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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.

Characterization of *Lactococcus* Strains Isolated from Artisanal Oaxaca Cheese

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ABSTRACT

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

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

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 López, 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).

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

2. Material and methods

2.1. Sampling and LAB isolation

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

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 95ºC for 7 min, followed by 40 denaturation cycles at 90ºC for 30 s, primer annealing at
113 42ºC (M13), 40ºC (BoxA2R) or 32ºC (OPA18) for 1 min, a first extension at 72ºC for 4
114 min, and then a final extension at 72ºC 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*

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

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

The antibiotic susceptibility against the antibiotics recommended by EFSA (2012) were tested using the Etest assay (AB BioDisk) in order to determine the minimum inhibitory concentrations (MIC) (Table 1). A 10^8 CFU/ml (100 µl) suspension was inoculated onto LSM agar plates (ISO, 2010). Afterwards (up to 15 min), two strips of the Etest were placed on the border of the plates and incubated for 24-48 h at 30ºC. *E. faecalis* ATCC 29212 was used as control. The breakpoints considered were those suggested by international organizations or research studies (Table 1).

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

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

2.4. Statistical analyses

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

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

3. Results and discussion

3.1. LAB population

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

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

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

3.2. Lactococcus *identification and typing*

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

The phylogenetic tree of partial 16S rRNA sequences using UPMGA algorithm was built with *L. lactis* subsp. *lactis* isolates and a variety of selected reference strains (Fig. 1). All *L. lactis* spp. were divided in three distant groups showing long branches: (i) 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

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

3.3. Phenotypic characterization of Lactococcus *strains*

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

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

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

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

because it would give cheeses with relatively high amount of 2-methylpropanal and 3-methylbutanal and thus high flavour intensity; the sensory acceptability of such a highly-flavoured cheese requires further study and it is far from the aim of this work. On the other hand, butane-2,3-dione, 3-hydroxy-2-butanone, ethanol, and acetic acid are products derived from pyruvate metabolism. The two former are typically produced via citrate metabolism and contribute to buttery and creamy flavours in dairy products (Marilley & Casey, 2004; Smit et al., 2005).

In a previous study (Sandoval-Copado, Orozco-Villafuerte, Pedrero-Fuehrer, & Colín-Cruz, 2016), the volatiles in the headspace of three Oaxaca cheeses –two made from pasteurized milk and one made from naturally acidified milk– were identified although not quantified. The authors reported a total of 14 volatiles from which 11 were present in the three cheeses. Four out of the 11 compounds were coincident with those of our study: 3-methylbutanal, butane-2,3-dione, 3-hydroxy-2-butanone, acetic acid, and 2-propanone. Discrepancies regarding the volatile profile among studies could be attributed to differences in the microbial species involved in fermentation, substrate (cheese vs acidified milk), and in the fibre type used in the SPME method.

4. Conclusions

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

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

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Table

Table 1. Concentration ranges of the antibiotic tested by the E-test method and break 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]

[1]EFSA, 2012

[2]Katla et al. (2001)

Table 2. Counts (log CFU/g; mean ± SD) of the viable lactic acid bacteria (LAB) in two 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}

[a, b, c]Mean values in columns with different number indicate significant difference ($p<0.05$; Tukey's test).

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
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

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.

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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

		pH at different acidifying times[1]				Phage resistance
Isolate	Making process[2]	6 h	12 h	24 h	Ability to acidify[3]	%
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 ≤ 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

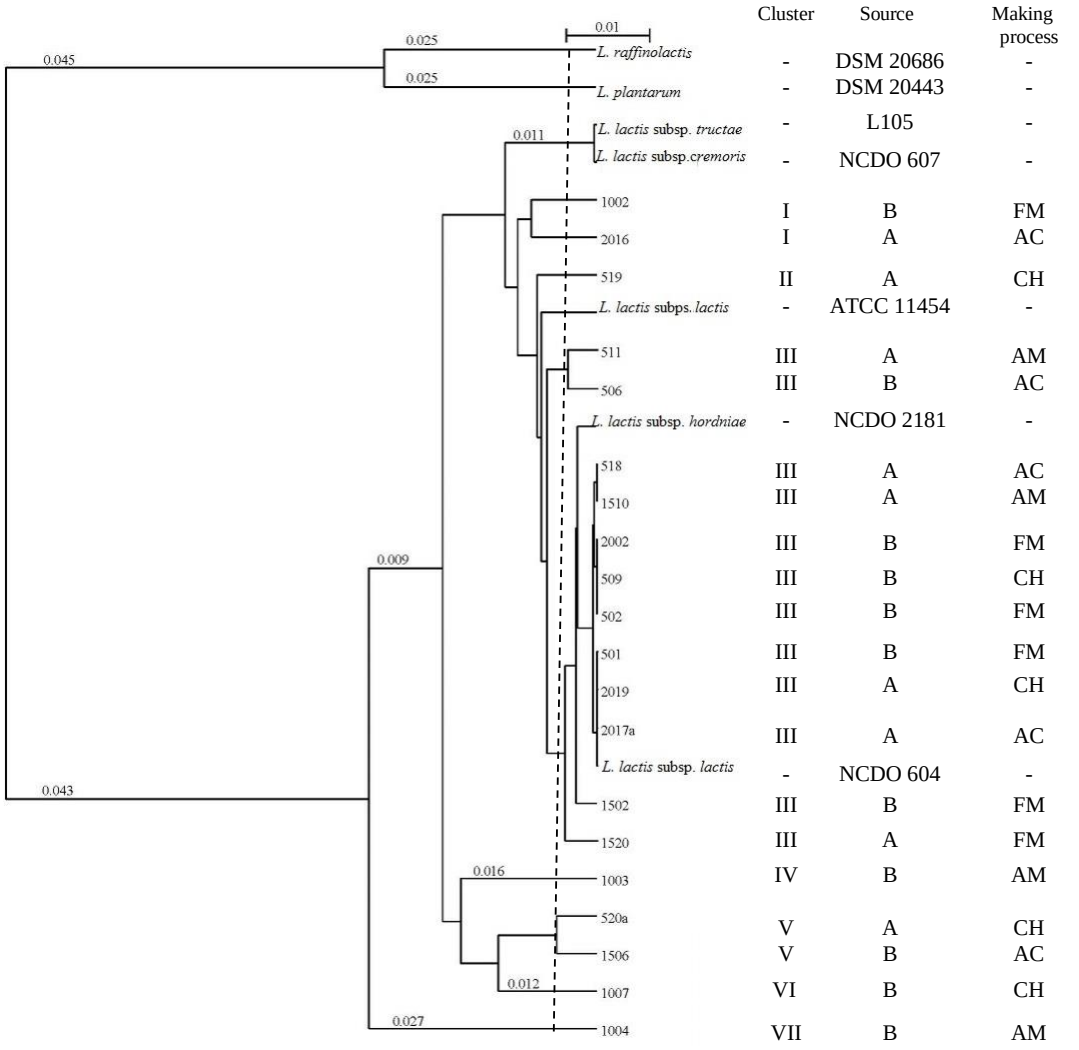

Figure 1. Phylogenetic tree of *Lactococcus lactis* subp *lactis* isolated from different dairy sources during Oaxaca cheese making process and a number of *Lactococcus* spp. reference strains based on their 16S rRNA sequences obtained, respectively, from 16S RNAr gene sequencing (670 bp) and the Ribosomal 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.

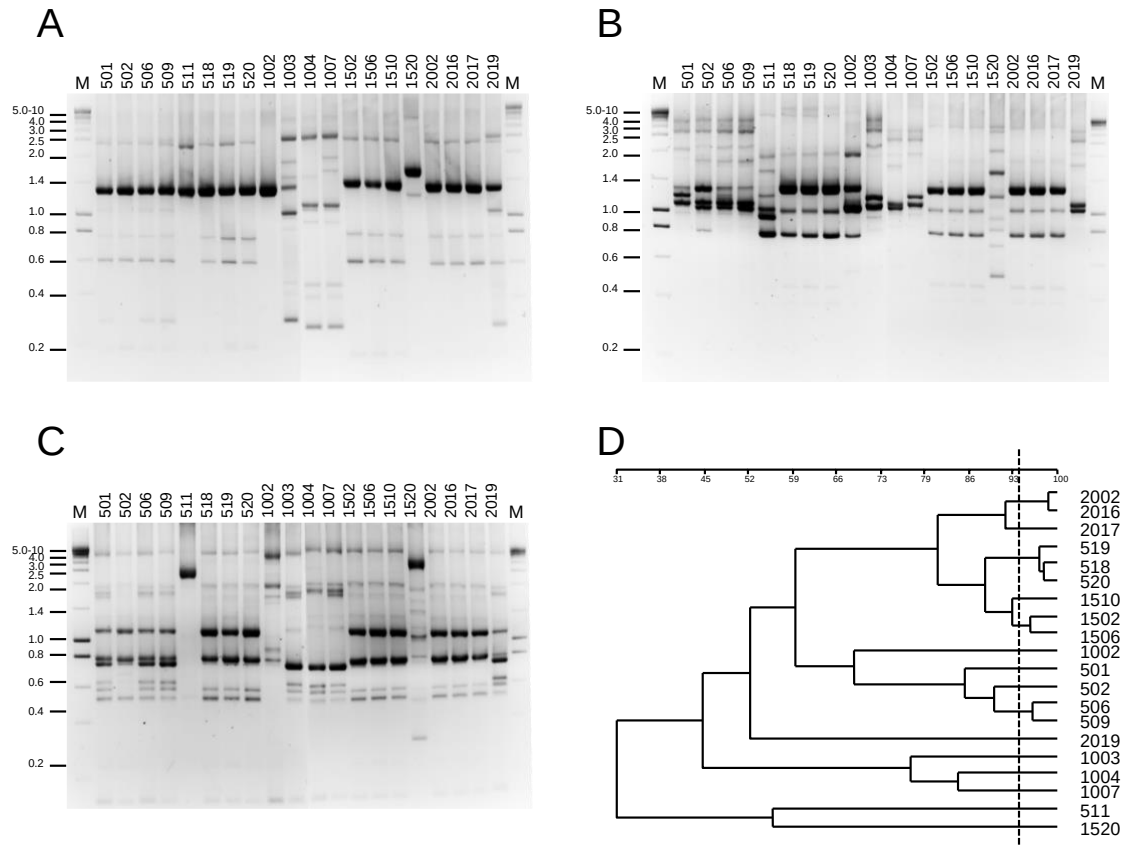

Figure 2.- RAPD and rep-PCR fingerprinting profiles obtained with primers OPA18 (Panel A), M13 (Panel B) and BoxA2R (Panel C), for the *Lactococcus lactis* isolates from Oaxaca cheese; strains from factory A: 2016, 2017, 519, 518, 520, 1510, 2019, 511 y 1520 and strains from factory B: 1502, 1506, 1002,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

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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.).