolates was verified by molecular methods, which included partial amplified ribosomal DNA restriction analysis (ARDRA), sequencing and sequence comparison. For this, total genomic DNA was purified from overnight cultures using the GenElute™ Bacterial Genomic DNA kit (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's recommendations. Electrophoresis was performed in 1% agarose gels, and the DNA stained with ethidium bromide (0.5 μg/ml) and photographed under UV light. ARDRA was performed after amplification of 16S rDNA genes with the bacteria-specific universal primer 27F (S-D-Bact-0008-a-S-20) (5'-AGAGTTTGATCCTGGCTCAG-3') and the bacteria/archaea-specific primer 1492R (S-*-Univ-1492R-b-A-21) (5'-GGTTACCTTGTTACGACTT-3'). Amplicons were purified using GenElute™ PCR Clean-Up columns (Sigma-Aldrich), digested with the restriction enzymes HaeIII and HhaI (Invitrogen Ltd., Paisley, UK), and

electrophoresed as above. Amplicons were double-strand sequenced using both 27F and 1942R primers; the sequences were then aligned and compared to those in databases.

Restriction Fragment Length Polymorphism (RFLP) typing by pulsed-field gel electrophoresis (PFGE). Intact genomic DNA from *L. lactis* strains was isolated and digested in agarose plugs as described by Howard et al. (26). Purified DNA was digested independently with 20 U of the restriction enzymes SmaI and SalI (Boehringer Mannheim, Mannheim, Germany) for 18 h at 37ºC in the restriction buffer recommended by the manufacturer. DNA digests were separated in a contour-clamped homogeneous electric field (CHEF) in a CHEF-DRII apparatus (Bio-Rad, Richmond, CA, USA). Low range and lambda ladder PFGE markers were obtained from New England Biolabs (Ipswich, MA, USA). Electrophoresis was carried out in 1% FastLane^{TM} agarose gels (FMC Corporation, Philadelphia, PA, USA) in 0.5 x TBE for 20-24 h at 14ºC and 6 V cm^{-1}. Pulse times ranged from 0.5 to 25 s for 12 h and from 25 to 50 s for 6 h for the SmaI digests, and from 0.5 to 5 s for 12 h and from 5 to 30 s for 8 h for the SalI digests. Similarity clustering was performed with the Multi-Variate Statistical Package (MVSP; Kovach Computing Services, Anglesey, Wales, UK) using the unweighted pair group method with arithmetic averages and the Sorensen's correlation.

Multilocus sequence typing (MLST) analysis. DNA sequence analysis of 350 to 861 bp of intragenic regions of the genes encoding the ATP synthase alpha subunit (*atpA*), the phenylalanyl-tRNA synthase alpha subunit (*pheS*), the RNA polymerase alpha subunit (*rpoA*), the branched-chain aminotrasferase (*bcaT*), the peptidase N (*pepN*), and the X-prolyl dipeptidyl aminopeptidase (*pepX*) was performed employing the oligonucleotides and PCR conditions reported by Rademaker et al. (2007). For MLST analysis, forward and reverse sequences were trimmed, aligned, and analyzed using MEGA 4 software (51).

165 Sequences were then compared to one another and the similarity of patterns analyzed by the
166 Neighbour-Joining method.
167 The partial sequences of the seven genes examined in the MLST analysis were
168 deposited in the GenBank database under accession numbers JF297335 through JF297474.
169 **Phenotypic characterization.** Phenotypic analysis of the strains was done in filter
170 sterilized media, as follows. Growth at 40 and 45ºC was tested in Elliker broth (Scharlau,
171 Barcelona, Spain) and examined daily for up to 5 days. Similarly, growth in 4% and 6.5%
172 NaCl and at pH 9.2 and 9.6 was assayed in Elliker broth at 30ºC and checked daily for up
173 to 5 days. To test for arginine hydrolase activity, strains were grown for 48 h at 30ºC in an
174 arginine broth composed of peptone 5%, tryptone 0.5%, yeast extract 0.5%, K_2HPO_4 0.2%,
175 L-arginine 0.5%, dextrose 0.05%, $MgSO_4$ 250 mg/l, ascorbic acid 0.5 g/l, pH 7.0. After
176 incubation, cells were removed by centrifugation and 10 µl of the supernatant was mixed
177 with a drop of Nessler's reagent (KI 5 g, $HgCl_2$, 5 g, NaOH 4 g, and 100 ml of filter-
178 sterilized H_20). Strains were recorded as negative or weakly or strongly positive, judged by
179 the intensity of their orange coloration.
180 The carbohydrate fermentation profiles of the isolates and control strains were
181 determined using the commercial PhenePlate™ system (Bactus, Stockholm, Sweden) as
182 recommended by the supplier. Additionally, strains were examined using the API 20 Strep
183 kit following the manufacturer's recommendations (bioMérieux, Montalieu-Vercieu,
184 France).
185 In addition to the Voges-Proskauer test of the API 20 Strep system, acetoin
186 (acetylmethylcarbinol) production was further analyzed in Clark and Lubs medium (casein
187 and meat peptone [3.5 g/L, each], dextrose 5 g/L, potassium phosphate 5 g/L, pH 6.9), with

188 incubation at 30ºC for 72 h. To a 2.5 ml aliquot of the cultures, 0.6 ml of Barritt's reagent
189 A (5% [w/v] α-naphthol in absolute ethanol) were added, followed by 0.2 ml of reagent B
190 (40% [w/v] KOH in water). Reagents were mixed and tubes were left to settle for 10 min.
191 Strains were recorded as negative or weakly or strongly positive, judged by the intensity of
192 their red coloration.

193 Citrate assimilation is also included among the API 20 Strep tests. Citrate utilization
194 was further assayed in Kempler and McKay medium (31) under anaerobiosis in the dark at
195 30ºC for 44-72 h.

196 Enzyme activities were measured using the commercial, semi-quantitative API-ZYM
197 system (bioMérieux) following the manufacturer's recommendations. Sixty-five µl of a cell
198 suspension corresponding to McFarland standard 5 (spectrophotometric equivalent of $3 \times$
199 10^9 cfu ml^{-1}) were inoculated into each well of the API-ZYM strips, incubated for 4 h at
200 30ºC, and developed as recommended.

201 Minimum inhibitory concentration (MIC) of antibiotics was determined by
202 microdilution in VetMIC™ plates for lactic acid bacteria (LAB) (National Veterinary
203 Institute of Sweden, Uppsala, Sweden) containing two-fold serial dilutions of 16
204 antibiotics. Colonies grown on LAB susceptibility test medium (LSM) (32) agar plates
205 were suspended in 2 ml of sterile saline solution (Oxoid, Basingstoke, Hampshire, UK) to
206 obtain a density corresponding to McFarland standard 1 (spectrophotometric equivalent $3 \times$
207 10^8 cfu ml^{-1}). The suspension was further diluted 1:1000 with LSM (final cell concentration
208 3×10^5 cfu ml^{-1}). One hundred microlitres of this inoculum were added to each well of the
209 VetMIC™ plate, which was incubated at 28ºC for 48 h. The MICs were defined as the
210 lowest antibiotic concentration at which no visual growth was observed. The presence of

211 tetracycline resistance genes was checked by PCR using the universal primers for genes
212 encoding the ribosomal protection proteins DI (5'-GAYACICCIGGICAYRTIGAYTT-3')
213 and DII (5'-G CCCARWAIGGRTTIGGIGGIACYTC-3') (10), and using the PCR
214 conditions described by the latter authors. Amplicons were purified, sequenced and the
215 sequences compared against those in GenBank.

216 **Growth and acidification of milk.** Acid production was determined in UHT milk
217 (CAPSA, Siero, Spain). A 1% inoculum from an overnight M17 culture was washed in
218 sterile water and used to inoculate the milk, which was then incubated at 22ºC; samples
219 were scored for clotting at 15, 18 and 24 h. The pH was measured at 24 h using a Crison
220 pH-meter (Crison Instruments S.A., Barcelona, Spain). The appearance of the coagulum
221 (whey drainage, curd firmness, presence of gas bubbles, curd breaking) was also recorded
222 by visual inspection.

223 **Production of volatile compounds in milk.** The volatile compounds produced in milk
224 were determined after growth of the strains in 10 ml of UHT milk (CAPSA) at 30ºC for 2,
225 5 and 21 days. Cultures were grown in screw-capped tubes with a rubber liner to prevent
226 the escape of volatiles, supplied with 100 µl of internal standard (cyclohexanone, 0.36 mg
227 mL^{-1}) and stored at -80°C until analysis. The separation and quantification of volatile
228 compounds was carried out by head space (HS)/gas chromatography (GC)/mass
229 spectrometry (MS) analysis using a combined system composed of the units G 1888 HS,
230 6890 GC and 5975B MSD (Agilent Technologies, Wilmington, DE, USA) equipped with
231 an HP-Innovax 60 m × 0.25 µm capillary column (Agilent). Sample preparation and gas
232 chromatographic separation were performed as described by Salazar et al. (44). Peaks were
233 quantified as the relative total ionic count abundance with respect to the internal standard.

The concentration (μg/mL) of some volatile compounds (acetaldehyde, diacetyl, 2-propanone, acetic acid, 2-butanone and ethanol) was calculated, using linear regression equations ($R^2 \geq 0.99$), from the standard curve obtained using five representative concentrations.

RESULTS

Partial ARDRA with the restriction enzymes HaeIII and HhaI, followed by sequencing, unequivocally identified all 20 strains as belonging to *L. lactis.* As expected from previous *in silico* analyses, 10 showed the *lactis* and 10 showed the *cremoris* genotype. Digestion profiles of amplicons of both genotypes were identical with HaeIII, but they gave distinct banding patterns with HhaI. The ribosomal sequences of the strains showing a *lactis* genotype were mostly identical, matching the 16S rRNA sequence of the *L. lactis* subsp. *lactis* type strain (ATCC 19435^T) and those of the sequenced strains *L. lactis* subsp. *lactis* IL 1403 and *L. lactis* subsp. *lactis* KF147, except for a single nucleotide change in the sequences of strains GE-1 and 2BA36 (adenine for guanine at positions 91 and 465 of the IL 1403 numbering, respectively). The sequences of the strains with the *cremoris* genotype differed in 10 positions from those of the *lactis* genotype, at positions 70, 76, 82, 87, 91, 93, 95, 98, 183 and 195. Two distinct sequences were found among the strains with the *cremoris* genotypes, varying in a single nucleotide at position 183, corresponding to cytosine in six strains (2A5, 2A22, 2A27, 3AA9, 3AA23, and 4AA28) and to thymine in four strains (1AA23, 3AA11, GE2-14, and LC44).

256 To assess the genetic diversity and relatedness of the strains, all were subjected to
257 RFLP-PFGE analysis. *L. lactis* subsp. *lactis* MG 1363 (phenotype *lactis*, genotype
258 *cremoris*), and *L. lactis* subsp. *lactis* IL 1403 (ex-phenotype *lactis* biovar *diacetylactis*,
259 genotype *lactis*) were included as a control. Fig. 1 shows the RFLP profiles obtained with
260 the enzyme SalI and the clustering of the strains in terms of the Sorensen's coefficient of
261 similarity. All *L. lactis* isolates of the *lactis* genotype were shown to be unrelated, as they
262 shared a low similarity index. On the contrary, two isolates of the *cremoris* genotype from
263 the same cheese sample (3AA9 and 3AA23) proved to be related (similarity index 0.89).
264 Moreover, three other *cremoris* isolates (2A5, 2A22, and 2A27) from a single cheese batch
265 showed identical digestion profiles, which indicates that all three could be replicates of the
266 same strain. Similar PFGE results were obtained after digestion with SmaI (data not
267 shown). However, the DNA of three strains (one belonging to the *lactis* genotype, GE-1,
268 and two of the *cremoris* genotype, 3AA9 and 3AA23) was shown to be resistant to
269 digestion with this last enzyme, which prevented a proper strain comparison. SmaI
270 digestions allowed the estimation of the size of the chromosomes, which were shown to
271 range approximately between 2250 and 2600 kbp for the *lactis* genotypes and between
272 2400 and 2650 for the *cremoris* genotypes. In conclusion, all 10 strains with the *lactis*
273 genotype and at least eight strains with the *cremoris* genotype could be considered
274 different. In spite of this, isolates were all independently subjected to further genetic
275 analyses and biochemical tests.
276 The genetic diversity of the *lactis* and *cremoris* genotype strains was further evaluated
277 by MLST using partial nucleotide sequences of six genes, *atpA, rpoA, pheS, pepN, bcaT,*
278 *pepX*, and the gene coding for 16S rRNA (Table 1). Amplicons and sequences from all
279 these genes of all strains were obtained and aligned. Nucleotide positions of the sequences

280 showing ambiguities were excluded from the analysis. Unique sequence types (STs) were
281 obtained for every strain. After individual analysis of the seven genes, and all genes
282 together, two consistent and distinct clusters with a similar topology were obtained for the
283 *lactis* and *cremoris* genotype strains (Fig. 2). The number of polymorphic sites varied
284 strongly from gene to gene, ranging from 12 in 16S rDNA up 94 in *pepX* (Table 1).
285 However, most of these nucleotide variations corresponded to nucleotide differences
286 among strains of the *lactis* and *cremoris* genotypes, indicating the actual number of alleles
287 in the two species to be much lower (from one to six; Table 1). Further, a majority of the
288 substitutions were synonymous (they did not result in amino acid changes), as inferred
289 from the low dN/dS ratio (particularly low for the *bcaT* gene), which indicates conservation
290 during evolution of amino acid sequences between members of the two subspecies.
291 Together, the above results strongly suggest that the two clusters are composed of
292 individual organisms showing a high degree of intra-cluster genetic similarity.
293 Since the same gene stretches as those reported by Rademaker et al. (40) were amplified
294 and sequenced, the sequences obtained in the present work were trimmed in the same
295 manner used by the latter authors. Sequences were then submitted to a recently developed
296 MLST database for *L. lactis* (http://www-mlst.biotoul.fr/). Composite sequences of all
297 seven genes were aligned with those of representative strains from the study of Rademaker
298 et al. (40). This allowed the relatedness of the present cheese isolates to be compared at the
299 DNA level with those of lactococci from dairy and non-dairy sources (Fig. 3). Most strains
300 in the present study of both the *lactis* and *cremoris* genotypes clustered together, indicating
301 greater similarity among themselves than to the dairy strains of Rademaker et al. (40).
302 Strains from different traditional cheeses grouped together, even though they were isolated
303 from geographical areas more than 200 km apart, indicating that related STs are

304 widespread. At the same time, some other strains from the same cheese batch clustered
305 separately, suggesting the presence of unrelated STs. The *cremoris* genotype strains were
306 split into two groups, one of which seems to be related to *L. lactis* subsp. *lactis* MG 1363
307 and some other strains with *lactis* phenotype and *cremoris* genotype (Fig. 3), and one
308 apparently closer to the true (phenotypic) *L. lactis* subsp. *cremoris* strains.
309 Table 2 summarizes the conventional tests used to distinguish the subspecies *L. lactis*
310 subsp. *lactis* and *L. lactis* subsp. *cremoris*, and the results obtained. All the strains studied
311 hydrolyzed arginine, grew in 4% NaCl, and at 40ºC. All but four of the strains hydrolyzed
312 hippurate and all but the *cremoris* type strain (*L. lactis* subsp. *cremoris* CECT 967^T) grew
313 at pH 9.2. In contrast, none of the strains used citrate, and single strains grew well at 45ºC
314 and in 6.5% NaCl. Strains did not give a positive reaction in the Voges-Proskauer test,
315 except for a weak reaction of the *L. lactis* subsp. *cremoris* type strain, suggesting that none
316 of the strains belonged to the biovar diacetylactis. Few phenotypic differences were seen
317 between strains belonging to the *lactis* and *cremoris* genotypes; the most notable was
318 growth at pH 9.6, for which all *cremoris* genotypes proved to be positive (although growth
319 was weak), whereas only two strains of the *lactis* genotype were positive. In conclusion,
320 according to the classical phenotypic criteria, the biochemical assays identified all 20 *L.*
321 *lactis* cheese strains as belonging to the *lactis* subspecies.
322 Carbohydrate fermentation profiles were analyzed by the combined use of the
323 PhenePlate and API 20 Strep systems, in which the utilization of 51 sugar and polyalcohols
324 was examined. Carbohydrates tested with the two systems always showed concordant
325 results. Table 3 shows the combined results. Strain to strain variations were found among
326 both *lactis* and *cremoris* genotypes. However, the fermentation profiles shown by the
327 strains of the *cremoris* genotype were wider owing to their use of L-arabinose, arbutin,

328 glycerol, inosine, mannitol, starch and D-xylose. Key sugars used for distinguishing strains
329 belonging to the *L. lactis* subsp. *lactis* (positive) and *L. lactis* subsp. *cremoris* (negative)
330 subspecies were in fact equally fermented by all the present strains (such as maltose and
331 ribose) or fermented for more strains of the *cremoris* genotype (mannitol).

332 To further compare the biochemical properties of the strains, all were subjected to
333 phenotypic profiling for enzyme activities using the API-ZIM and API 20 Strep systems,
334 and for antibiotic resistance-susceptibility via the MICs for 16 antibiotics using the
335 VetMIC^{TM} system. The results are shown in Tables 4 and 5, respectively. Only 10 of the 20
336 enzyme activities assayed with the two kits were positive for the strains studied. Activities
337 showed high variability among the strains and between genotypes (Table 4). The enzyme
338 profiles of the *cremoris* genotype were usually greater and/or the level of activity higher
339 than those of the *lactis* genotype; these showed moderate esterase (C4) activity, and high
340 esterase-lipase (C8), acid phosphatase, naphthol-AS-BI-phosphohydrolase, and α-
341 glucosidase activities. In contrast β-galactosidase and β-glucosidase activities were usually
342 stronger for the *lactis* genotype strains.

343 Little differences, if any, were observed in terms of the antibiotic MIC profiles among
344 the strains of the *lactis* and *cremoris* genotypes (Table 5). High MICs were observed in
345 both the *lactis* and *cremoris* genotypes for antibiotics to which lactococci have been
346 reported intrinsically resistant (aminoglycosides, trimethoprim, and rifampicin) (Ammor et
347 al., 2007). As an exception, the three identical strains of the *cremoris* genotype, 2A5, 2A22
348 and 2A27, showed atypical tetracycline MICs, compatible with the presence of dedicated
349 resistance mechanisms. Following standard gene amplification, sequencing and sequence
350 analysis, all three strains were shown to harbor a *tet*(S) gene, which is thought to be
351 responsible for strong resistance to this antibiotic.

352 Except for three *lactis* genotype strains (2BA36, 2A83 and 4AA10) all coagulated UHT
353 milk at 22ºC, reaching a final pH at 24 h ranging from 4.89 to 4.14 (Table 2). After 48 h in
354 milk at 30ºC, eleven volatile compounds were detected by HS/GC/MS, of which four were
355 quantified (Table 6). The repeatability of this analysis was high; the coefficient of variation
356 for the different volatile compounds and strains varied from 1 to 8%. Large strain to strain
357 variations in either absolute or relative abundance of most of the volatile compounds were
358 observed in strains of both the *lactis* and *cremoris* genotype, especially with respect to the
359 production of acetaldehyde, ethanol, and aldehyde- and alcohol-derived compounds from
360 the catabolism of branched-chain amino acids. The volatile compound profiles of the *lactis*
361 and *cremoris* genotype strains were qualitatively and quantitatively different. The *lactis*
362 genotype strains produced more diacetyl and marginal levels of acetoin, while the *cremoris*
363 genotype strains produced higher levels of acetaldehyde and all the known amino acid-
364 derived flavor compounds, especially 2-methyl propanal, 3-methyl butanal and 2- and 3-
365 methyl butanol. Prolonged incubation of milk for five and 21 days showed neither
366 significant difference in the volatile profiles of the strains nor in the abundance of the
367 volatile compounds detected (data not shown).
368
369
370 **DISCUSSION**
371 Traditional cheeses and non-dairy fermented products are considered a potential source
372 of new *L. lactis* strains with novel properties that might be able to replace or complement
373 currently used dairy starters (29, 30, 37, 54). Additionally, wild strains are a source of
374 phenotypic and genetic variability (13, 48) that might be used through genetic engineering
375 to enhance the activity and performance of current starter strains (55). Phenotypically, the

20 strains of this study belonged to *L. lactis* subsp. *lactis*, which agrees well with species identification of wild strains from milk (11, 18, 33) and traditional cheeses (14, 19, 34), as does the low percentage of *cremoris* genotypes.

In the past, the identification of *Lactococcus* species and subspecies was based entirely on phenotypic tests, primarily because species and subspecies are defined by their phenotypes (20). Phenotypic assays are sometimes ambiguous, can provide different results over the growth phase, and are dependent on culture conditions (19, 42, 52, 54). However, since the 1990s, identification has relied mostly on molecular genetic analyses (22, 42, 49), fuelled by the development of simple PCR-based methods (19, 36, 56). The use of molecular genetic techniques alone, however, has introduced some additional confusion into the taxonomy of *L. lactis*, complicating the unusual structure of this species (30, 40). Molecular genetic techniques allowed the recovery of strains with a *cremoris* genotype from different sources, including milk, dairy products and plant material. It is not clear whether these new *cremoris* isolates have biochemical properties similar to those used as starters in the dairy industry. In fact, the phenotypic and technological characterization of *cremoris* genotype isolates and the comparison of their properties with those of recognized starter strains of both the *lactis* and *cremoris* subspecies has only rarely been undertaken (18, 37, 43, 54). Such studies are critical, however, for the selection of the most suitable strains for each application. Replacing the unreliable, traditional phenotypic tests with other phenotypic assays and molecular genetic techniques such as those used in the present work could help to discover new traits for distinguishing between *L. lactis* subspecies.

The typing results obtained in this study by PFGE agreed well with those previously obtained by random amplification of polymorphic DNA (RAPD) and repetitive extragenic palindromic (REP) techniques (1). In general, cluster analysis of macro-restriction patterns

400 by PFGE showed less similarity between the strains than that obtained by PCR-based
401 typing methods. PFGE is a powerful means of assessing genetic relationships for bacteria
402 due to its larger genome coverage (greater than 90%) as compared to other typing
403 techniques (23). Cluster analysis of the typing results and sequencing of the 16S rDNA and
404 housekeeping genes consistently provided two clear-cut clusters -formed separately by the
405 strains of the *lactis* and *cremoris* genotypes- with only low level similarity to one another.
406 Similar results have been reported with other typing (11, 21, 37, 40) and sequencing
407 techniques (17, 40, 49, 54). The more robust MLST technique, which gathers together
408 several gene sequences, produced strongly separated *lactis* and *cremoris* genotypes in
409 deeply branched trees, as reported by other authors (39, 40, 50). Two major genomic
410 lineages for the *lactis* and *cremoris* genotypes have also been recently recognized using
411 pangenomic DNA array hybridization, determining the presence or absence of 4571 gene
412 orthologs (7). Proteomic analysis of the ribosomal proteins by matrix-assisted laser
413 desorption ionization-time of flight mass spectrometry has provided similar results (52).
414 This would all seem to indicate that, irrespective of their phenotype, the *lactis* and *cremoris*
415 lineages are phylogenetically related, but that they have long been on separate evolutionary
416 paths. In fact, based on 16S rRNA gene divergence (less than 0.8%), the deviation of the
417 *lactis* and *cremoris* genotypes has been estimated to have occured some 17 million years
418 ago (9). Though it is difficult to infer divergence times for the different evolutionary steps,
419 recent independent MLST analyses have confirmed an early separation of the *lactis* and
420 *cremoris* genotypes (39, 40).
421 Despite the similarity of the *lactis* and *cremoris* genotypes shown in the present
422 phenotypic assays, their member strains showed distinguishable carbohydrate utilization
423 and enzyme activity profiles. Surprisingly, the strains with a *cremoris* genotype showed

424 greater fermentation and enzyme activity profiles, even though true *L. lactis* subsp.
425 *cremoris* strains are reported to have extremely reduced fermentation and enzyme activity
426 profiles (20, 25, 45). The ability of *L. lactis* to ferment carbohydrates is thought to be
427 related to the degree of adaptation of the strains to the dairy environment (30), independent
428 of genotype. The genome sequence of two plant-associated *L. lactis* subsp. *lactis* strains
429 shows the largest number of genes in carbohydrate metabolism and transport category (46);
430 therefore, they ferment more carbohydrates than any of the three sequenced dairy strains
431 (MG 1363, IL 1403, and SK 11). Furthermore, the genome of *L. lactis* subsp. *lactis* MG
432 1363 (genotype *cremoris*) has been shown to encode more genes in these two categories
433 (57) than *L. lactis* subsp. *lactis* IL 1403 (genotype *lactis*) (8); consequently it can utilize
434 more sugars. Chromosome size has been shown to vary widely among *L. lactis* strains of
435 both subspecies (30, 39). However, the origin of the *L. lactis* strains has been shown to
436 correlate with chromosome size, particularly for the phenotypic *L. lactis* subsp. *cremoris*,
437 which have smaller chromosomes than the *L. lactis* subsp. *lactis* strains of both *lactis* and
438 *cremoris* genotypes (30). Chromosome sizes in this study fell within the normal range for
439 *L. lactis* of either *lactis* and *cremoris* genotype (30, 39). Adaptation of *L. lactis* to grow in
440 milk is thought to have occurred by gene decay and acquisition of key traits (30, 46, 57),
441 most probably under the high selective pressure imposed by cheese- and butter-making
442 technologies. The analysis of the implicated genes and the whole genome sequences of
443 more strains might give clues about the current phylogenetic position of the *lactis* and
444 *cremoris* genotypes and the evolutionary processes that gave rise to these dairy-adapted
445 starter strains.

446 In the present work, acid production in milk was variable among the strains of the two
447 genotypes; a few strains even failed to coagulate the provided UHT milk after 24 h of

448 incubation at 22ºC. In general, the *cremoris* genotypes usually caused the pH to decrease
449 more than the *lactis* genotypes, as reported elsewhere (37, 54).
450 Wild and non-dairy *L. lactis* strains have occasionally been associated with off-flavor
451 production (3, 54), which correlates with the formation of large amounts of volatile
452 compounds via the degradation of branched-chain amino acids (Leu, Ile, Val). These
453 compounds have a very low taste threshold and have been connected with malty and burnt
454 notes in dairy products (47). However, selected wild strains or combinations of wild and
455 starter strains have been shown to enhance the typical flavors and to increase ripening
456 indices (4, 5). Moreover, the same volatile compounds that caused the off flavors
457 mentioned above seem to be involved in the desired strong flavors of cheeses made from
458 raw milk (12); these flavors are strongly fostered in traditional cheese varieties, particularly
459 those with protected designation of origin (PDO) status. Starter candidates might therefore
460 be selected among wild *L. lactis* to ensure the production of intensely flavored cheeses.
461 In conclusion, the overall phenotypic and genotypic relatedness of the strains belonging
462 to the *lactis* and *cremoris* genotypes suggests they should be considered members of the
463 same species, as their properties meet the criteria presently used in the species concept for
464 prokaryotes (41). However, despite their similarity, the *lactis* and *cremoris* genotypes
465 consistently cluster separately when investigated with simple molecular genetic techniques,
466 suggesting they should be considered as true subspecies; a possibility contemplated in the
467 species definition referred to above (41), and on the more recent recommendations for the
468 taxonomy of prokaryotes (53). Separate or combined simple matching-based cluster
469 analysis of the phenotypic traits analyzed in the present study (carbohydrate fermentation,
470 enzyme activities, production of volatile compounds) consistently gave the same two well-
471 separated clusters as did the molecular genetic techniques (data not shown). Thus, in

disagreement with some other authors (16, 40), irrespective of their phenotype, the *cremoris* genotypes should all be considered as belonging to the *cremoris* subspecies.

Analysis of more *L. lactis* collections, including representative strains of all subspecies and biovars from different environments, using state-of-the-art high throughput phenotypic (Biolog, cheese models) and molecular genetic (genome sequencing, microarray hybridization, comparative genomic hybridization) screening techniques, should further help in assessing the diversity of the lactococci. These studies should be aimed at correlating genomic make-up and phenotypic traits with industrial performance, which has ultimately to be assessed by carefully controlled trials using defined mixtures of phenotypic and genotypic strains of both subspecies.

ACKNOWLEDGEMENTS

This research was supported by a project from the Spanish Ministry of Science and Innovation (MICINN) (AGL2007-61869-ALI). E. Fernández and A. Alegría were awarded a scholarship of the FPI program from MICINN (BES-2008-002031) and the Severo Ochoa program from FICYT (BP08-053), respectively. S. Delgado was supported by a research contract of MICINN under the Juan de la Cierva program (JCI-2008-02391). Dr. P. Le Bourgeois, Université Paul Sabatier de Toulouse, France, is acknowledged for the critical reading of the manuscript.

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Table 1.- Genetic diversity at seven loci based on the nucleotide sequences used for the MLST analysis and that of the 16S rRNA gene of 20 *L. lactis* subsp. *lactis* strains of both *lactis* and *cremoris* genotypes (ten each) used in this study.

Locus	Gene length (bp)	Amplified fragment length (bp)	Analyzed fragment length (bp)	G+C content (%)	No. of polymorphic sites	No. alleles *lactis*	No. of alleles *cremoris*	dN/dS ratio[a]
atpA	1,503	1,141	861	42	49	4	1	0.30
rpoA	939	814	721	39	15	4	4	0.26
pheS	2,533	618	477	42	45	6	3	0.13
bcaT	1,047	493	350	42	50	3	2	0.02
pepN	1,023	482	473	37	46	1	2	0.10
pepX	2,269	602	508	39	94	4	2	0.20
16S rRNA gene	1,548	1,465	605	49	12	3	2	NA

[a]The dN/dS ratio was calculated by dividing the number of non-synonymous substitutions by the number of synonymous substitutions.
NA, not applicable.

Table 2.- Phenotypic and biochemical properties of wild *L. lactis* subsp. *lactis* strains of *lactis* and *cremoris* genotypes isolated from starter-free, raw milk cheeses.

Genotype/strain	Phenotypic properties										
						Growth at or in					
	pH in milk 22ºC, 24 h	Hydrolysis of hippurate	Hydrolysis of arginine	Citrate utilization	Acetoin production	40ºC	45ºC	4% NaCl	6.5 NaCl	pH 9.2	pH 9.6
genotype *lactis*											
1AA59	4.19	-	+	-	-	+	-	+	-	w	-
CAS3	4.14	+	+	-	-	+	w	+	+	+	-
L39	4.69	+	+	-	-	+	-	+	-	+	w
3AA15	4.14	+	+	-	-	+	w	+	w	+	-
2BA36	6.17	+	+	-	-	+	+	+	-	+	-
1A38	4.89	+	+	-	-	+	-	+	w	+	+
2A83	6.37	+	+	-	-	+	-	+	-	+	-
GE-1	4.17	+	+	-	-	+	-	+	-	+	-
Q1-6	4.78	-	+	-	-	+	-	+	-	+	-
4AA10	5.76	-	+	-	-	+	-	+	-	w	-
CECT 185^T	4.24	-	+	-	-	+	-	+	-	+	-
genotype *cremoris*											
1AA23	4.24	+	+	-	-	+	-	+	w	+	w
2A5	4.30	+	+	-	-	+	-	+	w	+	+
2A22	4.29	+	w	-	-	+	-	+	w	+	w
2A27	4.27	+	+	-	-	+	-	+	-	+	w
3AA9	4.32	w	w	-	-	+	-	+	-	+	w
3AA11	4.23	+	+	-	-	+	-	+	-	+	w
3AA23	4.19	-	+	-	-	+	-	+	-	+	+
LC44	4.23	+	+	-	-	+	-	+	-	+	w
GE2-14	4.25	+	+	-	-	+	-	+	-	+	w
4AA28	4.25	+	+	-	-	+	-	+	-	+	w
CECT 967^T	4.28	+	-	-	w	-	-	-	-	-	-

w, weak reaction or growth.
CECT 185^T and CECT 976^T are the *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* type strains, respectively.

Table 3- Carbohydrate fermentation profiles of the wild *L. lactis* subsp. *lactis* strains of *lactis* and *cremoris* genotypes of this study.

Genotype/strain	Carbohydrate																
	Adonitol	**Amigdalin**	**L-arabinose**	**Arbutin**	**Gentibiose**	**Gluconate**	**Glycerol**	**Inosine**	**5-keto-gluconate**	**Maltose**	**Mannitol**	**β-methyl glucoside**	**Ribose**	**Salicine**	**Starch**	**Sucrose**	**D-xylose**
genotype *lactis*																	
1AA59	-	-	-	-	-	-	-	-	-	+	-	-	+	+	w	+	-
CAS3	-	+	-	-	+	-	-	w	-	+	+	+	+	+	+	+	-
L39	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-	-	-
3AA15	-	+	-	-	+	-	-	w	-	+	-	+	+	+	w	+	-
2BA36	-	+	-	-	+	-	-	+	-	+	w	+	+	-	+	+	-
1A38	w	-	-	-	+	w	-	-	-	+	-	+	+	+	w	w	-
2A83	-	-	-	-	+	-	-	-	-	+	-	+	+	+	w	-	-
GE-1	-	+	-	w	+	-	+/	-	-	+	w	+	+	+	w	+	-
Q1-6	-	+	-	-	-	-	-	-	-	+	w	+	+	+	-	-	-
4AA10	-	+	-	w	+	-	w	-	-	+	-	+	+	+	w	+	-
CECT 185^T	-	-	-	-	+	w	-	-	-	+	-	+	+	+	w	-	-
genotype *cremoris*																	
1AA23	-	+	w	-	+	-	w	w	-	+	+	+	+	+	+	+	+
2A22	-	+	w	w	+	-	w	w	-	+	+	+	+	+	+	+	+
2A27	-	+	-	w	+	-	-	+	-	+	+	+	+	+	+	+	+
2A5	-	+	-	w	+	-	-	w	-	+	+	+	+	+	+	+	+
3AA23	-	-	-	w	+	-	-	-	-	+	+	+	+	+	+	-	+
3AA11	-	-	+	w	w	-	w	w	w	+	+	w	+	w	+	+	-
3AA9	-	+	-	w	+	-	w	w	-	+	+	+	+	+	w	+	+
LC44	-	+	-	-	+	-	w	w	-	+	w	+	-	+	+	-	-
GE2-14	-	-	-	-	-	-	-	-	-	+	w	-	+	+	w	-	-
4AA28	-	-	+	-	+	-	-	-	-	+	w	-	+	+	w	-	-
CECT 967^T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

w, weak reaction.
CECT 185^T and CECT 976^T are the *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* type strains, respectively.
All strains fermented glucose, galactose, lactose, and lactulose, and all but *L. lactis* subps. *cremoris* CECT 967^T fermented cellobiose and trehalose.
None of the strains fermented D-arabitol, deoxy-ribose, doxy-glucose, dulcitol, D-fucose, L-fucose, fumarate, galacturonic-lactone, glycogen, inositol, inulin, malinate, malonate, maltitol, mannonic acid lactone, melbionate, melezitose, melibiose, ornithine, palatinose, pyruvate, raffinose, rhamnose, sorbitol, sorbose, tagatose, L-tartrate, urea, and under the assay conditions.

Table 4.- Enzymatic activities measured with the API ZYM and 20 Strep systems in wild *L. lactis* subsp. *lactis* strains of *lactis* and *cremoris* genotypes isolated from raw milk, starter-free cheeses.

Genotype/strain	Enzymatic activity[a]									
	Alkaline phosphatase	Esterase (C4)	Esterase lipase (C8)	Pyrrolidonyl -arylamidase	Leucine arylamidase	Acid phosphatase	Naphthol-AS-BI-phosphohydrolase	β-galactosidase	α-glucosidase	β-glucosidase
genotype *lactis*										
1AA59	2.5	0	5	30	10	2.5	0	40	2.5	0
CAS3	2.5	0	2.5	2.5	10	15	2.5	10	0	30
L39	2.5	0	5	30	15	20	10	2.5	15	40
3AA15	2.5	0	2.5	30	5	0	2.5	40	2.5	0
2BA36	2.5	0	5	20	5	5	5	40	5	0
1A38	2.5	0	5	2.5	20	10	2.5	0	0	40
2A83	2.5	0	5	5	5	5	5	0	0	0
GE-1	0	0	5	20	40	15	2.5	20	0	40
Q1-6	2.5	0	5	30	20	10	5	2.5	0	40
4AA10	2.5	0	5	20	10	10	2.5	0	0	15
CECT 185^T	2.5	0	5	20	30	20	5	2.5	40	40
genotype *cremoris*										
1AA23	2.5	2.5	5	20	10	30	10	2.5	10	0
2A22	2.5	5	10	30	10	40	15	2.5	20	20
2A27	2.5	5	10	30	10	40	15	0	10	10
2A5	2.5	5	10	30	10	35	15	0	20	20
3AA23	2.5	0	5	20	20	40	20	0	40	40
3AA11	2.5	10	10	30	5	20	15	10	15	0
3AA9	2.5	5	10	30	10	30	15	0	15	20
LC44	2.5	2.5	5	20	40	40	20	2.5	20	20
GE2-14	2.5	5	5	30	5	5	5	10	5	0
4AA28	2.5	2.5	2.5	5	5	30	20	2.5	10	10
CECT 967^T	0	2.5	5	20	5	10	5	0	0	0

[a]Units of activity are expressed as nanomol of substrate hydrolyzed under the assay conditions.
CECT 185^T and CECT 976^T are the *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* type strains, respectively.
Valine arylamidase, cysteine arylamidase, lipase (C14), trypsin, α-quimiotrypsin, α-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase activities were never detected.

Table 5.- Minimum inhibitory concentration (MIC) of 16 antibiotics to wild *L. lactis* subsp. *lactis* strains of *lactis* and *cremoris* genotypes.

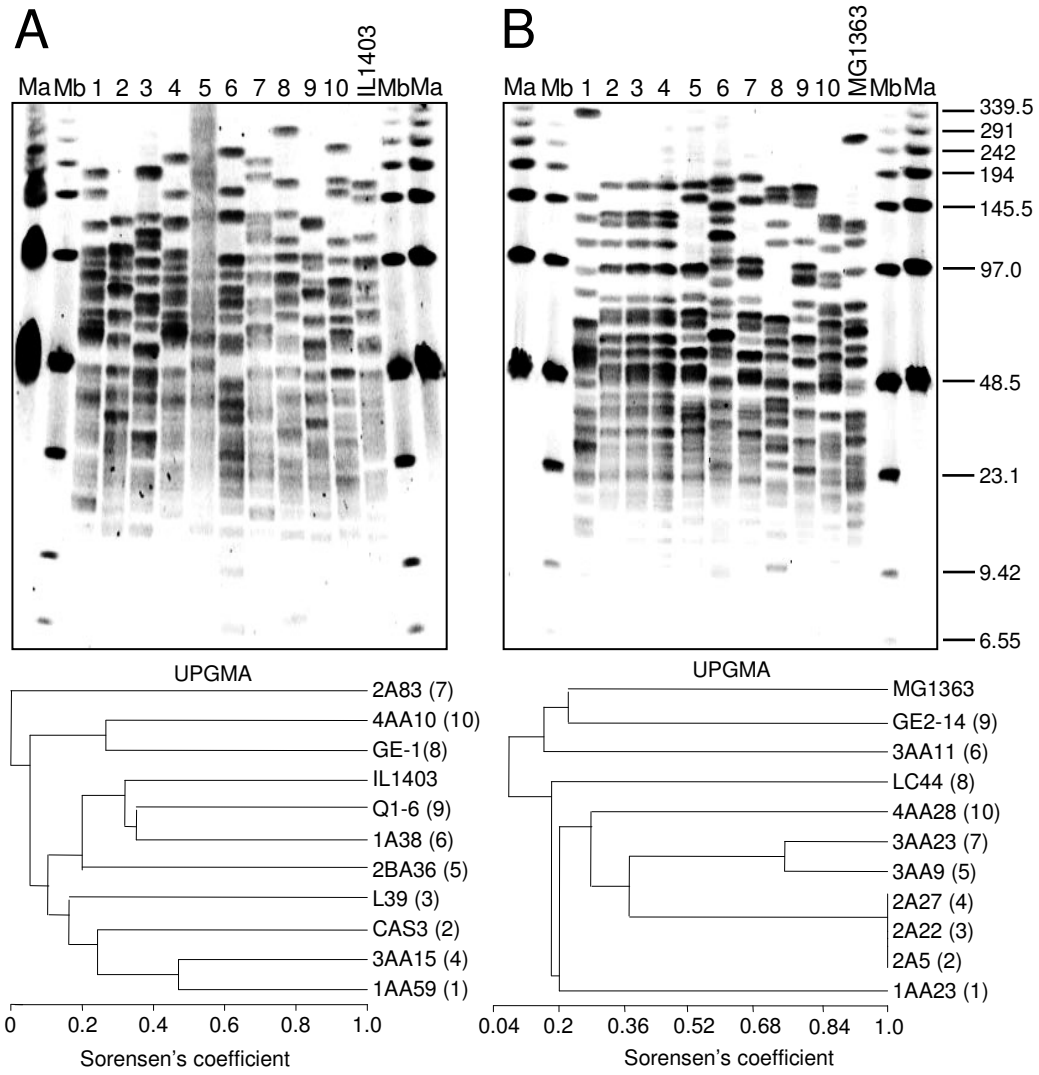
Genotype/strain	Antibiotic															
	Gm	Km	Sm	Nm	Tc	Em	Cl	Cm	Am	PG	Va	Vi	Lz	Tm	Ci	Ri
genotype *lactis*																
1AA59	4	8	32	8	0.50	0.25	0.12	8	0.50	0.25	0.50	1	2	>64	8	16
CAS3	4	16	32	8	0.25	0.25	0.12	2	0.25	0.25	0.50	2	2	>64	4	16
L39	1	8	16	2	0.50	0.25	0.25	4	0.25	0.25	0.50	2	4	>64	4	16
3AA15	2	16	16	4	0.50	0.25	0.12	4	0.25	0.25	0.25	1	2	>64	4	16
2BA36	2	8	32	2	0.50	0.25	0.25	8	0.12	0.25	0.25	2	4	>64	4	16
1A38	4	16	64	8	0.50	0.25	0.25	4	0.25	0.25	0.50	2	2	>64	2	32
2A83	2	8	32	8	0.50	0.50	0.12	4	0.25	0.12	0.50	0.50	2	>64	4	32
GE-1	4	16	32	8	0.50	0.25	0.12	8	0.25	0.25	0.25	1	4	>64	8	16
Q1-6	2	8	16	4	0.50	0.25	0.12	4	0.12	0.12	0.50	1	2	>64	4	16
4AA10	4	32	64	16	0.50	0.25	0.25	8	0.25	0.25	0.50	2	2	>64	4	16
CECT 185^T	16	32	64	32	0.50	0.25	0.12	8	0.50	0.50	0.50	8	4	>64	8	>64
genotype *cremoris*																
1AA23	1	8	16	16	0.50	0.25	0.25	4	0.25	0.25	0.50	2	2	>64	8	8
2A22	4	16	64	32	64	0.25	0.50	4	0.25	0.25	0.50	2	2	>64	16	32
2A27	8	32	64	32	64	0.25	0.50	4	0.25	0.25	0.50	2	2	>64	16	32
2A5	8	16	64	16	64	0.25	0.50	4	0.25	0.25	0.50	2	2	>64	16	32
3AA23	16	64	128	64	0.50	0.25	0.50	16	0.25	0.25	0.50	2	2	>64	8	8
3AA11	2	8	32	8	1	0.12	0.25	8	0.25	0.25	0.50	2	2	>64	8	32
3AA9	16	64	128	64	0.50	0.25	0.50	4	0.25	0.25	0.50	2	2	>64	8	8
LC44	2	16	32	8	0.50	0.25	0.25	8	0.25	0.50	0.50	2	2	>64	8	32
GE2-14	8	32	64	8	0.50	0.12	0.25	8	0.25	0.25	0.50	2	2	>64	4	32
4AA28	2	16	32	4	0.50	0.25	0.25	8	0.12	0.12	0.50	2	2	>64	8	16
CECT 967^T	0.50	2	2	1	0.50	0.25	0.12	8	0.25	0.25	0.50	1	2	64	4	>64

CECT 185^T and CECT 976^T are the *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* type strains, respectively.
Key of antibiotics: Gm, gentamicin; Km, kanamycin; Sm, streptomycin; Nm, neomycin; Tc, tetracycline; Em, erythromycin; Cl, clindamycin; Cm, chloramphenicol; Am, ampicillin; PG, penicillin G; Va, vancomycin; Vi, virginiamycin; Lz, linezolid; Tm, trimethoprim; Ci, ciprofloxacin; Ri, rifampicin.

Table 6.- Absolute or relative abundance of volatile compounds produced in UHT-milk at 30º for 48 h by the wild *L. lactis* subsp. *lactis* strains of the *lactis* and *cremoris* genotypes as detected by Head Space, Gas Chromatography, Mass Spectrometry (HS/GC/MS).

Genotype/strain	Volatile compound												
	µg/mL					Relative abundance[a]							
	Acetaldehyde	2-propanone	Ethanol	Diacetyl	Acetic acid	Acetoin	Methanethiol	2-methyl propanal	2-methyl propanol	2-methyl butanal	3-methyl butanal	2- and 3-methyl butanol
genotype *lactis*												
1AA59	6.41	11.47	802.33	7.31	-	0.06	0.88	3.60	-	3.19	15.49	8.10
CAS3	5.35	13.89	863.77	3.71	-	-	0.87	-	-	-	-	-
L39	6.06	13.55	586.84	-	-	-	0.79	6.30	3.35	2.99	32.89	34.71
3AA15	5.60	13.87	865.09	8.35	-	0.06	0.91	4.42	0.88	3.06	15.64	8.71
2BA36	5.88	12.05	650.70	9.27	-	0.08	0.87	0.95	-	0.66	6.34	5.19
1A38	27.57	9.03	140.84	2.55	-	0.19	-	-	-	-	-	-
2A83	6.41	11.47	802.33	7.31	-	0.05	0.96	0.28	-	-	0.98	4.97
GE-1	6.38	13.05	667.91	6.57	-	-	0.75	-	-	-	-	-
Q1-6	1.55	9.93	153.24	4.31	-	0.43	-	-	-	-	-	-
4AA10	20.89	8.63	1239.09	3.71	-	-	0.87	-	-	-	-	-
CECT 185^T	5.26	1.38	238.9	-	-	-	-	-	0.35	-	-	2.48
genotype *cremoris*												
1AA23	33.71	15.05	731.74	0.70	-	-	-	-	-	-	-	-
2A22	22.33	12.25	1146.28	4.80	-	-	0.85	14.33	5.75	3.25	84.50	36.13
2A27	21.89	11.50	1140.95	4.78	-	-	0.78	14.49	6.42	3.23	88.33	39.87
2A5	23.89	14.58	1287.38	5.52	-	-	0.81	15.13	5.91	3.53	87.65	37.10
3AA23	17.91	9.75	1170.95	4.84	-	-	0.46	19.76	9.10	2.79	89.76	53.99
3AA11	26.93	10.83	747.33	3.70	-	0.07	0.25	32.71	6.24	35.82	108.76	70.72
3AA9	79.56	10.76	823.03	2.40	-	-	0.38	17.38	9.76	0.95	58.39	66.17
LC44	25.00	9.31	402.08	-	-	-	0.26	-	-	-	-	-
GE2-14	33.81	12.46	679.72	2.56	-	-	0.24	35.74	6.06	35.09	113.76	83.26
4AA28	25.95	15.31	746.83	-	-	-	0.70	8.27	0.97	2.69	55.39	17.88
CECT 967^T	2.25	0.87	77.50	-	-	-	-	-	-	-	-	-

Average results of duplicated analysis are shown.
CECT 185^T and CECT 976^T are the *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* type strains, respectively.
[a]Relative abundance as compared to an internal control (cyclohexanone, 0.36 mg/mL).
-, not detected.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242
194
145.5
97.0
48.5
23.1
9.42
6.55
UPGMA
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4AA10 (10)
GE-1(8)
IL1403
Q1-6 (9)
1A38 (6)
2BA36 (5)
L39 (3)
CAS3 (2)
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Sorensen's coefficient
UPGMA
MG1363
GE2-14 (9)
3AA11 (6)
LC44 (8)
4AA28 (10)
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3AA9 (5)
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Sorensen's coefficient

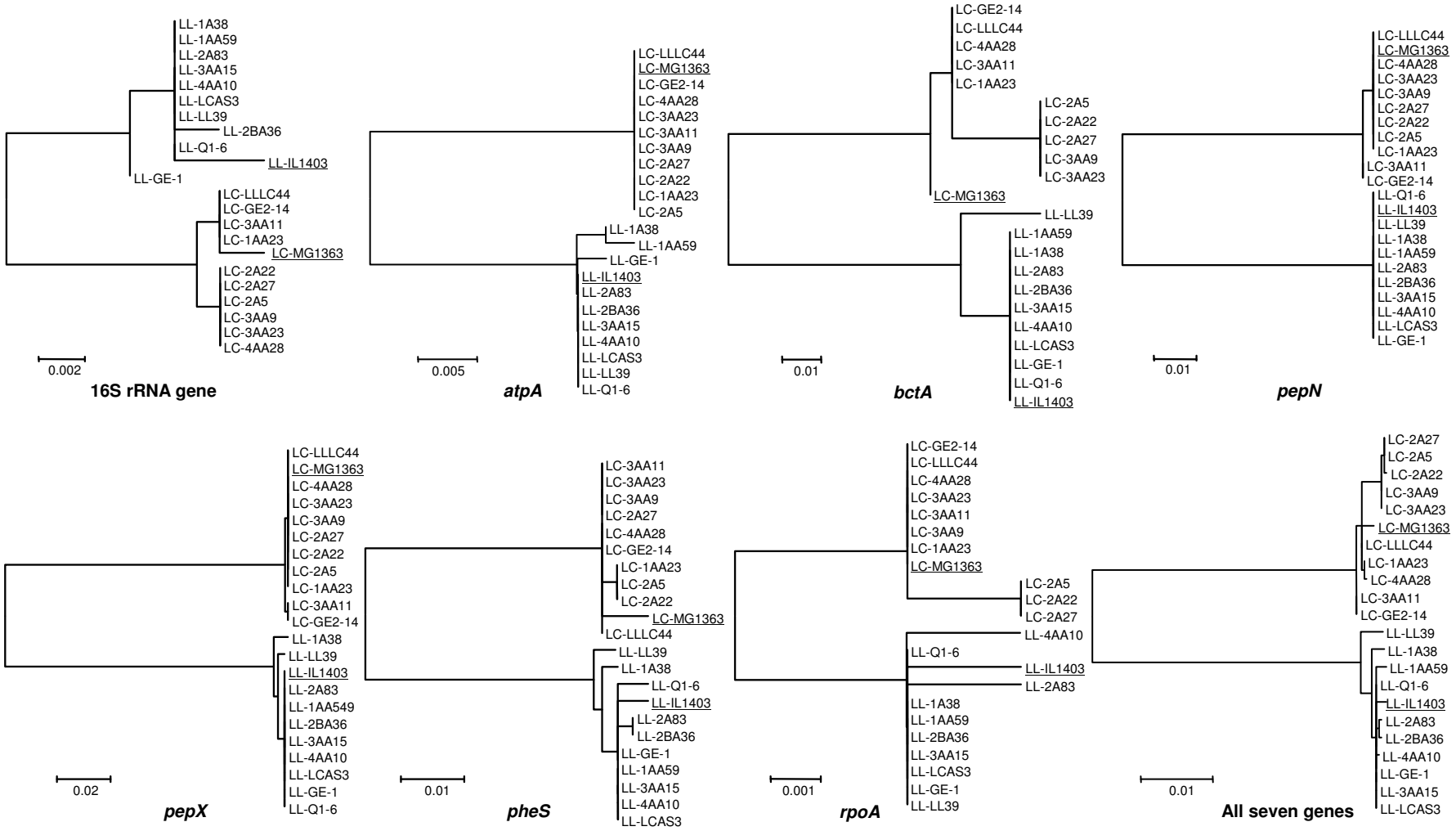
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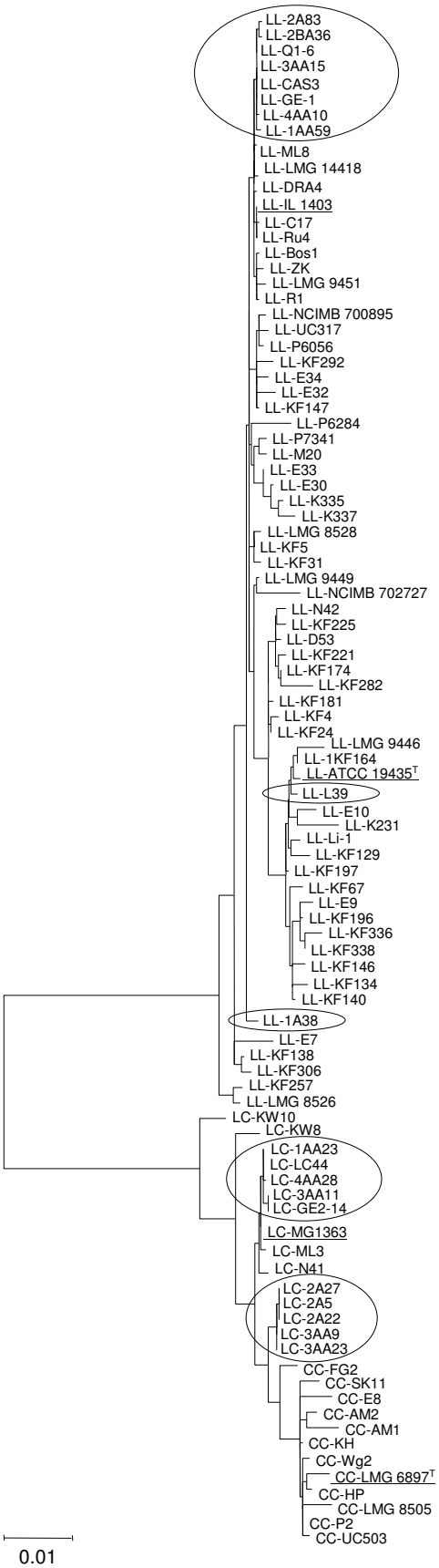
Figure 2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LC-3AA11
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LC-2A22
LC-3AA9
LC-3AA23
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CC-SK11
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CC-AM1
CC-KH
CC-Wg2
CC-LMG 6897T
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CC-LMG 8505
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CC-UC503
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Figure 3

FIGURE LEGENDS

Figure 1.- PFGE patterns of SalI-digested genomic DNA from *L. lactis* isolated from starter-free cheeses made of raw milk of *lactis* (Panel A) and *cremoris* (Panel B) genotypes. Order Panel A: Lines 1-10, *L. lactis* genotype *lactis* 1AA59, CAS3, L39, 3AA15, 2BA36, 1A38, 2A83, GE-1, Q1-6, and 4AA10. Order Panel B: Lines 11-20, *L. lactis* genotype *cremoris* 1AA23, 2A22, 2A27, 2A5, 3AA23, 3AA11, 3AA9, LC44, GE2-14, and 4AA28. IL 1403, *L. lactis* genotype *lactis* IL 1403; MG 1363, *L. lactis* genotype *cremoris* MG 1363; Ma and Mb, Low Range and Lambda ladder PFGE markers (New England Biolabs). Below the panels, dendogram of similarity of their respective profiles expressed by the Sorensen's coefficient. Clustering was performed by the unweighted pair group method using arithmetic averages (UPGMA). Figures in brackets after strains' code indicate their line in the gels.

Figure 2.- Neighbour-Joining cluster analysis of individual partial DNA sequences of the genes coding for 16S rRNA and the housekeeping protein-encoding genes *atpA, rpoA, bacT, pepN, pepX*, and *pheS* from 10 *L. lactis* of *lactis* genotype and 10 of *cremoris* genotype wild strains. As well as a seven-loci multi locus sequence typing (MLST) analysis based on the composite data set of the seven genes. Bootstrap percentage (μ50) after 500 simulations are shown for singly and composite sequence analyses. Sequences of the genome-sequenced *L. lactis* IL 1403 (genotype *lactis*) and MG 1363 (genotype *cremoris*) strains were used as a control and appeared underlined. Letters LL- and LC- in front of the

strains' code stands for *L. lactis* subsp. *lactis* having a *lactis* (LL-) or *cremoris* (LC-) genotype, respectively.

Figure 3.- Diversity analysis of the wild *L. lactis* subsp. *lactis* of *lactis* and *cremoris* genotypes studied in this work, compared with *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* strains of dairy (starter) and non-dairy origin (Rademaker et al., 2007). Neighbour-Joining cluster analysis of a seven-loci multi locus sequence typing (MLST) analysis based on a composite data set of partial DNA sequences of the 16S rRNA gene, *atpA, rpoA, bacT, pepN, pepX*, and *pheS*. For sequence analysis, bootstrap percentages (μ50) after 500 simulations are shown. Letters LL-, LC-, and CC- in front of the strains' code stands for *L. lactis* subsp. *lactis* of *lactis* (LL-) and *cremoris* (LC-) genotypes, and *L. lactis* subsp. *cremoris* (CC-). Codes of the strains of this study appear enclosed in ovals on the diagram; type strains of both subspecies, *L. lactis* subsp. *lactis* ATCC 19435^T (=CECT 185^T) and *L. lactis* subsp. *cremoris* LMG 6897^T (=CECT 967^T), and *L. lactis* laboratory strains IL 1403 (genotype *lactis*) and MG 1363 (genotype *cremoris*) are underlined.