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Research article

Microbiological and physicochemical characteristics of Chihuahua cheese manufactured with raw milk

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Abstract: Chihuahua cheese is traditionally produced in the State of Chihuahua, Mexico, with raw milk, which involves potential bacterial outbreaks. The objective of this work was to characterize the cheese-making process of Chihuahua cheese, including the determination of cheese composition and microbiological determination of pathogens, indicators and Lactic Acid Bacteria (LAB). Ten different cheese factories were sampled where cheese manufacturing protocols were documented. Also, samples of raw milk, curd, cheddaring, and final product were taken. Pathogens, indicators (total mesophilic bacteria, coliforms, coagulase-positive Staphylococci and *Salmonella*) and LAB were quantified in all samples. Proximal analysis of the final product was also completed. Although process steps were similar, there were significant variations in relevant parameters such as temperature, pH, and time. Regarding microbiological analysis, coliforms (5.9–7.4 Log10 CFU/g) and total mesophilic counts were high (8.8–9.2 Log10 CFU/g), while counts of *S. aureus* were within the range of 2.1–2.3 Log10 CFU/g. The microbial count of lactobacilli was lower in milk and higher in the final product. The highest counts of enterococci (6.97 Log10 CFU/g) and thermophilic lactobacilli (8.16 Log10 CFU/g) were observed in the final product. Microbial counts were different in all dairies and LAB groups varied during the manufacturing process. Principal Component Analysis grouped data by type of sample or process analyzed, rather than by dairy location. Manufacturing standardization, incorporation of good manufacturing practices, and milk pasteurization with the use of a starter culture, can improve the overall quality of Chihuahua cheese. The balance between the sensorial and physicochemical properties of the artisanal product will assure that Chihuahua cheese is not a risk of food-borne outbreaks for consumers.

Keywords: raw milk cheese; Chihuahua cheese; lactic acid bacteria; microbial quality; cheese characterization

1. Introduction

Cheese is a dairy product that provides proteins, carbohydrates, fats, and inorganic ions among other nutrients [1]. Modifications on the manufacturing process have led to the emergence of a wide variety of cheese, many of them closely related to the cultural and geographical conditions of their origin; currently, more than 500 cheese varieties are described, depending on their final characteristics [2]. Lactic Acid Bacteria (LAB) are fundamental for the development of specific flavor and odor in cheese, especially starter LAB (SLAB) during manufacturing and non-starter LAB (NSLAB) during ripening [3]. Traditional cheeses were initially manufactured with raw milk, and therefore many sensorial traits have been associated with the presence of autochthonous LAB. It is also considered that some rheological and sensorial properties are lost when pasteurized milk is used as a starting material in cheese manufacturing [4,5].

On the other hand, cheese nutrients can promote the growth of many microorganisms, including food-borne pathogens. Although LAB are predominant in cheese, other microorganisms are also present, including yeast, molds, spoilage bacteria, and food-borne pathogens (*Brucella* spp, *Staphylococcus aureus,* and *Listeria monocytogenes* among others) [6]. There are numerous reports of food-borne outbreaks related mainly to fresh cheese [6,7].

Chihuahua cheese is produced by the Mennonite community in the northern state of Chihuahua and is one of the most popular cheese products in Mexico. It is a semi-hard cheese whose manufacturing protocol includes cheddaring and salt addition before pressing. According to the manufacturing standard procedure, it has to be matured for 4–6 weeks before marketing (semi-matured), and it is usually consumed within three months of manufacturing [8,9]. Production of Chihuahua cheese in Chihuahua, Mexico, is of more than 60,000 kg per day [9], manufactured in different locations, from small farms to industrial-scale factories [10]. Chihuahua cheese has been manufactured using raw cow milk in an artisanal way, and according to manufacturers and consumers, pasteurization of milk affects the final flavor and texture. In small and medium-size cheese factories, Chihuahua cheese production is still done using raw milk as starter material. The maintenance of particular sensorial and physicochemical characteristic of traditional cheeses are among the technical challenges encountered in large scale cheese production [10]. As an initial phase in the scale-up of artisanal cheese manufacturing, the characterization of the process in small in medium-scale facilities will provide valuable information on Chihuahua cheese manufacturing standardization process.

In recent years, there has been an increase in consumption of traditional cheeses made with raw milk, along with consumer trends to "natural" and "healthy" choices [5]. However, dairy products manufactured with raw milk represent a high risk of infectious food-borne outbreaks. Pasteurization alone does not assure the microbial quality of the food product; post- contamination and inadequate manufacturing practices can also lead to unsanitary conditions [2]. Hygienic practices, from dairy cow health care, good milking practices, and good manufacturing practices, can lead to a microbiologically safe product. Health risks associated with pathogens present in cheese [11] has prompted the Mexican authorities to establish a national official criterion that requests that cheeses be manufactured with pasteurized milk [12].

Therefore, this work aimed to report variations in Chihuahua cheese manufacturing at medium-scale dairies across Chihuahua, Mexico, that used raw milk. The microbial profile of pathogens, indicator and LAB, as well as the proximal analysis of Chihuahua cheese can provide information on the distinctive characteristics of Chihuahua cheese, which can be used in the Protected Designation of Origin (PDO) certification.

2. Materials and methods

2.1. Sampling procedures

Ten dairies located at different geographical regions in the state of Chihuahua, Mexico, were sampled for this study. These factories were selected because they used raw milk for Chihuahua cheese manufacturing and no SLAB added. Composite samples taken during batch processing included stored milk (100 mL), curd (50 g), cheddaring (50 g) and the final product (1 kg) (Figure 1). All samples were transported to the laboratory in a cooler with frozen packs and analyzed within 48 h. Cheese samples were vacuum-packed and stored at 4 ℃ for 30 days, and cheese was also analyzed as the rest of the samples. The purpose of holding the cheese samples for 30 days at 4 ℃ was to simulate the ripening process done in the cheese factories, before marketing. During cheese manufacture, process variables were recorded, including temperature, pH (Hanna Model HI99161, Rumania) and time at each manufacturing step, as well as final pressing time.

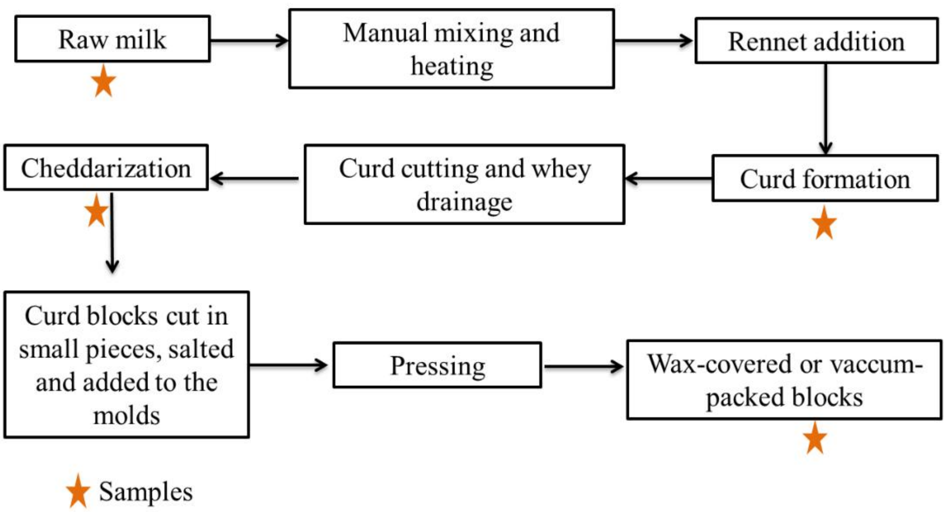

Figure 1. Chihuahua cheese manufacturing general procedure and samples analyzed.

2.2. Microbiological analysis of Chihuahua cheese

Samples (10 g or mL) were homogenized for 1 min in 90 mL sterile sodium phosphate buffer (PBS, pH 7.2) using BagMixer equipment (Model CC, Interscience, Saint Nom, France). Ten-fold dilutions were prepared in PBS and used for the different microbial group's enumeration.

Quantification of indicator and pathogenic microorganisms was done in all samples obtained during Chihuahua cheese manufacturing process. The total mesophilic aerobic count was done using Standard Count Agar (Bioxon, Mexico City, Mexico), incubated aerobically for 72 h at 37 ℃. For total coliforms enumeration, Violet Red Bile Agar (Bioxon, Mexico City, Mexico) was used, and plates were incubated aerobically for 24 h at 37 ℃. Quantification of coagulase-positive Staphylococci was done by spreading 0.1 mL of each dilution into Baird-Parker Agar (Bioxon, Mexico City, Mexico) enriched with egg yolk-tellurite emulsion, and incubated aerobically for 48 h at 37 ℃ [13]. Results are expressed as Log10 UFC/g or mL of samples.

For the enumeration of the main LAB groups, 0.1 mL of each serial dilution was dispersed into selective agar plates by spread plate technique. For lactobacilli, MRS Agar (BD, Bioxon, Mexico City, Mexico) was used and plates were incubated under a reduced oxygen atmosphere (BD Gas Pack CO_2, USA) for 72 h at 25 ℃ for presumptive mesophilic lactobacilli and 42 ℃ for presumptive thermophilic lactobacilli. For presumptive lactococci, M-17 Agar (BD, Bioxon, Mexico City, Mexico) was used, and plates were incubated at 25 ℃ for 72 h under a reduced oxygen atmosphere. Enumeration of presumptive enterococci was done using Kanamycin Esculin Azide Agar (BD, Bioxon, Mexico City,Mexico); plates were incubated aerobically for 72 h at 37 ℃ [14]. Results are expressed as Log10 UFC/g or mL of samples. LAB groups are identified as presumptive since the media used, although considered selective, allows the growth of other LAB. Colonies from each LAB growth were further isolated and tested for catalase and confirmed to be Gram-positive bacilli and cocci accordingly.

For the determination of *Salmonella*, the analysis was done in the cheese samples aged for 30 days, following Mexican official procedures [12]. A 25 g sample was homogenized for 1 min in 225 mL of buffered peptone water (BagMixer, Model CC, Interscience, Saint Nom, France), and then incubated at 37 ℃ for 24 h. After incubation, 1 mL was transferred into 10 mL of Tetrathionate Broth (Bioxon, Mexico City, Mexico) and to 10 mL of Selenite Cystine Broth (Bioxon, Mexico City, Mexico) and incubated aerobically for 18 h at 37 ℃. A loopful of each broth was then plated into a plate of Brillant Green Agar, Salmonella-Shigella Agar and Bismuth Sulfite Agar (all Bioxon, Mexico City, Mexico) for isolated colonies. Suspicious colonies were tested for biochemical differentiation using Lysin Iron Agar and Triple Sugar Iron Agar (Bioxon, Mexico City, Mexico) [13].

2.3. Chemical analysis of Chihuahua cheese

Chemical analysis of Chihuahua cheese was performed using AOAC methods [15] for moisture (method 926.08), protein (method 991.22), total fat (method 933.05) and total ash (method 935.42); carbohydrate content was obtained by difference. The determination of NaCl content was done with an electrode (HI, model 255, combined meter pH/ mV and EC/ TDS/ NaCl, Rumania) adapted to a pH meter (HI model 76310, Italy), using standard solutions to calibrate the electrode. One gram of cheese was homogenized in distilled water for 1 min and filtered to retain cheese particles. NaCl was determined in the solution and results were expressed as % NaCl.

2.4. Statistical analysis

Results of physicochemical analysis and microbial counts were subjected to Analysis of Variance, using the type of sample and dairies as independent variables. The comparison between samples was made using Tukey's test at 5% significance level. A Principal Component Analysis was done to evaluate the relationship between manufacturing conditions and the microbial groups at each sampled point in the different dairies. Statistical analysis was done using the statistical software Minitab 17 [16].

3. Results and discussion

3.1. Process variables and physicochemical analysis of Chihuahua cheese

As observed in Figure 2, cheese factories included in the study were located in different regions of the State of Chihuahua, Mexico.

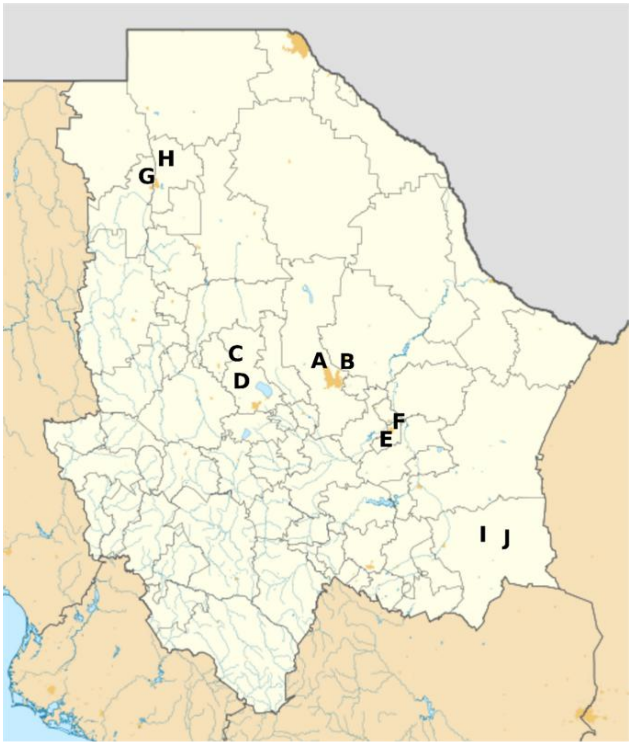

Figure 2. Location of the Chihuahua cheese manufacturing factories from the State of Chihuahua, Mexico, included in this study.

Dairies denominated A, B, E, F, I, and J are in non-Mennonite areas, while dairies G and H are located in traditional Mennonite communities. Cheese factories C and D are medium-scale cheese factories also owned and operated by Mennonites. Manufacturing of Chihuahua cheese has not been standardized, as is the case for many industrialized cheese types worldwide. Several references describe the manufacturing protocols of Chihuahua cheese in medium or large size factories, but the problem remains in small and medium-size dairies [9,10,17]. This information will provide critical parameters to standardize manufacturing practices. Results of the process variables recorded during Chihuahua cheese manufacture at the ten locations sampled are shown in Table 1.

Raw milk used is usually obtained from the same dairy where the cheese is produced, or is collected from neighboring farms; therefore, it is not refrigerated upon arrival to dairy premises. Milk temperature was between 14.4 and 31.7 ℃, and pH values were in the range of 6.2–6.8. It is important to consider that high temperatures can promote bacterial growth present in milk, and this will directly affect the microbial quality of cheese. Milk temperatures of 4–23 ℃ have been previously reported [17] in cheese factories located in the Mennonite area, where C, D, G, and H factories analyzed in this study are located. Regarding pH, only location B had a higher pH (6.82), which could interfere with curd formation.

Table 1. Time, temperature and pH values at different stages during Chihuahua cheese manufacturing process recorded in cheese factories located in different regions of the State of Chihuahua, Mexico.

Cheese factory	Process step									
	Raw milk		Curd Formation			Cheddaring			Cheese	
	Temperature (℃)	pH	Temperature (℃)	Time (min)	Final pH	Temperature (℃)	Time (min)	Final pH	Pressing time (h)	pH
A	18.6	6.2	39.2	25	6.2	29.7	10	6.0	0.5	5.5
B	14.4	6.8	40.0	15	6.3	31.0	10	6.1	1	5.5
C	19.4	6.6	39.0	15	6.3	29.6	15	5.9	24	5.6
D	23.3	6.5	35.1	15	6.3	28.6	10	6.0	18	5.4
E	31.7	6.5	38.4	60	6.1	26.9	10	5.9	1	5.5
F	23.4	6.7	40.0	10	6.3	33.7	10	6.0	1	5.2
G	22.9	6.5	40.0	25	6.1	29.3	40	5.4	24	5.5
H	27.3	6.6	36.2	25	6.2	32.0	20	5.9	2	5.8
I	29.3	6.5	40.7	25	6.0	30.0	15	5.8	4	5.0
J	27.3	6.5	29.9	25	6.5	29.9	15	6.4	1	5.3

Milk was heated to 30–35 ℃ before rennin addition for curd formation. During curd formation, the temperature was on the range of 29.9–40.7 ℃, and the time needed for curd formation ranged from 10 and 60 minutes. The most frequent time required for curd formation was 25 min; temperatures are within the range of curd formation for Cheddar cheese and those reported previously for Chihuahua cheese [9,17]. During curd formation, pH values were between 6.0 and 6.5, similar to values reported for Cheddar cheese. A slight decrease in pH was observed from milk to curd, as expected by microbial growth [18].

During cheddaring, curd slabs are stacked and overturned several times; during this step, pH value decreases and whey is expelled from curd. This step is of particular importance for the developing of some of the rheological properties of Chihuahua cheese. Curd temperature during cheddaring was within 26.9–37.7 ℃, with pH values of 5.4–6.4 and a cheddaring time of 10–40 min. Previous reports on Chihuahua cheese describe temperatures of 19–41 ℃ and 1–90 min cheddaring time [9,17]. Time spent in cheddaring is in direct relation to the lower pH reached, and correlated with desirable physicochemical properties of the final product. A cheddaring time of 15–90 min is recommended for Cheddar cheese manufacture [8], and of all the locations analyzed, those using traditional artisanal processes had the most extended cheddaring times (dairies G and H). The rest of the dairies had cheddaring times of 10–15 minutes, which is considered a short time that resulted in cheese pieces with high humidity and an evident syneresis.

Pressing time is directly related to humidity in the final product; 18–24 h are recommended for

Cheddar cheese [8]. According to our observations, pressing times were in the range of 0.5 to 24 h. Cheese factory A had a pressing time of 30 minutes and four dairies invested only one hour in this step. Dairies C, D, and G had press times of 18–24 h; Mennonites operated all three factories. Final product pH was between 5.0 and 5.8; except for location H, all cheese products were within the range required by the Mexican Standard of 5.0–5.5 [19].

Table 2 shows the compositional analysis of Chihuahua cheese manufactured with raw milk at the ten locations analyzed. The parameters established in the Mexican Standard for Chihuahua cheese [19] are also included at the end of the Table.

Table 2. Composition of Chihuahua cheese manufactured with raw milk at different locations of the state of Chihuahua, Mexico. Results are reported as mean +/− std deviation of three replicates for each cheese analyzed.

Cheese factory	Moisture (%)	Protein (%)	Fat (%)	Ash (%)	NaCl (%)
A	51.1 ± 0.3^{a}	22.9 ± 0.5^{bc}	25.2 ± 0.8^{g}	3.9 ± 0.1^{ab}	0.93 ± 0.06^{abc}
B	47.7 ± 0.4^{c}	25.5 ± 0.4^{a}	34.7 ± 0.2^{e}	2.8 ± 0.1^{fg}	0.73 ± 0.06^{d}
C	40.8 ± 0.03^{f}	25.3 ± 0.1^{a}	45.6 ± 0.9^{a}	3.5 ± 0.2^{cd}	0.87 ± 0.06^{cd}
D	43.2 ± 0.4^{e}	25.2 ± 0.3^{a}	40.7 ± 0.7^{c}	3.8 ± 0.2^{abc}	0.90 ± 0.00^{bc}
E	45.6 ± 0.1^{d}	22.8 ± 0.3^{c}	43.5 ± 0.5^{b}	4.0 ± 0.1^{a}	1.03 ± 0.06^{ab}
F	49.8 ± 0.1^{b}	20.4 ± 0.3^{d}	30.7 ± 0.8^{f}	3.2 ± 0.03^{de}	0.73 ± 0.06^{d}
G	40.1 ± 0.07^{f}	25.7 ± 0.3^{a}	40.7 ± 0.7^{c}	3.6 ± 0.02^{bc}	1.07 ± 0.06^{a}
H	47.1 ± 0.3^{c}	23.9 ± 0.2^{b}	34.8 ± 0.4^{e}	2.7 ± 0.2^{g}	0.83 ± 0.06^{cd}
I	45.6 ± 0.2^{d}	25.6 ± 0.4^{a}	38.2 ± 0.4^{d}	3.2 ± 0.05^{ef}	0.80 ± 0.01^{cd}
J	49.9 ± 0.5^{b}	20.9 ± 0.5^{d}	41.4 ± 0.3^{c}	2.8 ± 0.03^{fg}	0.83 ± 0.06^{cd}
Mean	46.1 ± 3.8	23.9 ± 1.80	36.9 ± 6.90	3.4 ± 0.50	0.87 ± 0.11
Mexican Std [19]	≤45.0	≥23.0	≥28.0	≤6.5	≤3.0

Regarding moisture content, values were between 40.1 and 51.1% with a mean value of 46.1%, which is higher than the recommended by the Mexican Standard. Cheese samples from locations A, B, E, F, H, I, and J were above the recommended moisture content and were also the dairies that had short pressing times during processing (1–4 h). On the other hand, cheese samples from locations C, D, and G had proper moisture content, in direct relationship with longer pressing times (18–24 h).

Protein content ranged from 20.4 to 25.7%, with a mean value of 23.9 ± 1.80%. The cheese manufactured in dairies F and J had protein values below the required percentage by the Mexican Standard for Chihuahua cheese. Previous studies in Chihuahua cheese have reported similar values as those presented in this study, with an average protein value of 26.4% [20] or a 23 to 27% protein content range in Mennonite cheese [17]. Differences in protein content can be attributed to variations in the chemical composition of milk, which can be influenced by animal feed as well as age and health conditions of the herd [18].

Average fat content in Chihuahua cheese samples was of 36.9 ± 6.90%, and considering the minimum 28% established by the Mexican Standard [19], only dairy A had a lower fat content. According to previous reports, fat content for Chihuahua cheese is in the range of 30–36% [17,21], while other authors had reported fat percentages of 21–30% [22]. As with protein content, variation in fat composition in Chihuahua cheese can be attributed to differences in the milk used for cheese manufacturing [18].

Ash content was on the range of 2.7 to 4.0%, and all cheese samples analyzed were within the limit established in the Mexican Standard (maximum value of 6.5%); regarding salt content, values were in the range of 0.73 to 1.07%; all within the limits (maximum 3%) [19]. The salt content in the samples analyzed in this study was lower since it has been reported salt content of 1–1.5% [17,21]. Differences observed can be related to variations in the manufacturing process in each cheese factory, as a consequence of not having a standardized protocol.

3.2. Determination of pathogens and indicator microorganisms during Chihuahua cheese manufacturing

Microbial quality of dairy products manufactured with raw milk as starter material has been a significant distress of public health risk. As such, there are concerns about the microbial quality of Chihuahua cheese manufactured with traditional methods, using raw milk as starting material. In order to evaluate the microbial quality of Chihuahua cheese manufactured in the ten dairies analyzed, indicator and pathogenic microorganisms were analyzed.

Average of microbial enumeration of total coliforms, total mesophilic aerobic microorganisms and coagulase-positive Staphylococci at four different steps during Chihuahua cheese-making process are shown in Table 3. The presence of *Salmonella* spp. was also tested in the final product, and none of the samples analyzed were positive. Total mesophilic aerobic counts were on the range of 8–9 Log10 CFU/g or mL of sample. According to the statistical analysis, there was a highly significant difference among dairies ($p < 0.001$), being dairy F the one with the lowest count. Regarding the processing step analyzed, curd and cheddaring samples were different from the cheese after 30 days of manufacturing ($p = 0.003$) (Table 3). Similar values of total mesophilic aerobic counts had been reported in Chihuahua cheese, with values on the range of 7.30 to 8.93 Log10 CFU/g [11] or 7.47–9.61 Log10 CFU/g [17] in samples manufactured with raw milk. It is important to consider that the conditions of analysis could have promoted the growth of some LAB, and are included in this microbial count.

Total coliform counts were on the range of 5–8 Log10 CFU/g or mL of sample, and highly significant differences were observed among dairies ($p = 0.003$). Differences among cheese factories can be related to adequate sanitary practices in some of the facilities, but still, raw milk used needs to be of better microbiological quality. Considering the sample analyzed, there was a significant difference ($p = 0.015$) among samples, being the cheddaring step the one that had the highest count and the sample of cheese after 30 days of ripening showed a reduction in the number of coliforms (Table 3). The high number of coliforms can be related to the quality of raw milk or inappropriate milk manipulation practices. The analysis of coagulase-positive Staphylococci showed highly significant differences among dairies ($p = 0.004$), with one of the dairies that had less than 10 CFU/g in all samples (not detected). Samples from dairies A, B, G, and H had high numbers of coagulase-positive Staphylococci in all samples from the manufacturing process. High counts of *S. aureus* in Chihuahua cheese manufactured with raw milk, has been previously reported, with values ranging from 4.25 to 6.86 Log10 CFU/g [9,11]. The presence of *S. aureus* is frequently reported in cheese manufactured with raw milk due to lousy milking practices [23]. In order to comply with Mexican sanitary requirements, the presence of *Salmonella* was tested in cheese after 30 days of ripening, which is when it is ready for distribution [12].

Table 3. Microbial quantification (Log10 CFU/g or mL of sample) of indicator and pathogenic microorganisms determined at the different stages of Chihuahua cheese manufacture. Results are reported as mean +/− std deviation of the samples from the ten dairies analyzed.

Process step	Microbial group			
	Total coliform	Mesophilic[1]	Staphylococci[2]	*Salmonella*
Raw milk	7.0 ± 0.03^{ab}	9.1 ± 0.1^{ab}	2.3 ± 0.7^{a}	−
Curd	6.8 ± 0.2^{ab}	8.9 ± 0.1^{b}	2.1 ± 0.6^{a}	−
Cheddaring	7.4 ± 0.6^{a}	8.8 ± 0.2^{b}	2.2 ± 0.8^{a}	−
Cheese	6.7 ± 0.2^{ab}	9.2 ± 0.1^{ab}	2.1 ± 0.7^{a}	−
Cheese 30d[3]	6.4 ± 0.9^{b}	9.3 ± 0.2^{a}	2.4 ± 2.5^{a}	ND
Mexican Norm [12]	−	−	<100 CFU/g	Absent in 25 g

[1]Total mesophilic count; [2]Coagulase positive Staphylococci counts; [3]Cheese samples after 30 days of ripening. ND: Not detected. Different letters in each microbial group (a–c) indicate different groups, according to Tukey test ($p < 0.05$).

Table 3 includes the information of the Mexican Standard for Chihuahua cheese, which includes Staphylococci and *Salmonella*. Although cheese can be manufactured using raw milk as starting material, the microbial quality of the milk will finally be reflected in the final product. It is important to consider that pathogens and indicator microorganisms can increase their number during the different steps of the manufacturing process, but the microbial quality will be related to the final product. Therefore, we tested *Salmonella* only in the final product, and it was not detected, as requested by the Mexican Norm [12]. In a very interesting report, Kim *et al.* [24] included a comparison of microbiological criteria for cheese products in different countries, including Mexico. In all cases, the criteria for indicator microorganisms is of a three-class type. FDA requires <100 MPN/g of *E. coli*, and <10,000 CFU/g of *S. aureus* [24]. We did not tested for *E. coli*, but rather for total coliforms, but the counts of coagulase positive Staphylococci is lower than the established by FDA. The European Community criteria for foodstuff [25] also include cheese products that can be prepared with raw milk or with pasteurized milk. As with FDA regulations, the microbiological criteria includes *E. coli* (n = 5 c = 2 m = 100 CFU/g M = 1000 CFU/g) for cheese manufactured with pasteurized milk, and coagulase positive Staphylococci for cheese manufactured with raw milk (n = 5 c = 2 m = 10^4 CFU/g M = 10^5 CFU/g). Considering the later criteria, Chihuahua cheese will comply with the criteria, since the results are in the range of 10^2 to 10^4 CFU/g. Still, the microbial quality of Chihuahua cheese can be improved by the establishment of good manufacturing practices in small and medium size dairies, as well as the implementation of milk pasteurization.

Chihuahua cheese producers claim that pasteurization can affect the sensorial or physicochemical characteristics of cheese. However, the microbiological quality of the milk used for cheese manufacturing was poor, with the consequent poor microbial quality of the final product. Lately, there has been an increase in food-borne outbreaks related to the consumption of raw milk in the United States, especially related to *Campylobacter* spp. [26]. The increased risk associated with cheese manufactured with raw milk can be 49 times higher for *Salmonella* spp. related infections or 39 for *Campylobacter* spp. related outbreaks [27]. The consumption of dairy products manufactured with raw milk has increased in many countries. However, there is an emphasis on safe milking conditions and good manufacturing practices, to decrease the chance of pathogen contamination in the final product. Most of the pathogens associated with dairy products, including *L. monocytogenes*, *Brucella*, *S. aureus*, *Salmonella* and *Campylobacter*, can grow or survive during cheese manufacture or ripening [28]. It is well known that pathogens are reduced during cheese maturation; Brooks *et al.* [29] determined that a ripening period of at least 60 days is sufficient enough to consider a cheese manufactured with raw milk as microbiologically safe. However, Chihuahua cheese is usually consumed within 2–6 weeks of manufactured, which is not enough time for pathogen elimination.

The microbial quality of artisanal European cheese types has also been reported. In a study of cheese identified as "Traditional Agri-Food Products" from Southern Italy, the presence of coagulase-positive Staphylococci was highlighted. The presence of enterotoxigenic genes in the isolates was determined by PCR; also, a positive cheese sample with staphylococcal toxin was reported [30]. Milk pasteurization does not eliminate the sanitary problems in traditional cheese manufacturing; a post pasteurization contamination can occur, as is the case with *S. aureus* that is associated with human contamination during processing. In a report of the microbial quality of Bryndza cheese manufactured in the Slovak Republic, *S. aureus* counts were different depending on the pasteurization of the starting material but were still high in the pasteurized product [31].

Effective educational programs have been implemented in Chihuahua with cheese producers to reduce contamination, and most have implemented the use of pasteurized milk to manufacture Chihuahua cheese. It is important to consider that pasteurization is not enough to maintain microbial quality in the final product, and good manufacturing practices need to be enforced throughout cheese production and storage [2]. It is important to create awareness of the risk to human health that a Chihuahua cheese of poor microbial quality can represent. It is also necessary to establish official procedures of microbiological analysis, as a tool for cheese producers to assess their compliance with international regulations, which can allow them to export their products.

3.3. Lactic Acid Bacteria during Chihuahua cheese manufacturing

Determination of LAB groups was done to characterize the predominant microbiota at each step of the manufacturing of Chihuahua cheese, as well as to establish differences among dairies analyzed. Starter and non-starter LAB are responsible for many of the physicochemical and rheological cheese properties, and also influences the sensorial profile [10]. Considering that the media used for the determination of the different LAB groups is not highly selective, they have been identified as presumptive enterococci, lactococci, mesophilic lactobacilli and thermophilic lactobacilli, as included in Table 4.

For the presumptive enterococci group, counts were on the range of 4 to 8 Log10 CFU/g or mL of sample; variations among dairies were significantly different ($p = 0.02$), where dairy E had the highest count and dairy G the lowest. Regarding the manufacturing step, there was a highly significant difference between samples ($p < 0.001$) with raw milk showing the lowest count of this microbial group (Table 4). Similar values have been reported previously in Chihuahua cheese samples manufactured with raw milk, but the microbial group was absent in cheese elaborated with pasteurized milk [11]. The absence of enterococci can indicate good milking and manufacturing practices [32]. Enterococci are usually present in the environment, and therefore can be found in raw milk; due to their capacity to tolerate high salt concentrations (6.5%), these microorganisms can be present in cheese even after 30 days of ripening [33].

Table 4. Microbial quantification (Log10 CFU/g or mL of sample) of Lactic Acid Bacteria groups determined at the different stages of Chihuahua cheese manufacture. Results are reported as mean +/− std deviation of the samples from the ten dairies analyzed.

Microbial group	Process step				
	Raw milk	Curd	Cheddaring	Cheese	[1]Cheese 30d
Presumptive enterococci	5.4 ± 0.3^{b}	6.4 ± 0.3^{a}	6.02 ± 0.2^{ab}	7.0 ± 0.2^{a}	6.8 ± 1.1^{a}
Presumptive thermophilic lactobacilli	6.4 ± 0.2^{b}	7.9 ± 0.4^{a}	7.5 ± 0.3^{a}	8.2 ± 0.2^{a}	8.1 ± 0.4^{a}
Presumptive mesophilic lactobacilli	8.5 ± 0.05^{c}	8.8 ± 0.1^{bc}	8.9 ± 0.2^{b}	9.1 ± 0.1^{ab}	9.4 ± 0.4^{a}
Presumptive lactococci	6.4 ± 0.4^{b}	8.5 ± 0.2^{a}	8.7 ± 0.2^{a}	8.9 ± 0.2^{a}	9.2 ± 0.3^{a}

[1]Cheese sample after 30 days of ripening. ND: Not detected. Different letters in each microbial group (a–c) indicate different groups, according to Tukey test ($p < 0.05$).

Presumptive lactococci counts showed a considerable variation, ranging from 3 to 9 Log10 CFU/g or mL of sample, but no statistical difference was observed among cheese factories ($p = 0.077$). Differences within the cheese process were highly significant ($p < 0.001$), with an increase in numbers from the initial sample (raw milk) to the final product (Table 4). *Lactococcus* are usually part of the SLAB, and conditions during Chihuahua cheese manufacturing are appropriate for the growth of this LAB group. Previous reports of *Lactococcus* in raw Chihuahua cheese were on the range of 7.5–8.7 Log10 CFU/g [11], or 6.–7.4 Log10 CFU/g [9]. During the 30 days ripening period analyzed in this study, the count of presumptive lactococci remained almost unaltered.

Presumptive mesophilic lactobacilli counts were similar between the different locations ($p = 0.125$), with values of 8–10 Log10 CFU/g or mL of sample. There was a highly significant difference ($p < 0.001$) between the sampling point during manufacturing, with lower counts in raw milk and higher counts in freshly prepared cheese. Differences can be attributed to the concentration of microorganisms in curd, but also to the promotion of microbial growth during cheese formation. A slight increase in the number of presumptive mesophilic lactobacilli was observed in cheese after 30 days of maturation. This increase was expected, since many NSLAB belong to the lactobacilli group and their number increase during ripening [34].

Finally, presumptive thermophilic lactobacilli showed highly significant differences among dairies ($p < 0.001$), with values between 5 and 9 Log10 CFU/g or mL of sample. Samples from dairy J had the highest values, while samples from dairies A and B had the lowest counts. Differences were also observed between the different points samples in the process ($p < 0.001$), with the lowest count found in raw milk, and the highest counts found in the cheese samples. The presence of thermophilic lactobacilli was expected in Chihuahua cheese, taking into consideration the temperatures used during the manufacturing process. Mesophilic and thermophilic lactobacilli, microbial counts obtained in this study were similar to previous reports [11].

The characterization of the microbiota present in artisanal cheese products can be valuable information used to claim originality in the “Designation of Origin” status [35]. Also, particular functions of LAB such as probiotic properties can be of particular interest for consumers. The determination of microbial counts of LAB groups has also been done for many traditional cheese types, including traditional Rugova cheese from Kosovo, where a predominance of *Lactococcus* spp. was suggested to be used in the preparation of a particular starter culture [36]. The microbial count of

Lactococci, Lactobacilli and Enterococci were also carried out in Bryndza cheese. No differences were observed in the LAB groups when pasteurized and unpasteurized milk was used in cheese manufacturing [31]. The vast diversity of LAB in artisanal cheese types provide unique characteristics to the food product; even though artisanal cheese is not as standardized in their composition and sensorial attributes, they have a wider variety of LAB species. In a study of industrial and artisanal Galotyri cheese samples from Greece, differences in microbial diversity were evident. In industrially produced cheese, there were always two LAB species recovered, while the diversity was more abundant in artisanal samples [37].

Further studies on LAB characterization can help with the establishment of the typical microbiota of Chihuahua cheese. The information can balance out the risks associated with the use of raw milk to manufacture traditional cheese products, with the benefits obtained from the autochthonous LAB microbiota [2,5]. Determination of the microbial dynamics, as well as identification of the strains present, can provide information to develop a starter LAB culture, which can be used to produce Chihuahua cheese using pasteurized milk [4,38]. These objectives, however, were out of the scope of this study.

The technological challenge is to scale-up the production of Chihuahua cheese, but to maintain its particular properties (physicochemical, rheological and sensorial characteristics) [10]. Consumer education on the benefits of consuming cheese elaborated with pasteurized milk is also important to promote the consumption of safe dairy products.

3.4. Relationship between microbiological data, cheese composition, and manufacturing conditions.

In order to relate the manufacturing conditions recorded at the different dairies, with the microbial data, including indicator and pathogenic microorganisms as well as LAB, a Principal Component Analysis (PCA) was done. The first three components explained 64.8% of data variation; 31.6% was accounted for by PC1, while PC2 explained 17.8% and PC3 15.4% of the variation. Figure 3 identifies the sample type of all dairies analyzed related to the three first components of the analysis. PC1 had a negative load for Raw Milk samples (coefficient values −1.4–−4.8) and positive load for Cheese (0.5–2.13). PC2 presented a negative load for Curd samples (−0.26–−2.9) and a positive load in Cheese samples (0.4–2.4). Finally, PC3 had positive or negative loads in all samples analyzed, with no tendency identified.

As part of data analysis, it was considered interesting to determine the relationship between manufacturing processing conditions in the different dairies and the microbial counts of indicators, pathogens and LAB groups. A correlation coefficient matrix did not show significant relationships (data not shown). PCA is used to identify the association of variables not easily identified, so it was used to test if microbial counts and manufacturing conditions were associated. When the load of the principal components was related to the manufacturing site, no association was observed. However, when the sample type or processing step was considered, an association was observed, as presented in Figure 3.

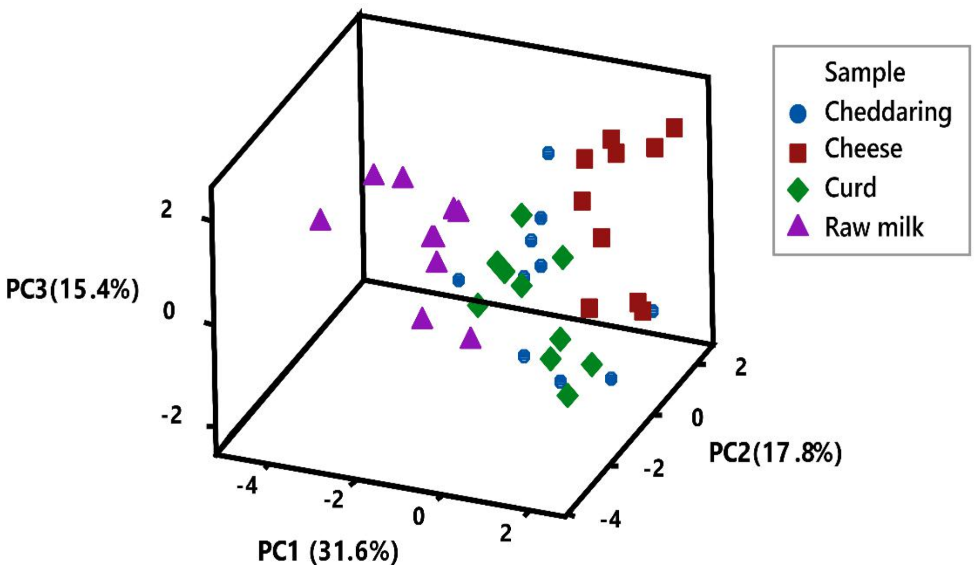

Figure 3. Principal Component Analysis for sample type and dairies using processing conditions and microbiological data. Scores of each sample for the three first Principal Component Analysis (PCA) are shown.

4. Conclusions

Chihuahua cheese manufacturing process is not standardized since conditions used by each cheesemaker are based on their experience and know-how knowledge. Those differences in manufacturing were reflected in cheese composition. On the other hand, the determination of indicator microbial groups can be related to poor sanitary conditions. LAB groups showed a similar tendency as other artisanal cheese products. Determination of specific LAB strains found in Chihuahua cheese can be valuable information for the Designation of Origin, and the preparation of a specific starter LAB culture.

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Conflict of interest

All authors declare no conflicts of interest in this paper.

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