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Diversity and evolution of the microbial populations during manufacture and ripening of Casín, a traditional Spanish, starter-free cheese made from cow's milk

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ABSTRACT

Classical culturing and denaturing gradient gel electrophoresis (DGGE) techniques have been used for studying the microbial diversity and dynamics of the traditional Spanish Casín cheese during manufacturing and ripening. As with other starter-free cheeses made from raw milk, the microbial diversity of Casín was shown to be high by both culturing and DGGE analyses. The culture technique showed that lactic acid bacteria (LAB) species constituted the majority of the microbial populations. Of the 14 bacterial species identified, *Lactococcus garvieae* was predominant in the three day-old cheese sample, although it was replaced by *Lactococcus lactis* subsp. *lactis* at day 30. As expected, the DGGE profiles obtained were complex, consisting, depending on the sample, in five to ten different amplification bands. Among these, a band corresponding to *Streptococcus thermophilus* was observed throughout the whole manufacturing process. This species had never been identified from traditional Spanish cheeses previously. Culturing and molecular methods showed high populations of undesirable microorganisms, arguing for a required improvement in the hygiene of Casín manufacture. Random amplification of polymorphic DNA (RAPD) profiling suggested that the *L. garvieae and L. lactis* populations were composed of one and five strains, respectively. In addition, only a single *L. lactis* RAPD pattern was stably maintained from day three through to day 30, indicating high succession of strains along ripening. After a thoroughly characterisation, strains of the two *Lactococcus* species could be used in designing specific starter cultures for Casín. Additional species (such as *Lactobacillus plantarum* and *Corynebacterium variabile*) might be included as adjunct cultures.

1. Introduction

Among the large list of Asturian Principality (Northern Spain) traditional cheeses, Casín, which wears a Protected Designation of Origin (PDO) label as of May 2008, is probably the one with more originality and typicity. Documents referring to this type of cheese date back as far as the XIII century, suggesting that it is amongst the oldest traditional cheeses in Spain. It is manufactured without a starter culture from raw cow's milk of the Casina breed (a kind of Scottish Highlander) in a small rural area surrounded by mountains. In such a secular isolated environment, manufacturers have maintained their traditional process through the ages. Figure 1 shows a detailed diagram of the manufacturing process of the cheese. In short, Casín cheese is still made by a mixed enzymatic (mostly) and acid curling of evening and morning milk mixtures at 35ºC. The coagulum is then cut into hazelnut-like grains, which are allowed to drain in a cheese cloth for 2–3 days. Then, salting is carried out by applying coarse salt to the cheese surface. Typical of Casín manufacture is a weekly manual kneading, which is maintained up to the end of ripening (Figure 1). Consequently, the cheese has no crust and its cylindrical or semi-spherical shape (12–15 cm diameter, 5–7 cm height) is formed by hand during the final kneading. At this point the upper surface is decorated for marketing by a wooden manufacturers' stamp.

As microbial studies on Casín have never been performed, the microbial typing of the cheese may serve the purpose of both evaluating its hygienic conditions and aiding in the design of specific starter and/or adjunct cultures. These starters are those respecting all technologically-relevant microorganisms found in traditional cheeses and their relative proportions (Parente and Cogan, 2004). The use of such cultures would insure to reproduce the

fermentation in a reliable manner, while preserving to some extent the typical intense flavour of the traditional cheeses (Albenzio et al., 2001).

In addition, interest in the microbiota of raw milk cheeses and other traditional dairy products is further maintained by a recognised need for new LAB strains to complement or replace those currently in-use industrial strains (Hansen, 2002; Wouters et al., 2002). Traditional dairy products harbour a huge recognized reservoir of phenotypic and genetic microbial diversity, which may have many potential biotechnological applications (Wouters et al., 2002; Topisirovic et al., 2006; van Hylckama Vlieg et al., 2006). Traits of LAB species of particular interest to the dairy industry include: bacteriophage resistance (Madera et al., 2003), production of antimicrobial substances (de Vuyst and Leroy, 2007) and unique flavour-forming potential (Ayad et al., 2001; Smit et al., 2005). At present, LAB strains are also analysed for their probiotic properties (Collado et al., 2007) and ability to form bioactive compounds (Siragusa et al., 2007; Guglielmetti et al., 2008). Strains with improved or new properties may be useful to fulfil the needs of traditional fermentations or be used for the formulation of new functional dairy products.

The microbial characterisation of dairy ecosystems is currently performed by using conventional culturing and culture-independent molecular techniques, as they both give complementary results (Giraffa and Neviani, 2001). Among the latter techniques, the denaturing gradient gel electrophoresis (DGGE) tracks compositional changes in the microbial communities via sequence-specific separation of PCR-amplified fragments (Muyzer et al., 1993). This technique has been used to characterise the microbial diversity in many dairy environments (Ercolini et al., 2001; Cocolin et al., 2002; Lafarge et al., 2004; Ogier et al., 2004). Moreover, it has also been used to follow the microbial population dynamics

90 throughout manufacture and ripening of several traditional cheeses (Coppola et al., 2001;
91 Randazzo et al., 2002; Ercolini et al., 2004; Flórez and Mayo, 2006).
92 The aim of the present study was to analyse the microbial diversity of major and indicator
93 populations of traditional Casín cheese and their evolution through manufacturing and
94 ripening by culturing and DGGE. For a complete microbial description of the cheese,
95 predominant microbial species detected by the two techniques were further identified by
96 molecular methods.
97
98 **2. Material and Methods**
99
100 *2.1. Sampling conditions*
101 Two batches of Casín cheese were made by two independent and geographically separated
102 manufacturers in June 2007. Milk, curd and cheese at three, seven, 15 and 30 days of ripening
103 were sampled according to FIL-IDF standard 50B and transported to the laboratory under
104 refrigerated conditions.
105
106 *2.2. Microbial counts*
107 Ten gram samples of milk, curd and cheese were homogenised with 90 ml of a 2% (w/v)
108 sodium citrate solution at 45ºC in a Colworth Stomacher 400 (Seward Ltd., London, UK) (for
109 3 x 1 min). Serial 10-fold dilutions were made in Maximun Recovery Diluent (Scharlau,
110 Barcelona, Spain) and plated in duplicate on to general and selective media.
111 2.2.1. Total Aerobic mesophilic

Aerobic mesophilic bacteria were grown on Plate Count Milk Agar (PCMA; Merck, Darmstadt, Germany) and enumerated after 72 h of incubation at 30ºC. Counts of total aerobic mesophilic bacteria were done on PCMA, Brucella Agar (BA, Merck) and Blood Agar (BLA, Merck) after 72 h of incubation in aerobiosis, microaerophilia, and anaerobiosis at 30ºC.

2.2.2. Lactococci

Lactococci were grown on M17 agar (Scharlau) and enumerated after 48 h of incubation at 32ºC.

2.2.3. Lactobacilli

Lactobacilli were grown on de Man, Rogosa and Sharpe agar (MRSA; Merck), adjusted to pH 5.4 and enumerated after 72 h of incubation at 32ºC in a Hera Cell 2400 (Thermo Fisher Scientific Inc., Waltham, Ma., USA).

2.2.4. Leuconostocs

Leuconostocs were grown on de Man, Sharpe and Elliker agar (MSEA; Biokar Diagnostics, Beauvais, France) containing 30 μg/mL vancomycin (Sigma) to inhibit lactococci and enterococci, and enumerated after five days of incubation at 25ºC.

2.2.5. Enterococci

Enterococci were grown on Slanetz and Bartley agar (S-BA; Merck) and enumerated after 24 h of incubation at 44ºC.

2.2.6. Enterobacteria and coliforms

Enterobacteria and coliforms were grown on Violet Red Bile Glucose agar (VRBGA) and Violet Red Bile Lactose agar (VRBLA) (both from Merck) respectively, using the pour-plate and overlay technique. In short, dilutions were mixed with 15 ml of agar and poured onto Petri

dishes. After solidification, a second agar layer of 10 ml was added. Bacteria were enumerated after 24–48 h of incubation at 37ºC.

2.2.7. Staphylococci

Dilutions were grown on Baird-Parker agar (B-PA; Merck) supplemented with egg yolk tellurite solution (Merck) and black colonies with, or without, egg yolk clearing were recorded after 24 h of incubation at 37ºC.

2.2.8. Yeasts and moulds

Dilutions of milk, curd and cheese samples were plated on Yeast-Extract Glucose Chloramphenicol agar (YGCA; Merck) and yeasts and moulds were enumerated after 3–5 days of incubation at 25ºC.

2.3 Chemical analysis

Standard FIL-IDF methods were used to determine basic chemical parameters. FIL-IDF Standards 21B and 4A were followed for examining total solids in milk and cheese respectively. pH was measured according to FIL-FID Standard 104A, and the NaCl and protein content was measured according to FIL-FID Standards 88A and 20B respectively. The water activity (a_w) was measured in duplicate using an AquaLab apparatus (Decagon Devices Inc., Pullman, Wa., USA).

2.4. Molecular identification of lactic acid bacteria

One hundred and eighty colonies from the PCMA, BA and BLA agar plates were purified by subculturing on the same media and pure cultures were stored frozen at -80ºC until analysis. Cultures were recovered in the corresponding media and isolated colonies were

157 suspended in milliQ water and heated for 10 min at 98ºC. After centrifugation for 10 min at
158 13,000 x g, cell free extracts were used as a source of DNA template to amplify a segment of
159 the 16S rRNA gene by the polymerase chain reaction (PCR) technique. The PCR primers
160 used, 27FYM (5'-AGAGTTTGATYMTGGCTCAG-3') and 1492R (5'-
161 GGTTACCTTGTTACGACTT-3'), were based on conserved regions of the 16S rRNA gene.
162 Amplicons were purified to remove unincorporated primers and nucleotides using Microcon
163 PCR filters (Millipore, Bedford, Ma., USA) and sequenced by cycle extension in an ABI 373
164 DNA sequencer (Applied Biosystems, Foster City, Ca., USA) with primer 27FYM. An
165 average of 850 bp were obtained per sequence and compared with those in the GenBank
166 database using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) and with those in
167 the Ribosomal Database Project database (http://rdp.cme.msu.edu/index.jsp). Sequences with
168 a percentage of identity of 97% or higher were allocated to the same species (Stackebrandt and
169 Goebel, 1994; Palys et al., 1997).
170
171 *2.5. Typing of Lactococcus spp. strains*
172 A representative number of *Lactococcus lactis* (45) and *Lactococcus garvieae* (25) isolates
173 were grouped by RAPD analysis using primer BoxA2R (5'-
174 ACGTGGTTTGAAGAGATTTTCG-3'), as reported by Koeuth et al. (1995). Total genomic
175 DNA was prepared by using a commercial kit (GenElute™ Bacterial GenomiC DNA; Sigma
176 Chemical Co., St. Louis, Miss., USA). The similarity of the patterns was expressed by the
177 Spearman moment correlation coefficient. Clustering was performed by the unweighted pair
178 group method using arithmetic averages (UPGMA).

179

180 *2.6. DGGE analysis*

181 2.6.1. Extraction of DNA from cheese samples

182 Homogenised milk, curd and cheese samples in 2% sodium citrate were used for isolation
183 of total microbial DNA. DNA extraction was accomplished essentially as described by
184 Ercolini et al. (2003) but with the following modification: cheese homogenates were treated
185 with pronase (2.5 mg/ml) (Sigma) for 1 h at 37 ºC before lysis of the cells.

186 2.6.2. PCR amplification

187 DNA was used as a template in PCR amplification of the V3 region of the bacterial 16S
188 rRNA gene by using the universal primers F357 (5'-TACGGGAGGCAGCAG-3' to which a
189 39 bp GC sequence was linked to give rise to GC-F357) and R518 (5'-
190 ATTACCGCGGCTGCTGG-3') (Muyzer et al., 1993). The D1 domain of the 26S rRNA
191 fungal gene was amplified by using the primers GC-NL1 (5'-
192 GCCATATCAATAAGCGGAGGAAAAG-3') and LS2 (5'-
193 ATTCCCAAACAACTCGACTC-3') (Cocolin et al., 2002). PCR was performed in 50 μL
194 volumes containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP,
195 0.2 mM of the primers, 1.5 U of Taq-polymerase (Roche Diagnostics, Barcelona, Spain) and
196 100 ng of extracted DNA. Amplification conditions of prokaryotic and eukaryotic sequences
197 were as described by Muyzer et al. (1993) and Cocolin et al. (2002), respectively.

198 2.6.3. Electrophoresis conditions

199 DGGE was performed using a DCode apparatus (Bio-Rad, Richmond, Ca., USA) at 60ºC
200 and 8% polyacrylamide gels with a denaturing range of 40–60% for bacteria and 30–50% for
201 fungi. Electrophoresis was carried out at 75 V for 17 h and at 130 V for 4.5 h for bacterial and

fungal amplifications, respectively. Bands were visualised after staining with 0.5 μg/mL ethidium bromide (Sigma).

2.6.4. Identification of DGGE bands

DNA bands in the polyacrylamide gels of the commonest species (*L. lactis, L. plantarum*) were assigned to species by comparison with a control ladder of known strains (Flórez and Mayo, 2006). All others were ascribed to species by sequencing and comparison of the sequences as detailed above, after isolation of DNA from the bands and reamplification with the same primers without the GC-clamps.

3. Results

3.1 Basic microbial and chemical parameters of Casín cheese

The basic microbial and chemical properties of two distinct batches of traditional Casín cheese made from raw milk by independent producers were analysed through manufacturing and ripening. Microbial and chemical values of the two batches were combined because we were more interested in discovering canonical aspects of Casín manufacture than in finding differences. Table 1 shows the composition and evolution of the predominant and indicator populations, while chemical gross composition is summarised in Table 2. Surprisingly, counts of the different microbial populations were rather similar between batches (Table 1), as shown by the low standard deviations; coliforms being the most variable population. Counts of total aerobic bacteria reached the highest value at around day seven, as did counts of lactococci, with maximal populations of around 10^9 colony forming units (cfu) per g of cheese. Initial

numbers of lactobacilli were around 10^3 cfu/g and attained their highest level at day 30 (near 10^9 cfu/g). Dextran-producing leuconostoc reached maximum numbers at day 15 ($2.75x10^6$ cfu/g). Numbers of hygienic-indicator populations were high throughout the whole process, reaching their highest levels (averaging $3.15x10^6$ cfu/g) from day seven to day 30, depending on the population. Of note was the continued growth of coliforms (which matched the population of *Enterobacteriaceae* from day seven onwards) until the end of ripening. Although numbers of staphylococci were also high, strains of *Staphylococcus aureus* were never detected in the B-PA counting plates. The population of yeasts reached a maximum level at day 15 (10^7 cfu/g), while moulds were never detected (detection limit two Log_{10} units lower than the corresponding yeast counts). Regarding chemical parameters (Table 2), normal trends during ripening were observed for most variables. In agreement with the highest population of lactococci at day seven, pH was the lowest at this time-point, increasing slowly thereafter. As humidity decreases during ripening, so the level of salt in moisture increases. Although this, the final content of salt in moisture was relatively low (2.30% at day 30). The water activity (a_w) also decreases through ripening, but the microbial growth is not compromised even at its lowest level (0.96).

3.2. Microbial diversity and dynamics of Casín cheese by DGGE

Samples of curd and cheese at days three, seven, 15 and 30 of ripening of the two batches were analysed by DGGE. In the ripened cheese, separated samples of the cheese interior and cheese surface (a rind of 0.3–0.5 cm) were examined. As an example, the results obtained for one of the batches are presented in Figure 2. Between five and ten different bands corresponding to the prokaryotic V3 variable region of the 16S rRNA were observed in the

247 distinct samples (Figure 2A). In total, 14 different bands were encountered, of which 13 were
248 identified by either comparison to bands from control strains or by isolation, reamplification,
249 sequencing and comparison against sequences in databases. The most prominent band in all
250 samples was that of *L. lactis* (band i). Two other bands were also present during both
251 manufacture and ripening; these corresponded to *Streptococcus parauberis* (band h) and
252 *Streptococcus thermophilus* (band k). In samples from curd and 3 day-old cheese, a weak band
253 was observed in the upper part of the gel, which was identified as *L. garvieae* (band a). Bands
254 corresponding to *Lactobacillus plantarum* (bands b) and *Enterococcus faecium* (band d) were
255 clearly visible in curd and the three- and seven-day old cheese samples. At around day seven,
256 five bands appeared, which corresponded to *Streptococcus uberis/Streptococcus iniae* (band
257 f), *Enterobacter* spp. (band l), *Corynebacterium variabile* (band m), and *Lactobacillus*
258 *casei/Lactobacillus paracasei* (bands n). Finally, a band matching the sequence of
259 *Macrococcus caseolyticus* (band e) was identified in the sample corresponding to the cheese
260 surface at day 30 (line 6 in Figure 2A). Similarly, nine bands were observed for the yeast D1
261 variable domain of the 26S rDNA (Figure 2B); these corresponded to only four species, as the
262 sequences of five bands matched those of a single species, *Geotrichum candidum* (bands a),
263 and two bands belonged to *Kluyveromyces lactis/Kuyveromyces marxianus* (bands b).
264 Furthermore, a faint band present in the seven day-old sample related to *Saccharomyces*
265 species (band c) and a weak band identified as *Trichosporum gracile* (band d) was observed
266 from day seven onwards.
267 Bacterial and yeast DGGE profiles of cheeses from the second producer were shown to be
268 highly similar, and a majority of identified bands coincided in the two batches. The exception
269 was the presence of a prominent band corresponding to *Acinetobacter johnsonii* in the three

day-old cheese sample from the second producer. Similarly, the bands of *K. lactis/K. marxianus* were more prominent and those of *G. candidum* were weaker (data not shown).

3.3. Microbial diversity and dynamics of Casín cheese by culturing

In order to maximize the recovery of microorganisms from Casín, which would improve the microbial description of the cheese, three different culture media (PCMA, BA, and BLA) and three different culture conditions (aerobiosis, microaerophilia, and anaerobisis) were assayed. Although statistically not significant, BA and BLA showed higher recovery numbers than PCMA from two seven day-old cheese samples of two independent batches, particularly under anaerobic conditions (data not shown).

Three- and 30-day old cheese samples from one of the producers were inoculated in these three media and incubated anaerobically at 30ºC for 72 h. These two sampling points were considered essential, since they roughly correspond to the end of acidification and the time at which the cheese is marketed. In total, 180 colonies (86 from day three and 94 from day 30) isolated from the different media were purified by subculturing and identified by molecular methods, as reported. The results are summarised in Table 3. Isolates of 14 different microbial types were detected, of which 11 could be identified to the species level. Despite different recovery rates, dominant species were detected in all three media; except for *Staphylococcus saprophyticus* and *Lb. plantarum*, which were only isolated from BA and BLA plates. The small number of isolates of most species makes it difficult to ascertain whether they have preferential recovery in the distinct media used.

L. garvieae was shown to be the dominant species at day three (46 isolates), followed by *L. lactis* subsp. *lactis* (15 isolates), *Staph. saprophyticus* (12 isolates) and *Klebsiella* spp. (7

293 isolates). The species distribution in this sample contrasts with that found in the mature cheese
294 (30 day-old sample), in which *L. lactis* isolates were dominant (82 isolates), followed by small
295 numbers of *Lb. plantarum* (5 isolates) and *Micrococcus luteus* (two isolates).
296 To assess the intra-species diversity, a representative group of *L. lactis* (45) and *L.*
297 *garvieae* (25) isolates were analysed by the RAPD typing technique. As *L. lactis* came from
298 both the three-day (15 isolates) and 30-day samples (30 isolates), the RAPD analysis may also
299 serve to address the evolution and/or stability of the *L. lactis* population during Casín ripening.
300 A single RAPD profile was obtained with primer BoxA2R for all *L. garvieae* isolates,
301 indicating that the acidification process was dominated by one strain. In contrast, eight distinct
302 RAPD patterns were found among the *L. lactis* isolates (Figure 3). Some of them resulted to
303 be related (Figure 3B), but as differences are shown in prominent bands (Figure 3A) they
304 could still belong to different strains. RAPD profiles from day three are different to those from
305 day 30, suggesting a certain degree of strain evolution. However, two isolates from day three
306 (Figure 3A, line 3) and three isolates from day 30 (Figure 3A, line 6) showed identical
307 patterns, which indicates that some *L. lactis* strains might be well adapted to the whole cheese
308 making process.
309
310 **4. Discussion**
311 The sensorial properties of cheeses depend on a large number of factors, among which the
312 qualitative and quantitative microbial composition is paramount (Smit et al., 2005). Microbial
313 types further determine hygienic conditions and shelf-life (Guinane et al., 2005). Thus, control
314 of the microorganisms through manufacturing and ripening is thought to be essential in
315 cheese-making. Not surprisingly, modern cheese manufacture relies upon pasteurisation and

316 the deliberate addition of carefully selected microorganisms. Depending on the main function,
317 added microorganisms are referred to as starters or primary cultures (if they participate in the
318 initial acidification) and adjunct, maturing or secondary cultures (if they influence flavour,
319 aroma and maturing activities) (Parente and Cogan, 2004). Primary and secondary cultures are
320 mainly composed of well-characterised strains of LAB species.

321 In this study, the basic microbial and chemical properties of two independent batches of
322 Casín cheese made by its traditional technology were analysed during manufacture and
323 ripening. Small differences were observed between batches in most variables measured, which
324 may reflect variations in uncontrolled environmental conditions, as well as differences in milk
325 composition and microbial load and composition among batches from the two producers. A
326 certain level of variation is typical of most artisan products, particularly in cheeses made from
327 raw milk without the addition of starters cultures (Poznansky et al., 2004; Flórez et al., 2006;
328 Randazzo et al., 2006; El-Baradei et al., 2007; Dolci et al., 2008).

329 Both conventional culturing and DGGE analysis were used in this work for the microbial
330 characterisation of Casín cheese. The combined use of culturing and culture-independent
331 techniques for the typing of complex microbial environments, including those of traditional
332 food fermentations, has been found to supply complementary data, as shown by the results
333 obtained in this work and those reported by others (Randazzo et al., 2002; Poznansky et al.,
334 2004; Flórez and Mayo, 2006). Therefore, the use of both approaches is considered more
335 comprehensive for a full description of the microbial populations in these environments. The
336 microbial diversity found using both techniques in Casín cheese was similar. At least 14
337 different bacterial types were determined from the 180 colonies identified from the culture
338 plates, and twelve distinct bands were identified by the DGGE technique. However, as

339 repeatedly reported for other cheeses (Randazzo et al., 2002; Ercolini et al., 2003; Ercolini et
340 al., 2004; Flórez and Mayo, 2006; El-Baradei et al., 2007), discrepancies in the
341 microorganisms detected by culture-dependent and culture-independent methods were also
342 noted. These differences could be attributed to some of the limitations of these two techniques.
343 On one hand, the presence of bacterial types in viable but not cultivable states and an
344 excessive selectivity of some media can cause a poor recovery of certain microorganisms by
345 culturing. On the other hand, differential lysis of the microbial populations, presence of
346 amplifiable DNA from dead microorganisms and differential amplification of some sequences
347 can bias the molecular culture-independent results.
348 The bacterial and fungal species detected by culturing and DGGE in Casín cheese have all
349 previously been isolated from dairy-related environments including traditional cheeses
350 (Randazzo et al., 2002; Ercolini et al., 2003; Ercolini et al., 2004; Flórez and Mayo, 2006; El-
351 Baradei et al., 2007). Despite this, the microbial characterisation of Casín cheese has provided
352 many differences in microbial composition and evolution as compared to other traditional
353 cheeses. It was surprising to find *L. garvieae* as the dominant species during acidification. In
354 agreement with culturing data, a noticeable (but diffuse) band corresponding to *L. garvieae*
355 was observed by DGGE in samples of curd and three day-old cheese (band a in Figure 2A).
356 This band, however, was absent in all other subsequent cheese samples; in accordance again
357 with culturing. *L. garvieae* is a well-recognised fish pathogen (Eyngor et al., 2004), and has
358 also been retrieved from subclinical mastitis in water buffalos (Teixeira et al., 1996) and from
359 many clinical human specimens (Fefer et al., 1998). Recently, *L. garvieae* has further been
360 reported as a common component of the autochthonous microbiota of dairy products
361 manufactured from raw milk (Fortina et al., 2007). Furthermore, DGGE analysis of Casín

362 from different producers has unambiguously determined the presence of *L. garvieae* strains in
363 the cheese milk (unpublished results). *L. garvieae* isolates from different sources have proven
364 to be genetically unrelated (Foschino et al., 2008), suggesting that niche-driven adaptations
365 allow this species to develop and persist in diverse environments. The study of several dairy
366 strains and their comparison to pathogenic counterparts has shown that the former do not
367 usually harbour virulence determinants (Fortina et al., 2007). It can therefore be deduced that
368 the presence of *L. garvieae* strains in artisan cheeses do not pose a serious health hazard. In
369 agreement, consumption of Casín and other similar cheeses has never been associated with a
370 food-borne disease. Furthermore, *L. garvieae* dairy strains have been found to present a series
371 of desirable technological properties and some authors propose the use of characterised strains
372 as part of the starter culture (Fortina et al., 2007), provided the absence of virulence factors
373 and pathogenicity has been unequivocally determined. *L. garvieae* cheese isolates have been
374 shown to present a slow rate of acidification (Fortina et al., 2007), but this is comparable to
375 wild lactococcal isolates from other cheeses (Delgado et al., 2002).
376 At day three, *L. lactis* isolates (15 isolates) constitute less than 18% of the dominant
377 population, while more than 53% of the microorganisms are *L. garvieae*. However, *L. lactis*
378 strains are clearly dominant at day 30, at which time only a single *L. garvieae* isolate was
379 found. This replacement in populations suggests that *L. garvieae* strains are more susceptible
380 to the stressful conditions (acidity, low temperature) of ripening. In this study, only one of the
381 batches was sampled by culturing, which raises the question of whether the data are
382 representative. However, the agreement between culturing analysis of one batch and DGGE
383 analysis of the two batches indicates that the data are likely to be typical.

384 Four different RAPD profiles were observed among the *L. lactis* isolates at day three and
385 five profiles were observed at day 30 (Figure 3). One of the profiles was present in the two
386 samples (day three and day 30), suggesting that at least some strains persist throughout
387 manufacture and ripening. High genetic variability in lactococcal strains from traditional
388 cheeses has been reported elsewhere (Corroler et al., 1998; Mannu et al., 2000; Delgado and
389 Mayo, 2004). In order to include unrelated strains in the design of specific starter cultures,
390 strains presenting early and late RAPD patterns will be selected. Less genetic variability was
391 observed in this study among the *L. garvieae* isolates, which showed a single RAPD profile
392 only. Although this, two clearly distinct strains could be distinguished by phenotypic tests
393 (unpublished data).
394 Of note from our findings is the presence of a DGGE band corresponding to *S.*
395 *thermophilus,* which was visible in the two batches analysed in this study and in batches from
396 other producers (data not shown). This species has never been isolated from traditional
397 Spanish cheeses (Cogan et al., 1997). The cultivation conditions used in this work (30ºC, 72 h)
398 did not allow *S. thermophilus* to form visible colonies on counting media after 72 h
399 incubation. Work is currently in progress to selectively isolate this species from Casín.
400 Also of interest is the presence of micrococci, staphylococci, microbacteria and
401 corynebacteria species within the cheese matrix, which might be a consequence of the
402 repeated kneading of the cheese mass, internalising surface-associated bacteria. Species from
403 these groups have recently been shown to dominate the surface microbial composition of
404 smear-ripened cheeses (Mounier et al., 2005), where they develop in higher numbers than
405 those attained by deliberately inoculated of commercial cultures (Goerges et al., 2008). The
406 typical flavour of Casín cheese is strong, pungent and spicy, indicative of a strong lipolysis.

Lipolysis may result from the action of native milk enzymes liberated from the fat globule during kneading, but it can further be enhanced by the action of microbial lipases. Strains of these species may certainly be useful as adjunct and maturing cultures.

The presence of high numbers of coliforms, enterococci and related organisms is also typical of cheeses made from raw milk. Species of these microbial types have been detected by both culturing and culture-independent techniques in this and many other raw milk cheeses (Poznansky et al., 2004; Flórez et al., 2006; Dolci et al., 2008). These populations are considered as indicators of faecal contamination and therefore also indicate poor manufacturing practices. The high counts observed in this work of species supposed to be opportunistic pathogens (such as *Staph. saprophyticus* and *Klebsiella* spp.), reinforces the need for improvement in hygiene conditions throughout Casín manufacture. However, it is worth noting that, as shown in Table 3, these undesirable microorganisms are not found among the major populations at day 30.

The results of this study present the first data on the microbial composition of Casín cheese and the dynamics of microbial diversity throughout ripening. A large collection of microorganisms have been gathered from two critical steps within the cheese manufacturing process (the end of acidification and ripened cheese). The technological characterisation of such isolates should permit the selection of appropriate strains for specific starter and adjunct cultures, which may be of help for standardisation and improvement of the overall cheese quality and safety.

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Table 1.- Average microbial counts (in Log_{10} cfu per g or mL) and standard deviation of diverse microbial groups along manufacturing and ripening stages of two independent batches of Casín cheese.

Microbial group (counting medium)	Stage of manufacturing or ripening					
	Milk	**Curd**	**3 day**	**7 day**	**15 day**	**30 day**
Total aerobic counts (PCA)	5.42±0.42	6.42±0.57	8.50±0.11	9.06±0.20	8.84±0.38	8.65±0.20
Lactococci (M17A)	5.01±0.38	6.40±0.57	8.58±0.07	8.93±0.20	8.74±0.32	8.54±0.19
Lactobacilli (MRSA, pH 5.4)	3.00±0.25	3.54±0.31	5.12±0.13	6.19±0.04	8.63±0.11	8.80±0.16
Leuconostoc (MSEA)	nd[a]	nd	3.02±0.02	4.58±0.11	6.44±0.33	6.24±0.52
Enterococci (S-BA)	3.20±0.27	4.91±0.57	4.23±0.15	5.71±0.17	6.56±0.10	6.33±0.21
Staphylococci (B-PA)	3.67±0.52	5.27±0.15	5.62±0.10	6.48±0.31	5.97±0.11	6.07±0.30
Enterobacteriaceae (VRBGA)	5.18±0.19	6.12±0.26	5.79±0.14	6.01±0.19	6.41±0.2	6.52±0.36
Coliforms (VRBLA)	3.73±0.80	5.15±1.20	5.54±0.09	6.13±0.10	6.36±0.18	6.47±0.47
Yeasts and moulds (YGCA)	nd	nd	3.38±0.26[b]	6.13±0.33	7.00±0.12	6.79±0.17

[a]nd, not detected; detection limit Log_{10} 2.0
[b]These numbers correspond to yeasts, as moulds were never recorder (detection limit two Log_{10} lower than that of yeast counts).

Table 2.- Average gross composition and physicochemical parameters of two independent batches of Casín cheese throughout manufacturing and ripening.

Chemical parameter	Stage of manufacturing or ripening					
	Milk	Curd	3 day	7 day	15 day	30 day
Total Solids (%)	11.28±0.87	35.37±0.56	53.19±0.83	55.28±1.29	58.47±1.80	61.85±1.35
Fat (%)	4,35±1.34	20.54±0.63	29.16±2.28	30.94±2.19	31.25±2.33	32.98±2.25
Total Protein (%)	3.30±0.53	12.07±0.46	19.30±0.65	21.68±0.58	24.18±0.28	25.14±0.41
pH	6.64±0.13	6.38±0.10	5.22±0.16	5.17±0.02	5.23±0.09	5.25±0.21
Salt in moisture (%)	0.13±0.03	0.18±0.04	1.65±0.07	1.68±0.04	1.85±0.09	2.29±0.12
a_w	0.999±0.01	0.997±0.02	0.993±0.02	0.987±0.03	0.983±0.02	0.962±0.03

Table 3.- Majority microorganisms identified from Casín samples of 3 and 30 day old cheeses isolated in three different culture media.

Species[a]	Stage of manufacturing and media of isolation						Total
	3 day old cheese			30 day old cheese			
	PCA	BA	BLA	PCA	BA	BLA	
Lactococcus lactis subsp. *lactis*	1	9	5	24	24	34	**97**
Lactococcus garvieae	17	20	9		1		**47**
Staphylococcus saprophyticus		3	9				**12**
Klebsiella spp.	3	3	1				**7**
Lactobacillus plantarum					1	4	**5**
Escherichia coli	1	1					**2**
Micrococcos luteus						2	**2**
Streptococcus spp.			1	1			**2**
Corynebacterium variabilis					1		**1**
Flavobacterium spp.			1				**1**
Leuconostoc mesenteroides			1				**1**
Microbacterium oxydans				1			**1**
Musa acuminata				1			**1**
Staphylococcus pasteuri		1					**1**
Total	**22**	**37**	**27**	**27**	**27**	**40**	**180**

[a]Isolates were all identified by partial amplification and sequencing of their 16S rRNA genes. Identical homology to two or more species impeded in some cases the accurately ascription of isolates to a specific species.

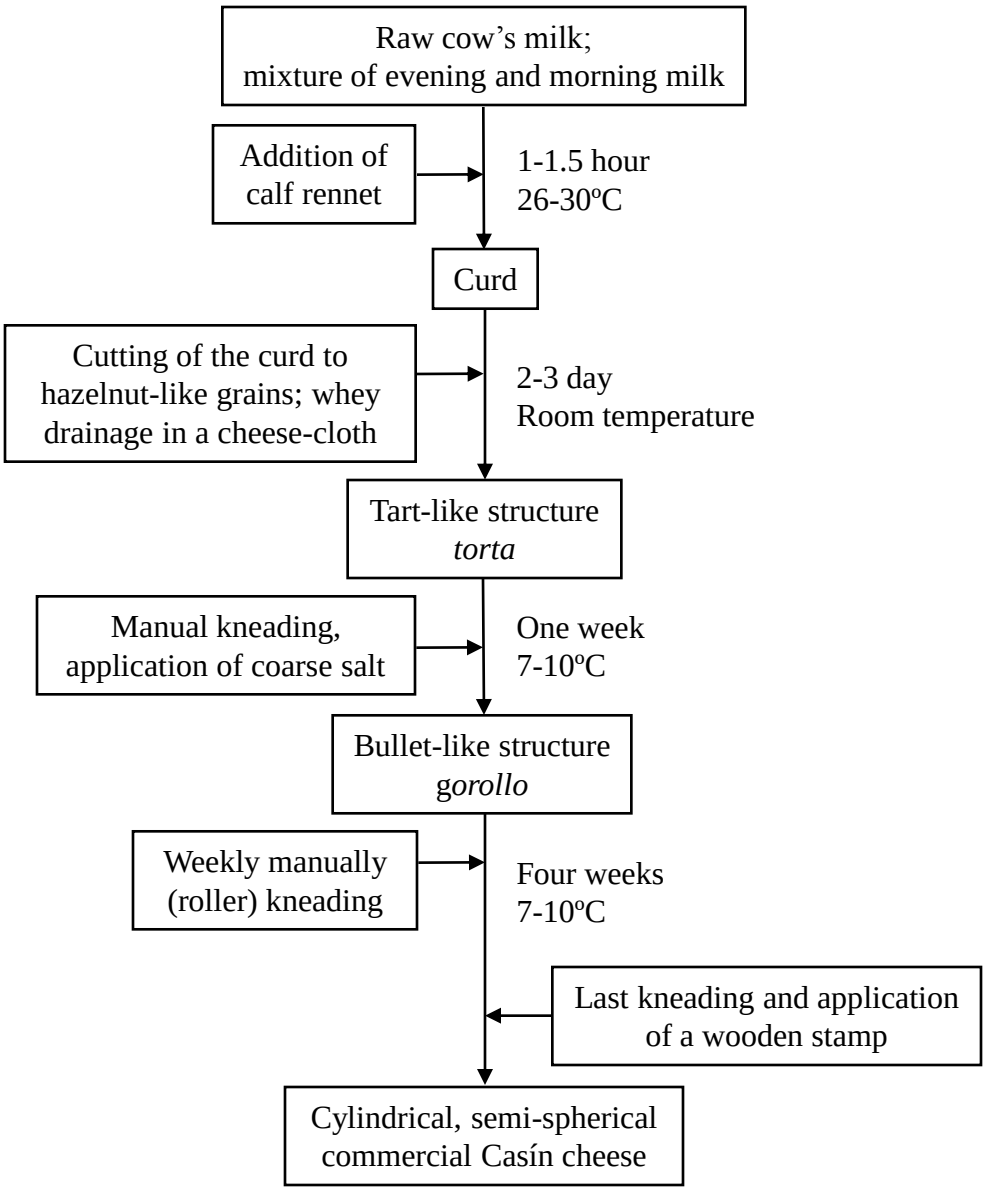
Raw cow's milk;
mixture of evening and morning milk
Addition of calf rennet
1-1.5 hour
26-30ºC
Curd
Cutting of the curd to hazelnut-like grains; whey drainage in a cheese-cloth
2-3 day
Room temperature
Tart-like structure
torta
Manual kneading, application of coarse salt
One week
7-10ºC
Bullet-like structure
gorollo
Weekly manually (roller) kneading
Four weeks
7-10ºC
Last kneading and application of a wooden stamp
Cylindrical, semi-spherical commercial Casín cheese

Figure 1

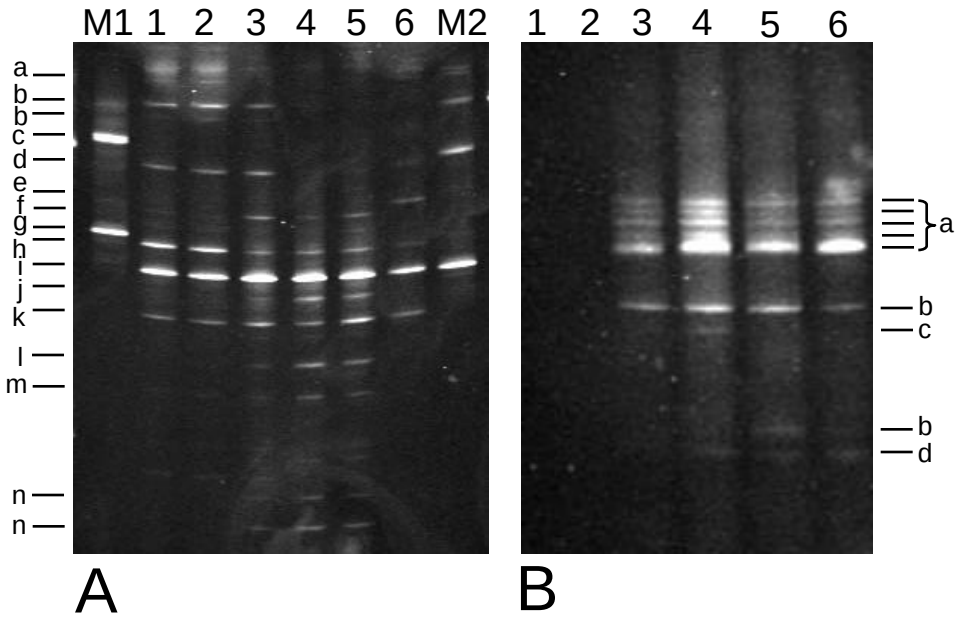

Figure 2

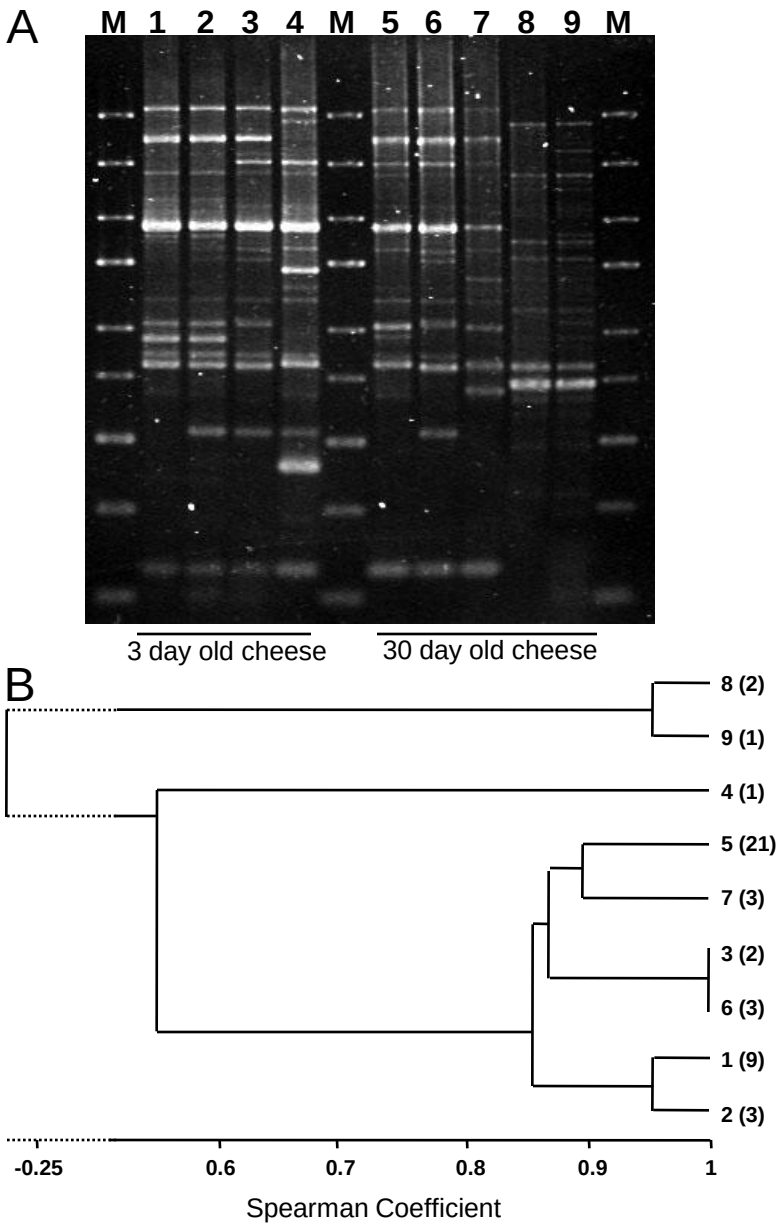
A
M 1 2 3 4 M 5 6 7 8 9 M
3 day old cheese
30 day old cheese
B
8 (2)
9 (1)
4 (1)
5 (21)
7 (3)
3 (2)
6 (3)
1 (9)
2 (3)
-0.25
0.6
0.7
0.8
0.9
1
Spearman Coefficient

Figure 3

FIGURE LEGENDS

Figure 1.- Flow scheme of the manufacturing process of Casín cheese. Approximate duration of manufacturing steps and temperature through the process is indicated. Words in italics are local terms for the successive forms of the cheese during ripening.

Figure 2. DGGE profiles of microbial populations from Casín cheese during manufacturing and ripening. Samples: 1, curd; 2, 3, 4, and 5, cheeses of 3, 7, 15 and 30 days of ripening; 6, cheese surface at day 30. **Panel A:** DGGE profiles of the V3 variable region of the bacterial 16S rRNA gene. M, combined amplicons of identified strains used as a control: M1, *Leuconostoc citreum* (c), *Lactobacillus brevis* (g); M2, *Lactobacillus plantarum* (b), *Enterococcus faecium* (d), *Lactococcus lactis* (i). Key of identified sequences different to those from the controls: a, *Lactococcus garvieae;* e, *Macrococcus caseolyticus;* f, *Streptococcus uberis/Streptococcus iniae;* h, *Streptococcus parauberis;* j, unidentified band; k, *Streptococcus thermophilus;* l, *Enterobacter* spp.; m, *Corynebacterium variabile;* n, *Lactobacillus casei/Lactobacillus paracasei.* **Panel B:** DGGE profiles of PCR amplicons of the eukaryotic domain D1 of 26S rDNA. Key of identified sequences**:** a, *Geotrichum candidum;* b, *Kluyveromyces lactis/Kluyveromyces marxianus*; c, *Saccharomyces* spp.; d, *Trichosporon gracile.*

Figure 3. Genotypic relationships among the *Lactococcus lactis* isolates from Casín cheese at day three (end of acidification) and day 30 (ripened cheese). **Panel A:** Distinct rapid amplification polymorphic DNA (RAPD) patterns obtained by PCR of 45 *L. lactis* isolates with primer BoxA2R (Koeuth et al., 1995). M, 100 bp molecular weight ruler (Bio-Rad, Richmond, CA., USA). **Panel B:** Dendogram of similarity of the RAPD patterns of all 55 strains clustered by the UPGMA method using the Spearman coefficient. In parenthesis, number of isolates having identical RAPD profiles.