



Microbial characterization and diversity of artisanal Ranchero cheese with emphasis in *Lactococcus* strains

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Abstract

The aim of this work is to characterize the natural microflora of artisanal Ranchero cheese and to identify *Lactococcus* isolates. Ten artisanal Ranchero cheese samples made with raw milk were obtained from local producers located in different geographical areas. Aerobic mesophilic, *Staphylococci*, *Psychrophilic*, total coliforms, molds, yeast and, lactic acid bacteria (LAB) (*Lactococcus*, *Streptococcus* mesophilic and thermophilic, *Lactobacillus*, *Enterococcus*, *Leuconostoc*) were numbered through agar plating. LAB isolates were classified by genus. Then, nineteen randomly selected presumptive *Lactococcus* isolates were assigned to specie by PCR amplification and DNA sequence. A high number of aerobic mesophilic *Staphylococci*, *Psychrophilic*, total coliforms, molds, and yeast were found. *Lactococci*, mesophilic and thermophilic *Streptococci* and *Lactobacilli* were the dominant LAB. *Enterococci* and *Leuconostoc* were also present. The isolates were identified as *L. lactis* subsp. *lactis* and *L. garvieae*. And both were found to be the dominant *Lactococcus* species that could be selected and used in a starter culture. Sanitation deficiencies in the production of artisanal Ranchero cheese were evident, which may translate into being a potential consumers health risk factor.

Keywords: *Lactococcus*; microbiota; artisanal Ranchero cheese.

Practical Application: The knowledge of the microbial quality and LAB involved in the elaboration of artisanal Ranchero cheese would be of great interest, not only to determine its sanitary quality but also to develop an appropriate starter culture that ensures optimum and consistent characteristics of the cheese.

1 Introduction

In Mexico, the dairy industry is one of the most important agricultural industries; as such, there is a wide variety of genuine artisan cheeses that represent different regions and their environment of origin. Chihuahua is a Mexican state with one of the highest production of milk and cheeses, where artisanal cheeses are made using traditional production techniques (Servicio de Información Agroalimentaria y Pesquera, 2017a). In 2016, the cheese industry produced 342 thousand tons with a market value of 15 thousand 823 million pesos, the main varieties of cheese produced were: Fresh (17.0%), Panela (14.0%) and Double cream (13.0%) (Servicio de Información Agroalimentaria y Pesquera, 2017b). Because Mexico has a deficit in milk production, it is not considered a cheese exporting country.

The artisanal Ranchero cheese made in Chihuahua is a semipressed dairy product elaborated from raw cow milk, and it is considered as a fresh cheese of bright, milky, and mild flavor, which is consumed within no more than five days after production (Villegas & Cervantes, 2011).

In cheeses made from raw milk, the contribution of autochthonous fermenting microbiota is prominent (Dagdemiç &

Ozdemir, 2008; Renye et al., 2008; Tunick et al., 2007); therefore, only specific bacterial combinations of strains isolated are used for cheese making. Artisanal Ranchero cheese is made with raw milk and without the addition of starter cultures; therefore, its organoleptic characteristics rely on the lactic microbiota presents in raw milk, which is dependent to the geographical area of production (Beresford et al., 2001). Also, the presence of yeasts and molds develop and enhance the texture and flavor of the cheese (Lavoie et al., 2012).

Currently, one of the challenges presented by the food industry in Mexico is to provide sufficient safe food for its population. In this context, and until now, artisan cheeses have been given little or no importance, so there is little research related to the quality and characterization of the same as well as a null regulatory regulation in practice. In general, the elaboration of cheese with raw milk is not permitted by Mexican health regulations, which dictate that raw milk can be used only if the cheese is consumed 100 days after its production (Norma Oficial Mexicana, 1994). Nevertheless, artisanal Ranchero cheese is still produced with raw milk because this regulation has not been enforced. An alternative to solve this issue is to develop a specific indigenous starter for

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use in the manufacture of artisanal Ranchero cheese. Therefore, it is important to know the microbial communities inherent in these cheeses. This knowledge can prove essential in dealing with product quality features, authenticity assurance, and traceability along the entire production chain.

Although researchers have studied the microbiota of Mexican cheese, mainly fresh cheese, there is still a need to study genuine Mexican handmade cheeses, such as Ranchero cheese because, to date, there is a lack of knowledge about many features of its genuineness and typicality, such as microbiological quality and biodiversity of the lactic microbiota. Hence, the aim of this work was to identify and characterize *Lactococcus* isolates present in artisanal Ranchero cheese elaborate with raw milk. This could be taken into consideration to select proper strains for a suitable starter culture for the manufacturing of this cheese.

2 Materials and methods

Ten traditional Ranchero cheeses made with raw milk were purchased from different local producers located in different geographical areas in the state of Chihuahua, Mexico (n = 10). It is known that the microbiological flora of milk changes when the type of food and climate changes. Chihuahua is the largest state of Mexico, it has mountains, plains and deserts, where temperatures and milk production systems vary considerably. Because of this, the ten samples of cheese were purchased from producers located in ten different geographic areas of the state. The cheeses were transported in sterile bags in a container at 4 °C, and they were examined before 24 h of chilled storage (4 °C).

For microbiological enumeration and isolation, a sample of 10 g from each cheese was aseptically homogenized in 90 mL of phosphate buffer and mixed for 2 min (Stomacher Lab Blender, London, UK). The homogenized sample was serially diluted (1:10) in sterile phosphate-buffered (Norma Oficial Mexicana, 2010). Each dilution was plated onto a specific media by triplicate. Psychrotrophs and aerobic mesophilic microorganisms were plated onto plate count agar (PCA; Oxoid, Basingstoke, U.K.) and incubated at 5 °C (Psychrotrophs) and 30 °C (Aerobic mesophilic). M17 agar (Oxoid, Basingstoke, U.K.), containing 1% lactose (M17L) at 30 °C and 45

°C, for mesophilic and thermophilic streptococci, respectively. M17 agar containing 1% lactose and ciclohexamida (100 µg/L) (M17LC) at 22 °C for *Lactococcus*; Rogosa Sharpe agar (MRS) (Oxoid, Basingstoke, U.K.) at 30 °C for Lactobacilli; MRS agar containing 500 µg/mL vancomycin (MRSV) at 30 °C for *Leuconostoc*; Baird-Parker agar (BP; BD Bioxon, Becton Dickinson and Company, Mex) supplemented with egg yolk tellurite (BD Bioxon, Becton Dickinson and Company, Mex) at 37 °C for *Staphylococcus*. Kanamycin Aesculin Azide agar (KAA; Oxoid, Basingstoke, U.K.) at 37 °C for *Enterococcus*; potato dextrose agar (PDA; BD Bioxon Becton Dickinson and Company, Mex) acidified with 10% tartaric acid (Merck, falta lugar) at 25 °C for molds-yeasts; and violet red bile agar (VRB; Oxoid, Basingstoke, U.K) at 32 °C for total coliforms. Media were inoculated by spreading 100 µL of diluted samples onto the agar, except in the case of the VRB media, which was inoculated by pour plate method (1000 µL). All inoculated agar media were incubated

anaerobically except PCA, PDA, and VRB media. KAA, VRB, and BP agars were incubated for 24 h; MRS, MRSV and M17L agars were incubated for 48 h; PCA agar at 30 °C agar was incubated for 72 h; M17LC and PDA were incubated for five days; PCA agar at 5 °C was incubated for 12 days (Renye et al., 2008). Numbers of colony-forming units (CFU) were counted on the plates with numbers ranging between 10-200 CFU. After incubation period, plates with 10 to 200 colonies were selected for isolation. Four to six colonies were randomly picked from each plate, except from PDA, VRB, and PCA agar at 5 °C. Colony morphology, gram, and catalase reactions were checked. All gram (+), catalase (-) colonies were streaked out three times on MRS or M17 media for rods and cocci, respectively, to assure purity. The isolates were stored at -80 °C in liquid medium (20% glycerol (Fermont, Monterrey, Nuevo León, México) and 80% of MRS or M17 broth (Oxoid, Basingstoke, U.K.) for rods and cocci, respectively) for further analysis.

PCR amplification and DNA sequence analysis were performed on randomly chosen isolates from the most significant genera. Genomic DNA was isolated from 1 mL of the overnight cultures using the DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany), according to the instructions of manufacturers. The obtained genomic DNA was used as template for PCR with the following oligonucleotides: forward primer G17: 5'GTGAAGTCGTAACAAGG'3 (Pangallo et al., 2008), and reverse primer GplusR: 5'GTGAAGTCGTAACAAGG'3 (Chebeňová-Turcovská et al., 2011) for amplification of 16S rRNA genes. The PCR mixture contained 12.5 µL Go Taq® Green Master Mix (Promega Corporation, USA), 1 mM each primer, 250 ng template DNA and HPLC-quality water up to the volume of 25 µL. The PCR conditions consisted in an initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min and polymerization at 72 °C for 3 min, followed by a final polymerization at 72 °C for 10 min. The PCR products were purified with the Wizard® SV Gel and PCR Clean-Up System (Promega Corporation, USA), according to the manufacturer's specifications, and sequenced. Sequencing was performed in the Institute of Biotechnology, UNAM in Cuernavaca, Morelos, Mexico. Sequences were analyzed using Sequencher 5.3 (Gene Codes Corp., Ann Arbor, MI) and compared with sequences available in GeneBank using the BLASTn algorithm (Altschul et al., 1997).

A multiple alignment of artisanal cheese clone sequences, identified as 16S rDNA, and 16S sequences obtained from GenBank database was performed using the PHYLIP (Felsenstein, 1989) and Clustal W 1.6 program (Thompson et al., 1994). In addition to the strains isolated in this study, 16S rRNA gene sequences from five closely related strains were included in the analysis: *Lactococcus chunganensis* (HM2419.1); *Lactococcus raffinolactis* (HM241918.1); *Lactococcus lactis* subsp. *lactis* (KC768838.1); and *Lactococcus garvieae* (HM241915.1). A phylogenetic tree was generated using the two parameters of Kimura for genetic distances (Kimura, 1980) and the neighbor-joining method (Saitou & Nei, 1987) with 1000 replications. To avoid any potential bias introduced as a result of sequence addition, the taxon addition order was randomized in the neighbor joining analysis.

Microorganisms genera were analyzed using basic statistics: $y = \mu + \varepsilon$, where μ = general average, and ε = random error. The significance level used for all statistical analyses was 5%. The analyzes were performed using the statistical package SAS version 9.1.3 (Statistical Analysis System, 2006).

3 Results

The microbial logarithmic colony forming units (\log_{10} CFU) mean counts for lactic and non lactic microorganisms of artisanal rancho cheese, are summarized in Tables 1 and 2.

Of the total of 320 isolates obtained, 45 (14.06%) were lost in the subculturing process, 103 (32.18%) could not be classified as LAB, and 172 (53.75%) were presumptively identified as

LAB genus, accordingly to its capacity for grow at different temperatures, NaCl concentrations, and its production of

CO₂ from glucose (Cogan et al., 1997). Of the 172 isolates, 73 (42.5%) were recognized as *Lactococcus*; 72 (41.8%) shown to be *Enterococcus*; 14 (8.1%) were considered as *Lactobacillus*; six isolates (3.5%) were Mesophilic *Streptococcus*; four isolates (2.3%) *Leuconostoc* and, three isolates (1.7%) were thermophilic *Streptococcus*. Randomly chosen *Lactococcus* isolates were subject to further identification.

PCR amplification and sequence analysis of 16S rDNA were performed on isolates randomly chosen from the *Lactococcus* genera and compared with sequences in GenBank. From that isolates, 13 (68.4%) were shown to be *Lactococcus lactis* subsp. *lactis*, with a 100% homology; while six isolates (26.3%) were identified as *Lactococcus garvieae* (five of them with a homology of 99%), and the other (5.2%), with a 95% homology, as is shown in Table 3.

Table 1. Microbial logarithmic counts (mean \pm SD) of non lactic acid bacteria of artisanal Rancho cheese.

Genera (\log_{10} CFU/g)				
Me	S	Co	M and Y	Ps
7.90 \pm 0.69 ^a	7.32 \pm 0.73 ^{ab}	5.90 \pm 1.06 ^b	4.12 \pm 1.42 ^c	6.23 \pm 0.85 ^b

Different lowercase letters indicates statistically significant difference between genera ($P < 0.05$). Me = Aerobic mesophiles; S = *Staphylococcus* spp; Co = Total coliforms; M and Y = Molds and yeast; Ps = Psychrotrophs.

Table 2. Microbial logarithmic counts (mean \pm SD) of lactic acid bacteria of artisanal Rancho cheese.

Genera (\log_{10} CFU/g)					
Lc	Sm	St	Lb	E	Le
8.14 \pm 0.22 ^a	7.79 \pm 0.65 ^a	7.65 \pm 0.71 ^a	7.65 \pm 0.55 ^a	6.30 \pm 1.33 ^b	5.45 \pm 1.47 ^c

Different lowercase letters indicates statistically significant difference between genera ($P < 0.05$). Lc = *Lactococcus*; Sm = Mesophilic *Streptococcus*; St = Thermophilic *Streptococcus*; Lb = *Lactobacillus*; E = *Enterococcus*; Le = *Leuconostoc*.

Table 3. Bacterial species identified in artisanal Rancho cheese.

Isolate ID	Taxonomic ID	% Identity*	GenBank Access Number*
2	<i>Lactococcus lactis</i> subsp. <i>lactis</i> strain II1403	100	KC768838.1
7	<i>Lactococcus lactis</i> subsp. <i>lactis</i> strain II1403	100	KC768838.1
18	<i>Lactococcus lactis</i> subsp. <i>lactis</i> strain II1403	100	KC768838.1
96	<i>Lactococcus lactis</i> subsp. <i>lactis</i> strain II1403	100	KC768838.1
107	<i>Lactococcus lactis</i> subsp. <i>lactis</i> strain II1403	100	KC768838.1
119	<i>Lactococcus lactis</i> subsp. <i>lactis</i> strain II1403	100	KC768838.1
120	<i>Lactococcus lactis</i> subsp. <i>lactis</i> strain II1403	100	KC768838.1
124	<i>Lactococcus lactis</i> subsp. <i>lactis</i> strain II1403	100	KC768838.1
128	<i>Lactococcus lactis</i> subsp. <i>lactis</i> strain II1403	100	KC768838.1
136	<i>Lactococcus lactis</i> subsp. <i>lactis</i> strain II1403	100	KC768838.1
137	<i>Lactococcus lactis</i> subsp. <i>lactis</i> strain II1403	100	KC768838.1
139	<i>Lactococcus lactis</i> subsp. <i>lactis</i> strain II1403	100	KC768838.1
140	<i>Lactococcus lactis</i> subsp. <i>lactis</i> strain II1403	100	KC768838.1
101	<i>Lactococcus garvieae</i> strain LMG 8501	99	HM241915.1
130	<i>Lactococcus garvieae</i> strain LMG 8501	99	HM241915.1
138	<i>Lactococcus garvieae</i> strain LMG 8501	99	HM241915.1
152	<i>Lactococcus garvieae</i> strain LMG 8501	99	HM241915.1
164	<i>Lactococcus garvieae</i> strain LMG 8501	99	HM241915.1
112	<i>Lactococcus garvieae</i> strain LMG 8501	95	HM241915.1

*Percent Identity and Accession Number of closest related species in GeneBank.

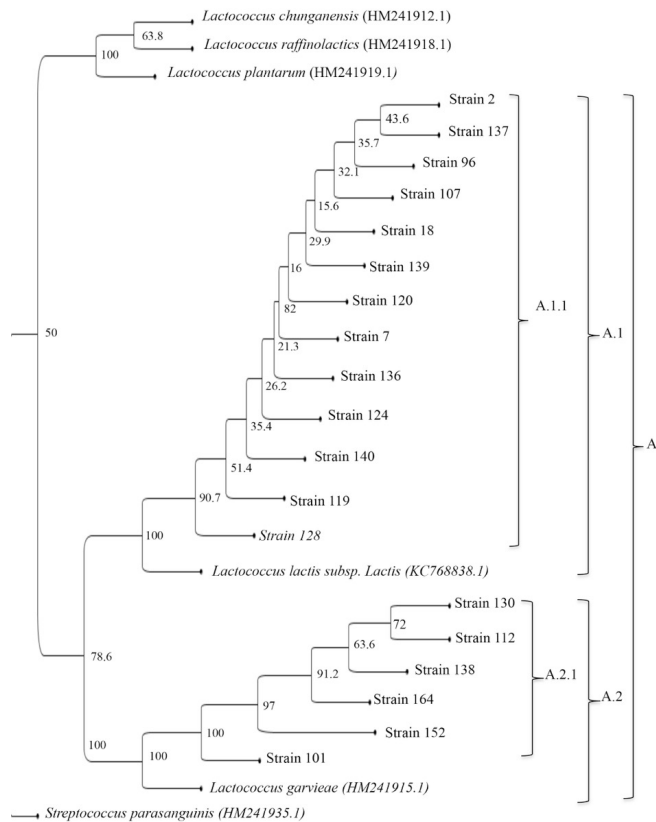


Figure 1. Phylogenetic tree of partial 16S rDNA from strains isolated from artisanal Ranchero cheese. The sequences were aligned by the Clustal W program. The distance matrix and phylogenetic tree were calculated with the neighbour-joining algorithms and the method of two parameters of Kimura. Artisanal ranchero cheese 16Sr rDNA sequences are represented by the strain number. The sequence of *Streptococcus parasanguinis* served as outgroup sequence. The number of 1000 bootstrap resamplings that support each topological element in the neighbour-joining analysis is indicated.

The phylogenetic analysis classified the strains in a single clade A with a bootstrap value of 78.6% (Figure 1). Clade A was divided into groups A.1 and A.2. The A.1 group had a bootstrap value of 100.0% and a sublevel A.1.1 with 90.7% bootstrap value. All the strains (2, 7, 18, 96, 107, 119, 120, 124, 128, 136, 137, 139, and 140) clustered into group A.1.1 were organized monophyletically with *Lactococcus lactis* subsp. *Lactis* (KC768838.1). The A.2 group had a bootstrap value of 100.0% and a sublevel, A.2.1 with 97.0% bootstrap value. These sublevels contained strains 112, 130, 138, 152, and 164 and all formed a monophyletic group with *Lactococcus garvieae* (HM241915.1).

4 Discussion

The average count of aerobic mesophilic bacteria ($7.90 \pm 0.69 \log_{10}$ CFU/g) was similar to that reported by Renye et al. (2008) and Caro et al. (2013) in fresh cheese (8.02 log CFU/g) and Oaxaca cheese (7.63 log CFU/g), respectively,

highlighting that both cheeses were made with raw milk. Mexican legislations do not have a regulation for this parameter (Norma Oficial Mexicana, 1994).

Count of *Staphylococcus* spp. was, on average, $7.32 \pm 0.73 \log_{10}$ CFU/g. *Staphylococcus* spp. is considered as microbiological safety only when it exceeds the $4 \log_{10}$ CFU/g or when it produces enterotoxins (Yoon et al., 2016). Moreover, Mexican official regulations stipulate a maximum permissible level of 1000 UFC/g ($3.00 \log_{10}$ CFU/g) in the specific case of *S. aureus* in fresh cheese.

Coliform counts were $5.90 \pm 1.06 \log_{10}$ CFU/g on average. Similar values were reported in fresh cheese elaborated with raw milk. Renye et al. (2008) observed counts of $5.90 \log$ CFU/g; while Torres-Llanez et al. (2006) registered elevated counts not only of total coliforms (around $4.25 \log$ CFU/g) but also fecal coliforms (around $4.5 \log$ CFU/g). The Mexican official regulations established a maximum permissible level for total coliform bacteria of $2.00 \log_{10}$ CFU/g. High numbers of coliforms could be associated with low hygiene and production process of the artisanal cheese (Solís et al., 2009).

Molds and yeast had an average count of $4.12 \log_{10}$ CFU/g. Higher values have been found in fresh cheeses elaborated from raw milk (Renyé et al., 2008; Torres-Llanez et al., 2006).

Coliforms, molds, and yeast can deteriorate sensorial characteristics and even became a real public health risk. It has been established that counts of coliforms, molds, and yeast in artisanal cheeses decrease within the maturation process (Alejo-Martínez et al., 2015). Nonetheless, the artisanal Ranchero cheese is commonly consumed fresh, within five days after processing, representing a risk for the health of the consumers.

As is shown in Table 2, *Lactococci* was the most abundant LAB isolated from artisanal ranchero cheese ($8.14 \log_{10}$ CFU/g), followed by mesophilic *Streptococci* ($7.79 \log_{10}$ CFU/g), thermophilic *Streptococci* ($7.65 \log_{10}$ CFU/g), and *Lactobacilli* microorganisms ($7.65 \log_{10}$ CFU/g), which could indicate that these microorganisms have important roles in odor and flavor characteristics of artisanal and fresh cheeses.

Lactococcus are an important part of the nonstarter microorganisms because they contribute to cheese flavor and texture (Tabak et al., 2012). *Lactococcus* from dairy products can be generally considered safe because they are consumed daily by humans, and only low incidence of human infections has been reported (Casalta & Montel, 2008). *Lactococcus* are also responsible for the initial acidification during the cheese-making process, which determines curd formation and whey expulsion and prevents the growth of undesirable bacteria due of the resulting low pH concomitant with the production of lactic acid (Karakuş & Alperden, 1995; Molloy et al., 2011; Renye et al., 2008). Therefore, in the present research, it has been proposed that the isolated *Lactococcus* could be used as part of starter culture for artisanal Ranchero cheese elaboration, preserving the sensorial characteristics and, according to official regulations, offer an innocuous product to consumers.

In this study, 19 randomly chosen presumptive *Lactococcus* isolates were further analyzed. Thirteen (68.42%) isolates were identified as *L. lactis* subsp. *lactis* and six (31.57%) as *L. garvieae*.

The majority of the isolates evaluated were recognized as *Lactococcus lactis* subsp. *Lactis* (68.42%). This LAB is the main component of dairy starter cultures used for the production of diverse fermented products (Cavanagh et al., 2015). However, it is a limited component of the starter cultures to produce cheddar cheese (Wouters et al., 2002). It also may serve as one of the principal acidifying agents for the production of fresh cheese because *L. lactis* subsp. *lactis* has been previously identified as part of its natural microbiota (Renyé et al., 2008; Torres-Llanez et al., 2006).

Meanwhile, six (31.57%) isolates were identified as *garvieae*. It has been isolated from diverse food sources such as fishes, crustaceans, raw milk, and raw milk cheeses (Foschino et al., 2008). At the same time, it has been identified as a fish pathogen and as the etiological agent of mastitis in cows and buffalos (Teixeira et al., 1996). This last may be the reason for its presence in cheeses made with raw milk and the fact that being recognized as a component of the native microflora of dairy products elaborated with raw milk (Fortina et al., 2007; Flórez et al., 2012), such as Ranchero cheese (Renyé et al., 2008). Further, it has been linked to human infections (Foschino et al., 2008); however, no association between raw milk cheeses and human infections has been established (Flórez et al., 2012; Foschino et al., 2008).

Even though scarce information is available on phenotypic and biochemical properties of *L. garvieae* isolated from dairy sources, it has been found that strains from dairy sources are lactose fermenters (Fortina et al., 2007; Teixeira et al., 1996), showing a slow rate of acidification, but still comparable with that presented by some *Lactococcus* isolates (Wouters et al., 2002). And have low proteolytic activity (Fortina et al., 2007), which make their use as starter or adjunct cultures feasible; however, technological characterization should be conducted on the isolates found in this study because some *L. garvieae* strains are found tetracycline resistance (Fernández et al., 2010).

5 Conclusions

Results obtained in the present work showed counts of total coliforms, molds, and yeasts that exceeded the maximum levels allowed by Mexican official regulations; in addition, high counts of aerobic mesophilic bacteria, psychrotrophs, and *Staphylococcus* spp. were observed. These results shows hygienic deficiencies in the production process, which translates into a potential risk factor for consumers' health. Likewise, no differences were found between counts of *Lactococcus*, *Streptococcus mesophilus*, *Streptococcus thermophilus*, and *Lactobacillus*. Both the GenBank database and the phylogenetic analysis achieved the identification of two *Lactococcus* species: *Lactococcus lactis* subsp. *lactis* and *Lactococcus garvieae*.

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