1 Microbial characterisation and stability of a farmhouse natural fermented milk

2 from Spain

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This work reports the microbial characterization of a farmhouse natural fermented milk (NFM) with good sensorial properties produced in Spain. Culturing and denaturing gradient gel electrophoresis (DGGE) analyses showed that *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* (approximate levels of 10^9 cfu ml^{-1}) were dominant in this NFM, while *Lactobacillus plantarum* appeared at a lower level (10^6-10^7 cfu ml^{-1}). Repetitive extragenic palindromic (REP)-PCR typing of the isolates identified single strains each of *Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris* and *Lb. plantarum*. These three strains formed a stable microbial association which has been maintained for at least some decades.

Key words: Natural fermented milk, lactic acid bacteria, traditional dairy products, *Lactococcus lactis* subsp. *lactis, Lactococcus lactis* subsp. *cremoris*, *Lactobacillus plantarum*

34 INTRODUCTION

35 Milk can be consumed in its fluid form or transformed into a variety of different products,
36 of which fermented milks are among the most important (de Ramesh et al. 2006; Robinson
37 and Tamime 2007). The first fermented milks were produced by accident via the
38 development –under serendipitously appropriate conditions– of indigenous lactic acid
39 bacteria (LAB). It is difficult to establish when the purposeful practice of fermenting milk
40 began, but it can be assumed that it was soon after the first human populations settled in the
41 Middle East some 15,000 years ago (Robinson and Tamime 2007; Tamime 2002). The
42 production of natural fermented milk (NFM) from raw milk later spread all over the world.
43 Evidence of NFMs can still be found in large areas of Africa, Middle East, Asia, and even
44 in Europe, such as *ergo* from Ethiopia, *amasi* (also known as *hodzeko* and *mukaka*
45 *wakakora*) from Zimbabwe, *roub* from The Sudan, *rayeb, lben, laban, kad, zabady* and *zeer*
46 from the Magreg, and *filmjölk* and *långfil* from Sweden (Kosikowski and Mistry 1997;
47 Robison and Tamime 2007; Tamang 2010). Traditional NFMs (such as *leite callado* and
48 *lleche presa*) are also still produced in rural areas of North-western Spain at a farmhouse
49 scale. In fact, more than 400 generic names of NFMs are registered throughout the world
50 (Kurmann et al. 1992), although the number of distinct varieties may be shorter (Robinson
51 and Tamime 1990; Robinson and Tamime 2007; Tamime 2002).

52 NFM relies on the growth of mesophilic LAB species, which lower the pH and
53 produce the most typical sensorial compounds of the products (FAO/WHO 2003). Two
54 different classes of NFM can be distinguished: inoculated and non-inoculated (Kosikowski
55 and Mistry 1997; Robinson and Tamime 2007). Non-inoculated NFMs are made by leaving
56 the raw milk at room temperature until it becomes sufficiently acidic for the coagulum to
57 appear. Inoculated NFMs are manufactured by adding a portion of a previous NFM batch to

58 a new milk substrate (backslopping). In either case, *Lc. lactis* strains are among the
59 dominant microbiota (Gonfa et al. 2001; Mathara et al. 2004; Patrignani et al. 2006). In
60 traditional products manufactured from raw milk it is also common to find species of
61 mesophilic lactobacilli such as *Lb. plantarum* and *Lactobacillus casei/Lactobacillus*
62 *paracasei*, as well as *Leuconostoc*, *Enterococcus* and *Pediococcus* species (Gonfa et al.
63 2001; Mathara et al. 2004; Patrignani et al. 2006). In warm climates, other lactobacilli such
64 as *Lactobacillus helveticus*, *Lactobacillus fermentum* and/or *Lactobacillus acidophilus* may
65 also develop. Moderate to high (up to 10^8 cfu g^{-1}) numbers of yeast species are also usually
66 present in NFMs (Benkerroum and Tamime 2004; Gadaga et al. 2000; Gonfa et al. 2001);
67 different types of yeast may cause its spoilage or enhance its flavour. The dominant yeast
68 species include *Saccharomyces cerevisiae, Kluyveromyces marxianus* and *Candida*
69 *lusitaniae* (Benkerroum and Tamime 2004; Gadaga et al. 2000). Micrococci, coliforms and
70 pathogens (*Staphylococcus aureus*, *Bacillus cereus*) are occasionally found in NFMs
71 (Gonfa et al. 2001), stressing the need for improving the microbial safety of these products.
72 NFMs can also be manufactured from pasteurised and/or sterilized milk (at both the
73 artisanal and industrial scale), which renders products safer. Industrial NFMs are inoculated
74 with acidifying and aromatic starter cultures, while artisanal products are usually inoculated
75 via backslopping techniques. Such transfers impose conditions that select strongly for
76 strains that grow rapidly in milk and that show strong resistance to high levels of lactic
77 acid.

78 This study reports the microbial characterization and development of a stable,
79 farmhouse NFM with good sensorial properties and commercial potential, produced by a
80 number of families in northwestern Spain. The original batch of NFM was of uncertain
81 origin, but the current product was the result of backslopping inoculation of sterilised UHT-

82 treated milk every three to five days followed by incubation at room temperature (20-25ºC)
83 for 18-24 h. Once coagulated, the milk was stored at 7ºC for consumption until the
84 manufacture of a new batch. The NFM was maintained for several years in the household
85 from which it was acquired without losing its original activity or sensorial properties.
86 Following acquisition and continued production at the laboratory using the same artisanal
87 methods, samples were analysed at 0, 3, 6, 12 and 15 months. The stable sensorial
88 properties of the product suggested the presence of a stable microbial community with
89 potential use as an industrial starter.
90
91 MATERIAL AND METHODS
92 **Sampling conditions**
93 Six batches of the NFM were sampled at the time of consumption (one to three days after
94 production) according to IDF Standard 50B (IDF, 1985) and transported to the laboratory
95 under refrigerated conditions. The pH of the milk before and after fermentation was
96 measured according to IDF Standard 104A (IDF, 1984). Duplicate culturing analyses were
97 performed on the day of sampling. For the isolation of DNA, samples were stored at –20ºC
98 until required.
99
100 **Microbial analyses**
101 Ten ml of NFM were homogenised with 90 ml of a 2% (w/v) sodium citrate solution at
102 45ºC in a Colworth Stomacher 400 (Seward Ltd., London, UK) (for 3 x 1 min). Ten-fold
103 serial dilutions were made in Maximum Recovery Diluent (Scharlau, Barcelona, Spain) and
104 plated in duplicate on general and selective media as follows.
105

106 *Aerobic mesophilic bacteria*

107 Aerobic mesophilic bacteria were enumerated on plate count agar with 1% skimmed milk

108 (PCAM; Merck, Darmstadt, Germany) after 72 h incubation under aerobiosis at 30ºC.

109

110 *Lactococci*

111 Lactococci were grown on M17 agar (M17A, Scharlau) and enumerated after 48 h

112 incubation at 30ºC.

113

114 *Lactobacilli*

115 Lactobacilli were grown on de Man, Rogosa and Sharpe agar (MRSA; Merck) adjusted to

116 pH 5.4, and enumerated after 72 h incubation at 32ºC in a 5% CO_2 atmosphere in a Hera

117 Cell 2400 incubator (Thermo Fisher Scientific Inc., Waltham, Ma., USA).

118

119 *Leuconostoc* spp.

120 Dextran-producing leuconostocs were grown on Mayeux, Sandine and Elliker agar (MSEA;

121 Biokar Diagnostics, Beauvais, France) and enumerated after five days incubation at 25ºC.

122

123 *Enterococci*

124 Enterococci were grown on Slanetz and Bartley agar (SBA; Merck) and enumerated after

125 24 h incubation at 44ºC.

126

127 *Enterobacteria and coliforms*

128 Enterobacteria and coliforms were grown on violet red bile glucose agar (VRBGA) and

129 violet red bile lactose agar (VRBLA) (both from Merck), respectively, using the pour-plate

130 and overlay technique. Dilutions were mixed with 15 ml of agar and poured onto Petri
131 dishes. After solidification a second agar layer of 10 ml was added. Bacteria were
132 enumerated after 48 h incubation at 30ºC.
133
134 *Staphylococci*
135 Dilutions were grown on Baird-Parker agar (BPA; Merck) supplemented with egg yolk
136 tellurite solution (Merck). Black colonies with or without egg yolk clearing were recorded
137 after 24 h incubation at 37ºC.
138
139 *Yeasts and moulds*
140 Dilutions of acidified milk samples were plated on yeast extract glucose chloramphenicol
141 agar (YGCA; Merck) and yeasts and moulds enumerated after 3–5 days incubation at 25ºC.
142
143 **Molecular identification**
144
145 *Molecular identification of bacteria*
146 From the different NFM samples, 104 colonies from the M17 (54), MRS (39) and MSE
147 (11) agar plates were purified by subculturing on the same medium from which they were
148 collected. Pure cultures were stored frozen at –80ºC until analysis. Total genomic DNA
149 from isolates was purified from overnight cultures using the GenElute Bacterial Genomic
150 DNA kit (Sigma-Aldrich Inc., St. Louis, Mo., USA) following the manufacturer's
151 recommendations. Total DNA from isolates was employed as a template to amplify a
152 segment of the 16S rRNA gene via the polymerase chain reaction (PCR) using the universal
153 prokaryotic primers S-D-Bact-0008-a-S-20 (27F) (5'–AGAGTTTGATCCTGGCTCAG–

154 3’) and S-*-Univ-1492R-b-A-21 (1492R) (5’–GGTTACCTTGTTACGACTT–3’). PCR
155 was performed in 50 µl volumes containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$,
156 0.2 mM of each dNTP, 0.2 mM of the primers, 1.5 U of Taq-polymerase (Ampliqon,
157 Skovlunde, Denmark) and 100 ng of extracted DNA. Purified amplicons were digested with
158 *Hae*III and *Hha*I restriction enzymes (Invitrogen Ltd., Paisley, UK) and electrophoresed in
159 2% agarose gels. These were visualised with ethidium bromide (0.5 µg ml^{-1} (Sigma-
160 Aldrich) and photographed under UV light.
161
162 *Molecular identification of yeasts*
163 Cell-free extract of yeasts, obtained by suspending a colony in water, boiling for 10 min
164 and centrifugation, were used as a template in PCR reactions to amplify a segment of the
165 eukaryotic rRNA operon encompassing the 5.8S rRNA gene and the flanking internal
166 transcribed spacers ITS1 and ITS2 using primers ITS4 (5’–
167 TCCTCCGCTTATTGATATGC–3’) and ITS5 (5’–GGAAGTAAAAGTGCTAACAAGG–
168 3’). The PCR conditions used were those reported by White et al. (1990).
169
170 *Sequencing and comparison of sequences*
171 Selected amplicons of bacteria and yeasts were purified in GenElute PCR Clean-Up
172 columns (Sigma-Aldrich) and sequenced by cycle extension in an ABI 373 DNA sequencer
173 (Applied Biosystems, Foster City, Ca., USA) using primer 27F or ITS5, respectively. On
174 average, 800 bp were obtained per sequence. These were compared with those in the
175 GenBank database using the BLAST program (National Center for Biotechnology
176 Information, 2009) and with those in the Ribosomal Database Project (RDP, 2009).

177 Sequences with a percentage similarity of 97% or higher were allocated to the same species
178 (Palys et al. 1977; Stackebrandt and Goebel 1994).
179
180 **Typing of isolates**
181 Isolates were grouped by repetitive extragenic palindromic PCR (REP-PCR) typing using
182 primer BoxA2R (5'–ACGTGGTTTGAAGAGATTTTCG–3'), as reported by Koeuth et al.
183 (1995). REP-PCR products were purified and electrophoresed in 1% agarose gels as above.
184
185 **Denaturing gradient gel electrophoresis (DGGE)**
186
187 *Extraction and purification of DNA.*
188 Homogenised NFM samples in 2% sodium citrate were used for isolation of total microbial
189 DNA. DNA extraction was accomplished using a commercial kit (QIAamp DNA Stool
190 Mini Kit; Quiagen, GmbH, Hilden, Germany) following the manufacturer's
191 recommendations. To confirm and quantify the bacterial populations identified by DGGE
192 in the NFM, DGGE analyses were also made of the total DNA extracted from enrichment
193 cultures (on M17 and MRS) of the 10-fold dilutions used for enumeration purposes. The
194 cells were pelleted by centrifugation and total microbial DNA isolated as reported above
195 for the purified cultures.
196
197 *PCR amplification*
198 DNA was used as a template in PCR amplification of the V3 region of the bacterial 16S
199 rRNA gene using the universal primers F357 (5'–TACGGGAGGCAGCAG–3'), to which a
200 39 bp GC sequence was linked to give rise to GC-F357, and R518 (5'–

201 ATTACCGCGGCTGCTGG–3') (Muyzer et al. 1993). The D1 domain of the 26S rRNA
202 fungal gene was amplified using primers GC-NL1 (5'–
203 GCCATATCAATAAGCGGAGGAAAAG–3') and LS2 (5'–
204 ATTCCCAAACAACTCGACTC–3') (Cocolin et al. 2002). The amplification conditions
205 for prokaryotic and eukaryotic sequences were those described by Muyzer et al. (1993) and
206 Cocolin et al. (2002), respectively.
207
208 *Electrophoresis conditions*
209 DGGE was performed using a DCode apparatus (Bio-Rad, Richmond, Ca., USA) at 60ºC
210 and employing 8% polyacrylamide gels with a denaturing range of 40–60% for bacteria and
211 30–50% for fungi. Electrophoresis was performed at 75 V for 17 h and at 130 V for 4.5 h
212 for bacterial and fungal amplifications respectively. Bands were visualised after staining
213 with 0.5 μg ml^{-1} ethidium bromide.
214
215 *Identification of DGGE bands*
216 DNA bands in the polyacrylamide gels were assigned to species by comparison with a
217 control ladder of known strains (Flórez and Mayo 2006), or, following isolation of DNA
218 from the bands and reamplification with the same primers without the GC-clamps, by
219 sequencing and comparison of the sequences as described above.
220
221 RESULTS
222 The pH of the NFM samples ranged from 4.1 to 4.4. Table 1 shows counts for the majority
223 and indicator microbial populations at the six sampling points, together with a diagram

224 indicating the origin of the different samples analysed. The total cultivatable aerobic counts
225 in PCMA matched those obtained in M17A for all six samples. This indicates that
226 lactococci were the dominant population, reaching around 1.0 x 10^9 cfu ml^{-1}. Lactobacilli
227 numbers were usually two logarithmic units lower than those for lactococci, although
228 sample-to-sample variations were noted (Table 1). Dextran producing leuconostocs were
229 occasionally observed at the very limit of detection (below 10^3 cfu ml^{-1}). No staphylococci,
230 enterococci, enterobacteria or coliforms were detected in any of the samples, except for
231 small numbers of the last three groups at the 6 month sample (t=6). In addition, a
232 homogeneous yeast population was recorded at 6 and 12a month samples, with counts of
233 2.8 and 5.0 x 10^5 cfu ml^{-1}, respectively.

234 Due to the relatively simple microbial composition of the samples, the DGGE profiles
235 were also expected to be rather simple. In fact, these varied between one or two bands. A
236 patent band corresponding to *Lc. lactis* was always present, while that of *Lb. plantarum*
237 was barely visible at time zero and 6 months. Figure 1 shows the DGGE results obtained at
238 3 months (t=3), in which its DGGE profile is shown in line 1. As for the NFM, the profiles
239 obtained from the cultures of the 10-fold dilutions involved either one or two bands.
240 Cultures from the MRS plates (growth was up to the –3 dilution) gave a single band which
241 migrated to the position of the *Lb. plantarum* control band. In contrast, profiles from the
242 enrichment cultures grown on M17A showed two bands corresponding to *Lb. plantarum*
243 and *Lc. lactis* up to the –3 dilution, followed by a band for *Lc. lactis* alone for the –4, –5
244 and –6 dilutions (Figure 1).

245 No DGGE profiles for eukaryotic organisms were recorded at time zero or at 3, 12b,
246 and 15 months, while two bands whose sequence showed identity to both *Kazachstania*

247 *unispora* and *Saccharomyces servazzii* were recorded at 6 and 12a months (data not
248 shown).

249 For the microbial characterization of the NFM, 104 colonies from the M17A (54),
250 MRSA (39) and MSEA (11) plates isolated from the six time points were purified by
251 subculturing, and identified by molecular methods. All colonies were subjected to PCR
252 amplification of the 16S rRNA genes with primers 27F and 1492R, followed by amplified
253 ribosomal DNA restriction analysis (ARDRA) with the restriction enzymes *Hae*III and
254 *Hha*I. Only two distinct ARDRA profiles were obtained with either *Hae*III or *Hha*I. As an
255 example, Figure 2 shows the profiles obtained with *Hae*III. All isolates from the M17A
256 plates gave the profiles depicted in Figure 2A, while Figure 2B shows that recorded for
257 isolates from the MRSA plates. Two isolates from the MSEA plates showed an ARDRA
258 profile identical to that shown by isolates from the M17A plates, while the remaining nine
259 showed a profile identical to that shown by the cultures from MRSA plates. These results
260 strongly suggest that the majority populations of the NFM recorded in M17A and MRSA
261 plates were represented by a small number of bacterial species. Sequencing of 15
262 randomly-chosen amplicons of colonies isolated from the three media identified these as
263 *Lc. lactis* and *Lb. plantarum* respectively. It is noteworthy that for *Lc. lactis*, members of
264 both the *lactis* and *cremoris* subspecies were detected during analysis of the sequences.
265 Indeed, *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* genotypes were represented as
266 two distinct colony morphotypes on the M17A plates. This allowed the differential
267 enumeration of *lactis* and *cremoris* subspecies in all NFM samples analysed. The two
268 subspecies were encountered in every sample, with dominance alternating between the
269 *lactis* and *cremoris*; e. g., percentages of *Lc. lactis* subsp. *lactis* ranged from 11-66%.

270 At 6 and 12a month samples, four yeast colonies each were identified using the
271 eukaryotic-specific primers ITS4 and ITS5. As expected from the single morphotype
272 observed on the enumeration plates, all sequences analysed corresponded to a single
273 species –*K. unispora* (formerly known as *Saccharomyces unisporus*).
274 All 94 bacterial isolates were subjected to REP-PCR typing to assess the strain
275 diversity of the *Lb. plantarum, Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris*.
276 Surprisingly, a single REP profile was obtained for each species (Figure 3), which indicated
277 a rather low genetic diversity at the strain level, similarly to that found at the species level.
278
279 DISCUSSION
280 Natural fermentation is one of the oldest methods of extending the shelf life of milk, and it
281 is still widely practised in many parts of the world. This work describes the microbial
282 characterisation of an NFM, analysed at six time points over one and a half years. The
283 acquired NFM of the present study was produced by backslopping inoculation of UHT-
284 sterilised milk, culturing at room temperature for 18-24 h, and storing under refrigeration
285 until use. Depending on consumption, the process was repeated every three to five days.
286 Both the temperature of incubation and the majority microorganisms identified (see below)
287 classify this NFM as of the mesophilic type (Robinson and Tamime 1990). This is probably
288 the largest group of fermented milks, into which fall many traditional products such as
289 cultured buttermilk, filmjölk, långfil, and many ethnic products from Africa, the Middle
290 East and Asia (Benkerroum and Tamime 2004; Beukes et al. 2001; Gadaga et al. 2000;
291 Gonfa et al. 2001; Tamang 2010). The original batch of this NFM could be not traced back.
292 It may well have come from somewhere in the Middle-East or the Balkans, but it has been

293 passed from one family to another in the producing area for more than ten years now, and it
294 is well appreciated for its agreeable sensory properties.

295 The dominant microorganisms on the culture plates were identified by partial ARDRA
296 and sequencing of the ribosomal amplicons, which where then compared against sequences
297 held in public databases. A large population of a single strain each of *Lc. lactis* subsp. *lactis*
298 and *Lc. lactis* subsp. *cremoris* formed the dominant cultivable population in the analysed
299 NFM. Some kind of proto-cooperation might maintain the two *Lc. lactis* strains at similar
300 numbers over consecutive inoculations. A single *Lb. plantarum* strain at around two $\log_{10}$
301 units lower numbers accompanied the two lactococcal strains. The same species were found
302 by the culture-independent technique of DGGE, by which no other organisms were
303 identified even after enrichment of the dilutions in M17 and MRS. These three strains were
304 shown to be present throughout the entire study period (15 months), suggesting they are
305 compatible and well adapted to one another. They also appear to be well adapted to the
306 restrictive conditions imposed by the manufacturing process; cells have to attain high cell
307 densities rapidly and to be resistant to the low pH (4.1-4.3) of the coagulated milk. These
308 restrictive conditions are surely responsible for the low species and strain diversity found in
309 this NFM.

310 *Lc. lactis* –both the *lactis* and *cremoris* subspecies– are among the dominant
311 microbiota of most mesophilic NFM types (Dewan and Tamang 2007; Gonfa et al. 2001;
312 Mathara et al. 2004; Patrignani et al. 2006). This is also the case of *Lb. plantarum,* which
313 has been reported in the literature as a usual component of traditional NFMs manufactured
314 from raw milk (Dewan and Tamang 2007; El-Baradei et al. 2008; Gonfa et al. 2001;
315 Mathara et al. 2004). Growth of these three LAB types to high cell densities during
316 fermentation produces lactic acid from lactose, causing the coagulation of milk when the

317 isoelectric point of the caseins is reached (around pH 4.6). In addition, LAB metabolism
318 modifies milk constituents (protein and fats) through their complex proteolytic and lipolytic
319 systems (Leroy and de Vuyst 2004; Topisirovic et al. 2006). These activities contribute to
320 the final sensorial characteristics of NFMs. Lactic acid further improves stability and safety
321 of NFMs by inhibiting spoilage and pathogenic microorganisms (Topisirovic et al. 2006).
322 A large population of *K. unispora* was observed in the 6 month sample, which was
323 maintained in the subsequent analysis at 12 months (t=12a). This non-lactose fermenting
324 species has been reported a common inhabitant of many dairy products, including kefir and
325 cheese (Callon et al. 2006; Wang et al. 2008). It is unknown how this yeast species entered
326 the present laboratory-produced NFM. At six months, small populations of enterococci and
327 coliforms were also noted, which disappeared at 12 months. All of these 'new
328 microorganisms' may have arise from a contaminated UHT-milk sample. Microbial
329 analysis of the mother NFM sample at 12 and 15 month (t=12b and t=15, respectively) and
330 showed no yeasts, which reinforces the contamination hypothesis. In any event, in the
331 laboratory-made NFM, the yeast seems to become a stable component of the microbial
332 association without perturbing the relationships between the other members or
333 impoverishing the sensorial properties of the NFM.
334

335 CONCLUSION

336 The results of this work provide a microbial characterization of an undefined NFM, the
337 fermentation of which appears to be accomplished by single strains of *Lc. lactis* subsp.
338 *lactis* and *Lc. lactis* subsp. *cremoris,* accompanied by a strain of *Lb. plantarum*. The
339 isolation and characterization of the component strains would allow a specific starter
340 culture to be produced, which would further allow manufacture of this NFM at an industrial

341 scale. This type of manufacture would contribute to the standardisation and marketing of
342 the product while assuring its safety. Due to its high activity and stability, the bacterial
343 combination might additionally be used as a starter culture for the manufacture of cheese
344 and other dairy products. Stable consortia of LAB and/or yeasts with a potential industrial
345 use as starter and/or adjunct cultures may be found in other NFMs.

346

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354

355 REFERENCES

356 Benkerroum N and Tamime A Y (2004) Technology transfer of some Moroccan traditional
357 dairy products (*lben, jben* and *smen*) to small industrial scale. *Food Microbiology* **21**
358 399-413.

359 Beukes E M, Bester B H and Moster J F (2001) The microbiology of South African
360 traditional fermented milks. *International Journal of Food Microbiology* **63** 189-197.

361 Callon C, Delbès C, Duthoit F and Montel M C (2006) Application of SSCP-PCR
362 fingerprinting to profile the yeast community in raw milk Salers cheeses. *Systematic*
363 *and Applied Microbiology* **29** 172-280.

364 Cocolin L, Aggio D, Manzano M, Cantoni C and Comi G (2002) An application of PCR-
365 DGGE analysis to profile the yeast populations in raw milk. *International Dairy*
366 *Journal* **12** 407-411.
367 de Ramesh C C, White C H, Kilara A and Hui Y H (2006) *Manufacturing Yogurt and*
368 *Fermented Milks*. Hoboken: Blackwell Publishing.
369 Dewan S and Tamang J P (2007) Dominant lactic acid bacteria and their technological
370 properties isolated from the Himalayan ethnic fermented milk products. *Antonie van*
371 *Leeuwenhoek* **92** 343-352.
372 El-Baradei G, Delacroix-Buchet A and Ogier A C (2008) Bacterial biodiversity of
373 traditional Zabady fermented milk. *International Journal of Food Microbiology* **121**
374 295-301.
375 FAO/WHO (2003) CODEX Standard for Fermented Milks. Codex Stan 243-2003.
376 Reviewed 2008. http://www.codexalimentarius.net/web/standard_list.jsp.
377 Flórez A B and Mayo B (2006) Microbial diversity and succession during the manufacture
378 and ripening of traditional, Spanish, blue-veined Cabrales cheese, as determined by
379 PCR-DGGE. *International Journal of Food Microbiology* **110** 165-171.
380 Gadaga T H, Mutukumira A N and Narvhus J A (2000) Enumeration and identification of
381 yeast isolates from Zimbabwean traditional fermented milk. *International Dairy*
382 *Journal* **10** 459-466.
383 Gonfa A, Foster H A and Holzapfel W H (2001) Field survey and literature review on
384 traditional fermented milk products in Ethiopia. *International Journal of Food*
385 *Microbiology* **68** 173-186.
386 IDF (1984) Determination of the pH of the serum. Potentiometric method. FIL-IDF
387 Standard104A.

388 IDF (1985) Milk and milk products. Methods of sampling. FIL-IDF Standard 50B.

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

392 Kosikowski F V and Mistry V V (1997) *Cheese and Fermented Milk Foods,* (3rd ed).
393 Connecticut: LLC Westport.

394 Kurmann J A, Rasic J Lj and Kroger M (1992) *Encyclopaedia of Fermented Fresh Milk*
395 *Products*. New York: Van Nostrand Reinhold.

396 Leroy F and de Vuyst L (2004) Lactic acid bacteria as functional starter cultures for the
397 food fermentation industry. *Trends in Food Science and Technology* **15** 67-78.

398 Mathara J M, Schillinger U, Kutima P M, Mbugua S K and Holzapfel W H (2004)
399 Isolation, identification and characterisation of the dominant microorganisms of kule
400 naoto: the Maasai traditional fermented milk in Kenya. *International Journal of Food*
401 *Microbiology* **94** 269-278.

402 Muyzer G, de Waal E C and Uitterlinden A G (1993) Profiling of complex microbial
403 populations by denaturing gradient gel electrophoresis analysis of polymerase chain
404 reaction-amplified genes encoding for 16S rRNA. *Applied and Environmental*
405 *Microbiology* **59** 695-700.

406 National Center for Biotechnology Information (2009) Basic Local Alignment Research
407 Tool (BLAST) URL http://www.ncbi.nlm.nih.gov/BLAST/. Accessed 17/12/2009.

408 Palys T, Nakamura L K and Cohan F M (1997) Discovery and classification of ecological
409 diversity in the bacterial world: the role of DNA sequence data. *International Journal*
410 *of Systematic Bacteriology* **47** 1145-1156.

411 Patrignani F, Lanciotti R, Mathara J M, Guerzoni M E and Holzapfel W H (2006) Potential
412 of functional strains, isolated from traditional Maasai milk, as starters for the
413 production of fermented milks. *International Journal of Food Microbiology* **107** 1-11.
414 RDP (2009) Ribosomal Database Project classifier. URL
415 http://rdp.cme.msu.edu/classifier/classifier.jsp. Accessed 17/12/2009.
416 Robinson R K and Tamime A Y (1990) Microbiology of fermented milks. In *Dairy*
417 *Microbiology,* (2nd ed.), pp 245-278. Robinson R K, ed. London: Applied Sciences
418 Publishers.
419 Robinson R K and Tamime A Y (2007) Types of Fermented Milks. In *Fermented Milks,* pp
420 1-10. Tamime A Y, ed. Hoboken: Wiley InterScience.
421 Stackebrandt E and Goebel B M (1994) Taxonomic note: a place for DNA-DNA
422 reassociation and 16S rRNA sequence analysis in the present species definition in
423 bacteriology. *International Journal of Systematic Bacteriology* **44** 846-849.
424 Tamang J P (2010) *Himalayan Fermented Foods: Microbiology, Nutrition, and Ethnic*
425 *Values.* New York: Taylor and Francis Group.
426 Tamime A Y (2002) Fermented milks: a historical food with modern applications–a review.
427 *European Journal of Clinical Nutrition* **56** S2-S15.
428 Topisirovic L, Kojic M, Fira D, Golic N, Strahinic I and Lozo J (2006) Potential of lactic
429 acid bacteria isolated from specific natural niches in food production and preservation.
430 *International Journal of Food Microbiology* **112** 230-235.
431 Wang S Y, Chen H C, Liu J R, Lin Y C and Chen M J (2008) Identification of yeasts and
432 evaluation of their distribution in Taiwanese Kefir and Viili starters. *Journal of Dairy*
433 *Science* **91** 3798-3805.

434 White T J, Bruns T, Lee S and Taylor J (1990) Amplification and direct sequencing of
435 fungal ribosomal RNA genes for phylogenetics. In *PCR protocols. A Guide to Methods*
436 *and Applications,* pp 315-322. Innis M A, Gelfand, D H, Sninsky J J and White T J,
437 eds. San Diego: Academic Press.

438

439 **Figure Captions**

440

441 **Figure 1.-** DGGE profiles of microbial populations from the NFM at 3 months (lane 1) and
442 those recovered after growth of the corresponding 10-fold dilutions on MRS and M17. M,
443 combined amplicons of identified strains used as a control: Ma, *Lactococcus garvieae* (a),
444 *Lactobacillus plantarum* (b), *Leuconostoc mesenteroides* subsp. *mesenteroides* (c), and
445 *Streptococcus parauberis* (d). Mb, *Enterococcus faecium* (e), *Enterococcus faecalis* (f),
446 *Lactococcus lactis* (g), *Escherichia coli* (h), and *Lactobacillus paracasei* (i).

447

448 **Figure 2.-** Partial ARDRA profiles of 15 colonies isolated from the NFM on M17A (**Panel**
449 **A**) and MRSA (**Panel B**). The 16S rRNA gene was amplified using primers S-D-Bact-
450 0008-a-S-20 (27F) and S-*-Univ-1492R-b-A-21 (1492R) and digested with the restriction
451 enzyme *Hae*III. M, molecular weight marker (GeneRuler1 kbp ladder; Fermentas GmbH,
452 St. Leon-Rot, Germany).

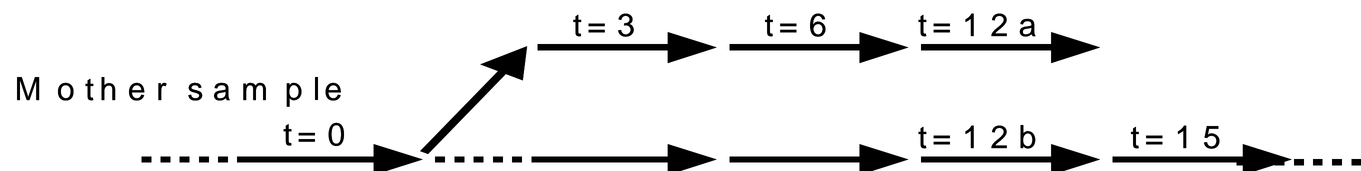
453

454 **Figure 3.-** REP-PCR typing of lactococci and lactobacilli isolates from the NFM with
455 primer BoxA2R. **Panel A.** Lanes 1-8, *Lc. lactis* subsp. *lactis* isolates; lanes 9-15 *Lc. lactis*
456 subps. *cremoris* isolates. **Panel B.** Lane 1-15 *Lb. plantarum* isolates. M, GeneRuler
457 molecular weight marker (Fermentas).

Table 1.- Diagram of sampling and average microbial counts (in Log_{10} cfu per ml) of different microbial groups in the natural fermented milk at four sampling times, and diagram of the sampling.

Microbial group (counting medium)	Sample[a] (month)					
	t=0	t=3	t=6	t=12a	t=12b	t=15
Total aerobic counts (PCAM)	8.90±0.31	8.49±0.42	9.18±0.28	8.65±0.18	9.03±0.35	8.68±0.41
Lactococci (M17A)	8.94±0.16	8.59±0.23	9.10±0.34	8.74±0.10	8.95±0.34	8.76±0.21
Lactobacilli (MRSA, pH 5.4)	7.04±0.36	5.70±0.67	7.40±0.46	5.84±0.68	6.94±0.72	6.54±0.56
Leuconostoc (MSEA)	≤ 3.00[b]	3.25±0.12	≤ 3.00	≤ 3.00	≤ 3.00	3.46±0.15
Enterococci (Slanetz-Bartley, SBA)	≤ 1.00	≤ 1.00	1.90±0.12	≤ 1.00	≤ 1.00	≤ 1.00
Staphylococci (Baird-Parker, BPA)	≤ 1.00	≤ 1.00	≤ 1.00	≤ 1.00	≤ 1.00	≤ 1.00
***Enterobacteriaceae* (VRBGA)**	nd	nd	1.30±0.23	nd	nd	nd
Coliforms (VRBLA)	nd	nd	1.08±0.18	nd	nd	nd
Yeasts and moulds (YGCA)	≤ 1.00	≤ 1.00	4.65±0.46	4.69±0.41	≤ 1.00	≤ 1.00

[a]Two replicates of each sample were analysed, from which counts were analysed in duplicate. Average results and standard deviation are indicated.
[b]The symbol ≤ indicates that colonies with the typical morphology of the microbial groups to be counted were not detected. If these were present, they must have been below the detection limit.
nd, not detected.



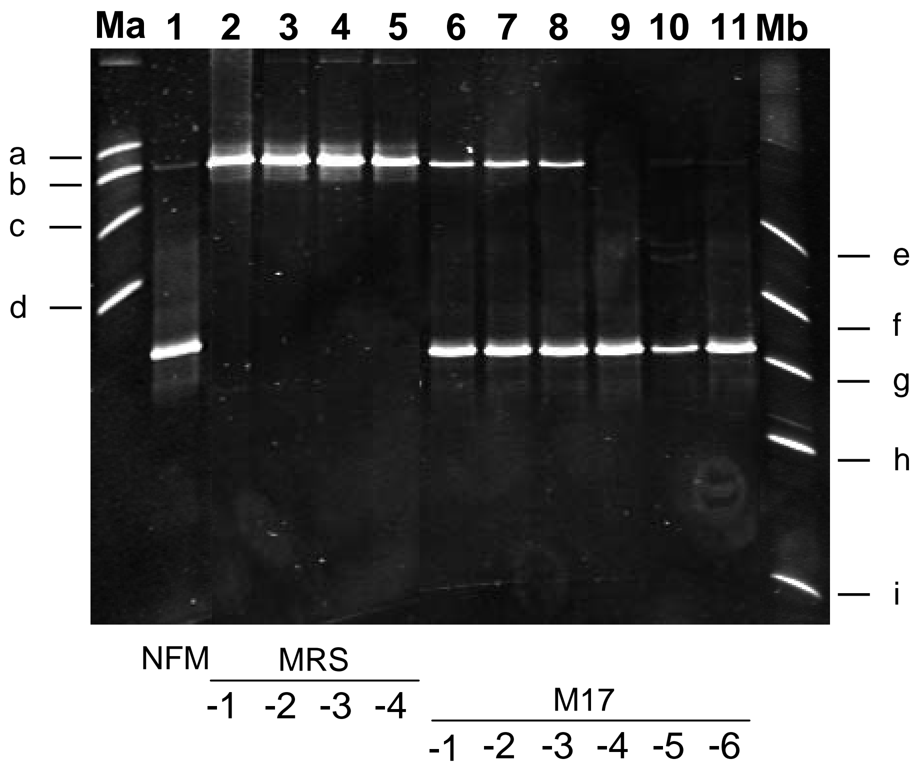


Figure 1

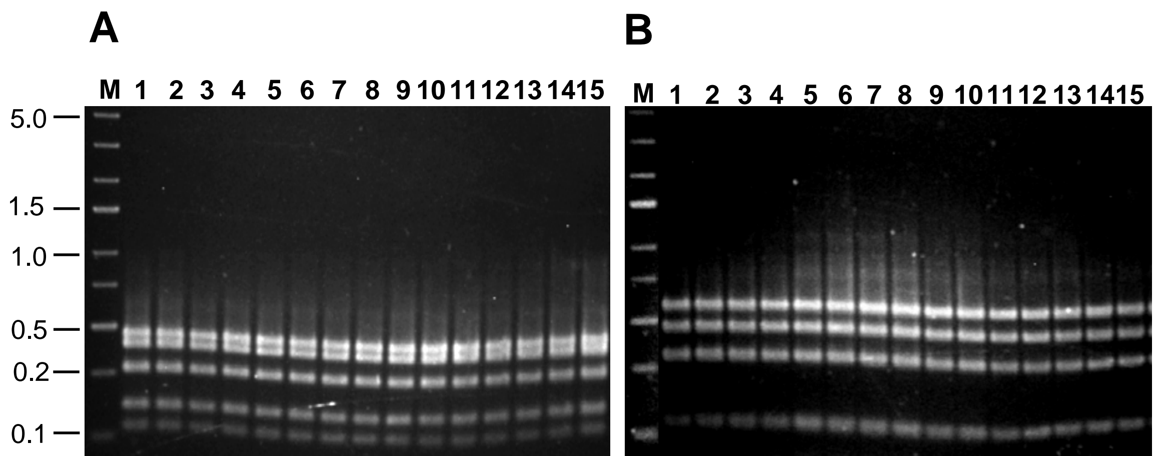


Figure 2

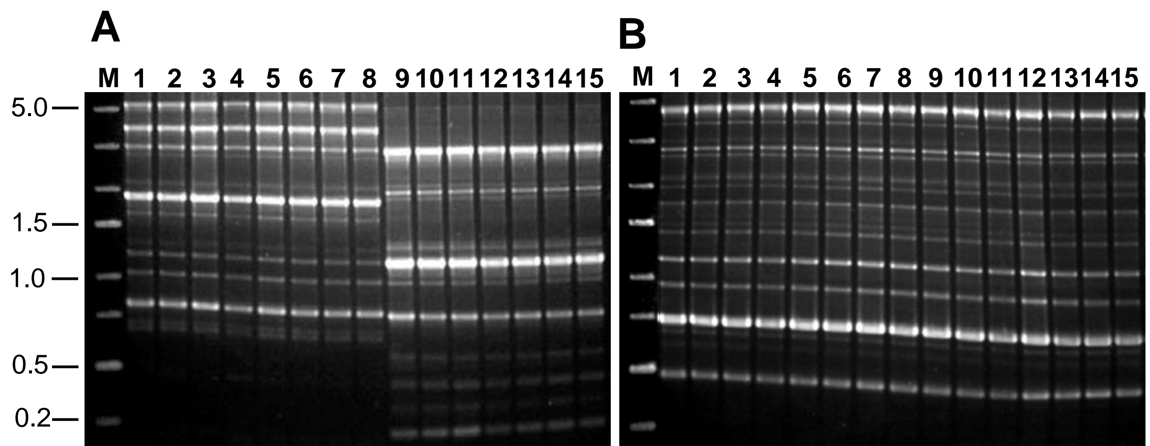


Figure 3