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Title: Characterization of Lactococcus Strains Isolated from Artisanal Oaxaca Cheese

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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**

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25

26 **ABSTRACT**

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.

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1. Introduction

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

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

3

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 4ºC 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 30ºC 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 ºC.

94

2.2. LAB identification and PCR typing

Isolates were recovered with MRS agar incubated at 30°C, 24 h. An initial

characterization of the isolates was performed to select the presumptive LAB using

Gram reaction, morphology, catalase and cytochrome-oxidase activities (Cowan & Steel,

1999; Harrigan, 1998).

A single presumptive-LAB isolate was collected from the recovery MRS agar and

incubated in TSB-YE (30°C, 24 h) for DNA isolation, PCR reaction, sequencing,

species identification, and phylogenetic analysis as described by Caro et al. (2015).

The RAPD and Rep-PCR analyses were performed from total genomic purified DNA

from overnight cultures using a GenElute bacterial genomic DNA kit (Sigma-Aldrich).

Isolates were typed according to their RAPD and rep-PCR fingerprinting profiles using

the primers OPA18 (5'-AGGTGACCGT-3'; Matto et al., 2004), M13 (5'-

GAGGGTGGCGGTTCT-3'; Rossetti & Giraffa, 2005), and BoxA2R (5'-

ACGTGGTTTGAAGAGATTTTCG-3'; Koeuth et al., 1995). RAPD and rep-PCR

amplifications were independently performed in 25 μl volume reactions containing 12.5

μl MasterMix (Ampliqon), 5 μl of either primer (10 μM), 3 μl of purified DNA, and

molecular grade water (Sigma-Aldrich). The DNA amplification involved one cycle at

95°C for 7 min, followed by 40 denaturation cycles at 90°C for 30 s, primer annealing at

42°C (M13), 40°C (BoxA2R) or 32°C (OPA18) for 1 min, a first extension at 72°C for 4

min, and then a final extension at 72°C for 10 min. Typing reaction products were

subjected to electrophoresis and recorded. GeneTools software v.4.03 (SynGene) was

used to compare the profiles.

2.3. Phenotypic characteristics of Lactococcus *strains*

119   The strains' acidification capacity was tested after 0, 6, 12, and 24 h at 30 ºC (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 ºC for 48 h under

127   anaerobiosis. *Staphylococcus aureus* CECT 5192 and *Enterococcus fecalis* 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 10^8 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 30ºC. *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 30ºC 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 18-28ºC and 17-22ºD. 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 $\geq$ 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)

8

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 ºC (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   amylum (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,

10

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 ($\leq$10 nmol).

11

Among the fast acidifying strains only one (number 520a) showed a relatively high

activity for β-glucosidase and N-acetyl- β-glucosaminidase (Table 6). These enzymatic

activities are not desirable for a starter as they might be associated with adverse effects

in the human intestinal tract by releasing aglycones from glycosides plants especially

dietary flavonoids (Bujnakova, Strakova, & Kmet, 2014; Parodi, 1999), although this

effect remains controversial due to reports of potential anti-carcinogenic and anti-

mutagenic effects, especially those derived from flavone C glycosides (Heavey &

Rowland, 2004; Xiao, 2017).

The distribution of *L. lactis* subsp. *lactis* isolates according to their MICs is shown in

Table 4S. None of the isolates was resistant to ampicillin, benzylpenicillin, vancomycin,

chloramphenicol, tetracycline and gentamicin. Resistance was found for streptomycin

(60%, 12 isolates), erythromycin (35%, 7), clindamycin (15%, 3), kanamycin (15%, 3)

and ciprofloxacin (5%, 1). High resistance of *L. lactis* to streptomycin has been reported

in several studies (Fernández et al., 2011; Katla, Kruse, Johnsen, & Herikstad, 2001;

Klare et al., 2007). In this study, 60% of the strains were resistant to streptomycin with

two of them showing a MIC higher than 512 μg/ml. This level appeared to be

intermediate in the studies by Katla et al. (2001) and Salem et al. (2018), who found a

resistance to streptomycin higher than 256 μg/ml in 90% of *L. lactis* strains. Only 15%

of the isolates were found resistant to erythromycin in this study. The results were

higher than those reported by Florez et al. (2005) and lower than those found by

Franciosi et al. (2009), who found that 1.5% and 57% of *L. lactis* subsp. *lactis* strains

were resistant, respectively.

The distribution of MICs allows the estimation of the isolated strains' resistance

breakpoints. The discrepancy between the experimental resistance and that obtained

from the literature (Table 4S) is for streptomycin only, with an experimental breakpoint

293   of ≥64 µg/ml instead of ≥32 µg/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   methybutanol, 3-methybutanal, 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**

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Table

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   [1]EFSA, 2012

4   [2]Katla et al. (2001)

5

6

7   Table 2. Counts (log CFU/g; mean ± 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 ± 0.45^b	6.58 ± 0.43^c
Acidified milk	7.96 ± 0.23^a	8.70 ± 0.83^a
Acidified curd	8.23 ± 0.29^a	7.62 ± 0.31^{ab}
Fresh cheese	7.64 ± 0.60^{ab}	6.63 ± 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

Table 3. Distribution of LAB isolates from different media and Oaxaca cheese

production stages as identified by partial 16S rRNA gene sequencing.

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

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.

Table 4. Taxonomic identification of Oaxaca cheese presumptive *Lactococcus* isolates based on partial 16S rRNA gene sequencing and 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	*L. lactis* subsp. *lactis*	99	1.000	0.991
502	NR_040955.1	DQ011898	*L. lactis* subsp. *lactis*	99	1.000	1.000
506	NR_040955.1	DQ011898	*L. lactis* subsp. *lactis*	99	0.997	0.980
509	NR_040955.1	DQ011898	*L. lactis* subsp. *lactis*	99	1.000	1.000
511	NR_040955.1	EU091387	*L. lactis* subsp. *lactis*	99	0.998	0.953
518	NR_040955.1	EU872263	*L. lactis* subsp. *lactis*	99	0.997	0.974
519	NR_040955.1	JF297355	*L. lactis* subsp. *lactis*	99	1.000	0.990
520a	NR_040955.1	EU091415	*L. lactis* subsp. *lactis*	99	0.996	0.977
1002	NR_040955.1	DQ011898	*L. lactis* subsp. *lactis*	99	0.997	0.969
1003	NR_040955.1	DQ011898	*L. lactis* subsp. *lactis*	98	1.000	1.000
1004	NR_103918.1	DQ255952	*L. lactis* subsp. *lactis*	97	1.000	0.971
1007	NR_040955.1	DQ173744	*L. lactis* subsp. *lactis*	99	0.984	0.976
1502	NR_040955.1	AF515224	*L. lactis* subsp. *lactis*	99	0.998	0.968
1506	NR_040955.1	DQ011898	*L. lactis* subsp. *lactis*	99	1.000	0.963
1510	NR_103918.1	DQ011898	*L. lactis* subsp. *lactis*	99	1.000	0.980
1520	NR_040955.1	EU872263	*L. lactis* subsp. *lactis*	99	1.000	0.964
2002	NR_040955.1	EU872263	*L. lactis* subsp. *lactis*	99	1.000	0.964
2016	NR_103918.1	DQ011898	*L. lactis* subsp. *lactis*	99	0.997	0.989
2017a	NR_103918.1	DQ011898	*L. lactis* subsp. *lactis*	99	1.000	1.000
2019	NR_103918.1	DQ011898	*L. lactis* subsp. *lactis*	99	1.000	0.991

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

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

S_ab scores indicate the degree of match of assembly consensus sequences to each named bacterial species in the RDP-II program.

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

Isolate	Making process[2]	6 h	12 h	24 h	Ability to acidify[3]	Phage resistance %
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)

[1] The initial pH of skimmed milk was 6.6.

[2] 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, Oaxaca cheese

[3] Groups established according to pH at 6 h of acidification at 30 ºC as reported by Roushdy et al. (1999): F, fast, pH $\leq$ 5.0; M, medium, pH between 5.0 to 5.5; and S, slow, pH >5.5.

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

Table 6. Enzymatic activity showed by the fast acidifying *Lactococcus lactis* subp. *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 ≤ 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 (µg/ml; Etest, AB BioDisk) against the fast acidifying *Lactococcus lactis* subsp *lactis* isolates[1].

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 ≤ 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 30ºC 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: $\sqrt{(\Sigma[y_1 - y_2]^2/N)}$, where y_1 and y_2 are duplicates of a strain and N is the total number of strains.

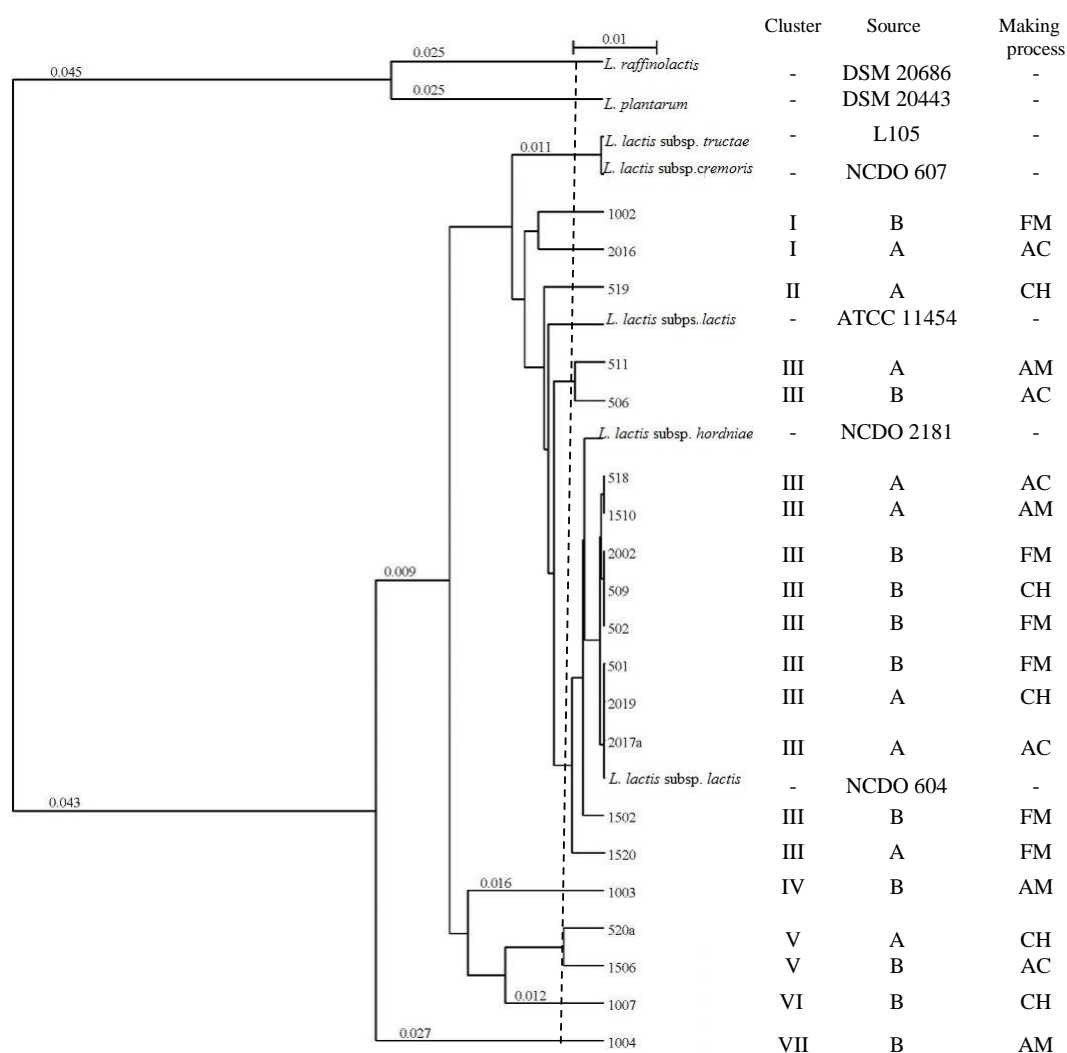-: not detected (below the quantification limit, 0.01 µg cyclohexanone eq. per ml of UHT milk).

Figure 1. Phylogenetic tree of *Lactococcus lactis* subp *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.
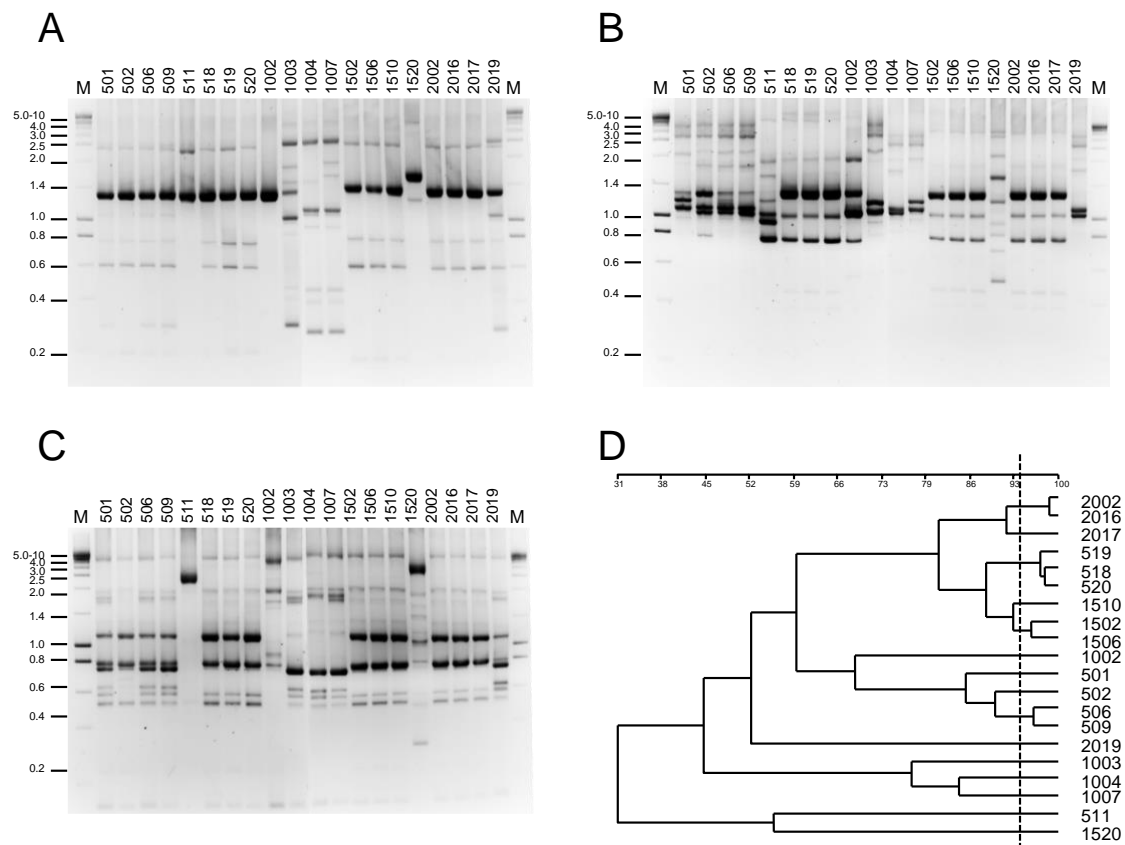
Figure 2.- RAPD and rep-PCR fingerprinting profiles obtained with primers OPA18 (Panel A), M13 (Panel B) and BoxA2R (Panel C), for the *Lactococcus lactis* isolates from Oaxaca cheese; strains from factory A: 2016, 2017, 519, 518, 520, 1510, 2019, 511 y 1520 and strains from factory B: 1502, 1506, 1002, 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, dendogram 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

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.).