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Microbiological Characteristics and Behavior of *Staphylococcus aureus*, *Salmonella* spp., *Listeria monocytogenes* and Staphylococcal Toxin during Making and Maturing Cotija Cheese

María de los Ángeles Olea-Rodríguez [1], Patricia Chombo-Morales [2], Karla Nuño [3], Olga Vázquez-Paulino [1], Zuamí Villagrán-de la Mora [4], Luz E. Garay-Martínez [1], Javier Castro-Rosas [5], Angélica Villarruel-López [1,*] and Ma. Refugio Torres-Vitela [1,*]

[1] Departamento de Farmacobiología, Centro Universitario de Ciencias Exactas e Ingenierías, Universidad de Guadalajara, Blvd. Gral. Marcelino García Barragán 1421, Olímpica, Guadalajara 44430, Mexico; mariangeles_olea@hotmail.com (M.d.l.Á.O.-R.); olga.vazquez@academicos.udg.mx (O.V.-P.); eduviges.garay@academicos.udg.mx (L.E.G.-M.)
[2] Food Technology Department, Centro de Investigacion y Asistencia en Tecnología y Diseño del Estado de Jalisco (CIATEJ), Av. Normalistas 800, Colinas de la Normal, Guadalajara 45019, Mexico; pchombo@ciatej.mx
[3] Departamento de Ciencias Biomédicas, Centro Universitario de Tonalá, Universidad de Guadalajara, Nuevo Perif. Ote. 555, Ejido San José, Tateposco, Tonalá 45425, Mexico; karlajanette.nuno@cutonala.udg.mx
[4] Departamento de Ciencias de la Salud, Centro Universitario de Los Altos, Universidad de Guadalajara, Av. Rafael Casillas Aceves 1200, Tepatitlán de Morelos 47620, Mexico; blanca.villagran@academicos.udg.mx
[5] Instituto de Ciencias Básicas e Ingeniería, Universidad Autónoma del Estado de Hidalgo, Carretera Pachuca-Tulancingo Km 4.5, Pachuca, 42183, Mexico; jcastro@uaeh.edu.mx
* Correspondence: angelica.vlopez@academicos.udg.mx (A.V.-L.); ma.torres@academicos.udg.mx (M.R.T.-V.)

Abstract: Cotija cheese is an artisanal matured Mexican cheese from unpasteurized milk. This work determined the microbiological characteristics and behavior of *Staphylococcus aureus*, *Salmonella* spp., *Listeria monocytogenes* and staphylococcal toxin during cheese elaboration and ripening. Sixteen 20-kg cheeses were used, eight inoculated with 6 log CFU/mL of each pathogen, and eight uninoculated, and samples were taken at each stage of the process. In the uninoculated samples, the survival of *S. aureus* and *L. monocytogenes* decreased after 30 days of ripening. The average counts of *S. aureus* in milk, curd, and serum were 7 log MPN /mL, and 8.7 log MPN /g in cheese, decreasing from day 15. *Salmonella* spp. counts (initial inoculum: 7.2 log MPN /mL) decreased after 24 h, and *L. monocytogenes* counts (8.7 log MPN/g at 24 h) decreased from day 15 in the cheese. *Salmonella* spp. and *L. monocytogenes* were not detected in any sample after 60 days of ripening, unlike *S. aureus*, which was detected at the end of the study. Lactic acid bacteria counts were 9 log CFU/mL in milk and whey and 7.2 log CFU/g in cheese. Pathogens behavior during the ripening process reduces the health risks by consuming products made with unpasteurized milk when subjected to ripening.

Keywords: Cotija; Staphylococcus aureus; Salmonella spp.; Listeria monocytogenes; maturing cheese

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1. Introduction

The prevalence of pathogens in milk is influenced by numerous factors, such as the size of the farm, the number of animals, hygiene, variability in the sampling, and the techniques used for sample analysis, geographic location, and the season of the year. However, within the variability that these factors may have, milk is one of the most important sources of pathogens that cause foodborne diseases [1]. In Mexico, most fresh cheeses are handmade using rustic methods in micro-industries located in small cities. These industries generally have little or no quality control and high composition and sensory parameters variability, leading to a limited shelf life [2–4]. However, artisanal matured cheeses show a different reality due to the fermentative processes during the maturation time, giving rise to the

formation of inhibitory compounds for certain microorganisms [5]. The microbiota is an essential component that plays an essential role during cheese maturation [6,7]. Interaction within the milk microbiota is vital for the characteristics of the final product. It participates from the acidification of the curd to the formation of compounds that determine the aroma, flavor, and texture during ripening [8,9]. Even raw milk is used in the elaboration of some matured cheeses. Therefore, the diversity of the native microbiota contributes to the final sensory properties and biopreservation [10,11].

Cotija cheese is a typical matured Mexican cheese that is artisanal-made and produced exclusively with milk from crosses of zebu cattle (*Bos taurus indicus*). It is manufactured from July to October in the Cotija area, located between the states of Jalisco and Michoacán. The salt added during the manufacturing process contributes to its flavor and firm consistency. During the aging process, salt concentration and other solutes produce a reduction in the water activity, promoting a selective medium in which halo-tolerant microorganisms like various genera of lactic acid bacteria dominate others, transform the physicochemical properties and composition of the cheese. Although the action of the different microbial populations from the milk used, the environment of the cheese factory and the production practices, the climatic conditions of the geographical area where the cheese is made and matured, favor the dynamics of the microbial populations, characteristics and, consequently, the distinctive characteristics of this cheese [5,12,13].

During the ripening process, adverse conditions are created for the development of pathogens. In the case of Cotija cheese, salt is added. However, some pathogenic bacteria may survive, causing health problems. An example occurred in Illinois due to the consumption of cheese produced in the USA, marketed as Cotija, contaminated with *Salmonella enterica* serotype Newport [14]. Another pathogen frequently recovered in Cotija cheese is *Staphylococcus aureus*, which can originate from the mammary glands of infected cows [15].

The objective of this work was to investigate microbiological characteristics and behavior of *Staphylococcus aureus*, *Salmonella* spp., *Listeria monocytogenes* and staphylococcal toxin during elaboration and maturation of Cotija cheese.

2. Results

Each 20-kg cheese was made using 180 L of unpasteurized milk. Cheeses were made immediately after the milk was obtained, and the main matrix was salted within the first 9 h. Sixteen cheeses were made for this work. Eight were left uninoculated (negative controls). Eight were inoculated with 6 log CFU/mL of *S. aureus* ATCC 13565, *S. Typhimurium* ATCC 14028, *L. monocytogenes* ATCC BAA-751, respectively, and staphylococcal toxin. The analysis of the cheese already molded and in the process of maturing began at 24 h. It is worth mentioning that at each sampling time, a complete cheese was taken. For sampling, each cheese was divided into three uniform vertical: (A) Core, (B) Main matrix, and (C) Surface (Figure 1).

2.1. Microbiological Parameters in Uninoculated Samples

Milk, Curd, and Whey Analysis

Before the beginning of the cheese production, the natural presence of *Staphylococcus aureus*, *Salmonella* spp., *Listeria monocytogenes* and staphylococcal toxin was analyzed, as well as lactic acid bacteria (LAB), aerobic mesophilic bacteria (AMB), coliform organisms (CO), molds and yeasts (M/Y) indicators in milk, uninoculated curd and whey. Only *S. aureus* and *Listeria* were recovered in milk, curd, and whey (Table 1), and two species were identified, *L. moncytogenes* and *L. ivanovii*. Enterotoxin (<1 ng/mL) and *Salmonella* were not detected in any non-inoculated samples.

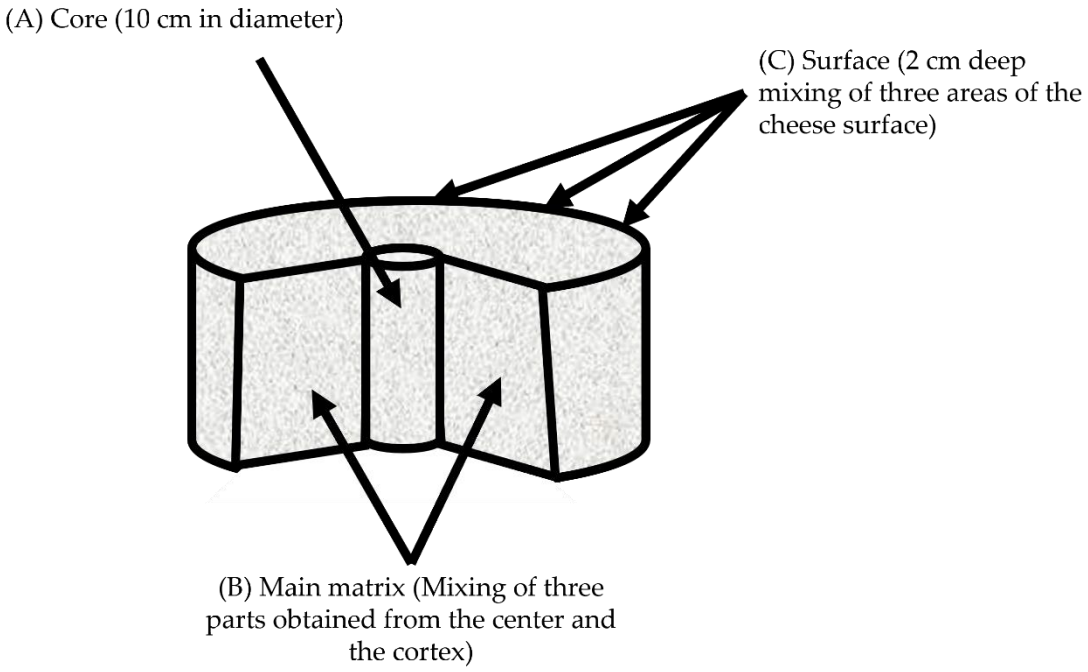

Figure 1. Revaluated sections of Cotija cheese (From Chombo-Morales et al., 2016 [12]).

Table 1. Behavior of pathogens in Cotija cheese made with uninoculated milk.

	Staphylococcus aureus			***Listeria monocytogenes***		
	(Log MPN/mL or g)			**(Log MPN/mL or g)**		
	Milk	**Curd**	**Whey**	**Milk**	**Curd**	**Whey**
0 *	5.9	6.4	5.9	5.9	7.5	7
Cheese	Surface	Main matrix	Core	Surface	Main matrix	Core
24 h		7.4			7.2	
7 d	6.5 ^{a}	6.1 ^{b}	4.9 ^{b}	3.4 ^{A}	4.3 ^{B}	4.1 ^{B}
15 d	6.7 ^{a}	6.5 ^{b}	5.6 ^{b}	3.1 ^{A}	2.7 ^{B}	2.9 ^{B}
30 d	4.5 ^{a}	3.9 ^{b}	ND	1 ^{A}	0.3 ^{B}	ND
45 d	1.1 ^{a}	ND	ND	ND	ND	ND
60 d	ND	ND	ND	ND	ND	ND
75 d	ND	ND	ND	ND	ND	ND
90 d	ND	ND	ND	ND	ND	ND

d = days of maturation; ND = not detected; * *Salmonella* and staphylococci enterotoxin were not detected. Values with different superscripts (^{a,b} or ^{A,B}) in the rows, are significantly different between adjacent columns of the same pathogen ($p < 0.05$).

Staphylococcus aureus. Once the cheese was molded, it was left in the ripening chamber for 24 h, and then the native *S. aureus* was enumerated, finding values of 7.4 log MPN/g in the non-inoculated cheese. Table 1 shows the behavior of *S. aureus* in the different sections and sampling times of the non-inoculated cheese.

The statistical analysis of the non-inoculated cheese did not show a significant difference in the distribution of *S. aureus* regarding the analyzed sampled area up to 45 days ($p = 0.343$), the last sample obtained in which the bacteria was recovered. In addition, during the ripening process, no traces of staphylococcal enterotoxin were detected (<1 ng/mL).

Listeria monocytogenes. In cheese made with non-inoculated milk, native *L. monocytogenes* counts increased at 24 h, reaching a population of 7.2 log MPN/g and then drastically decreased on day 15, when samples from the core had 2.9 log MPN/g, and later, no *L. monocytogenes* was detected. On the other hand, the main matrix and surface samples allowed

the pathogen to survive until day 30, with a recovery of 0.3 and 1 log MPN/g, respectively (Table 1).

2.2. Microbiological Parameters in Inoculated Samples

Staphylococcus aureus. In milk inoculated with 7 log CFU of *S. aureus* ATCC 13565 *per* mL, pathogen counts in curd and serum were 7.8 log MPN/mL and 7.1 log MPN/mL, respectively, while in the 24 h cheese, 8.7 log MPN/mL were recovered.

After seven days of maturation of the inoculated cheese, microorganisms from samples taken from the core survived until day 45 (0.15 log MPN/g), unlike the main matrix samples where it was only possible to detect them after 15 days (2.55 log MPN/g). The area where the longest survival time of *S. aureus* was observed was the surface, since counts were detected until day 90 with 3.6 log MPN/g. The statistical analysis of the inoculated cheese showed no significant differences regarding the recovery of the microorganisms on day seven (p = 0.326). From the second sampling time (15 days), a statistically significant difference was observed in the distribution of *S. aureus* (p = 0.001) in cheese.

All the samples, regardless of the sampling area, were negative for staphylococcal enterotoxin (≥1 ng/mL) in inoculated cheese after 24 h. Initially was detected in milk, rennet, and whey (Table 2).

Table 2. Behavior of pathogens in Cotija cheese made with inoculated milk.

	Staphylococcus aureus			***Salmonella* Typhimurium**			***Listeria monocytogenes***		
	(Log MPN/mL or g)			**(Log MPN/mL or g)**			**(Log MPN/mL or g)**		
Milk Inoculum	**7.3**			**7.2**			**7.3**		
Cheese	**Surface**	**Main Matrix**	**Core**	**Surface**	**Main Matrix**	**Core**	**Surface**	**Main Matrix**	**Core**
24 h		8.7			4.8			8.7	
7 d	6.7 ^{a}	6.42 ^{b}	5.45 ^{b}	3.55 ^{A}	2.82 ^{B}	3 ^{B}	7.4 ^{a}	7.4 ^{b}	6.9 ^{b}
15 d	7.2 ^{a}	3.77 ^{b}	5.05 ^{b}	3.85 ^{A}	2.4 ^{B}	4 ^{B}	7.1 ^{a}	5.6 ^{b}	5.3 ^{b}
30 d	6.4 ^{a}	ND	1.8 ^{b}	4.51 ^{A}	0.67 ^{B}	3.6 ^{B}	6.1 ^{a}	ND	ND
45 d	5.3 ^{a}	ND	0.15	1.85 ^{A}	0.72 ^{B}	ND	4.5 ^{a}	ND	ND
60 d	4.7 ^{a}	ND	ND	ND	ND	ND	ND	ND	ND
75 d	4 ^{a}	ND	ND	ND	ND	ND	ND	ND	ND
90 d	3.6 ^{a}	ND	ND	ND	ND	ND	ND	ND	ND

d = days of maturation; ND = not detected. Values with different superscripts (^{a,b} or ^{A,B}) in the rows, are significantly different between adjacent columns of the same pathogen ($p < 0.05$).

Salmonella Typhimurium. In the inoculated milk, *S. Typhimurium* ATCC 14028 showed a gradual and continuous decrease in all samples from the 7.2 log MPN/mL initial inoculum. After 24 h of storage, cheese samples had a reduction of 2.5 log (4.8 log MPN/g of cheese). In the following samplings, the concentration of the pathogen gradually decreased until it stopped recovering on day 45 (core) and 60 (main matrix and surface) (Table 2).

Listeria monocytogenes. Regarding cheese made with inoculated milk (7.3 log of MPN/mL), a similar behavior was observed compared to the uninoculated milk. In cheese, a pathogen concentration increase was observed (8.7 log of MPN/g) after 24 h of storage, then its presence decreased. In the core and the main matrix, only 5.3 and 5.9 log of MPN/g were recovered until day 15, respectively, while on the surface, *L. monocytogenes* ATCC BAA-751 survived longer since its last recovery was on day 45 with a 4.5 log of MPN/g. Table 2 shows the behavior of each pathogen in the different parts of Cotija cheese that were analyzed during its elaboration and maturation process.

Indicator groups. Lactic acid bacteria (LAB), aerobic mesophilic bacteria (AMB), coliform organisms (CO), molds and yeasts (M/Y) were quantified. Table 3 shows the microbiota content in milk, curd, whey, and cheese with 24 h of maturation, without inoculation. No significant differences were observed in LAB, CO, M/Y of milk, curd, and

whey ($p > 0.05$). Only the AMB had a higher content in milk and cheese ($p < 0.05$). The four types of samples showed <10 Log CFU/ g or mL of molds and yeasts (Figure 2).

Table 3. Indicator groups in milk, curd and whey not inoculated with pathogens.

	Lactic Acid Bacteria	Aerobic Mesophilic Bacteria	Coliforms	Molds and Yeast
Milk *	9.1 ^{a}	8.0 ^{a}	6.5 ^{a}	3.0 ^{b}
Curd **	9.4 ^{a}	6.9 ^{b}	6.1 ^{a}	3.1 ^{b}
Whey *	8.9 ^{a}	6.9 ^{b}	5.8 ^{a}	3.0 ^{b}
Cheese **	7.2 ^{a}	8.2 ^{a}	7.2 ^{a}	4.2 ^{b}

* Log of CFU/mL; ** Log of CFU/g. Values with different superscripts (^{a,b}) in the columns, are significantly different between rows ($p < 0.05$).

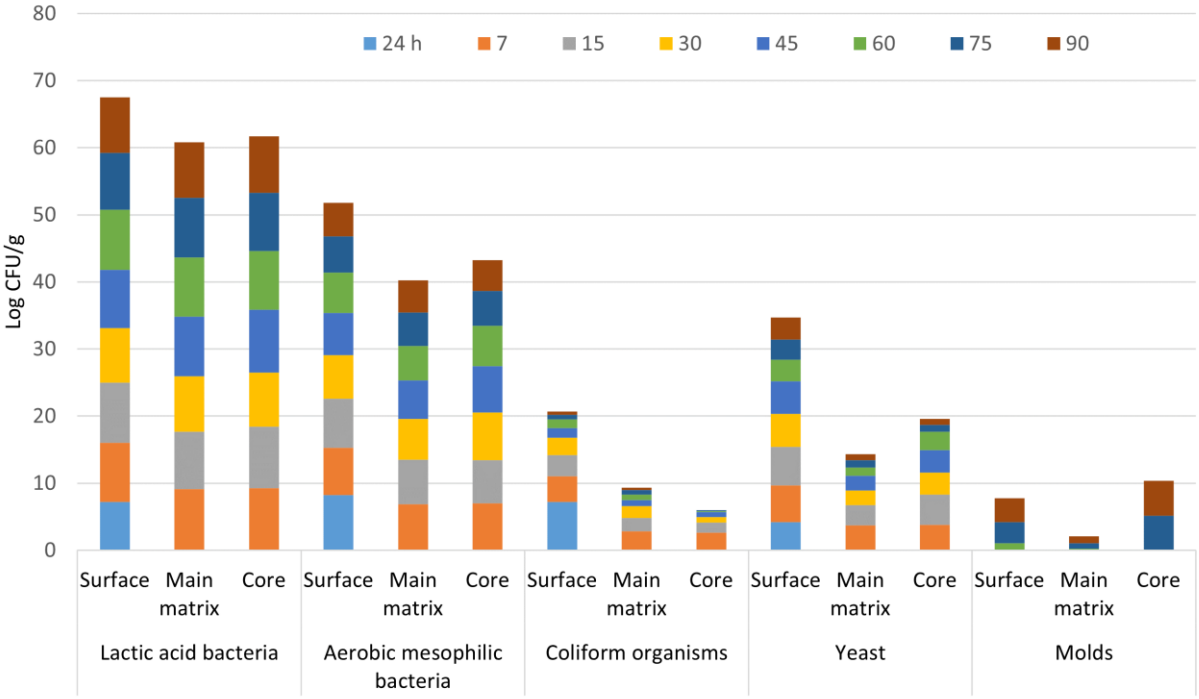

Figure 2. Behavior of indicator groups in Cotija cheese during its maturation process.

The indicator groups in cheese showed that after seven days of maturation, LAB continued to show a homogeneous behavior in the microbial content of the different areas analyzed (core, main matrix, and surface) and between the non-inoculated and inoculated cheese. In non-inoculated cheese, indicator groups maintained an average of around 9.0 log of CFU/g until day 45, then decreased to 1 log CFU/g from day 60, maintaining those count values until the end of this work. In the inoculated Cotija cheese, the LAB behavior was similar. However, it was maintained with an 8.8 log CFU/g until the end of the study.

The statistical analysis showed a significant difference between the main matrix and the core in the indicator groups at 15, 75 and 90 days ($p < 0.05$). There was no difference in either of the two groups, inoculated and not inoculated ($p > 0.05$).

From cheeses with seven days of maturation, main matrix and surface samples from days 7 and 15 had the highest content of indicator groups with an average of 5.9 and 6.4 log of CFU/g, respectively, while the core had 5.0 log of CFU/g. After 30 days, a gradual decrease in the microbial load was observed, more critical in the main matrix and the core. At 90 days, the AMB content was 2.4 log CFU/g in the core, 2.2 log CFU/g in the main matrix and 3.4 log CFU/g in the surface. Statistically, there was a difference in the AMB content in the surface regarding the main matrix and the core on days 7, 30, 45, 60,

75 and 90, while on day 15; the ABM content in the core, main matrix and surface was different ($p < 0.05$). The coliform content gradually decreased in the non-inoculated and the inoculated cheeses until it practically disappeared. In the non-inoculated cheese, the main matrix and the core had 3 log CFU/g at 7 days, ceasing to recover at 90 and 60 days in the main matrix and core, respectively, while in the surface, the CO content at 7 days was 5.8 log CFU/g, decreasing to 0.2 log CFU/g on day 90. In the inoculated cheese, the initial count of the core, main matrix and surface was 3 log CFU/g on day 7, and at the end of the study, the counts decreased to 0.3, 0.5, and <1 log CFU/g in the main matrix, surface, and core. A significant difference was observed on days 7, 30 and 60 between the surface, core and main matrix of non-inoculated cheese. Inoculated cheese showed a significant difference in surface compared to main matrix and core on days 7, 15 and 45; and between the core compared to the surface and main matrix on days 30 and 60 ($p < 0.05$).

In the case of yeasts, the content was heterogeneous in both types of cheese, non-inoculated and inoculated. The initial load (7 days) in the non-inoculated cheese was 1.4, 6.2 and 7.5 log CFU/g for the main matrix, surface, and core. At the end of the study, 1.4 log CFU/g were found in the main matrix, 4.4 log CFU/g in the surface and 1.2 log of CFU/g in the core. Yeast content in inoculated cheese was higher on the surface with 5.5 log CFU/g and lower in the main matrix and core (3.7 log CFU/g in both).

At 90 days, the content was higher in the surface (4.4 log CFU/g) and lower in the main matrix and core (1 log CFU/g). Statistically, all sampling times showed differences between the non-inoculated and the inoculated cheeses ($p < 0.05$). Finally, molds appeared after 60 days in not inoculated and inoculated cheeses. In non-inoculated cheese, no molds were recovered from the core in any sample (<1 log CFU/g). The main matrix had a mold content of 2.8, 2.1, and 0.67 log CFU/g on days 60, 75 and 90, respectively, while the surface presented 1.3, 2.3 and 2.9 log CFU/g on days 60, 75 and 90, respectively. In inoculated cheese, the main matrix had an increase from 0.3 log CFU/g at 60 days to 1 log CFU/g on days 75 and 90, while in the surface, mold increased by 1 log CFU/g on day 60 at 3.2 log CFU/g on day 75, to 3.5 log CFU/g on day 90. In the core, molds were only detected on days 75 and 90 (5.1 and 5.2 log CFU/g, respectively). The statistical analysis showed a significant difference between the three types of samples (main matrix, surface, and core) and the three sampling times in which the presence of molds in the non-inoculated cheese was detected. In contrast, in the inoculated cheese, there was only a significant difference on days 75 and 90 between the core, the main matrix, and the surface ($p < 0.05$).

2.3. Physicochemical Parameters

Changes were observed during the maturation time in titratable acidity, pH, water activity (Wa), and sodium chloride percentage (%) within the cheese's physicochemical parameters analyzed in different sections. The changes of each variable had a different dynamic and gave rise to a distinguishing composition between each cheese section: (A) Core (10 cm in diameter); (B) Main matrix (Section between the core and the surface), and (C) Surface (Obtained 2-cm deep from the surface) (Figure 1).

The surface had a constant increase of 0.22% of lactic acid, increasing to 1.1% at the end of the study (90 days); pH dropped from 5.9 after seven days of maturation to 5.5 at day 90; Wa decreased from 0.96 to 0.86, and sodium chloride increased from 3.2 to 4.06 due to moisture loss on the surface. In the cheese main matrix, the initial lactic acid was 0.4% and gradually increased to 0.9% at day 90, while the pH dropped slightly from 5.5 to 5.01; the Wa decreased from 0.91 to 0.89 and sodium chloride increased from 3 to 3.4. The samples from the core of the cheese showed the most contrasting changes compared to the surface; the acidity content increased from 0.32% (day 7) to 1.52% on day 90; the pH decreased from 5.9 at day 7 of maturation to 5.0 at day 90; Wa also decreased from 0.92 on day 7 to 0.89, and the sodium chloride content increased from 2.9 to 3.5 (Table 4).

Table 4. Physicochemical parameters during the cheese maturation process.

	Surface				Main Matrix				Core			
	Acidity	**Wa**	**pH**	**NaCl (%)**	**Acidity**	**Wa**	**pH**	**NaCl (%)**	**Acidity**	**Wa**	**pH**	**NaCl (%)**
7	0.22 ^{a}	0.92 ^{a}	5.9 ^{a}	3.2 ^{a}	0.32 ^{A}	0.921 ^{A}	5.5 ^{A}	2.89 ^{A}	0.4 ^{a}	0.916 ^{a}	5.01 ^{a}	3 ^{a}
15	0.48 ^{a}	0.9 ^{a}	6.2 ^{a}	3.21 ^{a}	0.43 ^{A}	0.918 ^{A}	5.6 ^{A}	3.04 ^{A}	0.42 ^{a}	0.916 ^{a}	5.2 ^{a}	3 ^{a}
30	0.76 ^{a}	0.87 ^{a}	6.2 ^{a}	3.23 ^{a}	0.92 ^{B}	0.899 ^{A}	5.6 ^{A}	3.12 ^{A}	0.47 ^{a}	0.910 ^{a}	5.09 ^{a}	3 ^{a}
45	0.9 ^{b}	0.87 ^{a}	5.5 ^{b}	3.87 ^{a}	1.21 ^{B}	0.899 ^{A}	5.1 ^{B}	3.32 ^{A}	0.64 ^{a}	0.907 ^{a}	5 ^{a}	3.26 ^{a}
60	1 ^{b}	0.83 ^{b}	5.5 ^{b}	4 ^{a}	1.32 ^{B}	0.866 ^{A}	5.0 ^{B}	3.45 ^{A}	0.85 ^{b}	0.897 ^{a}	4.9 ^{a}	3.29 ^{a}
75	1.19 ^{b}	0.84 ^{b}	5.5 ^{b}	3.85 ^{a}	1.4 ^{B}	0.870 ^{A}	5.0 ^{B}	3.49 ^{A}	0.89 ^{b}	0.891 ^{a}	5.0 ^{a}	3.07 ^{a}
90	1.1 ^{b}	0.86 ^{b}	5.5 ^{b}	4.06 ^{a}	1.52 ^{B}	0.885 ^{A}	5.0 ^{B}	3.52 ^{A}	0.9 ^{b}	0.895 ^{a}	5.01 ^{a}	3.4 ^{a}

Wa: water activity Values with different superscripts (^{a,b} or ^{A,B}) in the cheese section columns, are significantly different between rows ($p < 0.05$).

3. Discussion

Dairy production follows several stages, ranging from obtaining the milk on the farm to further processing in the dairy company or, in many cases, on the farm itself. During this process, the risk of contamination can enter at various points along the supply chain, compromising food safety [16]. During cheese making, microbial contamination can occur at any stage from cheese production to consumption, making cheese a significant vehicle for foodborne illness [4,17]. In the case of Cotija cheese, it is a Mexican artisan product made from raw cow milk without any heat treatment. The ripening process occurs spontaneously and is influenced by environmental conditions [12,18].

The fermentation of Cotija cheese is carried out by the native biota present in the milk; since starter cultures are not added, the initial microbiota is diverse and will influence its sensory characteristics and the safety of the final product. Because of the above, we decided to carry out this project in the region of Cotija. It was used the milk produced in the area. There was a space to work with inoculated milk, and the cheeses were stored for 90 days for their maturation, such as the traditional process of Cotija cheesemaking.

Therefore, the native biota as control (not inoculated) and inoculated cheeses had the same diversity of microorganisms. This allowed us to know the natural behavior that the microbiota has throughout the cheese maturation process and the microbial diversity in the milk obtained in the Cotija region, Michoacán, to the final product generated after 90 days of maturation.

The use of unpasteurized milk has represented a debate in the dairy industry due to the risk posed by the possibility that it is contaminated with pathogens, causing health problems for consumers. There are multiple reports of products made with raw milk in various outbreaks [19–21]. However, this does not mean that all products, particularly cheeses made with raw milk, pose a health risk to consumers. The presence of pathogens in cheese depends on several factors: the type of cheese, the microbiological quality of the milk used, and the storage conditions. Fresh cheeses made with unpasteurized milk represent a greater risk because nothing inhibits the pathogen that may be present in the raw milk, favoring the development and/or survival of pathogens [2,4,20]. The difference with a fermented cheese is that the same fermentation process causes the production of compounds and changes in some parameters that can inhibit the development of the pathogens, like pH, lactic acid and the bacteriocins produced by some of the lactic acid bacteria that participate in the fermentation and cheese ripening [18]. It is important to mention that this study was done in the Cotija region using milk sourced from the dairy herd, which is regularly used for cheesemaking under the same environmental conditions as Cotija ripening.

In the present work, the inoculated pathogens: *S. aureus*, *S. Typhimurium* and *L. monocytogenes* were no longer detected in the different sections of the cheese after day 45 of ripening. However, *S. aureus*, was detected until the end of the maturation process (90 days) in surface samples, probably because of their halotolerant characteristics [22]. Cheese native *S. aureus* from uninoculated cheese was not detected at 45 days. Meanwhile, a different behavior was observed in *S. aureus* (ATCC 13565) from inoculated cheese which was still

being detected until the end of the study (90 days). In both cases, they remained longer on the surface of the cheese. It may be due to the increased sensitivity of the native strain to the metabolites of cheese microbiota or the lower halotolerance in comparison to ATCC strain. *S. aureus* was recovered in the surface in concentrations significantly higher than in the core and the main matrix ($p < 0.05$). The behavior of the distribution of *S. aureus* in the inoculated and non-inoculated cheese did not show differences ($p > 0.05$); both were decreasing as time passed. On the surface of both cheeses, inoculated and non-inoculated, always remained in higher concentration (2 log MPN/g). Behavior of native *L. monocytogenes* of no inoculated cheese was like inoculated strain; they were not detected to 30 and 40 days, respectively. On the other hand, the distribution of *L. monocytogenes* in the inoculated cheese showed significant differences ($p = 0.0045$). The surface showed 3 log more with respect to the core and the main matrix. The distribution of *L. monocytogenes* in the inoculated and non-inoculated cheese did not show significant differences ($p > 0.05$). In both cheeses, inoculated and non-inoculated, the surface had the highest concentration of this pathogen. While the distribution of *S.* Typhimurium in the inoculated cheese, showed significant differences ($p = 0.0005$). It was observed that it was similar in the main matrix and the core, meanwhile, the surface showed 1.5 log more than the main matrix.

The inhibition of pathogens in ripened cheeses made with raw milk, like Cotija, has also been reported by other authors [23] who have shown the gradual demise of pathogens such as *L. monocytogenes* [24], and the inhibition of other pathogens in fermented cheeses [25,26]. The absence of inoculated pathogens is associated with the metabolic processes that occurred during cheese maturation and the inhibitory compounds that the other microorganisms have, such as lactic acid bacteria [18,26,27]. García-Cano et al. [25], reported production of bacteriocins from *Enterococcus faecium* and *E. faecalis* recovered from Cotija cheese. This author showed inhibition of *S. aureus, Yersinia enterocolitica*, *S.* Typhimurium and *Pseudomonas aeruginosa.* Escobar-Zepeda et al. [18] did not detect pathogenic bacteria like *Salmonella*, *Listeria monocytogenes*, *Brucella*, or *Mycobacterium* in a metagenomic analysis performed on Cotija cheese. They reported three predominant phyla (*Lactobacillus*, *Leuconostoc* and *Weissella*) associated with the production of various metabolites with antimicrobial activity. Within the complex microbiota in Cotija cheese, the presence of lactic acid bacteria can inhibit the inoculated pathogens, and the yeast present could have participated in this process [28–30]. Chombo et al. [12] observed that surface of Cotija cheese showed higher counts of yeasts, which is like our study. These microorganisms could contribute to the inactivation of pathogens observed in cheese's surface, in conjunction with physicochemical factors. Also, yeasts can be present in different cheeses [28,31].

As previously described, the metabolic activity of the microbiota present during the fermentation process generates many compounds ranging from peptides, like bacteriocins, to peroxide and organic acids, among other metabolites that determine the predominance of some microbial groups such as lactic acid bacteria, responsible for the synthesis of these compounds, as well as the typical organoleptic characteristics of Cotija cheese [7,18,32]. Even though only the production of lactic acid was quantified in the present investigation, it should be noted that the absence of pathogens coincided with the increase of lactic acid and the consequent drop in pH. Water activity and its decrease also influenced, although to a lesser degree, to the inactivation of the inoculated pathogens. Lactic acid concentration and pH had the highest impact on the decrease of *S. Typhimurium* and *S. aureus*. Both pathogens showed a gradual decrease associated with increasing acidity and decreasing pH. On the other hand, the inactivation of *L. monocytogenes* showed no relationship with the parameters determined since it occurred drastically.

The present study shows the importance of interaction between microorganisms that constitute the complex microbiota ecosystem of cheese made with no pasteurized milk. The same microbiota present in cheese is used for its production. In Cotija cheese, the robustness of its native ecology is decisive to ensure its quality. Quality control considers the maturity stage, which ensures the absence of pathogens and takes advantage of the

characteristics *per se* of Cotija cheese, including its functional properties provided by its characteristic microbiota [12]. Likewise, the quality of raw material in Cotija cheese is important because it is the one that will determine the type of microorganisms that will carry out the fermentation process and give the desired sensory characteristics. However, the raw material and milk must be free of pathogens like *Brucella* and *Mycobacterium* [18].

4. Materials and Methods

4.1. Bacterial Strains

Strains used in inoculated cheese were typified: *Staphylococcus aureus* ATCC 13565, *Salmonella* Typhimurium ATCC 14028 and *Listeria monocytogenes* ATCC BAA-751. In uninoculated cheese, native bacteria were isolated from fresh cheese and biochemically characterized.

4.2. Inoculum Preparation

All strains were separately reactivated in trypticase soy broth with yeast extract (CSTEL) twice a row. Subsequently, each bacterium was inoculated in Roux bottles containing 200 mL of trypticase soy agar with yeast extract and 20 mL of CSTEL, then incubated at 35 °C for 18 h. The biomass obtained was separated from the agar using sterile glass beads and extracted with a pipette. Physiological saline solution was added to the biomass obtained to a final volume of 40 mL. From this suspension, the count was carried out in selective agar for each microorganism (Bright Green Agar, Oxford Agar and Baird Parker agar, respectively) to know the bacterial concentration. Additionally, in the culture of *S. aureus*, the presence of staphylococcal toxin was verified by visual immunoassay (Tecra 3M).

4.3. Cheesemaking

The fresh raw milk (180 L) was filtered through a clean gauze cloth to remove foreign matter, and once it reached a temperature between 30 to 33 °C, rennet was added, leaving it to rest until it formed a firm curd, which was cut using lira to obtain approximately 1 cm^3 cube.

After a few minutes of standing, the whey was drained, and the curd was transferred to a stainless-steel table and mixed with 5–10% NaCl until forming a homogeneous mass. The molding of the dough was done in previously washed and disinfected cylindrical hoop-shaped stainless-steel molds, provided with an ixtle or cotton blanket. The mass was pressed for 24 h, and the main matrix was removed from the press. The mold and the blanket with which the main matrix was wrapped allow it to give the traditional cylindrical shape of the cheese and print the characteristic mark on the rind. The blanket was removed, and the hoop was left for the next 15 days. Every 18–24 h, the cheese was removed from the mold, turned over, and the surface cleaned with a clean cotton cloth. This process was carried out daily for the first eight days and then alternates every third day until 15 days were completed; the goal was to have complete drainage and a sturdy piece that could be handled without the shaping ring. Finally, the cheese was stored to begin its ripening process. Ripening was carried out for three months within the same geographical area of Cotija, Michoacán, Mexico, to provide the environmental conditions (Temperature < 28 °C, Relative humidity: 60 to 95% and Altitude: 700 to 1700 m above the level of the sea) that give the cheese its specific organoleptic characteristics (Figure 3).

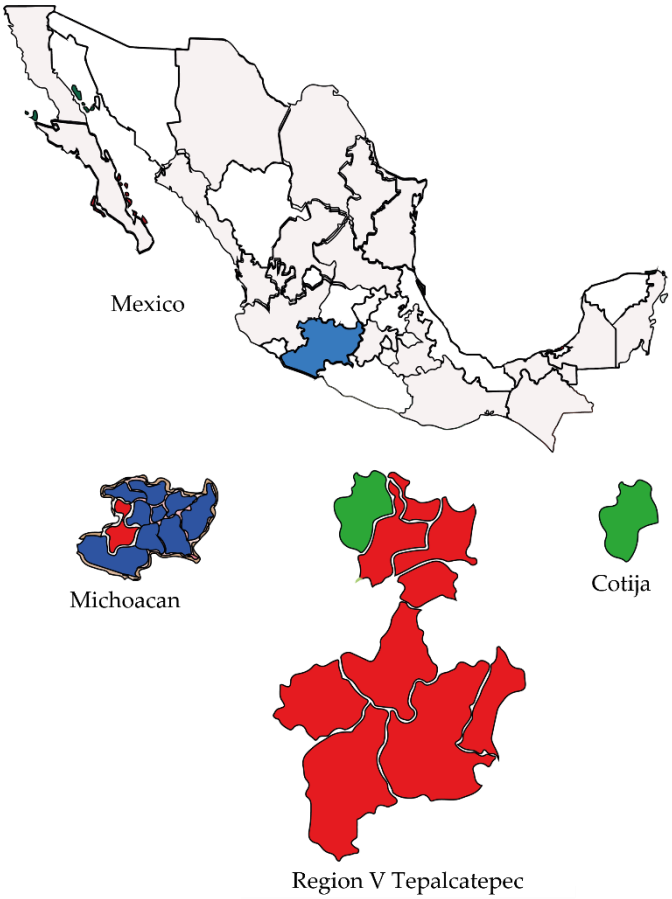

Figure 3. Cotija region in the State of Michoacan, Mexico.

4.4. Microbiological Parameters

During Cotija cheese production, samples were taken in each process: milk, curd, whey, and cheese during the aging process (NMX-F-735-COFOCALEC-2011). For the cheese analysis during aging, samples were taken directly in the ripening chambers, and seven sampling times were considered (1, 7, 15, 30, 45, 60, 75 and 90 days).

4.5. Milk and Cheese Sampling

4.5.1. Milk

The uninoculated milk was analyzed for *S. aureus*, staphylococcal toxin, lactic acid bacteria, aerobic mesophilic bacteria, coliforms, molds, yeasts, pH, and titratable acidity. In the case of the previously inoculated milk, each pathogen was quantified, and the presence of staphylococcal toxin was confirmed.

4.5.2. Cheese

For the first sampling (24 h of maturation), only one sample was taken that ranged from the core to the cheese surface. From the 7th ripening sampling: each cheese was divided into three uniform vertical sections to take a sample from each section: (A) core (10 cm in diameter); (B) main matrix (Section between the core and the surface), and (C) surface (obtained 2-cm deep from the surface) (Figure 1). The samples were placed in separate sterile bags (A, B and C, respectively) where they were homogenized manually.

4.6. Evaluation of Microbiological Parameters

4.6.1. Pathogen Count in Cheese

Each pathogen was counted on days 1, 7, 15, 30, 45, 60, 75 and 90, employing traditional counting per culture. In addition, at time 7, lactic bacteria, aerobic mesophilic, coliforms,

molds, yeasts, pH, and titratable acidity were quantified in both cheeses, inoculated and non-inoculated. In all cases, the samples were analyzed in duplicate.

The most probable number technique (MPN) was used to enumerate the pathogens in the cheese. It was weighed 25 g of each sample, then 225 mL of CSTEL were added, homogenized for 1 min in a Stomacher (Lab Blender 400), and subsequently seven dilutions were made. Tubes were inoculated with 9 mL of CSTEL and incubated at 35 °C for 24 h from each dilution. Baird Parker agar (ABP) plates for *S. aureus* were inoculated from the tubes that showed bacterial growth; Bright green agar (AVB) for *Salmonella* and Oxford agar (OXA) for *Listeria monocytogenes* to confirm the development of each pathogen.

The growth of each pathogen on selective agar was first confirmed by Gram staining and the biochemical tests corresponding to each microorganism through the API system. Additionally, specific tests were carried out for each bacterium. For *Salmonella* strains, serological tests were performed with antiserum O Anti-serum Poly A–I & Vi (BD Difco TM), and the hemolysis test and CAMP (Cristie-Atkins-Munch-Peterson Test) for *L. monocytogenes*.

4.6.2. Staphylococcal Toxin

The presence of staphylococcal toxin was investigated by the visual immunoassay method (Tecra 3M). For which 10 mL of milk or 10 g of cheese were taken and homogenized with 20 mL of sterile water for 3 min, a 3 mL aliquot was taken. The pH was adjusted to 4; next, the aliquot was centrifuged at 1000× *g*, and then, the liquid was passed through a 45 µm filter, and the pH was adjusted between 6–7. Subsequently, 1 mL was placed in an Eppendorf tube, and 50 µL of the additive was added and shaken manually 200 µL of this mixture were plated in 96-well plates, incubated at 35 °C for 2 h. The liquid was discarded, and four washes were performed with the washing solution. Plates were allowed to dry, and 200 µL of the conjugate was added to each well and incubated for 1 h at room temperature (20–25 °C). The liquid was poured out, and they were carefully washed. Finally, 200 µL of substrate were added to each well and incubated at room temperature (20–25 °C) for 30 min and then the samples were read. The support was placed on a white background, and the reading was done visually by comparing it with the color card.

4.6.3. Lactic Bacteria Count

To 25 g of sample, 225 mL Man-Rogosa-Sharpe (MRS) broth were added twice the concentration with aerobic lactic acid bacteria broth (3M) supplement and homogenized for 1 min in a Stomacher (Lab Blender 400). Subsequently, seven decimal dilutions were made. The Petri film plates (3M) for anaerobic bacteria were inoculated with 1 mL of each dilution and incubated at 37 °C for 48–72 h in anaerobic jars provided with a gas pack.

4.6.4. Indicator Groups

The determination of indicator groups (aerobic mesophilic bacteria, coliforms, molds, and yeasts) was made from 25 g of each sample and homogenized with 225 mL of peptone diluent for 2 min in a Stomacher (Lab Blender 400). From here, seven decimal dilutions were made. From each dilution, Petri-film plates (3M) were inoculated with Aerobic Count and *E. coli*/Coliform Count, both incubated at 35 °C for 24 h, and Yeast and Mold Count incubated at 20 °C for 5 to 7 days.

4.7. Physicochemical Parameters

In addition to the microbiological determinations, pH, titratable acidity, water activity and chlorides were quantified from non-inoculated cheese samples. The pH was measured in 10 g of sample, placing them in a 100 mL volumetric flask with distilled water; subsequently, it was filtered, and pH was determined with an Orion3 Star pH Benchtop meter (Thermo Fisher Scientific, Waltham, MA, USA. In acidity, this was determined by titration from 10 g of sample, using 0.1 N NaOH and phenolphthalein as indicators. The test was performed by titration in triplicate. Water activity determination was carried

out using 3TE Series AquaLab equipment (Decagon Device Inc., Pullman, Washington, USA). To determine sodium chloride, the AOAC 983.14 "Chloride in Cheese" method was used, weighing 2 g of ground cheese, and titrating the samples with a standard solution of 0.0856 M $AgNO_3$.

4.8. Statistical Analysis

A one-factor analysis of variance (ANOVA) with an alpha level of $\alpha = 0.05$ was performed on the results of the indicator groups, behavior, and distribution of pathogen in cheese. LSD Fisher method (Least significant difference) was performed for homogeneous groups. Statistical analysis was performed with Statgraphics Centurion XIX version 19.2.01 software.

5. Conclusions

This work shows important results on the behavior of pathogens and the dynamics that follow during the ripening process directly in the place where Cotija cheese is traditionally made. In addition, it shows the reduction of the risk represented by the consumption of products made with unpasteurized milk by being subjected to a maturation process, without neglecting good hygiene practices from obtaining the milk to the end of the cheese fermentation process and its subsequent marketing.

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