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RESEARCH ARTICLE

Characterization of Microbial Traits Involved with the Elaboration of the Cotija Cheese

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Abstract Physicochemical and microbial analyses were conducted for artisanal and semi-industrial manufacturing processes of the Cotija cheese. Differences between manufacturing processes in Cotija cheese affect the microbial composition in the final product. Lactic acid bacteria are important microorganisms in the artisanal Cotija cheese manufacturing. Thirty-one different microorganisms were isolated and identified by ribosomal sequencing analysis during the production of this artisanal cheese. The yeast isolates comprised the following species: *Kluyveromyces lactis*, *Kluyveromyces marxianus, Pichia guillermondii*, *Rhodotorula mucilaginosa*, *Galactomyces reessii*, and *Galactomyces geotrichum*. Bacterial isolates were members of the Lactobacillaceae family with the follow species: *Pediococcus pentosaceus*, *Lactobacillus brevis*, *Lactobacillus plantarum*, and *Lactobacillus casei*. The possible role of these microorganisms in the final flavor and taste, and their utility in the control of undesirable microorganisms in artisanal Cotija cheese is discussed.

Keywords: Cotija cheese, microbial profile, lactic acid bacteria, artisanal cheese, microbial diversity

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Introduction

Cotija cheese is a Mexican ripened white cheese made of strained curds of cow's milk obtained mainly from zebu crossbred cattle (1). It is produced during the months of July through October in the province of Cotija, which is located in the Mexican states of Jalisco and Michoacán. Artisanal manufacturing of Cotija cheese is carried out in batches without pasteurization of the milk. Coagulation (curding) takes place at 27-30ºC for 1-2 h. Once a certain degree of consistency is attained, the curd is crushed, poured into cylinders to obtain blocks weighting approximately 20 kg, and then pressed and aged. The Cotija cheese is traditionally salted several times for preservation purposes and to provide a distinctive color and specific taste. Artisanal cheeses are ripened for 3 months whereas semi-industrial manufactured cheeses are ripened between 1 to 3 months, and it is worth noting that pasteurized milk is often used. In both cases, the final product has a sand- or grain-like consistency. In recent years, the market for Cotija cheese has increased considerably, probably due to the promotion from the Intellectual Institute of Rights of Mexico conferring the region of Cotija a collective trademark to produce this cheese (1).

The province of Cotija represents a specific ecological region of 400 km² in the center of Mexico (19º15'N; 102º30'W) with specific secondary deciduous vegetation (1). Such characteristics must have an impact on the formation of the specific micro-flora found in milk. The importance of particular microorganisms has been widely related to the specific flavor and taste properties found in other artisanal manufactured cheeses (2-4). Within the complex bacterial community of traditional raw milk cheeses, lactic acid bacteria (LAB) are considered to be the dominant microflora. However, many genera and species of these microorganisms are necessarily involved in the

curd ripening process in order to assure the typical taste and aroma of the final cheese through appropriate acidifying, proteolytic, and flavoring activities (5). The variety of these organisms in cheeses depends on the starter used and could therefore represent a good marker to discriminate traditional from semi-industrial products. Despite the great demand of Cotija cheese and the possible importance of the microorganisms involved during its production, to our knowledge there are no reports for their occurrence during the manufacturing of this particular cheese. Therefore, the present study is focused on the characterization of the main groups of microorganisms involved in the manufacturing of the artisanal Cotija cheese.

Materials and Methods

Cheese sampling Artisanal Cotija cheese was obtained from Lourdes village and the semi-industrially manufactured Cotija cheese was obtained from La Troja village, both communities of the province of Cotija. The samples were collected through 4 elaboration stages; curding, salt-addition to the curd, cheese ripened for 1 month, and cheese ripened for 3 months. Two replicates were taken from the center portion of the samples and fractioned for physiochemical and microbiological analysis. After a portion (25 g) of Cotija cheese was taken at various times, cheese were homogenized in sterile physiological solution (0.9% NaCl) for approximately 5 min. Homogenized was serially diluted in 0.9% NaCl to assay the colony forming unit (CFU) and kept in 40% glycerol stock at 70ºC for the further study.

Physicochemical analysis and CFU assay Physico-chemical analyses were conducted in different Cotija cheese samples and included temperature and conductivity. Moisture and pH were determined by standard methods (6). Microbial composition was determined by method of colonies total number in specific culture broths: aerobic bacteria in agar standard count (Bioxon, Beckton Dikinson, Monterrey, Mexico), LABs in Lactobacilli MRS agar (Difco, Becton Dickinson), and yeast in potato dextrose agar adjusting pH at 3.5 (Difco, Becton Dickinson). After 48 h incubation at 37ºC, the numbers of colonies appearing on the media were counted and the morphology of each colony was observed under a microscope.

Microbial isolation and preliminary identification A fraction of 10 g of the artisanal Cotija sample was homogenized in 90 mL of 1% peptone water and then aliquots of serial dilutions were inoculated onto plates with MRS agar (Difco, Becton Dickinson) at 30ºC or potato dextrose agar (PDA, Difco, Becton Dickinson) at 30ºC, for bacteria and yeasts, respectively. Individual colonies were picked, and then re-isolated on the same medium. The strains were maintained in 80%(v/v) of glycerol and stored at −70ºC.

Yeasts were first identified by means of their macroscopic characteristics and several biochemical tests were performed afterwards. These tests included growth on acetic acid, development at 37ºC, and test of urea and carbohydrate fermentation patterns (7). The experiments were performed in duplicate. Likewise, bacteria were first identified by morphological and physiological features. LABs isolates were selected on based of their morphological and physiological differences. The analysis included as follow: Gram staining, cell morphology, motility, catalase test, gas (CO_2) production from glucose and carbohydrate fermentation patterns (8). The growth was measured at various concentrations of pH and NaCl.

Acidification potential The selected bacterial isolates were analyzed by means of their potential of acidification as follow; cells of LAB strains were washed twice in peptone H_2O and inoculated (1%,v/v) in pasteurized milk for 48 h to evaluate the acidification kinetics. Finally, lactic acid accumulation was estimated by the Mexican norm (9).

Preparation of total DNA Genomic DNAs were extracted from bacteria isolates grown on Luria-Bertani broth (Difco, Becton Dickinson) at 37ºC overnight with a rotary shaking at 100 rpm and from yeast isolates grown on 100 mL of PDA for 5 days at room temperature, with a rotary shaking at 250 rpm. Bacterial cells were harvested by centrifugation at 5,000×g at room temperature. Yeast cells were collected through filtration with filter paper and washed with distilled water. In both microbial fractions, the fresh biomass was homogenized in liquid nitrogen and transferred to a 1.5-mL tube containing 1 mL of TEN [100 mM Tris-HCl, 50 mM EDTA, 500 mM NaCl, pH 8.0] buffer and vortexed for 1 min. The centrifugation took place for 10 min at 10,000×g at room temperature, and the pellet was then re-suspended in 1 mL of TEN buffer. Subsequently, the cells were transferred to a fresh 1.5-mL tube in order to carry out the silica-based protocol without modifications as described by Rojas-Herrera *et al*. (10).

Molecular identification Microbial genetic identification was supported by the alignment of the 16S rDNA and ITS1-5.8S-ITS2 rDNA for bacteria and most yeast, respectively. Two yeast isolates (FCOT-4 and -30) were identified using the 26S rDNA region since these were not responsive to the ITS1-5.8S-ITS2 primers. Primers 16S-For/16S-Rev (11), ITS1/ITS4 (12), and NL1/NL4 (13) were used to amplify the desired fragment of rDNA. All

amplifications were performed in 50 μL of PCR reaction mix containing 5 μL of reaction buffer, 5 units of Taq polymerase (Gibco-BRL, Rockville, MD, USA), 1.5 mM $MgCl_2$, 50 pmol of each primer, 120 μM dNTPs, and 50 mg of template cDNA. Amplification was done with an initial denaturation of 5 min at 94ºC, followed by 30 cycles of 1 min at 94ºC, 45 s at 60ºC for bacteria and 58ºC for yeast, and 1 min at 72ºC with a final extension of 5 min at 72ºC. Amplicons were checked by electrophoresis with 1% agarose gel in TBE buffer (89 mM TrisHCl, 89 mM boric acid, 2 mM EDTA, pH 8.0). The PCR products were gel purified with a GeneClean II kit (Bio 101; Vista, CA, USA) and eluted in sterilized distilled water and then re-amplified using the conditions of initial amplification. PCR products were directly sequenced (ABI 3130 DNA sequencer; Applied Biosystems, Foster City, CA, USA) by using the BigDye terminator v3.1 cycle sequencing ready reaction kit according to the instructions of the manufacturer (Applied Biosystems). The resulting sequences were compared with the data in the GenBank (http://www.ncbi.nlm.nih.gov/) and nearest neighbors were calculated for phylogenetic inferences. Sequence-based phylogenetic inference was carried out following the minimum-evolution method by using bootstrap values based on 1,000 replications with MEGA 3.1 program (freely available at http://www.megasoftware.net/).

Results and Discussion

Physicochemical analysis Cotija cheese is supplied by various farmhouses located in the Cotija province, which all have very similar condition. The manufacturing processes of the cheese result in some differences between the artisanal and semi-industrial products (Table 1). Physicochemical parameters between both cheeses vary slightly with exception of the protein content, which varied greatly from 30 to 24% for artisanal and semi-industrial Cotija cheese, respectively. The major protein value found in artisanal Cotija ripened cheese is perhaps from their extended ripening period.

CFU changes during Cotija cheese elaboration Composition in the Cotija cheese varied between their manufacturing processes (Table 2). Artisanal cheese showed a higher occurrence of microorganisms during their first manufacturing stage (curd) reaching values of 5.9 log CFU/g for mesophilic aerobic bacteria, 4.0 log CFU/g for LAB, and 5.0 log CFU/g for yeast. An exception occurs with the filamentous fungi, which were not present during this initial curd stage. On the contrary, semi-industrial cheese exhibited a higher concentration of filamentous fungi with values of 7.8 log CFU/g and the lowest values for other microbial descriptors in the initial curd. In general, final product cheeses showed lower microbial count values regardless of their manufacturing process; however, artisanal Cotija cheese exhibited higher LABs count values in relation to their semi-industrialized counterpart, reaching count values of 2.9 log CFU/g.

The high yeast values found in the initial stage are frequently observed in other cheeses and are believed to make a significant contribution to the maturation process (14,15). Some authors reported that yeasts are involved in the ripening process of cheese, participate in microbial interactions and contribute to texture changes and the biosynthesis of aromatic compounds (14,16). Occurrence of high count yeast values in the initial manufacturing

Table 1. Mean values for the gross composition of Cotija cheese of according to their manufacturing process

Cotija cheese	pH	Ash (mg)	Conductivity (μS)	Moisture	Fat	Protein
				%(w/w)		
Artisanal	5.0±0.00	0.5±0.03	16.0±0.00	30.0±0.07	34.0±0.00	30.1±0.07[1)]
Semi-industrial	5.3±0.07	0.7±0.01	16.0±0.00	33.7±0.01	31.3±0.07	24.6±0.28

[1)]Metabolite concentrations are presented as the mean±SD of 3 replicates.

Table 2. Mean values for the microbial composition of the Cotija cheese of according their manufacturing process

Cotija cheese	Elaboration stage	Microbial count values (log CFU/g)			
		Aerobic bacteria	Lactic acid bacteria	Fungus	Yeast
Artisanal	Curd	5.9±0.00[1)]	4.0±0.01	ND	5.0±0.04
	Curd salted	2.4±0.07	4.1±0.21	ND	2.3±0.00
	Ripening 1 month	5.3±0.07	2.6±0.06	1.5±0.07	4.1±0.24
	Ripening 3 months	1.8±0.07	2.9±0.09	2.3±0.00	2.3±0.00
Semi-industrial	Curd	3.2±0.14	2.5±0.00	7.8±0.06	1.8±0.07
	Curd salted	5.1±0.14	1.8±0.14	2.1±0.24	8.7±0.03
	Ripening 1 month	2.8±0.05	1.7±0.00	3.3±0.10	3.5±0.07

[1)]Mean±SD of 3 replicates; ND, not detected

Table 3. Phenotypic characteristics of fungi isolated and taxonomical classification according to ribosomal sequencing

Strain FCOT	Accessions no.	Obtained from	Identity	Ureasa	Growth		
					On acetic acid	at 37ºC	at 48ºC
-4	HQ436413	Curd	*Kluyveromyces marxianus*	-		+	-
-20	HQ436454	Curd salted	*Rhodotorula mucilaginosa*			+	-
-21	HQ436455	Ripening 1 month	*Galactomyces geotrichum*			+	-
-22	HQ436456	Ripening 1 month	*Pichia guillermondii*			+	-
-27	HQ436457	Curd salted	*Kluyveromyces lactis*		+	+	-
-28	HQ436460	Curd salted	*Galactomyces geotrichum*			+	-
-30	HQ436414	Ripening 1 month	*Kluyveromyces marxianus*				+
-36	HQ436458	Curd	*Kluyveromyces lactis*		+		+
-37	HQ436461	Curd	*Galactomyces geotrichum*			+	-
-43	HQ436459	Ripening 1 month	*Galactomyces* sp.	+	+		-
-45	HQ436462	Ripening 1 month	*Galactomyces geotrichum*	+	+		-

stages can be explained due to yeast tolerance to low pH values and high NaCl concentrations (17). Furthermore, in artisanal cheese, a reduction in yeast counts was found towards the end of the ripening period. Similar yeast counts have been well documented in other artisanal cheeses where it has been suggested that the utilization of lactic acid by the yeasts leads to an increase in pH that encourages bacterial growth and contributes to the ripening process of the cheese (15,18). In this study, the semi-industrial Cotija cheese presented a high yeast count and a reduced LABs count towards the end of the ripening 1 month period. The latter could be due to its shorter ripening period (1 month), which could imply that the yeasts do not have time to liberate enough growth factors or amino acids contents from their autolysis, which are known to promote growth of the LABs (19). Consequently, in the semi-industrialized Cotija cheese, aerobic bacteria which grow without these nutritive compounds supplied by yeast are maintained until the end of the ripening period. The effect of the sensory characteristics of semi-industrial Cotija cheese as consequence of its particular microbial profile remains to be explored.

In initial manufacturing stages (curd and salted-added curd), artisanal Cotija cheese showed LABs counts values similar to other artisanal cheese (20-22). On the contrary, semi-industrial Cotija cheese exhibited lower LABs counts in all of their manufacturing stages. During artisanal Cotija cheese making, the composition of LAB microflora undergoes several changes, according to modifications of environmental conditions, such as it has previously been reported by Di Cagno *et al*. (23). Higher LABs counts in relation to mesophilic aerobic bacteria towards the end of the ripening period in artisanal Cotija cheese might be a consequence of the production of antimicrobial substances. Many of these substances as hydrogen peroxide, diacetyl, and bacteriocins are antagonistic to spoilage and pathogenic organisms (24, 25), even though more research must be done to clarify this.

Yeast identification A total of 11 isolates were selected from around 30 isolates based on their different phenotypic characteristics (Table 3). Most isolates thrive at 37ºC although 2 *Kluyveromyces* strains (FCOT-30 and -36) also grown at 48ºC. Physiological parameters and fermentation patterns (data not shown), assigned a probable identity in agreement with phylogenetic analysis (Table 4). The comparison of the ribosomal regions to a database showed a total of 6 species: *Kluyveromyces lactis*, *Pichia guillermondii*, *Rhodotorula mucilaginosa*, *Galactomyces geotrichum, Galactomyces reessii*, and *Kluyveromyces marxianus* (Table 4). Some yeasts isolates such as *K. lactis* or *Geotricum* sp. have been documented in other artisanal manufacturing cheeses such as Fiore Sardo, Cabrales, Camambert, and Robiola di Roccaverano (3,18,26,27).

LABs identification A total of 16 bacterial isolates were selected from a total of around 200 isolates based on their different phenotypic characteristics (Table 4). All bacteria were Gram-positive and most were catalase negative although some isolates identified as BCOT-7 and BCOT-13 showed a very weak catalase activity. Growth at 10ºC was the most frequent phenotypic characteristic, although certain isolates such as BCOT-9 and -14 strains can also grow at 45ºC. Five bacteria isolates (BCOT-3, -4, -9, -10, and -11) can grow at high saline concentrations (6.5 mM NaCl), as it might be expected due to the high salinity of these chesses. Fermentation patterns complemented the identity obtained with ribosomal sequence analysis (data not shown) and the comparison of the 16S rDNA sequences to the database showed that all the isolated strains belonged to the Lactobacillaceae family with the follow species: *Pediococcus pentosaceus*, *Lactobacillus*

Table 4. Phenotypic characteristics of bacteria isolated and taxonomical classification according to 16S rRNA sequencing

Strain BCOT	Accession no.	Obtained from	Identity	Catalase	Gram	Cell shape	Motility	CO_2 glucose	Grown at					
									Temp. (°C)		NaCl (mM)		pH	
									10	45	1.8	6.5	6.4	8.6
-1	HQ616499	Curd	Lactobacillales bacterium	+	+	Rods	-	-	+	-	-	-	-	-
-2	HQ616500	Curd	Lactobacillales bacterium	+	+	Cocci	+/-	+	+	+	+	-	-	+
-3	HQ616501	Curd salted	*Pediococcus* sp.	+	+	Cocci	-	-	+	+	-	+	+	+
-4	HQ616502	Ripening 1 month	*Lb. plantarum*	+	+	Cocci	+	-	+	+	-	+	-	-
-5	HQ616503	Ripening 1 month	*Lb. casei*	+	+	Rods	-	-	+	-	-	-	-	-
-6	HQ616504	Ripening 3 month	Lactobacillales bacterium	+	+	Rods	-	-	+	-	-	-	-	-
-7	HQ616505	Ripening 3 month	*P. pentosaceus*	+/-	+	Rods	-	-	+	-	-	-	-	-
-8	HQ616506	Curd	*Lb. casei*	+	+	Rods	+	+	+	-	-	-	-	-
-9	HQ616507	Curd	Lactobacillales bacterium	+	+	Cocci	-	+	+	+	-	+	-	+
-10	HQ616508	Curd salted	Lactobacillales bacterium	+	+	Cocci	-	+	+	+	+	+	+	-
-11	HQ616509	Curd salted	Lactobacillales bacterium	+	+	Cocci	-	-	+	+	-	+	+	-
-12	HQ616510	Ripening 1 month	Lactobacillales bacterium	+	+	Cocci	-	-	+	+	-	-	-	-
-13	HQ616511	Ripening 1 month	*P. pentosaceus*	+/-	+	Rods	-	-	+/-	+/-	-	-	+	+
-14	HQ616512	Ripening 3 month	Lactobacillales bacterium	+	+	Cocci	+/-	+	+	+	-	-	-	-
-15	HQ616513	Ripening 3 month	Lactobacillales bacterium	+	+	Rods	-	-	+	-	-	-	-	-
-16	HQ616514	Ripening 3 month	*Lb. brevis*	+	+	Rods	-	-	+	-	-	-	-	-

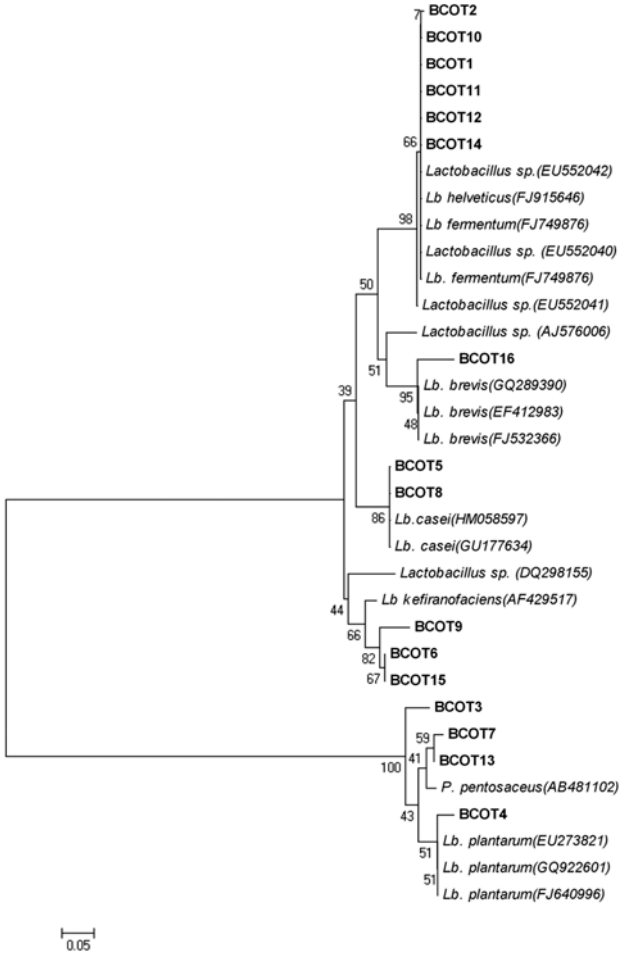

Fig. 1. Phylogenetic relationship from BAL isolates with the nearest neighbors inferred using the minimum evolution method. Numbers over the branches are bootstrap values (over 1,000 repetitions). BAL isolates of this study are represented in bold.

brevis, *Lactobacillus plantarum*, and *Lactobacillus casei* (Table 3).

A phylogenetic analysis was conducted to clarify the identity of some LAB isolates. This analysis showed 2 main clades (Fig. 1). The more separated clade included organisms closely related to *Pediococcus pentosaceus* (BCOT-3, -7, and -13) and *Lb. plantarum* (BCOT-4). The second clade contained 4 sub-clades. The most abundant includes 6 isolates (BCOT-1, -2, -10, -11, -12, and -14), which belonged to the *Lactobacillus* species and related to *Lb. helveticus* or *Lb. fermentus* species. However, genetic distances within this sub-clade are short and therefore all isolates were classified as Lactobacillales bacterium (Table 3). In addition, this clade includes a sub-clade with an isolate (BCOT-16) well separated and closely related to *Lb. brevis*, a third sub-clade with isolates (BCOT-5 and -8) closely related to *Lb. casei*, and a fourth sub-clade with Isolates (BCOT-6, -9, and -15) weakly related to *Lb. kefiranofaciens*. All isolates of this last sub-clade exhibit differences in their 16S rDNA nucleotide-base composition and were only classified as Lactobacillales bacterium. An additional genetic marker might be necessary to reliable clarify the identity of this sub-clade. Finally, many of LABs characterized in this study as *P. pentosaceus*, *Lb. plantarum*, and *Lb. casei* have been reported by other authors to be involved in the ripening of other important artisanal cheeses (28,29).

LABs acidification potential The 16 isolates representative for LABs species in the artisanal Cotija cheese were characterized for their acidifying capacity in pasteurized milk, resulting in around pH 5.5 values after 8 h, and a

Table 5. Acidification potential of dominant LABs in the artisanal Cotija cheese

Isolate	Identity	Initial pH	pH after 8 h	Lactic acid production (g/L)[1)]
BCOT-1	Lactobacillales bacterium	6.67	5.28	90
BCOT-2	Lactobacillales bacterium	6.66	5.18	70
BCOT-3	*Pediococcus* sp.	6.65	5.60	80
BCOT-4	*Lb. plantarum*	6.63	5.38	70
BCOT-5	*Lb. casei*	6.62	5.06	100
BCOT-6	Lactobacillales bacterium	6.63	5.30	70
BCOT-7	*P. pentosaceus*	6.63	5.69	80
BCOT-8	*Lb. casei*	6.61	5.20	80
BCOT-9	Lactobacillales bacterium	6.62	5.40	90
BCOT-10	Lactobacillales bacterium	6.62	5.20	80
BCOT-11	Lactobacillales bacterium	6.62	5.37	80
BCOT-12	Lactobacillales bacterium	6.61	5.15	90
BCOT-13	*P. pentosaceus*	6.66	5.25	90
BCOT-14	Lactobacillales bacterium	6.60	5.47	110
BCOT-15	Lactobacillales bacterium	6.54	5.30	70
BCOT-16	*Lb. brevis*	6.54	5.45	90

[1)]All data are the mean of 2 replicates.

good lactic acid accumulation (Table 5), with values that ranged from 70 to 110 g/L. The LAB isolates with the highest acid lactic production were BCOT-5 (*Lb. casei*) and BCOT-14 (Lactobacillales bacterium). LAB microflora plays a fundamental role in the acidification of curd, as well as in other physical and chemical transformations that affect the development of curd and flavor (2).

The results obtained in the present study suggest that artisanal manufacturing of Cotija cheese might contribute to some extent to a high occurrence of LABs in the initial curd stage that is higher than the semi-industrial Cotija cheese. Higher LABs occurrence might be implied in the specific aroma and taste as well as in the lower levels of spoilage microorganism found in the artisanal Cotija cheese.

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