



Bacterial evaluation of Zacazonapan artisanal cheese matured under non-controlled conditions in two production periods



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Abstract:

Traditional Zacazonapan cheeses have unique organoleptic characteristics and are characterized by being linked to the territory of origin. In the maturation process, there are many interactive variables that are responsible for physical, chemical, biological and structural changes. In order to evaluate the bacteriological evolution of artisanal cheeses during their maturation under non-controlled conditions in two production periods, samples of raw milk and cheese were collected at 0, 30, 60, 120 and 150 d of maturation. The

presence of molds and yeasts (MaY), mesophilic aerobic bacteria (MAB), *Staphylococcus* spp. (Staph), total coliforms (TC), fecal coliforms (FC), *Salmonella* spp. (Salm) and *Listeria* spp. (List) was determined. The average microbial load was 9.68, 9.38, 8.55 and 8.10 log₁₀ CFU/g of cheese for MaY, MAB, Staph and TC respectively, as well as 2.68 log₁₀ MPN/g of cheese for FC. Salm was not detected but List was. The microbiological evolution of Zacazonapan matured cheese had counts that exceed the maximum levels of the Official Mexican Standard 243 SSA1 2010.

Key words: Environmental maturation, Aging, Microbiological evolution, Raw milk.

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Traditional cheeses are produced from a complex system that gives rise to unique organoleptic characteristics and are characterized by strong links with their territory of origin⁽¹⁾. In the process, there are several interactive variables that are responsible for physical, chemical, biological and structural changes. Their quality depends on environmental factors, and on interactions between inoculated microorganisms and curd substrates that result from variations in the quality of raw milk and processing conditions⁽²⁾. Lactic microflora is of particular interest because the biochemical activities of these organisms are involved in cheese making and may play a role in the development of organoleptic characteristics during maturation⁽³⁾; however, due to their process of preparation and use of raw milk, they can generate outbreaks of food poisoning⁽⁴⁾.

Zacazonapan cheese is handmade with raw milk from Creole cattle from the southern region of the State of Mexico in the rainy season (July-November). In this season, the feeding of the cattle is based on grazing and the cheeses are consumed after four months of maturation at environmental temperature and relative humidity. This cheese has also been described as Zacazonapan aged cheese⁽⁵⁾.

Knowing the evolution of the main microbial groups during the maturation of this cheese and the final microbiological quality can suggest modifications to the maturation process that improve the quality without losing any of its characteristics. Therefore, the objective of the study was to evaluate the bacteriological evolution of artisanal cheeses during their maturation under non-controlled conditions in two production periods.

The work was carried out in seven cheese factories in the municipality of Zacazonapan (19° 07' 27" N, 100° 02' 57" W and 1,470 m asl). Its average annual temperature and precipitation is 23.0 °C and 1,041.8 mm respectively. The study was carried out at the end of the cheese production season (from November 2010 to April 2011), which was called a batch of "*dry season cheese*" due to the environmental conditions in which it is matured. In the middle of the following year's production season (September 2011 to February 2012), the batch was called "*rainy season cheese*". Before the cheese was made, a milk sample was taken from each production site according to the Official Mexican Standard (NOM, for its acronym in Spanish) 109 (handling and collection of samples)⁽⁶⁾. The samples were placed in closed sterile containers and transported at 4 °C for their analysis in the microbiology laboratory the next day. Two pieces of fresh cheese weighing approximately 2.0 kg were acquired in both sampling seasons and taken to a cheese factory in the area, where they were left to mature for 150 d under normal conditions of temperature and relative humidity. Using a cork borer, two samples of 50.0 g were collected from each piece of cheese at 0, 30, 60, 120 and 150 d of maturation. Campeche wax was used to seal the holes in the place where the sample was taken. From each sample of cheese, the presence of molds and yeasts (MaY), mesophilic aerobic bacteria (MAB), *Staphylococcus* spp. (Staph), total coliforms (TC), fecal coliforms (FC), *Salmonella* spp. (Salm) and *Listeria* spp. (List) was determined. To determine MaY, 10 g of the central part of the cheese + 10 ml of milk from each sample obtained were homogenized in 90 ml of 0.1 % peptone water (in duplicate) and decimal dilutions were prepared from this first dilution, obtaining dilutions 10⁻² to 10⁻⁷. Subsequently, they were incubated for 24 h at a temperature of 25 °C⁽⁷⁾.

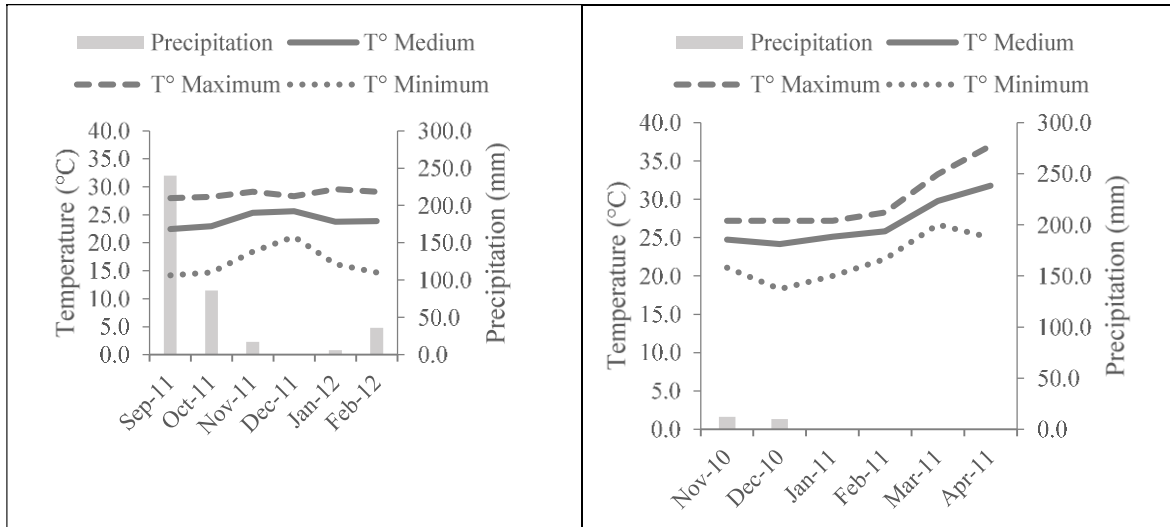
To determine MAB, Staph, TC, FC, Salm and List, 25 g from the central part of the cheese + 25 ml of milk from each sample were collected and homogenized in 225 ml of 0.1 % peptone water (in duplicate). Decimal dilutions up to 10⁻⁷ were made from this solution, then incubated for 24 h at a temperature of 35 °C⁽⁷⁾.

According to NOMs, the presence of MaY was determined by plate counting with potato dextrose agar after incubation at 25 °C for 48 h⁽⁸⁾. For MAB, tryptone-yeast extract agar was used, incubating at 35 °C for 48 h⁽⁹⁾. TCs were determined in plate by violet red bile agar after incubation at 35 °C for 24 h⁽¹⁰⁾. The presence of FC was determined by the most probable number technique, using lauryl sulfate tryptose broth, after incubating at 35 °C for 24 h⁽¹¹⁾. The determination of Staph with Baird-Parker agar and the addition of egg yolk tellurite, incubated at 37 °C for 48 h after growth⁽¹²⁾. Analysis for Salm in Salmonella-Shigella agar incubated for 48 h at 35 °C⁽¹³⁾ and List incubated at 35 °C for 48 h in Oxford agar⁽¹⁴⁾. The data obtained were normalized by log₁₀, an experimental design of completely randomized blocks was used and analyzed with the command of the general linear model of

minitab V.14⁽¹⁵⁾. When significant differences were observed ($P < 0.05$), the Tukey test was applied.

The evolution of temperature and precipitation in the study region is shown in Figure 1. The maturation temperature of dry season cheeses starts at 24.8 °C in November and ends with 31.8 °C in April; rainfall is almost zero (<12 mm/mo), typical characteristics of the dry season (Figure 1a). In rainy season cheeses, the maturation temperature begins with 22.1 °C in September and ends with 22.7 °C in February, rainfall was 240 mm at the beginning of maturation and 36 mm at the end (Figure 1b).

Figure 1: Precipitation and temperature during the environmental maturation of Zacazonapan cheese



a) Dry season cheese

b) Rainy season cheese

Relative humidity, temperature and time are important factors during cheese maturation⁽¹⁶⁾. The maturation temperatures observed in this work favored the development of microorganisms and rainfall affected the final texture of the cheeses; the absence of rains produced drier cheeses, which showed cracked crusts, and the presence of rains produced softer cheeses with wet crusts, which prevented the cheeses from bursting due to the production of gas. However, these cheeses had holes and putrefactive areas inside, a phenomenon known as late swelling⁽¹⁷⁾.

The counts made on the milk used for cheese making (Table 1) were 7.03, 7.02 and 4.9 (\log_{10} CFU/ml of milk) for MaY, MAB and TC. The use of poor microbiological quality milk is a common practice during the production of artisanal raw milk cheeses and is attributed to poor milking practices. Similarly, the absence of a cold chain and poor transport conditions leads to the counts of MaY, MAB and TC⁽¹⁸⁾.

Table 1: Microbiological quality of milk and Zacazonapan cheese during maturation

Milk / Cheese	MaY ¹	MAB ¹	Staph ¹	TC ¹	FC ²
Milk	7.03	7.02	ND	4.9	ND
Cheese maturation days					
0	9.250 ^b	9.499 ^a	9.090 ^{ab}	9.468 ^a	3.040 ^a
30	9.632 ^b	9.882 ^a	9.827 ^a	9.854 ^a	3.040 ^a
60	9.628 ^b	9.403 ^a	7.133 ^b	7.276 ^b	3.040 ^a
120	9.908 ^{ab}	8.858 ^b	8.438 ^b	6.680 ^b	2.340 ^b
150	10.102 ^a	8.900 ^b	7.797 ^b	7.147 ^b	1.767 ^c
Average	9.704	9.308	8.457	8.085	2.645
Cheese production season					
Dry	9.722	8.912 ^b	7.992 ^b	8.189	3.040 ^a
Rainy	9.648	9.865 ^a	9.123 ^a	8.022	2.195 ^b
Average	9.685	9.388	8.558	8.106	2.618
SEM	0.21	0.26	0.529	0.947	0.264

MaY= molds and yeasts; MAB= mesophilic aerobic bacteria; Staph= *Staphylococcus* spp. TC= total coliforms; FC= fecal coliforms.

¹ = \log_{10} colony-forming units / g of cheese or ml of milk.

² = \log_{10} most probable number / g of cheese.

ac= different letters within a column indicate differences ($P < 0.05$). SEM= standard error of the mean. ND: Not determined

In both batches of cheese, it is observed that, in the first days of maturation, there is an increase in microbial counts (d 30), which decrease throughout the maturation process due to the biochemical and microbiological processes that occur inside the cheese, such as the reduction of water content, the concentration of solids, the increase in acidity and a reduction in pH caused by the action of lactic bacteria. This causes a microbial competition for nutrients⁽¹⁹⁾.

The group of MaY (Table 1) had significant differences ($P < 0.05$) between days of maturation. This is due to the physical retention of microorganisms in rennet and microbial multiplication during milk curdling and whey draining⁽¹⁸⁾. The reduction of MaY from day

60 is attributed to the fact that, as the maturation progresses, the center of the cheese becomes compressed, and the oxygen needed to microbial reproduction reduces⁽²⁰⁾.

The MaY counts observed in this study are consistent with those found in similar cheeses. For example, the Tepeque aerated cheese had counts of 7.6 log₁₀ CFU/g of cheese in the dry season and 7.7 log₁₀ CFU/g of cheese in the rainy season; and when the Tepeque cheese was taken to maturity, it had 6.2 log₁₀ CFU/g in the dry season and 6.4 log₁₀ CFU/g in cheese matured in the rainy season⁽²¹⁾.

During the maturation of Kurdish cheese, fresh cheese starts with 5.6 log₁₀ CFU/g of cheese, after 20 d it presents 5.95 log₁₀ CFU/g of cheese, until reaching 9.28 log₁₀ CFU/g of cheese at 40 d, then the load begins to decrease, reaching 9.06 log₁₀ CFU/g of cheese on d 60⁽²²⁾. The same population dynamics were observed in the present study and are related to the depletion of lactose content due to the simultaneous use of lactic acid bacteria^(18,20,22).

NOM 243 SSA1, which indicates the sanitary provisions and specifications for milk and milk products⁽²³⁾, does not specify the determination of MAB as quality indicator microorganisms in cheeses, because this group includes lactic acid bacteria, which are desirable microorganisms in cheese maturation⁽²⁴⁾, which, due to their long-term proteolytic and lipolytic activities, contribute to the development of flavor and aroma^(18,20,22), providing the typical characteristics to the product of a region. In this study, MAB had significant differences ($P < 0.05$) between days of maturation and between production periods. In this regard, the counts of the dry season cheese were 8.91 log₁₀ CFU/g of cheese and that of the rainy season was 9.86 log₁₀ CFU/g of cheese (Table 1). Microbiological counts increased in the first 30 d of maturation due to the presence of inherent lactic acid bacteria in the milk or rennet used to make the cheese, obtained from young calves from the region.

The decrease in MAB counts from d 60 is attributed to the fact that the growth of these organisms during cheese maturation is controlled by some physicochemical factors such as water activity, salt concentration, pH, organic acids, temperature during maturation, oxidation-reduction potential and presence of nitrates⁽²⁵⁾.

The population dynamics of MAB in this study are consistent with those of other cheeses. In artisanal pore cheese three days after being made, MAB were present with a concentration of 6.77 log₁₀ CFU/g of cheese and after 12 d of being made, they increased to 7.44 log₁₀ CFU/g of cheese⁽¹⁶⁾. In the Tepeque aerated cheese, concentrations of 7.9 log₁₀ CFU/g of cheese were reported in the dry season and 7.6 log₁₀ CFU/g of cheese in the rainy season. In Tepeque cheese taken to maturity, the concentration in both the cheese produced in the dry season and in the rainy season was 6.1 log₁₀ CFU/g of cheese⁽²¹⁾. In the study

carried out by Hernández *et al*⁽²⁶⁾ on the Zacazonapan aged cheese, the cheeses were matured at a temperature of 24 °C and at a relative humidity of 65 % and at 27 d of maturation, MAB were present at a concentration of 1.5 to 7.8 log₁₀ CFU/g of cheese.

The group of Staph (Table 1) had differences ($P<0.05$) between days of maturation and between production periods. The average of the counts made to the dry season cheese was 7.99 log₁₀ CFU/g of cheese and that of the rainy season was 9.12 log₁₀ CFU/g of cheese. The presence of staphylococci and the appearance of enterotoxins in foods are important parameters in the evaluation of food safety⁽²⁷⁾. *Staphylococcus aureus* is often found in raw milk and in the environment of cheese plants (equipment and staff), is salt tolerant and can grow under a wide range of conditions; low acid production can allow staphylococci to grow and produce enterotoxins^(16,27).

In artisanal pore cheese, it was found that *S. aureus* was present with an average concentration of 5.91 log₁₀ CFU/g of cheese in three-day old cheese and 6.29 log₁₀ CFU/g of cheese with 12 d of maturation⁽¹⁶⁾. In Tepeque aerated cheese, the microbial load was 7.9 and 7.7 log₁₀ CFU/g of cheese in cheese from the dry season and the rainy season, respectively⁽²¹⁾. In Kurdish cheese, the concentration was 3.06 log₁₀ CFU/g of cheese in fresh cheese and 1.12 log₁₀ CFU/g of cheese at 40 d of maturation⁽²²⁾. Results similar to those found in this study indicate serious failures in the hygienic-sanitary conditions of cheese factories⁽²⁸⁾ and, consequently, the consumption of this cheese can cause staphylococcal poisoning, representing a danger to the consumer⁽²⁹⁾.

Significant differences ($P<0.05$) were observed in the TC content between days of maturation, decreasing throughout the process. The average of both batches of cheese was 8.10 log₁₀ CFU/g of cheese (Table 1). TCs are a good indicator of hygienic quality, their presence is undesirable because it causes structural defects in the cheese and are an indicator of fecal contamination and reflect lack of hygiene during the preparation or handling of the product and warn of the possible presence of other pathogens⁽³⁰⁾. During salting, there is a slow process of natural dehydration of cheeses, favoring the survival of these bacteria for longer⁽¹⁶⁾.

The bacterial load of TC in cheeses at day zero (Table 1) was similar to that reported in fresh cheeses^(28,29). The minimum load observed in this study was 6.68 log₁₀ CFU/g of cheese at 120 d, similar to matured Tepeque cheese⁽²¹⁾. However, it exceeds the counts reported in aerated or controlled maturation cheeses, such as artisanal pore cheese⁽¹⁶⁾, Zacazonapan aged cheese⁽²⁶⁾ and Corrientes cheese⁽³¹⁾, which makes clear the importance of controlling the maturation conditions of this type of cheese.

The counts for FC (Table 1) showed significant differences ($P < 0.05$) between production seasons, being higher in dry season cheeses ($3.04 \log_{10}$ MPN/g of cheese). The results of this study were lower than those found in Mexican tropical cream cheese made with unpasteurized milk from the region of Tonalá, Chiapas⁽²⁴⁾ and their presence in cheese indicates contamination by feces⁽³²⁾. The decrease in counts after 120 d of maturation in rainy season cheeses is due to the action of lactic acid bacteria⁽²⁷⁾.

The results of the analyses carried out for Salm and List during the maturation of the cheeses (Table 2) indicate that Salm was only detected in the cheeses of d 0 (fresh cheeses) and List was present throughout the maturation.

Table 2: *Salmonella* spp. and *Listeria* spp. during the maturation of Zacazonapan cheese

Microbial group	Batch	Days of maturation				
		0	30	60	120	150
Salm	Dry season	P				
	Rainy season	P				
List	Dry season	P	P	P	P	P
	Rainy season	P	P	P	P	P

Salm= *Salmonella* spp.; List= *Listeria* spp.; P= Present in 25 g of sample.

The presence of Salm in cheeses is related to the preparation of products with unpasteurized milk and it has been detected in fresh cheeses⁽²⁹⁾, in the first days of maturation^(22,27,32). In cheeses with 7 d of maturation, Salm was not detected, and this is attributed to the accumulation of lactic acid and the decrease in pH (< 4.7)⁽²⁷⁾.

The presence of List in cheeses (Table 2) suggests contamination when using milk contaminated by cows that suffer from subclinical mastitis⁽³³⁾. Bacteria of the genus List have been detected in equipment used in the manufacture of cheeses⁽³⁴⁾, in aerated and matured cheeses^(21,28). Although the species was not determined, the risk to consumer health that List represents must be considered, since it can produce important alterations to the central nervous system and even death⁽³⁴⁾.

The results obtained in this research; the bacterial count of the studied groups exceeds what is allowed by NOM 243 SSA1⁽²³⁾. However, there is a worldwide trend of consuming artisanal products that are sought after for their taste and quality linked to the place of origin. Microbial diversity as well as interactions between populations are the main factors that contribute to the taste of traditional cheeses^(16,20).

It is concluded that the maturation for 150 d of both batches of cheeses, as is traditionally done, is insufficient to inhibit the development of pathogenic microorganisms, although the rainy season cheese had a lower microbial load. The presence of bacteria of the genus *List* and *Salm* indicated a greater health risk, and it is not suitable for consumption. It is necessary to carry out actions throughout the cheese process, and it is suggested to implement good manufacturing practices for the production of cheese, and that a space of the cheese factory be adapted as a maturation chamber.

Acknowledgements and conflicts of interests

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