


Changes in the count of four bacterial groups during the ripening of Prensa (Costeño) Cheese from Cuajinicuilapa, Mexico



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Abstract:

Prensa Cheese, also called Costeño, is made in an artisanal way from raw cow's milk in the Costa Chica region of the state of Guerrero. In order to know the characteristics of Mexican artisanal cheeses, the objective of this research was to analyze the changes in the count of aerobic mesophilic bacteria (AMB), total coliform (TCs) microorganisms, lactic acid bacteria (LAB) and coagulase-positive staphylococci (CPS), during the ripening (5, 30, 60 and 90 d) of Prensa cheeses, made by four different cheese factories (A, B, C and D) of Cuajinicuilapa, Guerrero, Mexico. A portion (25 g) of each cheese sample was homogenized with peptone

diluent (225 mL) and dilutions from 10^{-1} to 10^{-6} were prepared with which 3M™ Petrifilm™ plates were sown. After incubating under different conditions, depending on the type of microorganism, AMB, TCs, LAB and CPS counts were made. The results showed that as the ripening time of the Prensa Cheese progressed, the microbial load decreased: AMB from 4 to 2, TCs from 6 to 3, LAB from 6 to 2 and CPS from 5 to 2 \log_{10} CFU g^{-1} . The changes in the counts of the bacterial groups studied can be attributed to the physicochemical and microbiological transitions typical of cheese maturation and to the characteristics of the microbiota present in each of the cheese factories. The results of this research provide elements for the microbial characterization of Mexican artisanal cheeses.

Key words: Lactic acid bacteria, Aerobic mesophilic bacteria, Coagulase-positive staphylococci, Raw milk, Microbiota, Total coliform microorganisms, Artisanal cheeses.

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Introduction

Around the world, cheese, in addition to being a rich source of nutrients, is an essential food used in the local gastronomy of different societies^(1,2). Currently, around 1,833 varieties of cheese located in 74 countries are known⁽³⁾; this diversity is determined by the technological processes used for its preparation, such as the origin of milk, fat-protein ratio, types of cultures and coagulating agents; the shape, size of the cheese and the maturation conditions^(4,5,6).

Cheese ripening consists of its storage, under certain conditions of temperature and moisture, for a period of time that can range from 3 to 7 d, up to 2 yr^(5,7). The maturation process, in addition to providing sensory characteristics, is a method of conservation^(5,6,8). In this stage, biotic and abiotic changes that have a direct impact on the microbiota present in the cheese occur^(5,7,9).

Most artisanal cheeses are made from raw milk, with spontaneous fermentation, non-technified preparation processes and varied maturation times^(5,7,10).

In Mexico there are about 40 artisanal cheeses⁽⁷⁾, among them are matured cheeses such as Cotija from the Sierra JalMich, Añejo cheese from Zacazonapan, Maduro cheese from Veracruz, Chihuahua cheese and artisanal cheese from the Ojos Negros region of Baja

California, of which some aspects of their microbiology have been published⁽¹¹⁻¹⁴⁾. Prens cheese (PC) is made with unpasteurized cow's milk, commercial liquid rennet and salt; it goes through a pressing stage whose duration varies at the discretion of the manufacturer from 1 to 3 days, then it is left to mature for periods of up to three months. The cheese thus obtained has color variations between white and yellow⁽¹⁵⁾. It is generally rectangular or circular in shape, its consistency is firm, and its weight is 1 to 14 kg per piece (Figure 1)⁽¹⁵⁾.

Figure 1: Prens cheese in rectangular and cylindrical shapes



PC has been produced for more than 100 years in southwestern Mexico, mainly in the Costa Chica region of the state of Guerrero in the municipalities of Cuajinicuilapa and Ometepec, as well as in the municipality of Pinotepa Nacional, on the coast of the state of Oaxaca⁽¹⁵⁾. According to INEGI⁽¹⁶⁾, the climate of the Costa Chica region is warm subhumid, and its temperatures range from 22 to 28 °C.

Studies are currently being carried out to identify the characteristics of artisanal cheeses from Mexico^(7,15), the objective of this research was to analyze the changes that occur in the count of aerobic mesophilic bacteria, total coliform microorganisms, lactic acid bacteria and coagulase-positive staphylococci, during the ripening (5, 30, 60 and 90 d) of prens cheeses made by four cheese factories (A, B, C and D) of Cuajinicuilapa, Guerrero, Mexico.

Material and methods

Cheese samples

Samples of PC made in an artisanal way in the municipality of Cuajinicuilapa, Guerrero, Mexico (16°28'18' N, 99°24'55' W), were analyzed in July 2018. Based on a targeted

sampling, four cheese factories were selected as sampling units, which will henceforth be named A, B, C and D. Four freshly made cheeses, weighing 1 kg, were purchased from each cheese factory. The samples were moved to the municipality of San Marcos, Guerrero (16°47'46' N, 99°23'05' W) to a space with characteristics similar to those of the cheese factories of Cuajinicuilapa. In this place, the samples of the cheeses from the cheese factories A, B, C and D (four of each cheese factory) were left to mature for 5, 30, 60 and 90 d. After the ripening time, each batch was transferred in polyethylene bags, inside coolers with refrigerant, to the laboratory. The samples were kept in refrigeration at 4 °C until analysis. The maximum ripening time was 90 d because after that period the flavors intensify, and local consumers avoid it because they prefer softer flavors.

Sample preparation

Each of the cheese samples (25 g) was mixed with 225 mL of peptone diluent, the mixture was homogenized for 2 min (VWR® symphony D S41 Vortex, VWR International) and dilutions from 10^{-1} to 10^{-6} were made by transferring 1 mL of the sample to vials containing 9 mL of peptone diluent⁽¹⁷⁾.

Microorganism count

The following culture media (3M Petrifilm™ plates) were used: aerobe count (AC No. of catalog 6400), coliform count (TC No. of catalog 6410), lactic acid bacteria (No. of catalog 6461) and staph express for coagulase-positive staphylococci (No. of catalog 6493); 1 mL of the corresponding dilution was placed in each of the plates⁽¹⁸⁻²¹⁾.

All counts were done in duplicate. For AMB, dilutions 10^{-3} and 10^{-4} were sown and the medium was incubated at 35 ± 2 °C for 48 ± 3 h⁽¹⁸⁾. TC microorganisms were studied based on dilutions 10^{-2} and 10^{-3} , being incubated at 35 ± 1 °C for 24 ± 2 h⁽¹⁹⁾. The determination of LAB was made by inoculating the media with dilutions from 10^{-3} to 10^{-6} and incubating at 35 ± 2 °C for 48 ± 3 h⁽²⁰⁾. The CPS study was conducted from dilutions 10^{-2} to 10^{-4} and an incubation at 37 ± 1 °C for 24 ± 3 h⁽²¹⁾.

Once the incubation time was completed, the growth was reviewed and the plates containing between 15 and 300 colonies were counted, the mean of the two repetitions was obtained and this average was multiplied by the inverse of the dilution with which the plate was inoculated⁽²²⁾. The result of the count was reported as \log_{10} of the number of colony-forming units per gram (\log_{10} CFU g⁻¹)⁽²³⁾.

Statistical analysis

The statistical analysis was based on a design with repeated means and completely random distribution of treatments, tested over time, whose probabilistic model corresponds to:

$$Y_{ijk} = \mu + \alpha_i + \gamma_k + (\alpha\gamma)_{ik} + e_{ijk}^{(24)}$$

Where:

$\mu + \alpha_i + \gamma_k + (\alpha\gamma)_{ik}$ is the mean of treatment i at time k , which contains the effects of treatment, time, and the time \times treatment interaction;

e_{ijk} is the random error associated with the measurement at time k on j assigned to treatment i .

The effect of the treatments (cheese factories A, B, C and D) was evaluated through the ripening time (5, 30, 60 and 90 d) with four repetitions, generating 64 experimental units, each consisting of 25 g of cheese.

The response variables evaluated were: total count of aerobic mesophilic bacteria (AMB), total coliforms (TCs), lactic acid bacteria (LAB) and coagulase-positive staphylococci (CPS). The data were analyzed using a mixed model^(24,25) whose random effect corresponds to the maturation time and the fixed effect to the cheese factories. The Tukey-Kramer method ($P < 0.05$) was applied to identify the effect of the treatments. Analyses were performed in the SAS package version 9.1 (SAS Institute, Inc., Cary, NC, USA).

Results and discussion

During the time of ripening, a decrease in the count of the different microorganisms was observed. The highest counts of all microbial groups were reached at 5 d of ripening while the minimum values occurred at 90 d (Table 1).

Table 1: Count of bacterial groups, \log_{10} CFU g^{-1} , during the ripening of Prensa cheese made in four cheese factories (A, B, C and D) of Cuajinicuilapa, Guerrero, Mexico

Bacterial group	Time (days)	Cheese factories			
		A	B	C	D
Aerobic mesophilic bacteria (\log_{10} CFU g^{-1})	5	6.3143 \pm 0.2973 Cc	4.6802 \pm 0.2973 Ba	5.8948 \pm 0.2973 Cb	5.9077 \pm 0.2973 Cb
	30	5.1156 \pm 0.2973 Bb	4.3884 \pm 0.2973 Ba	4.4981 \pm 0.2973 Ba	5.1043 \pm 0.2973 Bb
	60	4.0021 \pm 0.2973 Ab	3.1505 \pm 0.2973 Aa	4.4047 \pm 0.2973 Bbc	4.6703 \pm 0.2973 ABc
	90	4.3178 \pm 0.2973 Ab	2.5638 \pm 0.2973 Aa	2.4005 \pm 0.2973 Aa	4.3597 \pm 0.2973 Ab
Total coliforms (\log_{10} CFU g^{-1})	5	4.1093 \pm 0.1002 Cb	2.1505 \pm 0.1002 Aa	2.4203 \pm 0.1002 Aa	4.7211 \pm 0.1002 Db
	30	3.7726 \pm 0.0541 Cc	2.8838 \pm 0.0541 Ba	3.7916 \pm 0.0541 Cc	3.0878 \pm 0.0541 Cb
	60	2.3138 \pm 0.2854 Bb	2.3763 \pm 0.2854 ABb	2.2698 \pm 0.2854 Ab	1.5753 \pm 0.2854 Ba
	90	<1 \pm 0.0440 Aa	2.3451 \pm 0.0440 Ab	2.0753 \pm 0.0440 Ab	<1 \pm 0.0440 Aa
Lactic acid bacteria (\log_{10} CFU g^{-1})	5	6.5457 \pm 0.0651 Dc	5.9454 \pm 0.0651 Cb	5.5084 \pm 0.0651 Ba	6.7366 \pm 0.0651 Cc
	30	5.8336 \pm 0.0651 Cb	5.8231 \pm 0.0651 Cb	5.1193 \pm 0.0651 Ba	6.1241 \pm 0.0651 Cb
	60	3.5524 \pm 0.0651 Ba	3.7918 \pm 0.0651 Ba	3.1945 \pm 0.0651 Aa	5.0914 \pm 0.0651 Bb
	90	<1 \pm 0.0651 Aa	<1 \pm 0.0651 Aa	3.2258 \pm 0.0651 Ab	4.2394 \pm 0.0651 Ac
Coagulase-positive staphylococci (\log_{10} CFU g^{-1})	5	5.6562 \pm 0.0540 Cb	3.7456 \pm 0.0540 Ba	5.6918 \pm 0.0540 Cb	5.8746 \pm 0.0540 Cb
	30	3.6276 \pm 0.3811 Bb	2.2500 \pm 0.3811 Aa	3.7271 \pm 0.3811 Bb	3.8389 \pm 0.3811 Bb
	60	2.6945 \pm 0.0438 Aa	2.7143 \pm 0.0438 Aa	2.7311 \pm 0.0438 Aa	2.8063 \pm 0.0438 Aa
	90	2.5951 \pm 0.0524 Aa	2.4203 \pm 0.0524 Aa	2.6945 \pm 0.0524 Aa	2.5951 \pm 0.0524 Aa

Means with lowercase letter in rows and means with uppercase letter in columns, followed by different letter, indicate statistical significance (Tukey, $P < 0.05$).

Aerobic mesophilic bacteria

In the AMB counts, there were significant differences ($P<0.05$) between the cheese factories (Table 1). The gradual decrease in AMB from day 5 to 90 was close to $2 \log_{10} \text{CFU g}^{-1}$, for cheese factories A, B and D; while for cheese factory C, it was $3.49 \log_{10} \text{CFU g}^{-1}$.

Most of the AMB values found in the PC are within the range of 4 to 9 logarithms CFU g^{-1} , reported for cheeses made from raw milk and matured for 60 or more days⁽²⁶⁾. Since the maturation process of cheeses involves the multiplication of the microorganisms present, AMB concentrations of 4 to 9 logarithms (10^4 to 10^9CFU g^{-1}) are expected in this type of products, without this implying a deterioration of the food or suggesting that non-sanitary conditions occurred during its preparation or storage^(26,27,28). In the region of Ojos Negros in the state of Baja California, based on a study that included matured cheeses from 22 cheese factories, made with raw milk, it is reported that AMB were found in a range of 4.6 to 7.2 $\log_{10} \text{CFU g}^{-1}$ ⁽¹⁴⁾.

In studies on the ripening of artisanal Cotija cheese, salted and matured at temperatures of 14 °C to 32 °C, Chombo⁽¹¹⁾ reports the following variations in AMB: 8.3, 7.0, 3.5 and 4.7 $\log_{10} \text{CFU g}^{-1}$, on d 8, 30, 60 and 90, while Magallón⁽²⁹⁾ found 5.3 and 1.8 \log_{10} on d 30 and 90, respectively. The counts found in the PC at 30 and 90 d are very close to those reported for Cotija cheese that is ripened in temperature ranges similar to those of PC.

The decrease in AMB was common in the cheeses from the four cheese factories (Table 1), reflecting a certain homogeneity in the preparation processes and the ripening conditions of the cheeses. The statistical difference ($P<0.05$) between cheese factories suggests quantitative or qualitative variations in the microbiota of cheese generated by milk and the microenvironments of each cheese factory. It should be noted that, between d 5 and 60, it is observed that the AMB of cheeses from cheese factory A descend $2.31 \log_{10} \text{CFU g}^{-1}$, however, between d 60 and 90 they remain unchanged; while the AMB of cheeses from cheese factory C, from d 60 to 90, show a reduction of $2 \log_{10} \text{CFU g}^{-1}$.

The development of AMB in cheeses from cheese factory A shows the ability of bacteria to adapt and survive, while the decrease in AMB in the cheeses from cheese factory C exhibits a loss of viability with the release of enzymes that contribute to the generation of flavors and textures⁽⁵⁾. This suggests that it is appropriate to study the relationship of the organoleptic characteristics of prensa cheese, between cheese factories and between different concentrations of AMB over maturation time, as well as the relationship of AMB with the shelf life of cheese at room temperature. On the other hand, AMB studies are useful as an initial stage in the search for starter cultures from artisanal cheeses. Variations in salt concentration and moisture may have influenced the survival of AMB. The results suggest

that, in cheeses from cheese factories A and D, there are bacteria adapted for long-term survival. While in cheeses from cheese factories B and C, bacteria of short survival may be present, and therefore can contribute more quickly to the production of tastes, smells and textures (pleasant or unpleasant)⁽⁵⁾. Another possibility is that the reduction of AMB in cheeses from cheese factories B and C is due to the presence of substances with antimicrobial action, caused by the metabolism of microorganisms, or by the biochemical changes that occur in the cheese, derived from the proteolysis of casein to give rise to peptides with antimicrobial activity^(5,30).

Total coliform bacteria

Total coliform counts in the cheeses showed significant differences ($P < 0.05$) between cheese factories during the ripening period (Table 1). The highest counts were found on d 5 ($4.72 \log_{10} \text{CFU g}^{-1}$) and the lowest on d 90 ($< 1 \log_{10}$). Cheese, being a solid sample, hinders a direct count, so it is necessary to make an initial dilution that leads to the minimum detection level being 10CFU g^{-1} , so the absence of growth was reported as $< 1 \log_{10}$.

Two different dynamics were observed, cheeses from cheese factories A and D had the highest initial TC loads, which decreased at 30 d of maturation to reach 3.72 to $3.10 \log_{10} \text{CFU g}^{-1}$, respectively (Table 1). In cheeses from cheese factories B and C, the TCs showed initial levels of 2 logarithms, which rose during the first month and remained with slight variations to coincide on d 90 with values very close to each other.

The dynamics of TCs in cheeses from cheese factories A and D have been reported in semi-hard cheeses and are characterized by a progressive decrease in coliforms as ripened progresses⁽³¹⁾, which is attributed to the decrease in pH due to the fermentation of lactose⁽³²⁾. In ripened cheeses such as Cheddar, coliforms die at a rate of $0.3 \log_{10} \text{CFU g}^{-1}$ per week and in Gouda cheese at $0.7 \log_{10}$ per week⁽³³⁾. Therefore, the dynamics observed in cheeses from cheese factories B and C is atypical, because between d 5 to 30, there is an increase of 0.73 and $1.37 \log_{10}$, respectively, followed on d 60 to 90 by very small decreases, 0.03 and $0.19 \log_{10}$, respectively (Table 1). This suggests that there are common aspects between the two cheese factories that favor the selection of bacteria that persist during ripening, such as water and milk quality, personnel or variations in the preparation or cleaning processes.

The accepted levels of total coliforms in matured cheeses are less than 100CFU/g ($< 2 \log_{10} \text{CFU g}^{-1}$)⁽³⁴⁾, values that were reached between d 60 and 90 (Table 1) in cheese factories A and D. According to Metz⁽³²⁾, cheeses made with good quality raw milk, under hygienic sanitary conditions, applying good manufacturing practices and properly ripened, will have

low levels of total coliform bacteria, fecal coliforms, enterococci, *Enterobacteriaceae* and *Escherichia coli*.

The activity of coliform organisms in cheeses can adversely affect their sensory characteristics^(28,35). However, it has been observed that certain genera of coliforms contribute positively to the texture and sensory characteristics of the product; in addition to the fact that some strains of *Hafnia* contribute to the accumulation of aromas, and generation of flavors^(32,35,36). The persistence of coliforms suggests the possibility of the participation of these microorganisms in the organoleptic characteristics of cheeses from cheese factories B and C, an aspect that has not been addressed in studies of Mexican cheeses.

Lactic acid bacteria

During the ripening period, the LAB counts of the cheeses from the cheese factories studied showed significant differences ($P < 0.05$) (Table 1). This microbial group had the highest counts of the entire study. On d 5 the counts ranged between 5.50 and 6.73 \log_{10} CFU g^{-1} , these values decreased from day 30. From day 5 to 60, the reductions were 2.99, 2.15, 2.31 and 1.77 \log_{10} , for cheeses from cheese factories A, B, C and D, respectively.

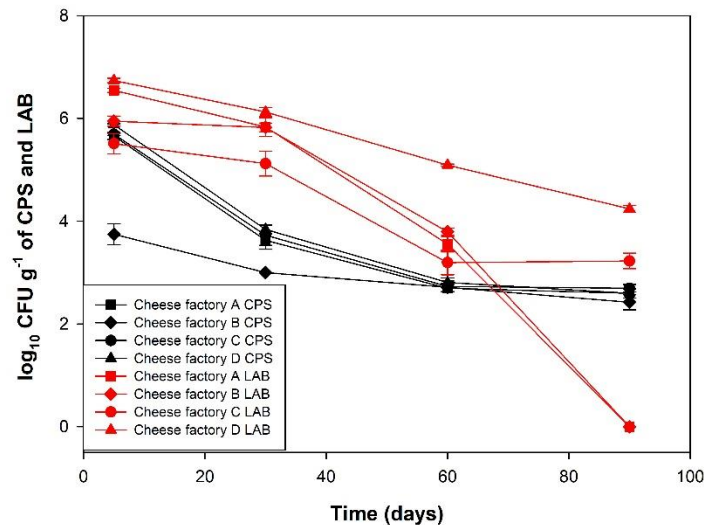
During the ripening of the Spanish artisanal cheeses Casar de Cáceres, Afuega'l Pitu and Cabrales, decreases in lactococcal counts of 2 to 3 \log_{10} CFU g^{-1} were reported between d 0 and 60, while in "La Serena" cheese there was only a reduction of less than one logarithm, this is attributed to the fact that this cheese had a low salt content during the first weeks of ripening⁽⁵⁾. This suggests that the reductions observed in the PC from d 5 to 60 are consistent with what happens with homofermentative lactic acid bacteria in cheeses made in an artisanal way with native microbiota⁽⁵⁾. The concentrations of LAB found in the PC from d 60 to 90 are lower than those reported in ripened cheeses from Europe, which have values of 7 to 9 \log_{10} CFU g^{-1} ^(5,37,38).

With differences in the type of cattle and geographical areas, Cotija cheese and PC share temperatures, preparation and ripening processes. In Cotija cheese, small increases and decreases have been found in LAB counts; one study reports 2.6 \log_{10} CFU g^{-1} on d 30 with an increase to 2.9 \log_{10} on d 90⁽²⁹⁾, another study indicates 5.9 \log_{10} on d 60, which decreases to 5.0 \log_{10} on d 90⁽¹¹⁾. The above data suggest that, in both PC and Cotija cheese, LAB counts tend to be lower than in other ripened cheeses; this could be explained by the temperatures at which they are ripened, which favors a greater loss of moisture that generates values of water activity and moisture/salt ratio that are inhibitory for LAB⁽⁵⁾.

Coagulase-positive staphylococci

The CPS counts of the cheeses from the cheese factories studied showed significant differences ($P < 0.05$) during the ripening period (Table 1). From d 5 to 60, a continuous decrease in CPS was observed in cheeses from cheese factories A, C and D, followed by a stabilization from d 60 to 90. In cheese factory B, there was a decrease from d 5 to 30 followed by an increase from d 30 to 60 and a stabilization from d 60 to 90 (Figure 2). The death rates of CPS (average decrease \log_{10} CFU g^{-1} divided between week of ripening)⁽³⁹⁾ in cheeses from cheese factories A and C were 0.30 \log_{10} and 0.23 and 0.31 \log_{10} in cheese factories B and D, respectively.

Figure 2: Antagonism and change in lactic acid bacteria (LAB) concentration with respect to coagulase-positive staphylococci (CPS)



For cheeses made from raw milk, the accepted limits of coagulase-positive staphylococci are 10^4 to 10^5 CFU g^{-1} , which is equivalent to 4-5 \log_{10} CFU g^{-1} ⁽⁴⁰⁾, limits that were exceeded on day 5 in cheese factories A, C and D but that were reached again on day 30 and remained until day 90 (Table 1). CPS counts greater than 4 \log_{10} show the need to apply corrective measures in the hygiene of the processes of milk collection, cheese making and the selection of raw materials⁽⁴⁰⁾. Values of 10^5 CFU g^{-1} or higher lead to study the presence of staphylococcal toxin in cheeses⁽⁴⁰⁾, since being thermostable, it can persist even when staphylococci have died. Concentrations of 10^6 CFUs g^{-1} are usually needed to produce enough toxin (one nanogram per gram of cheese) to cause a disease outbreak⁽⁵⁾.

Reductions of *Staphylococcus aureus* of 1 to 3 logarithms have been reported in different cheeses^(41,42,43), figures that coincide with reductions in CPS during PC ripening.

The death rate of *S. aureus* in Manchego cheese made with raw milk from d 1 to 60 is 0.404 \log_{10} CFU g^{-1} ⁽³⁹⁾, the death rates of CPS in cheese factories A, C and D (0.49, 0.50 and 0.52) were close to this value, suggesting that the decrease was as expected for this type of cheese (matured Prensa paste cheeses). No factors that explain the lower death rate observed in cheese factory B (0.36) were identified.

The development and survival of *S. aureus* are affected by factors such as: physicochemical changes that occurred during the ripening process, secondary metabolites generated by LAB, as well as the composition of the product, storage period and temperature^(44,45). *Staphylococcus aureus* is inhibited by LAB through nutrient competition, production of lactic acid, hydrogen peroxide and production of antimicrobial substances⁽⁴⁶⁾, which may explain the decrease in CPS in the first 30 d of ripening, a period in which LAB levels were higher (Figure 2). Between d 30 and 60, storage conditions at room temperature could increase moisture loss, which changes the moisture/salt ratio, being inhibitory for LAB⁽⁵⁾, this favors *S. aureus*, which could explain its slight increase in cheese factory B and the suspension of its decrease in cheese factories A, C and D.

Conclusions and implications

Prensa cheese is a cheese made in an artisanal way, ripened in warm subhumid climate with the participation of native lactic acid bacteria, whose concentrations are lower than those reported for European cheeses, but similar to those reported in Cotija cheese. Statistical differences in microbial counts at different times show the changes that occur as cheese matures. Meanwhile, the statistical differences between the cheese factories suggest the existence of microbiomes specific to each cheese factory, which could be able to generate variants of PC among different artisanal producers, even when they have similar production processes. The changes in the counts of the bacterial groups studied can be attributed to physicochemical changes and successions in the bacterial populations typical of the maturation of the cheese and to the characteristics of the microbiota present in each of the cheese factories. It is convenient to explore whether the shelf life of this cheese extends beyond 90 days. The finding of coliforms that persist during ripening shows the need to investigate whether this is an exceptional case, and bacteria from this group contribute to the pleasant characteristics of the cheese or are related to its deterioration. The data on reduction and survival of coagulase-positive staphylococci generated in this research can serve as a reference to initiate and evaluate improvement programs in this type of cheese factories. Although this research included four cheese factories in the main PC-producing municipality, the information generated can serve as a reference for the characterization of this artisanal cheese.

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