

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

Characterization of Lactococcus strains isolated from artisanal Oaxaca cheese

This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1734855> since 2020-04-02T11:18:35Z

Published version:

DOI:10.1016/j.lwt.2020.109041

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

Manuscript Number: LWT-D-19-04782R1

Title: Characterization of Lactococcus Strains Isolated from Artisanal Oaxaca Cheese

Article Type: Research paper

Keywords: Oaxaca cheese; Lactococcus; acidifying capacity; antibiotic resistance.

Corresponding Author: Dr. Emiliano J Quinto, Ph.D.

Corresponding Author's Institution: Universidad de Valladolid

First Author: Irma Caro

Order of Authors: Irma Caro; Emiliano J Quinto, Ph.D.; Lucía Fuentes; Valentina Alessandria; Luca Cocolin; María P Redondo-Del-Río; Baltasar Mayo; Ana Flórez; Javier Mateo

Abstract: The aim of this work was to identify and characterize lactococci strains from Mexican Oaxaca cheese. A total of 120 autochthonous isolates were obtained from Oaxaca cheese along its production. Cheese samples were collected from three industries in the Tulancingo Valley of Hidalgo State. Twenty lactococci strains were identified and characterized by molecular and phenotypic methods. Isolates were screened, among others, for their acidifying capacity, antibiotic resistance and production of volatile compounds. High phenotypic diversity was observed among the *Lactococcus lactis* spp. isolates and confirmed by rep-PCR fingerprints. Nine of the 20 strains reached a pH below 5.0 in milk and they were considered as fast fermenting strains. Fifty percent of the strains were resistant to streptomycin and thirty-five were resistant to erythromycin. 3-methylbutanol, 3-methylbutanal and butane 2,3-dione were the predominant volatile compounds produced by *L. lactis*. Some strains isolated in this work have good technological properties to be used as starters for the industrial production of Oaxaca cheese.

Highlights

- Lactococci strains from Mexican Oaxaca cheese were identified and characterized.
- *Lactococcus lactis* spp. isolates showed high phenotypic and molecular diversity.
- Nine fast-fermenting strains (pH < 5.0 in milk) were found.
- Strains have technological properties as starters for industrial cheese production.

1 **Characterization of *Lactococcus* Strains Isolated from Artisanal Oaxaca Cheese**

2 I. Caro*[†], E. J. Quinto*¹, L. Fuentes[‡], V. Alessandria[§], L. S. Cocolin[§], M. P. Redondo-
3 del-Río*, B. Mayo[&], A. B. Flórez[&], J. Mateo[†]

4

5 * Department of Nutrition and Food Science, Faculty of Medicine, University of
6 Valladolid, 47005 Valladolid, Spain

7 [†] Department of Food Hygiene and Technology, University of León, Campus de
8 Vegazana s/n, 24071 León, Spain

9 [‡] Instituto Tecnológico Superior del Oriente del Estado de Hidalgo, 43900 Apan, México

10 [§] Department of Agricultural, Forest and Food Science, University of Torino, 10095
11 Grugliasco, Italy

12 [&] Department of Microbiology and Biochemistry, Instituto de Productos Lácteos de
13 Asturias (IPLA-CSIC), Paseo Río Linares s/n, 33300-Villaviciosa, Asturias, Spain

14

15 ¹ Corresponding author: E. J. Quinto, Department of Nutrition and Food Science,
16 Faculty of Medicine, University of Valladolid, 47005, Valladolid, Spain, Phone: +34-
17 983-184943 (equinto@ped.uva.es)

18

19 I. Caro (icarc@unileon.es), E. J. Quinto (equinto@ped.uva.es), L. Fuentes
20 (lfuentesjimenez@yahoo.com.mx), V. Alessandria (valentina.alessandria@unito.it), L.
21 S. Cocolin (lucasimone.cocolin@unito.it), M. P. Redondo-del-Río (pazr@ped.uva.es),
22 B. Mayo (baltasar.mayo@ipla.csic.es), A. B. Flórez (abflorez@ipla.csic.es), J. Mateo
23 (jmato@unileon.es)

24 Declarations of interest: None.

25

ABSTRACT

26

27

28 *Lactococci* strains obtained from raw-milk Oaxaca cheese through its production process
29 in two factories from the Tulancingo Valley, Mexico, were isolated and characterized. A
30 total of 120 colonies were selected from the growth in M17 and MRS agars. Twenty
31 were identified as *lactococci* strains, all *Lactococcus lactis*, and were characterized by
32 molecular and phenotypic methods including carbohydrate use, enzymatic profile,
33 acidifying capacity, and antibiotic and phage resistance. High phenotypic diversity was
34 observed and confirmed among the *Lactococcus lactis* strains by rep-PCR fingerprints.
35 Fifty percent of the strains were resistant to streptomycin and 35% to erythromycin.
36 Nine isolates were considered as fast acidifying strains. The predominant volatile
37 compounds produced were 3-methylbutanol, 3-methylbutanal and butane-2,3-dione. A
38 selection of strains isolated in this study has shown satisfactory characteristics to be used
39 as potential starters for the industrial production of Oaxaca cheese.

40

41 **Key Words:** Oaxaca cheese; *Lactococcus*; acidifying capacity; antibiotic resistance.

42

43

44 1. Introduction

45

46 Oaxaca cheese is one of the most popular Mexican cheeses with a production of about
47 14,700 tons (SIAP, 2016); it has becoming increasingly produced in the United States
48 and other countries as well. It is considered a soft pasta filata cheese (Caro et al., 2014)
49 and its making process involves curd acidification (until pH of 5.3) kneading in hot
50 water (72°C) and stretching, forming long and thin strips of curd, which are cooled in
51 chilled water, salted, cut into 0.2-2 kg segments and moulded with a ball shape (Caro et
52 al., 2011; De Oca-Flores, Castelán-Ortega, Estrada-Flores, & Espinoza-Ortega, 2009).
53 These authors described the main quality attributes of this cheese, such as a fibrous
54 structure, acidic taste, mild flavour, high creaminess, and good meltability. Two types
55 of Oaxaca cheeses are recognized: Those produced on medium or large factories using
56 pasteurized milk acidified either with starters –not specifically designed for this cheese–
57 or organic acids (Colín-Cruz, Dublán-García, Espinoza-Ostega, & Domínguez López,
58 2012), and those manufactured in small factories with naturally fermented raw milk
59 (Caro et al., 2011).

60 Soft cheeses should be produced with pasteurized milk for health reasons. In order to
61 maintain the sensorial properties of artisanal cheese, a suggested approach in pasteurized
62 milk cheeses is to select indigenous microorganisms for the design of specific starter
63 cultures (Cogan et al., 1997). *Lactococcus* strains have been widely used as starters;
64 selected indigenous strains intended to be used as starters should produce acid quickly
65 and specific flavour and texture (Leroy & De Vuyst, 2004). Moreover, they should not
66 carry virulence factors or other risk factors such as antibiotic resistance, high amino
67 acid-decarboxylase activity, etc. Furthermore, they must be identified and characterized
68 for their technological properties (Randazzo, Caggia, & Neviani, 2009).

69 The aim of this study was to identify and characterize *Lactococcus* spp. strains from
70 artisanal raw-milk Oaxaca cheeses in order to select potential candidate strains for the
71 design of a suitable starter culture to be used in pasteurized-milk Oaxaca cheese
72 production.

73

74 **2. Material and methods**

75

76 *2.1. Sampling and LAB isolation*

77 Samples of fresh milk (FM) at arrival to the cheese factory, acidified milk (AM) before
78 renneting, acidified curd (AC) before kneading, and fresh cheese (CH) after salting were
79 collected from two artisanal raw-milk cheese factories (Tulancingo Valley, Mexico) on
80 three working days. FM and AM (250 ml), AC (500 g), and CH (500 g) samples were
81 transported into sterile screw-capped flasks or sterile containers at 4°C to the laboratory
82 and analyzed within 4 h after sampling. Representative portions (10 ml: FM or AM; 10
83 g: AC or CH) were homogenized with 90 ml of buffered peptone water (peptone 0.1%,
84 NaCl 0.85%) using a Stomacher blender (Seward). Decimal dilutions were prepared and
85 pour plated using the two-layer method in M17 agar (Oxoid), and in Man, Rogosa, and
86 Sharpe agar (MRS; Oxoid) previously acidified (pH 5.5) with lactic acid (Panreac); the
87 plates were incubated at 30°C for 48-72 h. Four colonies were randomly selected from
88 FM, AM and AC, and three colonies from CH, reaching a total of 180 isolates (90 per
89 medium). Isolates were recovered in Tryptone Soy Broth (TSB; Bacto) with 0.5% (w/v)
90 of yeast extract (YE; Difco) (TSB-YE) at 37°C for 24 h. Aliquots (1 ml) were
91 centrifuged (12,000 rpm, 3 min) in Eppendorf tubes; the supernatants were discarded
92 and the pellets were suspended in 1 ml of MRS broth with 50% (v/v) of glycerol
93 (Acofarma) and stored at -40 °C.

94

95 *2.2. LAB identification and PCR typing*

96 Isolates were recovered with MRS agar incubated at 30°C, 24 h. An initial
97 characterization of the isolates was performed to select the presumptive LAB using
98 Gram reaction, morphology, catalase and cytochrome-oxidase activities (Cowan & Steel,
99 1999; Harrigan, 1998).

100 A single presumptive-LAB isolate was collected from the recovery MRS agar and
101 incubated in TSB-YE (30°C, 24 h) for DNA isolation, PCR reaction, sequencing,
102 species identification, and phylogenetic analysis as described by Caro et al. (2015).

103 The RAPD and Rep-PCR analyses were performed from total genomic purified DNA
104 from overnight cultures using a GenElute bacterial genomic DNA kit (Sigma-Aldrich).

105 Isolates were typed according to their RAPD and rep-PCR fingerprinting profiles using
106 the primers OPA18 (5'-AGGTGACCGT-3'; Matto et al., 2004), M13 (5'-
107 GAGGGTGGCGGTTCT-3'; Rossetti & Giraffa, 2005), and BoxA2R (5'-
108 ACGTGGTTTGAAGAGATTTTCG-3'; Koeuth et al., 1995). RAPD and rep-PCR
109 amplifications were independently performed in 25 µl volume reactions containing 12.5
110 µl MasterMix (Ampliqon), 5 µl of either primer (10 µM), 3 µl of purified DNA, and
111 molecular grade water (Sigma-Aldrich). The DNA amplification involved one cycle at
112 95°C for 7 min, followed by 40 denaturation cycles at 90°C for 30 s, primer annealing at
113 42°C (M13), 40°C (BoxA2R) or 32°C (OPA18) for 1 min, a first extension at 72°C for 4
114 min, and then a final extension at 72°C for 10 min. Typing reaction products were
115 subjected to electrophoresis and recorded. GeneTools software v.4.03 (SynGene) was
116 used to compare the profiles.

117

118 *2.3. Phenotypic characteristics of Lactococcus strains*

119 The strains' acidification capacity was tested after 0, 6, 12, and 24 h at 30 °C (IDF,
120 1995) and were classified into three categories (Roushdy, 1999). The strains were tested
121 for phage sensitivity against a laboratory phage bank composed by 12 purified industrial
122 phages and 25 infective whey samples following Estepar, Sánchez, Alonso, & Mayo
123 (1999).

124 *Lactococcus* strains were biochemically characterized using API-CH50 and API-ZYM
125 (bioMérieux) galleries; haemolytic (Smith, Gordon, & Clark, 1952) and proteolytic
126 (Facklam & Wilkinson, 1981) activities were studied at 37 °C for 48 h under
127 anaerobiosis. *Staphylococcus aureus* CECT 5192 and *Enterococcus faecalis* ATCC 29212
128 were respectively used as positive controls.

129 The antibiotic susceptibility against the antibiotics recommended by EFSA (2012) were
130 tested using the Etest assay (AB BioDisk) in order to determine the minimum inhibitory
131 concentrations (MIC) (Table 1). A 10⁸ CFU/ml (100 µl) suspension was inoculated onto
132 LSM agar plates (ISO, 2010). Afterwards (up to 15 min), two strips of the Etest were
133 placed on the border of the plates and incubated for 24-48 h at 30°C. *E. faecalis* ATCC
134 29212 was used as control. The breakpoints considered were those suggested by
135 international organizations or research studies (Table 1).

136 The production of volatile compounds from the fastest acidifying strains was tested
137 using solid-phase micro extraction (SPME) and gas chromatography coupled with mass
138 spectrometry (GC/MS). Cells suspensions (100 µl) from the MRS broth-glycerol cryo-
139 conservation media were grown in 5 ml of TSB-YE at 30°C for 24 h, inoculated in UHT
140 milk in duplicate adding cyclohexanone as internal standard (0.4 mg/ml), and incubated
141 at 30°C for 2 days in leak-tight screw-cap vials (Fernández, Alegría, Delgado, Martín, &
142 Mayo, 2011). Two vials containing milk plus cyclohexanone were used as controls. The
143 SPME extraction was carried out using 2 g of the fermented milk as described by Soto et

144 al. (2015), and the chromatographic separation and identification of volatile compounds
145 according to Carballo et al. (2018). Results were calculated as µg of cyclohexanone
146 equivalent/g of milk.

147

148 *2.4. Statistical analyses*

149 M17 and MRS counts were statically analysed using general lineal model analysis of
150 variance with the production stage as a fixed factor followed by the post hoc Tukey's
151 test (SPSS Statistics software, version 23, IBM).

152 Typing reaction patterns from the RAPD and Rep-PCR analyses were clustered using
153 the unweighted pair group with arithmetic mean (UPGMA) method, and pattern
154 similarity expressed via the simple matching (SM) coefficient.

155

156 **3. Results and discussion**

157

158 *3.1. LAB population*

159 M17 and MRS agar LAB counts are shown in Table 2. FM presented relatively high
160 counts, which could be attributed to temperature abuse of milk before processing. De
161 Oca-Flores et al. (2009) have reported temperature and acidity of milk at arrival to
162 artisanal Oaxaca dairy factories of 18-28°C and 17-22°D. The highest LAB counts
163 ($p<0.05$) were found in both AM and AC for both M17 and MRS media. CH showed
164 lower LAB mean counts than AM, although differences were found only for MRS
165 counts ($p<0.05$). This decrease could be attributed to the kneading of curd in hot water.
166 CH's MRS counts were similar to those found in previous studies (Caro et al., 2009).
167 Among the 180 isolates obtained from M17 and MRS plates, 121 isolates proved to be
168 presumptive LAB (Table 3). *Enterococcus* spp., mainly *E. faecalis*, were the most

169 abundant LAB in all production stages followed by *Lactobacillus* spp. (mainly
170 *Lactobacillus plantarum*) and *Lactococcus* spp. (all *L. lactis* subsp. *lactis*). The number
171 of *Enterococcus* spp. isolates was similar for both media. The prevalence of enterococci
172 in dairy products has been associated with poor hygienic conditions during production
173 and processing of milk (Giraffa, 2003). Survival of *Enterococcus* spp. in Oaxaca cheese
174 could be explained by their high thermal resistance and acid tolerance.

175 No literature was found on LAB species in raw-milk Oaxaca cheese. Saxer,
176 Schwenninger, & Lacroix (2013) studied the LAB population in pasteurized-milk
177 Oaxaca cheese; in contrast with our results, low presence of *Lactococcus* spp. (4% of
178 total LAB isolates) and the predominance of *Lactobacillus* spp. (41%) and
179 *Streptococcus thermophilus* (20%) were found, suggesting the relevance of designing
180 specific starters for this cheese.

181

182 3.2. *Lactococcus* identification and typing

183 Table 4 shows that *Lactococcus* spp. isolates were assigned to *L. lactis* subsp. *lactis*
184 with an identity percentage $\geq 99\%$ using BLAST, with the exception of the strains 1004
185 (97%) and 1003 (98%). The suggested criterion for the species level is the range 97-
186 99% of similarity (Stackebrandt & Goebel, 1994; Tindall, Rosselló-Móra, Busse,
187 Ludwig, & Kämpfer, 2010) although some authors consider $<0.5\%$ of divergence
188 (Janda & Abbott, 2007). With regard to RDP-II identity scores, 7 isolates showed an
189 S_{ab} score ≥ 0.99 , and the remaining presented scores between 0.964 and 0.989.

190 The phylogenetic tree of partial 16S rRNA sequences using UPMGA algorithm was
191 built with *L. lactis* subsp. *lactis* isolates and a variety of selected reference strains (Fig.
192 1). All *L. lactis* spp. were divided in three distant groups showing long branches: (i)
193 including the reference strains belonging to other *Lactococcus* species than *L. lactis*, (ii)

194 with the 1004 isolate showing the lowest BLAST identity (Table 4), and (iii) including
195 the rest of the *L. lactis*-identified isolates. Into the last group, *L. lactis* subsp. *tructae*
196 and *L. lactis* subsp. *cremoris* reference strains were assigned in a separate subgroup,
197 while the other subgroup was composed by all the *L. lactis* subsp. *lactis* isolates and the
198 *L. lactis* subsp. *hordniae* reference strain. In partial agreement with these results, Kim
199 (2014) found members of *Lactococcus* spp. forming two distant and separate groups:
200 The first formed by *L. raffinolactis*, *L. plantarum*, *Lactococcus chungangensis* and
201 *Lactococcus piscium* with 95.5% and 98.1% sequence similarities; and the second
202 formed by *L. garvieae*, *L. lactis* and *Lactococcus fujiensis* with 93.1% and 94.6%
203 sequence similarities.

204 The Oaxaca cheese isolates were grouped in seven clusters (Fig. 1). The major cluster
205 (cluster III) contains 60% of the isolates and the reference strain NCDO 604; the other
206 clusters contain only a maximum of two isolates each. When comparing the alignments
207 of 16s rRNA sequences of all the *L. lactis* isolates and the sequence of the *L. lactis*
208 subsp. *lactis* NCDO604 reference strain (Accession number AB100803), the 1004 strain
209 showed the major difference. According to Janda & Abbott, (2007), gene sequence data
210 from an individual strain with a nearest neighbour exhibiting a similarity score <97%
211 could represent a new species.

212 RAPD and rep-PCR fingerprinting profiles are shown in Fig. 2. Fifteen clusters were
213 formed with a coefficient of similarity >94%, suggesting a low homology of the isolated
214 *L. lactis* subsp. *lactis*. Dal Bello et al. (2010) also found a high biodiversity of
215 *Lactococcus lactis* in raw-milk cheeses. The low homology in the present study might
216 be explained, at least partially, because the milk used in the factories was collected from
217 different regions. The use of rep-PCR plus RAPD and several primers was capable of

218 grouping most of the strains according to factory (Fig. 2), i.e. only strains 2002 (factory
219 A) and 2016 (factory B) were placed in the same cluster (cluster I).

220

221 3.3. Phenotypic characterization of *Lactococcus* strains

222 According to their acidification activity, the *L. lactis* subsp. *lactis* were grouped as fast,
223 medium or slow acidifiers (Table 5). A 45% of the strains were considered as fast
224 acidifiers reducing the pH of milk from 6.6 to 5.3 in less than 6 h at 30 °C (Cogan et al.,
225 1997). One of the key issues in the Oaxaca cheese making process is to achieve a short
226 length of milk acidification period (Caro et al., 2014).

227 High percentage of industrial phage resistance was found: 45% of the strains showed
228 resistance to more than 60% of the phage tested. Strains 520a and 2002 were low
229 resistant (to less than 30% of the phages). Among the fast acidifying strains, 1002 and
230 1003 showed the highest resistance (to 67 and 60% of the phage, respectively). More
231 importantly, strains showed different resistance profiles to the phage collection, which
232 allow designing complementary starter mixes or the use of strains in an alternation
233 strategy.

234 The isolates showed variability in their ability to use some carbohydrates (Table 2S).
235 Most of them could ferment D-galactose, D-sorbitol, amygdalin, aesculin, D-melizitose,
236 amyllum (starch) and D-tagatose. Seven of the 20 isolates fermented glycerol and
237 potassium gluconate, and only 4 were capable of using L-arabinose, L-sorbose, D-
238 melibiose, D-raffinose and D-turanose. These results were similar to those found by
239 Delgado and Mayo (2004) in wild lactococci isolates. It is possible that the
240 carbohydrate profiles are related to the habitat (Kelly et al., 2010); wild *L. lactis* strains
241 tend to ferment sugars that are present in plants and vegetables (Díaz-Ruiz et al., 2003;
242 Fernández et al., 2011). In our study, the strains isolated were able to ferment starch,

243 sucrose and mannitol at ratios of 70, 60, and 45%, respectively, probably related to the
244 geographical region and the cattle feeding.

245 Enzyme activity of the *L. lactis* subsp. *lactis* strains is shown in Table 3S (medium and
246 low acidifying activity) and Table 6 (fast acidifying activity), showing no activity for
247 trypsin, alkaline phosphatase, α -galactosidase and α -fucosidase, β -glucuronidase, and
248 α -mannosidase (data no shown). Results were similar to those found by Nomura et al.
249 (2006).

250 The β -galactosidase activity is important from the technological point of view. Thirteen
251 *L. lactis* strains showed this activity with 1.7 (8.5 nmol) and 0.8 (4 nmol) scale points in
252 the fast and medium acidifying group, respectively. Only 4 out of 9 fast acidifying
253 strains showed an activity of 10 nmol. The β galactosidase activity of *L. lactis* strains
254 isolated from Oaxaca cheese was lower than that found by Fernández et al. (2011) in
255 raw-milk cheeses: 3 scale points (20 nmol). *L. lactis* isolates showed lower α - and β -
256 glucosidase activities (3 and 2 nmol on average, respectively) as compared to those
257 studied by Nomura et al. (2006) and Fernández et al. (2011).

258 Aminopeptidase activity was also moderate. It was especially high for leucine
259 arylamidase with mean values higher than 3 (20 nmol) for all isolates, followed by the
260 α -chymotrypsin activity showed by 15 out of 19 strains with mean values of 1.7 and 1.3
261 (8.5 nmol and 6.5 nmol) for fast and medium acidifying groups, respectively.

262 The main differences in enzymatic activities between the fast and medium acidifying
263 groups were a higher activity (from 4.5 to 3 nmoles) for β -galactosidase and α -
264 glucosidase, respectively. The presence of β -galactosidase in *L. lactis* strains is
265 important for their use as dairy cultures for both the acidification of milk and probiotic
266 use (Monteagudo-Mera et al., 2011). However, the strains 520a, 1003, and 2002 show
267 low activity (≤ 10 nmol).

268 Among the fast acidifying strains only one (number 520a) showed a relatively high
269 activity for β -glucosidase and N-acetyl- β -glucosaminidase (Table 6). These enzymatic
270 activities are not desirable for a starter as they might be associated with adverse effects
271 in the human intestinal tract by releasing aglycones from glycosides plants especially
272 dietary flavonoids (Bujnakova, Strakova, & Kmet, 2014; Parodi, 1999), although this
273 effect remains controversial due to reports of potential anti-carcinogenic and anti-
274 mutagenic effects, especially those derived from flavone C glycosides (Heavey &
275 Rowland, 2004; Xiao, 2017).

276 The distribution of *L. lactis* subsp. *lactis* isolates according to their MICs is shown in
277 Table 4S. None of the isolates was resistant to ampicillin, benzylpenicillin, vancomycin,
278 chloramphenicol, tetracycline and gentamicin. Resistance was found for streptomycin
279 (60%, 12 isolates), erythromycin (35%, 7), clindamycin (15%, 3), kanamycin (15%, 3)
280 and ciprofloxacin (5%, 1). High resistance of *L. lactis* to streptomycin has been reported
281 in several studies (Fernández et al., 2011; Katla, Kruse, Johnsen, & Herikstad, 2001;
282 Klare et al., 2007). In this study, 60% of the strains were resistant to streptomycin with
283 two of them showing a MIC higher than 512 $\mu\text{g/ml}$. This level appeared to be
284 intermediate in the studies by Katla et al. (2001) and Salem et al. (2018), who found a
285 resistance to streptomycin higher than 256 $\mu\text{g/ml}$ in 90% of *L. lactis* strains. Only 15%
286 of the isolates were found resistant to erythromycin in this study. The results were
287 higher than those reported by Florez et al. (2005) and lower than those found by
288 Franciosi et al. (2009), who found that 1.5% and 57% of *L. lactis* subsp. *lactis* strains
289 were resistant, respectively.

290 The distribution of MICs allows the estimation of the isolated strains' resistance
291 breakpoints. The discrepancy between the experimental resistance and that obtained
292 from the literature (Table 4S) is for streptomycin only, with an experimental breakpoint

293 of ≥ 64 $\mu\text{g/ml}$ instead of ≥ 32 $\mu\text{g/ml}$ (EFSA, 2012). The resistant population for
294 streptomycin was 5 strains, 8 for erythromycin, 3 for clindamycin and 3 for kanamycin.
295 The MIC showed by the fast acidifying strains is shown in **Table 7**. Almost all of the
296 isolates proved to be susceptible to the tested antimicrobial agents except for strain 1004
297 –which showed resistance to clindamycin, erythromycin, kanamycin and streptomycin–
298 and strain 1003 –resistant to erythromycin.

299 A total of 14 volatile compounds were identified in the head space of acidified milk by
300 the fast acidifying *L. lactis* strains (**Table 8**). The six major compounds were 3-
301 methylbutanol, 3-methylbutanal, butane-2,3-dione, 3-hydroxy-2-butanone, 5-hydroxy-
302 2,7-dimethyl-4-octanone and butanoic acid. Profiles from 1002 strain deviated from all
303 others by producing twice or more than the mean value of 3-methylbutanal, 3-
304 methylbutanol, and 5-hydroxy-2,7-dimethyl-4-octadione. The high production of acetic
305 acid by 1004 strain is also outstanding. All the compounds detected, except for 4-
306 methyl-2-oxopentanoic acid, have been previously reported in milk cultures of
307 *Lactococcus* spp. strains. The 2- and 3-methyl-aldehydes, alcohols and acids are
308 considered to be derived from the breakdown of branched amino acids (Marilley &
309 Casey, 2004) by the transaminase pathway which is highly active in the *Lactococcus*
310 species (Smit, Smit, & Engels, 2005). Those volatiles are formed via oxoacids (α -keto
311 acids), such as 4-methyl-2-oxopentanoic acid which originated from leucine and was
312 detected in this study. The 2- and 3-methylaldehydes have a low odour threshold and
313 seem to play a key role in the flavour of cheeses, being responsible of positive overall
314 flavour in balance with other volatile compounds (Morales, Fernández-García, Gaya, &
315 Nuñez, 2003). The importance of controlling the decarboxylating activities of selected
316 strains due to their flavour potential has been remarked (Smit et al., 2005). In this
317 context, the use of the strain 1002 might have an advantage over the other strains

318 because it would give cheeses with relatively high amount of 2-methylpropanal and 3-
319 methylbutanal and thus high flavour intensity; the sensory acceptability of such a
320 highly-flavoured cheese requires further study and it is far from the aim of this work.
321 On the other hand, butane-2,3-dione, 3-hydroxy-2-butanone, ethanol, and acetic acid are
322 products derived from pyruvate metabolism. The two former are typically produced via
323 citrate metabolism and contribute to buttery and creamy flavours in dairy products
324 (Marilley & Casey, 2004; Smit et al., 2005).
325 In a previous study (Sandoval-Copado, Orozco-Villafuerte, Pedrero-Fuehrer, & Colín-
326 Cruz, 2016), the volatiles in the headspace of three Oaxaca cheeses –two made from
327 pasteurized milk and one made from naturally acidified milk– were identified although
328 not quantified. The authors reported a total of 14 volatiles from which 11 were present
329 in the three cheeses. Four out of the 11 compounds were coincident with those of our
330 study: 3-methylbutanal, butane-2,3-dione, 3-hydroxy-2-butanone, acetic acid, and 2-
331 propanone. Discrepancies regarding the volatile profile among studies could be
332 attributed to differences in the microbial species involved in fermentation, substrate
333 (cheese vs acidified milk), and in the fibre type used in the SPME method.

334

335 **4. Conclusions**

336

337 *Lactococcus lactis* subsp. *lactis* is the predominant species in raw-milk Oaxaca cheese.
338 Significant genotypic and phenotypic differences among the studied *L. lactis* strains
339 suggest high interspecies variability. Six strains are proposed as potential starter culture
340 for pasteurized milk Oaxaca cheese mainly due to their high acidifying activity and
341 antibiotic susceptibility. Among them, 1002 strain, due to its higher production of 2-
342 methylpropanal and 3-methylbutanal, would be recommended to improve flavour.

343 Further studies are needed to evaluate the performance of the strains on Oaxaca cheeses
344 making process.

345

346 **Acknowledgements**

347

348 Lucía Fuentes thanks the Secretaría de Educación Pública, México, for the fellowship
349 granted. The authors thank Mr. Julio Armando Claro for his technical assistance.

350

351 **References**

352

353 Bujnakova, D., Strakova, E., & Kmet, V. (2014). Invitro evaluation of the safety and
354 probiotic properties of Lactobacilli isolated from chicken and calves. *Anaerobe*,
355 29, 118–127. <https://doi.org/10.1016/j.anaerobe.2013.10.009>

356 Carballo, D. E., Caro, I., Andrés, S., Giráldez, F. J., & Mateo, J. (2018). Assessment of
357 the antioxidant effect of astaxanthin in fresh, frozen and cooked lamb patties. *Food*
358 *Research International*, 111. <https://doi.org/10.1016/j.foodres.2018.05.054>

359 Caro, I., Soto, S., Franco, M. J., Meza-Nieto, M., Alfaro-Rodríguez, R. H., & Mateo, J.
360 (2011). Composition, yield, and functionality of reduced-fat Oaxaca cheese:
361 Effects of using skim milk or a dry milk protein concentrate. *Journal of Dairy*
362 *Science*, 94(2). <https://doi.org/10.3168/jds.2010-3102>

363 Caro, I., Soto, S., Contreras, I. L., & Mateo, J. (2009). Thermal inactivation of
364 *Escherichia coli* and coliform in Oaxaca cheese curd during a simulated kneading
365 process. In *Current Research Topics in Applied Microbiology and Microbial*
366 *Biotechnology* (pp. 412–415). WORLD SCIENTIFIC.
367 https://doi.org/10.1142/9789812837554_0086

368 Caro, Irma, Bécáres, G., Fuentes, L., Garcia-Armesto, M. R., Rúa, J., Castro, J. M., ...
369 Mateo, J. (2015). Evaluation of three PCR primers based on the *16S rRNA* gene for
370 the identification of lactic acid bacteria from dairy origin. *CyTA - Journal of Food*,
371 *13*(2), 181–187. <https://doi.org/10.1080/19476337.2014.934297>

372 Caro, Irma, Soto, S., Fuentes, L., Gutiérrez-Méndez, N., García-islas, B., Monroy-
373 gayosso, K. E., & Mateo, J. (2014). Compositional , Functional and Sensory
374 Characteristics of Selected Mexican Cheeses. *Food and Nutrition Sciences*,
375 *5*(February), 366–375. <https://doi.org/http://dx.doi.org/10.4236/fns.2014.54044>

376 Cogan, T. M., Barbosa, M., Beuvier, E., Bianchi-Salvadori, B., Cocconcelli, P. S.,
377 Fernandes, I., ... Rodriguez, E. (1997). Characterization of the lactic acid bacteria
378 in artisanal dairy products. *Journal of Dairy Research*, *64*(3), 409–421.
379 <https://doi.org/10.1017/S0022029997002185>

380 Colín-Cruz, M. de los Á., Dublán-García, O., Espinoza-Ostega, A., & Domínguez
381 López, A. (2012). The effect of varying fat content on the microstructure of
382 Oaxaca cheese, a typical pasta filata cheese. *International Journal of Dairy*
383 *Technology*, *65*(1), 71–80. <https://doi.org/10.1111/j.1471-0307.2011.00735.x>

384 Cowan, S. T., & Steel, K. L. (1999). Theory and practice of bacterial identification. In
385 G. I. Barrow & R. K. A. Feltham (Eds.), *Manual for the identification of medical*
386 *bacteria* (pp. 1–6). Cambridge: Cambridge University Press.
387 <https://doi.org/10.1017/CBO9780511527104.007>

388 Dal Bello, B., Rantsiou, K., Bellio, A., Zeppa, G., Ambrosoli, R., Civera, T., & Cocolin,
389 L. (2010). Microbial ecology of artisanal products from North West of Italy and
390 antimicrobial activity of the autochthonous populations. *LWT - Food Science and*
391 *Technology*, *43*(7), 1151–1159. <https://doi.org/10.1016/j.lwt.2010.03.008>

392 De Oca-Flores, E. M., CastelÁN-Ortega, O. A., Estrada-Flores, J. G., & Espinoza-

393 Ortega, A. (2009). Oaxaca cheese: Manufacture process and physicochemical
394 characteristics. *International Journal of Dairy Technology*, 62(4), 535–540.
395 <https://doi.org/10.1111/j.1471-0307.2009.00533.x>

396 Delgado, S., & Mayo, B. (2004). Phenotypic and genetic diversity of *Lactococcus lactis*
397 and *Enterococcus* spp. strains isolated from Northern Spain starter-free farmhouse
398 cheeses. *International Journal of Food Microbiology*, 90(3), 309–319.
399 [https://doi.org/10.1016/S0168-1605\(03\)00323-4](https://doi.org/10.1016/S0168-1605(03)00323-4)

400 Díaz-Ruiz, G., Guyot, J. P., Ruiz-Teran, F., Morlon-Guyot, J., & Wachter, C. (2003).
401 Microbial and physiological characterization of weakly amylolytic but fast-
402 growing lactic acid bacteria: A functional role in supporting microbial diversity in
403 pozol, a Mexican fermented maize beverage. *Applied and Environmental*
404 *Microbiology*, 69(8), 4367–4374. [https://doi.org/10.1128/AEM.69.8.4367-](https://doi.org/10.1128/AEM.69.8.4367-4374.2003)
405 [4374.2003](https://doi.org/10.1128/AEM.69.8.4367-4374.2003)

406 Dominguez, A. (2011). Alimentos artesanales y tradicionales: El queso Oaxaca como
407 un caso de estudio del centro de Mexico. *Estudios Sociales*, 19(38), 165–193.
408 Retrieved from [http://www.ciad.mx/coordinaciones/desarrollo-regional/revista-](http://www.ciad.mx/coordinaciones/desarrollo-regional/revista-estudios-sociales/numeros-revista-electronica.html%5Cnhttp://search.ebscohost.com/login.aspx?direct=true&db=ecn&AN=1301765&site=ehost-live&scope=site)
409 [estudios-sociales/numeros-revista-](http://www.ciad.mx/coordinaciones/desarrollo-regional/revista-estudios-sociales/numeros-revista-electronica.html%5Cnhttp://search.ebscohost.com/login.aspx?direct=true&db=ecn&AN=1301765&site=ehost-live&scope=site)
410 [electronica.html%5Cnhttp://search.ebscohost.com/login.aspx?direct=true&db=ecn](http://www.ciad.mx/coordinaciones/desarrollo-regional/revista-estudios-sociales/numeros-revista-electronica.html%5Cnhttp://search.ebscohost.com/login.aspx?direct=true&db=ecn&AN=1301765&site=ehost-live&scope=site)
411 [&AN=1301765&site=ehost-live&scope=site](http://www.ciad.mx/coordinaciones/desarrollo-regional/revista-estudios-sociales/numeros-revista-electronica.html%5Cnhttp://search.ebscohost.com/login.aspx?direct=true&db=ecn&AN=1301765&site=ehost-live&scope=site)

412 EFSA. (2012). Technical specifications on the harmonised monitoring and reporting of
413 antimicrobial resistance in *Salmonella*, *Campylobacter* and indicator *Escherichia*
414 *coli* and *Enterococcus* spp. bacteria transmitted through food 1. *EFSA Journal*,
415 10(6), 64. <https://doi.org/10.2903/j.efsa.2012.2742>.

416 Estepar, J., Del Mar Sánchez, M., Alonso, L., & Mayo, B. (1999). Biochemical and
417 microbiological characterization of artisanal “Penamellera” cheese: Analysis of its

418 indigenous lactic acid bacteria. *International Dairy Journal*, 9(10), 737–746.
419 [https://doi.org/10.1016/S0958-6946\(99\)00141-7](https://doi.org/10.1016/S0958-6946(99)00141-7)

420 Facklam, R. R., & Wilkinson, H. (1981). The family Streptococaceae (medical aspects).
421 In B. and S. Stolp, Trüper (Ed.), *The prokaryotes: A Handbook on Habitants.*
422 *Isolation and Identification of Bacteria* (1st ed., pp. 1572–1597). Verlag, Berlin:
423 Springer.

424 Fernández, E., Alegría, A., Delgado, S., Martín, M. C., & Mayo, B. (2011).
425 Comparative phenotypic and molecular genetic profiling of wild *Lactococcus lactis*
426 subsp. *lactis* strains of the *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*
427 genotypes, isolated from starter-free cheeses made of raw milk. *Applied and*
428 *Environmental Microbiology*, 77(15), 5324–5335.
429 <https://doi.org/10.1128/AEM.02991-10>

430 Florez, A. B., Delgado, S., & Mayo, B. (2005). Antimicrobial susceptibility of lactic
431 acid bacteria isolated from a cheese environment. *Can.J.Microbiol.*

432 Franciosi, E., Settanni, L., Cavazza, A., & Poznanski, E. (2009). Biodiversity and
433 technological potential of wild lactic acid bacteria from raw cows' milk.
434 *International Dairy Journal*, 19(1), 3–11.
435 <https://doi.org/10.1016/j.idairyj.2008.07.008>

436 Giraffa, G. (2003). Functionality of enterococci in dairy products. *International Journal*
437 *of Food Microbiology*, 88(2–3), 215–222. [https://doi.org/10.1016/S0168-](https://doi.org/10.1016/S0168-1605(03)00183-1)
438 [1605\(03\)00183-1](https://doi.org/10.1016/S0168-1605(03)00183-1)

439 Harrigan, W. F. (1998). Laboratory methods in food microbiology. UK: Academic
440 Press.

441 Heavey, P. M., & Rowland, I. R. (2004, April). Gastrointestinal cancer. *Best Practice*
442 *and Research: Clinical Gastroenterology.*

443 <https://doi.org/10.1016/j.bpg.2003.10.003>

444 IDF (International Dairy Federation). (1995). Milk and milk products—Guidance on
445 methods of sampling. *Standard 50C, IDF, Brussels, Belgium.*

446 International Organization for Standardization. (2010). ISO 10932:2010 - Milk and milk
447 products - Determination of the minimal inhibitory concentration (MIC) of
448 antibiotics applicable to bifidobacteria and non-enterococcal lactic acid bacteria
449 (LAB). *ISO 10932/IDF 233 Standard, 2010(Cmi)*, 38. Retrieved from
450 <https://www.iso.org/standard/46434.html>

451 Janda, J. M., & Abbott, S. L. (2007, September). 16S rRNA gene sequencing for
452 bacterial identification in the diagnostic laboratory: Pluses, perils, and pitfalls.
453 *Journal of Clinical Microbiology*. American Society for Microbiology (ASM).
454 <https://doi.org/10.1128/JCM.01228-07>

455 Katla, A.-K. K., Kruse, H., Johnsen, G., & Herikstad, H. (2001). Antimicrobial
456 susceptibility of starter culture bacteria used in Norwegian dairy products.
457 *International Journal of Food Microbiology*, 67(1–2), 147–152.
458 [https://doi.org/10.1016/S0168-1605\(00\)00522-5](https://doi.org/10.1016/S0168-1605(00)00522-5)

459 Kelly, W. J., Ward, L. J. H., & Leahy, S. C. (2010). Chromosomal diversity in
460 *Lactococcus lactis* and the origin of dairy starter cultures. *Genome Biology and*
461 *Evolution*, 2(1), 729–744. <https://doi.org/10.1093/gbe/evq056>

462 Kim, W. (2014). The genus *Lactococcus*. *Lactic Acid Bacteria: Biodiversity and*
463 *Taxonomy*, 429–443. <https://doi.org/10.1002/9781118655252.ch26>

464 Klare, I., Konstabel, C., Werner, G., Huys, G., Vankerckhoven, V., Kahlmeter, G., ...
465 Goossens, H. (2007). Antimicrobial susceptibilities of *Lactobacillus*, *Pediococcus*
466 and *Lactococcus* human isolates and cultures intended for probiotic or nutritional
467 use. *Journal of Antimicrobial Chemotherapy*, 59(5), 900–912.

468 <https://doi.org/10.1093/jac/dkm035>

469 Koeuth, T., Versalovic, J., & Lupski, J. R. (1995). Differential subsequence
470 conservation of interspersed repetitive *Streptococcus pneumoniae* BOX elements
471 in diverse bacteria. *Genome Research*.

472 Leroy, F., & De Vuyst, L. (2004, February 1). Lactic acid bacteria as functional starter
473 cultures for the food fermentation industry. *Trends in Food Science and*
474 *Technology*. Elsevier. <https://doi.org/10.1016/j.tifs.2003.09.004>

475 Marilley, L., & Casey, M. G. (2004). Flavours of cheese products: Metabolic pathways,
476 analytical tools and identification of producing strains. *International Journal of*
477 *Food Microbiology*, 90(2), 139–159. [https://doi.org/10.1016/S0168-](https://doi.org/10.1016/S0168-1605(03)00304-0)
478 [1605\(03\)00304-0](https://doi.org/10.1016/S0168-1605(03)00304-0)

479 Mättö, J., Malinen, E., Suihko, M. L., Alander, P., Palva, A., & Saarela, M. (2004).
480 Genetic heterogeneity and functional properties of intestinal bifidobacteria.
481 *Journal of Applied Microbiology*, 97(3), 459–470. [https://doi.org/10.1111/j.1365-](https://doi.org/10.1111/j.1365-2672.2004.02340.x)
482 [2672.2004.02340.x](https://doi.org/10.1111/j.1365-2672.2004.02340.x)

483 Monteagudo-Mera, A., Caro, I., Rodríguez-Aparacio, L. ., Rúa, J., Ferrer, M. A., &
484 García-Armesto, M. R. (2011). Characterization of Certain Bacterial Strains for
485 Potential Use as Starter or Probiotic Cultures in Dairy Products. *Journal of Food*
486 *Protection*, 74(8), 1379–1386. <https://doi.org/10.4315/0362-028X.JFP-10-392>

487 Morales, P., Fernández-García, E., Gaya, P., & Nuñez, M. (2003). Formation of volatile
488 compounds by wild *Lactococcus lactis* strains isolated from raw ewes' milk
489 cheese. *International Dairy Journal*. [https://doi.org/10.1016/S0958-](https://doi.org/10.1016/S0958-6946(02)00151-6)
490 [6946\(02\)00151-6](https://doi.org/10.1016/S0958-6946(02)00151-6)

491 Nomura, M., Kobayashi, M., Narita, T., Kimoto-Nira, H., & Okamoto, T. (2006).
492 Phenotypic and molecular characterization of *Lactococcus lactis* from milk and

493 plants. *Journal of Applied Microbiology*, 101(2), 396–405.
494 <https://doi.org/10.1111/j.1365-2672.2006.02949.x>

495 Parodi, P. W. (1999). The role of intestinal bacteria in the causation and prevention of
496 cancer: modulation by diet and probiotics. *Australian Journal of Dairy*
497 *Technology*, 54(2), 103–121.

498 Randazzo, C. L., Caggia, C., & Neviani, E. (2009). Application of molecular
499 approaches to study lactic acid bacteria in artisanal cheeses. *Journal of*
500 *Microbiological Methods*, 78(1), 1–9. <https://doi.org/10.1016/j.mimet.2009.04.001>

501 Rossetti, L., & Giraffa, G. (2005). Rapid identification of dairy lactic acid bacteria by
502 M13-generated, RAPD-PCR fingerprint databases. *Journal of Microbiological*
503 *Methods*. <https://doi.org/10.1016/j.mimet.2005.03.001>

504 Roushdy, I. (1999). Caracterización molecular y fenotípica de bacterias de ácido láctico
505 aisladas de raban de laban. *Annals of Agricultural Science*, 44, 617–630.

506 Salem, A. Z. M., Khusro, A., Elghandour, M. M. Y., Olivares-Pérez, J., Rojas-
507 Hernandez, S., & Jiménez-Guillén, R. (2018). Susceptibility of ruminal bacteria
508 isolated from large and small ruminant to multiple conventional antibiotics.
509 *Microbial Pathogenesis*, 121(April), 93–99.
510 <https://doi.org/10.1016/j.micpath.2018.05.013>

511 Sandoval-Copado, J., Orozco-Villafuerte, J., Pedrero-Fuehrer, D., & Colín-Cruz, M. A.
512 (2016). Sensory profile development of Oaxaca cheese and relationship with
513 physicochemical parameters. *Journal of Dairy Science*, 99(9), 7075–7084.
514 <https://doi.org/10.3168/jds.2015-10833>

515 Saxer, S., Schwenninger, S. M., & Lacroix, C. (2013). Characterization of the
516 microflora of industrial Mexican cheeses produced without added chemical
517 preservatives. *LWT - Food Science and Technology*, 53(1), 314–320.

518 <https://doi.org/10.1016/j.lwt.2013.01.016>

519 SIAP. (2016). *Panorama de la Lechería en México*. Retrieved from

520 [http://infosiap.siap.gob.mx/opt/boletlech/Brochure leche_Septiembre2016.pdf](http://infosiap.siap.gob.mx/opt/boletlech/Brochure%20leche_Septiembre2016.pdf)

521 Smit, G., Smit, B. A., & Engels, W. J. M. (2005). Flavour formation by lactic acid

522 bacteria and biochemical flavour profiling of cheese products. *FEMS Microbiology*

523 *Reviews*. <https://doi.org/10.1016/j.femsre.2005.04.002>

524 Smith, R. N., Gordon, E. R., & Clark, F. G. (1952). *Aerobic sporeforming bacteria*.

525 Washington, D.C: U.S Dept. of Agriculture. Retrieved from

526 https://archive.org/stream/aerobicsporeform16smit/aerobicsporeform16smit_djvu.t

527 xt

528 Soto, S., Serrano, E., Humada, M. J., Fernández-Diez, A., Caro, I., Castro, A., & Mateo,

529 J. (2015). Volatile compounds in the perirenal fat from calves finished on

530 semiextensive or intensive systems with special emphasis on terpenoids. *Grasas y*

531 *Aceites*, 66(4). <https://doi.org/10.3989/gya.0244151>

532 Stackebrandt, E., & Goebel, B. M. (1994). Taxonomic Note: A Place for DNA-DNA

533 Reassociation and 16S rRNA Sequence Analysis in the Present Species Definition

534 in Bacteriology. *International Journal of Systematic and Evolutionary*

535 *Microbiology*. <https://doi.org/10.1099/00207713-44-4-846>

536 Tindall, B. J., Rosselló-Móra, R., Busse, H. J., Ludwig, W., & Kämpfer, P. (2010).

537 Notes on the characterization of prokaryote strains for taxonomic purposes.

538 *International Journal of Systematic and Evolutionary Microbiology*.

539 <https://doi.org/10.1099/ijs.0.016949-0>

540 Xiao, J. (2017, July 15). Dietary flavonoid aglycones and their glycosides: Which show

541 better biological significance? *Critical Reviews in Food Science and Nutrition*.

542 <https://doi.org/10.1080/10408398.2015.1032400>

- 1 Table 1. Concentration ranges of the antibiotic tested by the E-test method and break
 2 points considered for resistance in *L. lactis*

Antibiotic	Category	Concentration range (µg/mL)	Breaking point
Ampicillin	Beta lactam	0.016-256	2 ¹
Benzylpenicillin	Beta lactam	0.002-32	4 ²
Chloramphenicol	Chloramphenicol	0.016-256	8 ¹
Ciprofloxacin	Fluoroquinolone	0.002-32	4 ²
Clindamycin	Lincosamide	0.016-256	1 ¹
Erythromycin	Macrolide	0.016-256	1 ¹
Gentamicin	Aminoglycoside	0.016-256	32 ¹
Kanamycin	Aminoglycoside	0.016-256	64 ¹
Streptomycin	Aminoglycoside	0.064-1024	32 ¹
Tetracycline	Tetracycline	0.016-256	4 ¹
Vancomycin	Glycopeptide	0.016-256	4 ¹

3 ¹EFSA, 2012

4 ²Katla et al. (2001)

5

6

7 Table 2. Counts (log CFU/g; mean \pm SD) of the viable lactic acid bacteria (LAB) in two
8 media from samples collected from different Oaxaca cheese production stages

Production stage	M17 (n=3)	MRS (n=3)
Fresh milk	6.46 \pm 0.45 ^b	6.58 \pm 0.43 ^c
Acidified milk	7.96 \pm 0.23 ^a	8.70 \pm 0.83 ^a
Acidified curd	8.23 \pm 0.29 ^a	7.62 \pm 0.31 ^{ab}
Fresh cheese	7.64 \pm 0.60 ^{ab}	6.63 \pm 0.65 ^{bc}

9 ^{a, b, c}Mean values in columns with different number indicate significant difference
10 ($p < 0.05$; Tukey's test).

11

12

13 Table 3. Distribution of LAB isolates from different media and Oaxaca cheese
 14 production stages as identified by partial 16S rRNA gene sequencing.

Species	Isolates	Medium		Cheese production stage			
		M17	MRS	FM	AM	AC	CH
<i>Lactobacillus</i> spp.							
<i>Lb. plantarum</i>	20	6	14	7	6	5	2
<i>Lb. paracasei</i> subsp. <i>paracasei</i>	5	4	1	1	1	2	1
<i>Lb. rhamnosus</i>	1	-	1	-	-	-	1
<i>Lactococcus</i> spp.							
<i>L. lactis</i> subsp. <i>lactis</i>	20	13	7	6	4	5	5
<i>Leuconostoc</i> spp.							
<i>Le. lactis</i>	1	1	-	-	1	-	-
<i>Enterococcus</i> spp.							
<i>E. faecalis</i>	68	37	31	20	22	20	6
<i>E. faecium</i>	6	2	4	1	1	1	3
Total	121	63	58	35	35	33	18

15 FM, milk at arriving to the cheese factory; AM, acidified milk at the moment of
 16 renneting; AC, acidified curd at the moment of kneading; CH, cheese just after salting.

17

18

19 Table 4. Taxonomic identification of Oaxaca cheese presumptive *Lactococcus* isolates based on partial 16S rRNA gene sequencing and
 20 comparison of the sequences with two software programs.

Strain	Most homologous sequence (Accession no.)		Species ¹	Statistics		
	BLAST (NCBI)	Classifier (RDP-II)		BLAST	RDP-II	
				Identity (%)	Similarity	Sa_b score
501	NR_103918.1	DQ011898	<i>L. lactis</i> subsp. <i>lactis</i>	99	1.000	0.991
502	NR_040955.1	DQ011898	<i>L. lactis</i> subsp. <i>lactis</i>	99	1.000	1.000
506	NR_040955.1	DQ011898	<i>L. lactis</i> subsp. <i>lactis</i>	99	0.997	0.980
509	NR_040955.1	DQ011898	<i>L. lactis</i> subsp. <i>lactis</i>	99	1.000	1.000
511	NR_040955.1	EU091387	<i>L. lactis</i> subsp. <i>lactis</i>	99	0.998	0.953
518	NR_040955.1	EU872263	<i>L. lactis</i> subsp. <i>lactis</i>	99	0.997	0.974
519	NR_040955.1	JF297355	<i>L. lactis</i> subsp. <i>lactis</i>	99	1.000	0.990
520a	NR_040955.1	EU091415	<i>L. lactis</i> subsp. <i>lactis</i>	99	0.996	0.977
1002	NR_040955.1	DQ011898	<i>L. lactis</i> subsp. <i>lactis</i>	99	0.997	0.969
1003	NR_040955.1	DQ011898	<i>L. lactis</i> subsp. <i>lactis</i>	98	1.000	1.000
1004	NR_103918.1	DQ255952	<i>L. lactis</i> subsp. <i>lactis</i>	97	1.000	0.971
1007	NR_040955.1	DQ173744	<i>L. lactis</i> subsp. <i>lactis</i>	99	0.984	0.976
1502	NR_040955.1	AF515224	<i>L. lactis</i> subsp. <i>lactis</i>	99	0.998	0.968
1506	NR_040955.1	DQ011898	<i>L. lactis</i> subsp. <i>lactis</i>	99	1.000	0.963
1510	NR_103918.1	DQ011898	<i>L. lactis</i> subsp. <i>lactis</i>	99	1.000	0.980
1520	NR_040955.1	EU872263	<i>L. lactis</i> subsp. <i>lactis</i>	99	1.000	0.964
2002	NR_040955.1	EU872263	<i>L. lactis</i> subsp. <i>lactis</i>	99	1.000	0.964
2016	NR_103918.1	DQ011898	<i>L. lactis</i> subsp. <i>lactis</i>	99	0.997	0.989
2017a	NR_103918.1	DQ011898	<i>L. lactis</i> subsp. <i>lactis</i>	99	1.000	1.000
2019	NR_103918.1	DQ011898	<i>L. lactis</i> subsp. <i>lactis</i>	99	1.000	0.991

21 BLAST: Basic Local Alignment Search Tool (NCBI database). Classifier (RDP-II: The Ribosomal Database Project).

22 ¹ Bacterial species assigned based on the highest percentage of coincidence or similarity obtained with both programs.

23 S_{ab} scores indicate the degree of match of assembly consensus sequences to each named bacterial species in the RDP-II program.

24 Table 5. Acidifying activity and phage resistance of *Lactococcus lactis* subsp. *lactis*
 25 isolates cultured on skimmed milk at 30 °C

Isolate	Making process ²	pH at different acidifying times ¹			Ability to acidify ³	Phage resistance
		6 h	12 h	24 h		%
501	FM	5.2	4.8	4.7	M	74.3 (35) ⁴
502	FM	5.3	4.8	4.6	M	42.8 (35)
506	AC	5.1	4.7	4.6	M	74.3 (35)
509	CH	5.5	4.7	4.6	M	52.3 (36)
511	AM	5.1	4.7	4.6	M	66.6 (36)
518	AC	4.9	4.5	4.4	F	38.9 (36)
519	CH	5.3	4.6	4.6	M	80.5 (36)
520a	CH	5.0	4.6	4.4	F	27.0 (37)
1002	FM	4.9	4.5	4.4	F	67.6 (37)
1003	AM	5.0	4.5	4.4	F	60.0 (37)
1004	AM	4.9	4.6	4.5	F	44.4 (37)
1007	CH	5.1	4.7	4.6	M	41.6 (36)
1502	FM	4.9	4.4	4.3	F	48.6 (37)
1506	AC	4.5	4.4	4.3	F	32.4 (37)
1510	AM	5.1	4.7	4.6	M	83.3 (36)
1520	FM	5.1	4.4	4.2	M	57.1 (35)
2002	FM	5.0	4.4	4.3	F	29.7 (37)
2019	CH	4.9	4.5	4.3	F	51.3 (37)
2016	AC	6.3	5.8	5.8	S	72.2 (36)
2017a	AC	5.8	4.6	4.5	S	45.9 (37)

26 ¹ The initial pH of skimmed milk was 6.6.

27 ² Making process (cheese production stages): FM, milk at arriving to the cheese factory;
 28 AM, acidified milk at the moment of renneting; AC, acidified curd at the moment of
 29 kneading; CH, Oaxaca cheese

30 ³ Groups established according to pH at 6 h of acidification at 30 °C as reported by
 31 Roushdy et al. (1999): F, fast, pH ≤ 5.0; M, medium, pH between 5.0 to 5.5; and S,
 32 slow, pH >5.5.

33 ⁴ Between brackets is the number of phage examined for each strain

34

35

Table 6. Enzymatic activity showed by the fast acidifying *Lactococcus lactis* subsp. *lactis* isolates using the API-ZYM system (values between 0 and 5)²

Isolate	C4 ³	C8	LI	LA	VA	CA	CH	ACP	PHO	β-Gal	α-Glu	β-Glu	AGS
518	0	0	0	4	1	1	0	4	1	0	0	0	0
520	1	1	0	4	1	1	1	4	1	4	2	3	3
1002	1	0	0	3	1	1	1	4	1	0	0	0	0
1003	2	1	0	3	0	1	2	3	1	1	1	0	0
1004	3	2	0	4	1	2	4	3	2	2	0	0	0
1502	1	1	0	3	0	0	1	4	1	0	0	0	0
1506	1	1	0	2	0	1	1	4	2	1	0	0	0
2002	0	0	0	4	3	2	2	4	1	5	2	1	0
2019	0	1	0	3	1	1	3	3	2	2	0	0	0
Mean	1.0	0.8	0.0	3.3	0.9	1.1	1.7	3.7	1.3	1.7	0.6	0.4	0.3

¹ pH of milk at 6 h of acidification $\leq 5,0$ (see Table 5).

² Values ranging from 0 to 5 correspond to the nmol of the substrate hydrolyzed: 0, 0 nmol; 1, 5 nmol; 2, 10 nmol; 3, 20 nmol; 4, 30 nmol; 5, ≥ 40 nmol. Activities with values of 0 for all the isolates were not shown in the table.

³ C4, Esterase; C8, Esterase lipase; LI, Lipase; LA, Leucine arylamidase; VA, Valine arylamidase; CA, Cystine arylamidase; CH, α -Chymotrypsin; ACP, Acid phosphatase; PHO, Naphthol-AS-BI-phosphohydrolase; α -Gal, α -Galactosidase; β -Gal, β -Galactosidase; α -Glu, α -Glucosidase; β -Glu, β -Glucosidase; AGS, N-acetyl- β -glucosaminidase;

⁴ Enzymatic activity of the isolate 2017a could not be determined.

Table 7. Minimum inhibitory concentrations of antimicrobial agents ($\mu\text{g/ml}$; Etest, AB BioDisk) against the fast acidifying *Lactococcus lactis* subsp *lactis* isolates¹.

Antimicrobial agents	Strains								
	518	520	1002	1003	1004	1502	1506	2002	2019
Ampicillin	0.38	0.25	0.25	0.25	0.25	0.19	0.25	≤ 0.02	0.50
Benzylpenicillin	0.25	0.19	0.19	0.25	0.50	0.125	0.125	0.19	0.25
Vancomycin	0.09	0.19	0.25	0.19	0.75	0.38	0.25	0.38	0.38
Chloramphenicol	0.75	1.0	0.5	2.0	1.0	2.0	1.0	1.5	1.0
Clindamycin	0.19	0.38	0.38	0.06	12.0 ^R	0.05	0.16	0.05	0.05
Erythromycin	0.75	0.50	0.75	1.5 ^R	2.4 ^R	0.125	0.032	0.125	0.03
Tetracycline	0.05	0.05	0.06	0.38	0.03	0.125	0.25	0.125	0.125
Gentamicin	3.0	3.0	0.75	1.5	16.0	0.75	3.0	0.75	0.75
Kanamycin	3.0	3.0	3.0	1.5	≥ 256 ^R	2.0	1.5	6.0	2.0
Streptomycin	12.0	12.0	12.0	8.0	384 ^R	8.0	6.0	24.0	16.0
Ciprofloxacin	1.5	1.0	2.0	1.5	1.0	2	1.5	2.0	1.5

^R Resistant according to EFSA (2012) and Katla et al. (2001) (see Table 1 for the breakpoints).

¹ pH of milk at 6 h of acidification $\leq 5,0$ (see Table 5).

Table 8. Amounts of the volatile compounds produced by the *L. lactis* subsp. *lactis* isolates from Oaxaca cheese in UHT milk at 30°C for 48 h expressed as µg cyclohexanone equivalent/g milk).

Volatile compound	RRT	Strains									Mean ± SD	SEL
		518	520	1002	1003	1004	1502	1506	2002	2019		
Ethanol	<600	0.07	0.05	0.06	0.03	0.14	0.08	0.10	0.07	0.04	0.07 ± 0.03	0.001
Propanone	<600	0.04	0.09	0.02	0.04	0.08	0.07	0.18	0.09	0.04	0.07 ± 0.05	0.007
2-Methylpropanal	<600	0.01	0.05	0.22	-	-	0.04	0.04	0.04	0.02	0.05 ± 0.07	0.002
Butane-2,3-dione (diacetyl)	613	1.62	1.70	1.32	1.32	1.52	1.53	1.46	1.96	1.40	1.54 ± 0.20	0.334
2-Methylpropanol	622	-	-	0.07	-	-	-	-	-	0.01	0.01 ± 0.02	0.000
3-Methylbutanal	652	1.77	1.97	3.11	0.62	1.52	2.03	2.04	1.93	1.12	1.79 ± 0.69	0.076
3-Methyl-2-butanone (acetoin)	657	0.01	0.10	0.01	0.03	0.02	0.02	-	-	0.02	0.02 ± 0.03	0.006
Acetic acid	661	0.06	-	-	0.89	0.07	0.01	-	0.01	0.25	0.14 ± 0.29	0.012
3-Hydroxy-2-butanone	722	0.72	0.68	0.28	0.72	1.34	0.61	0.67	0.83	0.83	0.72 ± 0.28	0.147
3-Methylbutanol	743	2.94	2.17	7.69	2.51	1.97	2.22	1.95	1.95	1.53	2.77 ± 1.89	0.326
2,3-Heptanedione	838	0.04	0.10	0.58	0.14	0.15	0.11	0.09	0.17	0.05	0.16 ± 0.16	0.013
Butanoic acid	825	0.65	0.40	0.60	0.39	0.52	0.27	0.21	0.14	0.20	0.37 ± 0.18	0.022
4-Methyl-2-oxopentanoic acid	950	0.21	0.12	0.58	0.19	0.19	0.14	0.12	0.14	0.13	0.20 ± 0.15	0.003
5-Hydroxy-2,7-dimethyl-4-octanone	954	0.68	0.30	1.55	0.61	0.46	0.47	0.38	0.47	0.27	0.58 ± 0.39	0.069

RRT: Relative retention time.

SEL: Standard error of the laboratory: $\sqrt{(\sum[y_1 - y_2]^2/N)}$, where y_1 and y_2 are duplicates of a strain and N is the total number of strains.

-: not detected (below the quantification limit, 0.01 µg cyclohexanone eq. per ml of UHT milk).

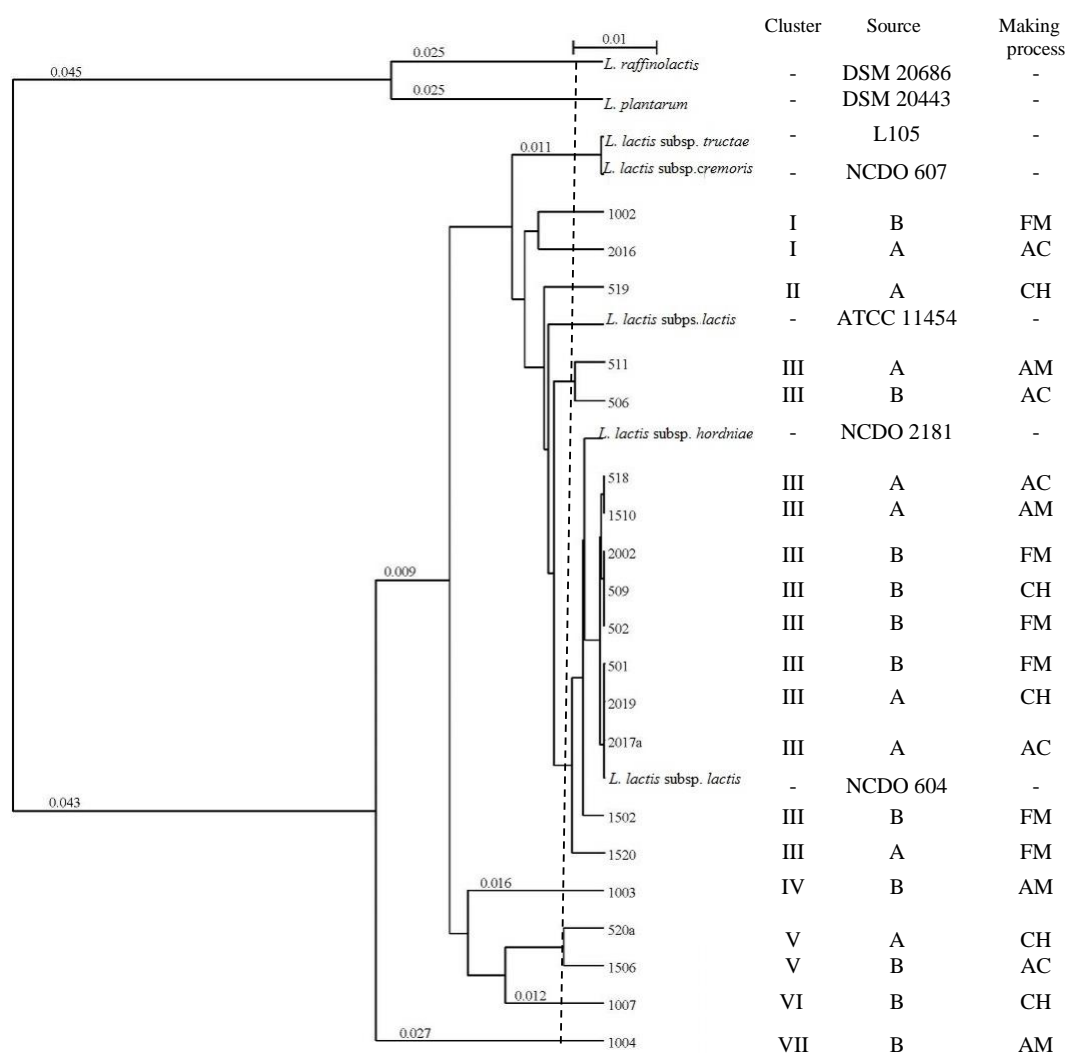


Figure 1. Phylogenetic tree of *Lactococcus lactis* subsp. *lactis* isolated from different dairy sources during Oaxaca cheese making process and a number of *Lactococcus* spp. reference strains based on their 16S rRNA sequences obtained, respectively, from 16S RNAr gene sequencing (670 bp) and the Ribosomal Database Project (Cole et al., 2014). Sequences were aligned using the Clustal W program. The genetic distances (see the scale at the top) were calculated by the UPMGA algorithm.

Source: A and B, factory code; ATCC, American Type Culture Collection; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen; L 105, Velazquez Collection number.

NCDO, National Collection of Dairy Organisms.

Making process (cheese production stages): FM, milk at arriving to the cheese factory; AM, acidified milk at the moment of renneting; AC, acidified curd at the moment of kneading; CH, cheese just after salting.

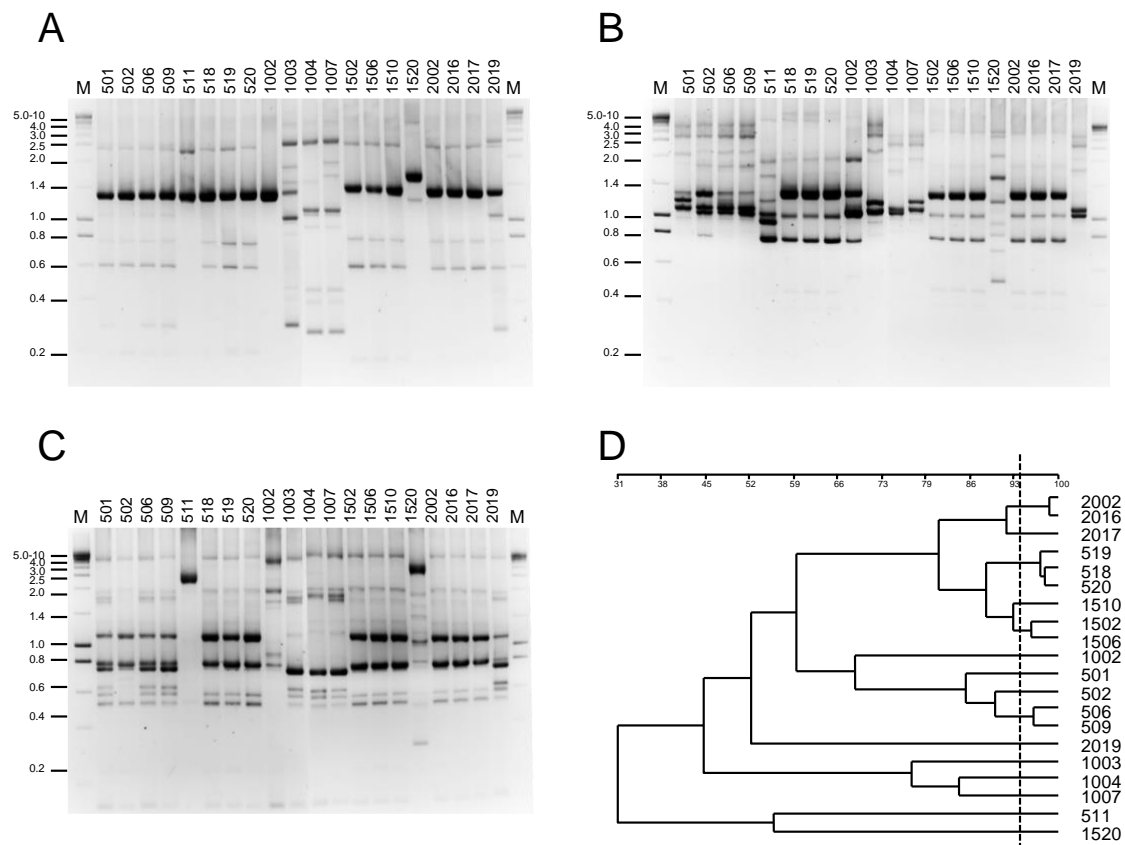


Figure 2.- RAPD and rep-PCR fingerprinting profiles obtained with primers OPA18 (Panel A), M13 (Panel B) and BoxA2R (Panel C), for the *Lactococcus lactis* isolates from Oaxaca cheese; strains from factory A: 2016, 2017, 519, 518, 520, 1510, 2019, 511 y 1520 and strains from factory B: 1502, 1506, 1002, 501, 502, 509, 1003, 1004, 1007. M, molecular weight marker; on the left of the panel, the size of the fragments in kbp is indicated. Panel D, dendrogram of similarity of the combined typing profiles expressed by the Simple Matching (SM) coefficient. Clustering was performed by the unweighted pair group method using arithmetic averages (UPGMA). The dotted line indicates the repeatability of the combined typing method (94%).

Supplementary Material

[Click here to download Supplementary Material: SUPPLEMENT MATERIALfinal-2.docx](#)

Credit Author Statement

Conceptualization (I.C. and J.M.); methodology (I.C., V.A., L.S.C., B.M. and A.B.F.); investigation (I.C. and L.F.); resources (J.M., V.A., L.S.C., B.M. and A.B.F.); writing – original draft (E.J.Q., M.P.R.-d.-R. and J.M.); writing – review & editing (I.C., E.J.Q. and J.M.); and funding acquisition (I.C. and J.M.).