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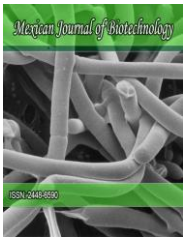

ORIGINAL RESEARCH

Genetic characterization of the microbiota of artisan fresh cheese from the Papaloapan region

Caracterización genética de la microbiota del queso fresco artesanal de la región del Papaloapan

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ABSTRACT

The population of the Papaloapan region consume artisan fresh cheeses and no pathogen outbreaks have been reported recently. The microbiota is responsible to develop desirable characteristics of cheeses and undesirable characteristics due to the presence of certain pathogens microorganisms. Therefore, to identify the microorganisms of fresh cheeses is an important issue for the producers, consumers, and authorities. 11 Artisan fresh cheese samples from the Papaloapan region were collected in the summer and 11 samples in winter to characterize their microbiota. Traditional microbial techniques were used to identify the fungus and the amplification of the 16S rRNA gene and PCR-denaturing gradient gel electrophoresis (DGGE)

method was used for bacteria identification. For all the samples, the presence of aerobic mesophiles, *Streptococcus* mesophiles and thermophiles, *Lactobacillus* mesophiles, *Leuconostoc*, total coliforms, *Staphylococcus aureus*, molds, and yeasts were identified. The complexity and variety of microorganisms in the summer and winter seasons samples were not significantly different. In conclusion, all samples of fresh artisan cheeses were under high microbial loads. Lactic Acid Bacteria (LAB) were in a typical load, as established by the quality and safety standards in the food industry. Conversely, pathogenic bacteria exceeded this limit.
The microorganisms present in the fresh artisanal cheeses of the Papaloapan region were identified with precision, regarding the count and their diversity. A recommendation for the cheese manufacturers is to prepare starter cultures by selecting the appropriate microorganisms to produce the desirable characteristics such as aroma and flavor and reduce the risk of microbial infections by using pasteurized milk.

Keywords: Fresh artisan cheese, cheese microbiota, lactic acid bacteria, genetic characterization, starter culture, raw milk.

RESUMEN

La población de la región de Papaloapan consume quesos frescos artesanales y sin brotes de patógenos recientemente. La microbiota es responsable del desarrollo de características deseables de los quesos e indeseables debido a microorganismos patógenos. Identificar estos microorganismos es un tema importante para los productores, consumidores y autoridades. Se recolectaron 11 muestras de queso fresco artesanal de la región de Papaloapan en verano y 11 muestras en invierno para caracterizar su microbiota. Se utilizaron técnicas microbianas tradicionales para identificar los hongos y se utilizó la amplificación del gen 16S rRNA y el método de electroforesis en gel de gradiente desnaturalizante por PCR (DGGE) para identificar bacterias. Se identificaron mesófilos aeróbicos, *Streptococcus* mesófilos y termófilos, *Lactobacillus* mesófilos, *Leuconostoc*, coliformes totales, *Staphylococcus aureus*, mohos y levaduras. La complejidad y variedad de microorganismos identificados en verano y en invierno no fueron significativamente diferentes. En conclusión, todas las muestras presentaron alta carga microbiana. Las bacterias ácido láctico (LAB) mostraron una carga típica, de acuerdo con estándares de calidad y seguridad de la industria alimentaria. Contrariamente, las bacterias patógenas superaron este límite. Los microorganismos presentes en los quesos frescos artesanales fueron identificados con precisión, en su conteo y diversidad. Una recomendación para los fabricantes es utilizar cultivos iniciadores apropiados y leche pasteurizada para producir las características deseables, como aroma y sabor, y reducir riesgos de infecciones microbianas.

Palabras clave: Queso artesanal Fresco, microbiota de queso, bacterias ácido lácticas, caracterización genética, cultivo iniciador, leche bronca.

1. INTRODUCTION

Cheese is one of the oldest and most popular food products made by humankind. Cheeses are globally well accepted as a classic product of the human diet. The nutritional, functional, sensorial, and texture characteristics of cheeses differ in each type. There are about 2,000 cheese varieties worldwide and they can be classified from a number of viewpoints, e.g. according to: milk origin, curd formation, texture or consistence, and fat content (Belitz *et al*., 2004). The cheese making process has undergone relevant changes throughout history, from an empirical art to an industrial technology with strong scientific bases. Cheese processing is an important study area to elucidate and understand the phenomena that have an impact during its production, formulation, and storage. Besides, the effects such as taste, aroma, color, and texture caused by microorganisms on the quality of the final product are also essential. In general, raw milk is the primary ingredient used to produce artisan cheeses in many regions. The microbial group of cheeses that has a significant presence or greater industrial interest is Lactic Acid Bacteria (LAB). Some other genera like pathogenic microorganisms are also present (Cremonesi *et al*., 2020) (SIAP, 2019).

In 2019, Mexico produced 476,927 tons of cheese of which 89,557 tons were fresh cheeses (SIAP, 2020). The Papaloapan region has potential as a milk producer due to a lower production cost, as compared to those of temperate zone milk-based livestock production intensive systems. The Papaloapan region produces raw cow's milk based products for the local and regional markets. Cheese agribusiness stems from the need to preserve the milk, which is affected by the content of dry matter and fat (Popović-Vranješ *et al*., 2018). The microbiota present in artisan cheeses is extraordinarily complex and occasionally, the presence of pathogenic microorganisms affects their hygienic and sensorial quality (Yeluri Jonnala *et al*., 2018). As a result, there are variations between cheeses, although they originate from the same geographical area. To avoid this problem, manufacturers have started to use pasteurized milk. However, industrial cheeses develop less intense flavors than those made with raw milk. Pasteurized milk directly influences the cheese's sensorial characteristics such as taste, aroma, color, and texture (Tadjine *et al*., 2020). The modification of the characteristics, impaired by the pasteurized milk, results in less acceptance by the consumer. The local population prefer the characteristics produced by the natural microorganism. Therefore, industrial cheeses are less attractive and less accepted by consumers in the region, even when the standardization of the product assures the enhancing of sanitary conditions.

Most of the small enterprises use raw milk to produce artisanal fresh cheeses (Torres-Llanez *et al*., 2006). There is not a characterization of the microorganisms present in the cheeses manufactured in Papaloapan region, where the raw milk is also used. However, there are reports on the characterization of cheeses from other regions. For instance, Torres-Llanez, *et al*. (2006), used raw milk to produce fresh cheese in Hermosillo, Sonora. LAB was the major microbial group in artisanal Mexican Fresh cheeses at the three incubation temperatures studied. The temperature is an important parameter that

promotes the proliferation of microorganism. Research works reported the analysis of the microbiota of cheeses produced during different seasons. During summer and winter seasons to effect on the microorganism count and the diversity could be significant. Aldrete-Tapia, *et al*., (2018) reported the characterization of bacterial communities in Mexican artisanal raw milk "Bola de Ocosingo" Cheese by High-Throughput Sequencing. They analyzed Twenty-four samples from three producers in the state of Chiapas, Mexico, collected at dry (March-June) and rainy seasons (August-November). In the Bola de Ocosingo cheese production, *S. thermophilus, L. lactis, L. helveticus, L. delbrueckii* and *L. plantarum* dominated during the cheese processing, all reported with potential probiotic effect. In addition, they found that prevalence of these bacteria differed across manufacturers and seasons which could account to differences in final product quality (Aldrete-Tapia *et al*., 2018). In similar study, the bacterial community of the artisanal Adobera cheese from Los Altos de Jalisco was described through high-throughput sequencing of 16S rRNA gene libraries. Samples were collected in two different seasons (dry and rainy). Firmicutes and Proteobacteria were the most abundant phyla, strongly represented by the *Streptococcaceae, Enterobacteriaceae* and *Lactobacillaceae* families, and core bacteria genera such as *Streptococcus* spp., *Lactococcus* spp., and *Lactobacillus* spp. Undesirable bacteria, including *Pseudomonas* spp. and *Acinetobacter* spp., were also detected in raw milk but almost undetectable at the end of the cheese manufacturing process (Ruvalcaba-Gómez *et al*., 2020). Similarly, the season for sampling was considered to characterize the artisanal Aro cheese. Then, the samples were collected in April, May and June, since the highest temperatures in the region are recorded at this time of year (González-Montiel & Franco-Fernández, 2015).

As a result of the above stated, it is valuable to identify the microorganisms that make up the fresh cheese microbiota in the Papaloapan region. Adversely, the pathogenic strains that incur affect not only the sanitary quality of the cheese but also the beneficial properties (Murphy *et al*., 2016). The isolation and characterization of the latter could allow the addition of pasteurized milk for cheese making, thus providing the sensorial characteristics of fresh artisan cheese.Therefore, this work aimed to characterize the microbiota of fresh cheeses produced in the Papaloapan region and referred exclusively to cheeses made from cow's raw milk. Although is not an objective of the research, the results could help to enhance the manufacturing process and at the same time, to inform about the existing risk to consuming this kind of products.

2. MATERIALS AND METHODS

Disregarding the season and place of sampling, all the protocols applied were the same for the 22 samples studied.

2.1. Cheese Sampling

A total of 22 artisan fresh cheese samples made with raw milk were collected from four places in the Papaloapan region. The samples were taken in two different seasons, 11

cheese samples were collected in summer in July (2014) and 11 in winter in January (2015). All the analysis in the samples were performed by two replications.

Farms with the highest cheese production were selected as sampling sites according to statistical analysis. The samples collected were as follows: two cheese samples from Tuxtepec (TUX1 & TUX2), one sample, from Chiltepec (CHIL), four samples from Benemérito Juárez (BJ1, BJ2, BJ3, & BJ4) and four samples from Loma Bonita (LB1, LB2, LB3, & LB4). The samples were obtained directly from the producers with a period of no more of one day of storage prior to collection. The temperature of the sampling places varied between 36-40ºC. The sampling of the fresh cheese was carried out by the method indicated in the NMX-F-718-COFOCALEC-2006 (DOF, 2006). The samples were stored at 4ºC and transported to the laboratory for analysis the next day in the Faculty of Zootechnics and Ecology at the Autonomous University of Chihuahua. The samples from both seasons were treated with the same protocols and all the analysis were performed by duplicate.

2.2. Microbial counts and selective media

A suspension of 10 g of cheese in 90 mL of phosphate buffer solution was homogenized for two minutes in a blender (Laboratory Blender, UK). The homogenate was left to rest for one minute to settle down the large particles. Then, 1 mL of the supernatant was used to perform serial decimal dilutions for plating in selective media for microbial counts.

Different selective media and growth conditions such as temperature and incubation time for counting the microorganisms by agar plates such as: Plate count agar (PCA; Oxoid), M17 agar (Oxoid) lactose (M17L), De Man, Rogosa, and Sharpe agar (MRS agar) MRSV, Baird Parker Agar (BPA; BD Bioxon), azide agar (KAA; Oxoid), Potato Dextrose Agar (PDA; BD Bioxon), and Violet Red Bile Lactose Agar (VRBLA; Oxoid). Colony-forming units (CFU) were determined by surface seeding with 100 µL of the respective dilution. Only the VRBLA plates were inoculated with 1000 µL per mass seeding.

The plates were incubated for 24-48 h, at a temperature appropriated for the microorganisms. All the media were done in duplicate and incubated under anaerobic conditions except BPA, PCA, PDA, and VRBLA.

2.3. Microbial identification

2.3.1. DNA extraction.

The DNA extraction from the pellet was carried out according to the specifications of the Macherey-Nagel Nucleospin Tissue extraction kit with a single modification. Briefly, an aliquot of 1 mL of sample thawed at room temperature, was transferred in a new sterile tube of 1.5 mL. The sample was centrifuged at 5000 rpm for 5 min to obtain the pellet of

microorganisms and the kit extraction was applied. To enhance the enzyme hydrolysis, a second addition a new pre-lysis solution containing 20 mM Tris-HCl; 2 Mm EDTA; 1% Triton X-100 pH 8 and 2 mg / ml of lysozyme was performed.

2.3.2. PCR amplification of 16s rRNA

Amplification of genomic DNA was performed according to the method reported elsewhere (Godálová *et al*., 2016). The temperature program consisted of initial denaturation at 94 °C for 2 min, 40 cycles (94 °C for 1 min, 54 °C for 1min, 72 °C for 1 min) and a final polymerization step at 72 °C for 5 min, all amplifications being carried out in a Corbett Thermocycler (Corbett Research, Australia). The oligonucleotides used to amplify a 16S rRNA fragment present in the bacteria were those designed by Godálová *et al*., (2016). Analysis of the 16s region was performed and it was determined that the oligonucleotides span from region V1 to V3 and more specifically it amplifies the V3 region and only 10 bp of the V4 region.

All the amplification reactions for PCR-DGGE were carried out in a final volume of 50 µL with 200 ng of DNA. GoTaq Green Master Mix (Promega Corp.), and 100 ng of each oligonucleotide B = GplusR (5'-CGT CCT TCA TCG GCT – 3') and F = G17-CG (5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GGT GAA GTC GTA ACA AGG - 3') were used for the amplification of the 16S rRNA gene. Reactions were carried out in a Corbett thermocycler under the following parameters: initial denaturation at 94°C for 2 min, followed by 30 cycles, each denaturing cycle at 93°C for 30 seconds and alignment at 78°C for 30 seconds and an extension at 72°C for 30 seconds, ending with a final extension of 72°C for 2 min.

2.3.3. Denaturing gradient gel electrophoresis (DGGE)

DGGE was performed using a D-Code Universal Mutation Detection System (Bio-Rad, Richmond, Calif.) on 16 cm×16 cm×1 mm gels, following the method reported by Walter *et al*., 2000. From the banding profiles of the DGGE gels, the elution method used for the DNA fragments was done according to the technique proposed by Karnati *et al*., (2009). Once completing the PCR process, a 1.5% agarose gel electrophoresis was run, then the amplicons underwent a sequencing process.

Wizard ® SV Gel and PCR Clean-Up System kit (Promega Corporation) was the cleaner solution for the PCR products and in accordance with the specifications of the supplier (Jiang *et al*., 2011).

2.3.4. Cutting, purification, and sequencing of DGGE products

The amplicons embedded within the matrix of the agarose gel were cut directly from the gel under UV light and placed in a 1.5 mL microtube with 110 µL of membrane binding solution added, and incubated at 65°C until the complete dissolution of the gel. Thereafter, the solution was placed inside the column and followed by the next steps: A

volume of 25 µL of membrane binding solution was added to the PCR reaction product and then gently mixed.

The total solution was transferred inside a minicolumn previously mounted in a 1.5 mL microtube, incubated for 1 min at room temperature, and then centrifuged at 13,000 rpm for 1 min. The supernatant was discharged from the microtube and refilled with 700 µL of membrane wash solution, then centrifuged again at 13000 rpm for 1 min. The washing process and centrifugation process were repeated but the volume of washing solution was only 500 µL and the centrifugation time was 5 min. The supernatant was then discharged and refilled with 50 µL of nuclease-free water, transferred into a new column, and incubated for 1 min, then centrifuged at 13,000 rpm for 1 min to obtain a clean DNA eluate. DNA yield and purity parameters were obtained by using the NanoDrop kit (Thermo Scientific) and stored at -20 °C for later use.

From the purified products, 15 µL was sent for automatic sequencing in the ABI PRISM® 3100 Genetic Analyzer kit (Perkin Elmer) to the Molecular Diagnostic and Analysis Unit of the National Institute of Public Health located at Cuernavaca city, Morelos, Mexico.

2.4. Statistical analysis

An analysis of variance (ANOVA) and a post-Tukey test described the data to contrast the seasons (summer and winter) count of the samples collected. A linear model with two explanatory factors of fixed effects at a level of $\alpha = 0.05$, with the statistical program SAS/STAT® (SAS Institute Inc., 2004, version 9.0).

3. RESULTS

3.1. Microbiologic analysis

Firstly, it is important to declare that this work does not pretend to influence or interact with the owners and their processes. We agreed only to receive the samples and we cannot comment about the processes used to produce the cheeses. The cheese producers clearly explained that all materials that come into contact with the processing milk are cleaned to the best standards. They further iterated that until now, they have not received any complaints regarding cheese contamination by bacterial infection.

Microbiological analysis of artisan fresh cheese collected from eleven different producers in two seasons (summer and winter) based on specific culture media is reported in Table 1 as Log_{10} CFU/mL (CFU to abbreviate).

The samples from BJ2 and LB2 showed less cell count concerning *Enterococcus* since they have an average from 4.58±0.15 to 6.16±0.16 CFU for summer and winter, respectively. The other samples maintained an average from 6.51 to 7.88 CFU for both seasons. Regarding *Lactobacillus* mesophilic, the samples from LB1 and LB2 have less cell count of microorganisms, with a mean of 5.45 to 6.57 CFU. The samples obtained

from CHIL had a wider variety of strains with a mean of 8.62 to 9.16 CFU for both seasons. The mean in CFU for *Leuconostoc*, in the samples from LB3, was the less diverse for this group of bacteria. with an average of 4.62 CFU in winter, and there was no count of microorganisms in summer. This effect was due to the *Leuconostoc* species growth better in media containing sucrose, like in sugar cane juice, to produce dextrans. It is reported that *Leuconostoc* does not grow in temperatures higher than 40 °C (Vos *et al*., 2011). In summer, the temperature in the Papaloapan region exceeds 45 °C. Samples from CHIL showed a greater cell count for this group, with an average of 7.78 to 8.02 CFU. The statistical analysis showed that samples from different producers collected in summer and winter were not significantly different ($p > 0.05$) regarding the CFU count.

LB1 and LB2 samples showed less cell count concerning *Streptococcus* mesophilic, with a mean of 6.64 to 7.38 CFU, while the others maintained an average of 7.41 to 9.43 CFU for the two seasons. Regarding *Streptococcus* thermophiles (Table 1), the samples from LB2 have less cell count, with an average of 5.19 to 5.36 CFU, while the others maintained an average of 7.25 to 8.89 CFU for the two seasons. The mean in CFU for mesophilic from LB1 and LB2 samples were the least count for this group of bacteria, with an average of 6.66 to 7.43 CFU, while for the other samples, an average from 7.54 to 9.73 CFU for both seasons was obtained. *Lactococcus* count in samples from LB1 and LB2 contained the lowest count, with a mean of 6.54 to 7.69 CFU. The samples from CHIL and BJ1 showed the highest count with a mean of 8.86 to 9.56 CFU for the two seasons. The LB2 samples showed the lowest count of *S. aureus*, with an average of 5.71 CFU in summer and 6.06 CFU in winter. The remaining values for the other samples showed higher means from 6.48 to 8.93 CFU for both seasons. LB2 and BJ2 samples showed the lowest count of microorganisms, with an average of 4.85 to 6.72 CFU. The rest of the samples showed similar means of 6.43 to 8.67 CFU for both seasons.

Analysis of molds and yeasts of samples from TUX1, TUX2, BJ1, BJ4, LB1, LB3, and LB4 show mold counts with averages from 3 to 3.8 CFU in both seasons. The rest of the samples (CHIL, BJ2, BJ3, and LB2) were almost free of these kinds of microorganisms. The samples from LB3 were the only ones that did not show yeasts in the summer. The other sample showed a mean of 3.53 to 5.07 CFU in both seasons.

Results showed that all the samples collected in the Papaloapan region are in the permissible range for LAB, as established by the Official Mexican Standard NOM-243-SSA1-2010. However, the results of total coliforms, *S. aureus*, as well as molds and yeasts, were above the limits allowed in the standard (2, 3, and 2.69 CFU, respectively). *S. aureus* was one of the microorganisms most present in microbial count in all cheese samples, the presence of this microorganism is due to contamination of the material and work equipment or the milk as raw material. Contamination by *S. aureus* could also be infected by the workers (from the skin, mouth, and nostrils) during the process management and final product. The results suggested that there could be staphylococcal enterotoxins capable of causing poisoning to the consumer.

Table 1. Microbial load found in samples of artisan fresh cheese from the Papaloapan Basin Region (Log_{10} CFU / mL).

Microorganism	Season	TUX 1	TUX 2	CHIL	BJ1	BJ2	BJ3	BJ4	LB1	LB2	LB3	LB4
S. aureus *(1000 UFC/g)*	S[a]	7.93±0.02	8.39±0.02	8.93±0.05	8.81±0.04	6.86±0.05	7.85±0.07	7.78±0.03	6.58±0.03	5.71±0.02	8.50±0.10	7.46±0.06
	W[b]	7.84±0.03	8.40±0.11	8.85±0.07	8.78±0.06	6.97±0.02	7.89±0.07	7.69±0.01	6.48±0.05	6.06±0.8	8.50±0.06	7.80±0.03
Enterococcus *(<100 UFC/g)*	S	7.39±0.02	7.59±0.03	7.52±0.06	7.75±0.08	5.03±0.05	7.70±0.06	6.89±0.19	6.64±0.02	4.58±0.15	7.65±0.05	6.89±0.01
	W	7.36±0.01	7.41±0.12	7.56±0.06	7.69±0.04	6.03±0.11	7.75±0.04	7.88±0.07	6.51±0.01	6.16±0.16	7.50±0.01	6.72±0.02
Total Coliforms (<100 UFC/g)	S	7.33±0.05	7.88±0.09	7.19±0.69	8.67±0.16	5.58±0.01	8.35±0.04	7.79±0.02	6.97±0.04	4.85±0.09	8.13±0.05	8.46±0.05
	W	7.26±0.05	7.73±0.11	7.61±0.05	8.65±0.00	6.72±0.02	8.63±0.01	7.69±0.10	6.43±0.13	5.73±0.06	7.88±0.07	7.56±0.06
Lactobacillus mesophiles	S	7.57±0.16	8.65±0.3	9.16±0.22	8.62±0.02	7.61±0.05	7.67±0.08	7.09±0.26	6.57±0.01	5.45±0.10	8.22±0.26	7.61±0.00
	W	7.41±0.12	8.42±0.09	8.62±0.04	8.62±0.09	7.52±0.09	7.60±0.01	7.27±0.3	6.30±0.03	5.61±0.06	8.27±0.06	7.46±0.07
Leuconostoc	S	5.59±0.03	7.34±0.09	8.02±0.02	6.52±0.08	5.32±0.07	6.79±0.12	5.61±0.5	4.95±0.06	5.09±0.02	0.00±0.00	5.64±0.05
	W	5.43±0.10	7.28±0.04	7.78±0.02	5.16±0.12	5.17±0.14	6.24±0.10	5.37±0.03	4.92±0.03	5.03±0.11	4.62±0.21	5.22±0.07
Enterococcus mesophiles	S	7.80±0.07	8.85±0.00	9.07±0.00	8.67±0.17	7.41±0.22	8.60±0.0**3**	8.29±0.16	6.75±0.3	6.80±0.01	8.43±0.04	7.74±0.06
	W	7.88±0.01	8.77±0.04	9.01±0.02	8.67±0.07	7.55±0.07	8.46±0.03	8.42±0.14	7.38±0.3	6.64±0.11	9.43±0.07	8.58±0.02
Streptococcus thermophilus	S	7.53±0.25	8.00±0.05	8.89±0.03	8.36±0.30	7.25±0.03	7.87±0.07	7.40±0.11	6.82±0.05	5.36±0.05	7.40±0.11	7.78±0.03
	W	7.72±0.14	7.98±0.05	8.46±0.03	8.51±0.10	7.82±0.10	7.67±0.02	7.63±0.13	6.77±0.06	5.19±0.11	7.32±0.05	7.37±0.10
Fungi (500 UFC/g)	S	3.53±0.8	3.50±0.28	0.00±0.00	3.38±0.55	0.00±0.0	0.00±0.0	3.53±0.8	3.38±0.12	0.00±0.00	0.00±0.00	0.00±0.00
	W	0.00±0.00	3.00±0.00	0.00±0.04	3.38±0.12	0.00±0.0	0.00±0.0	0.00±0.00	0.00±0.0	0.00±0.00.	0.00±0.00	3.00±0.00
Yeast (500 UFC/g)	S	4.79±0.01	4.46±0.06	4.77±0.04.	4.22±0.7	3.97±0.9	5.07±0.1	4.66±0.01	4.44±0.12	4.77±0.08	0.00±0.00	4.61±0.08
	W	4.35±0.04	4.21±0.14	4.39±0.16	4.07±0.17	3.80±0.14	4.50±0.6	3.99±0.12	4.06±0.02	4.36±0.19	3.53±0.08	4.22±0.07
Mesophiles (200,000 UFC/g)	S	7.82±0.03	8.54±0.03	8.91±0.02	8.80±0.04	8.42±0.09	8.65±0.04	7.54±0.05	6.75±0.24	6.87±0.24	8.48±0.05	7.67±0.09
	W	7.68±0.08	8.75±0.09	9.46±0.06	9.73±0.07	8.74±0.01	8.65±0.10	7.57±0.17	6.66±0.17	7.43±0.16	8.61±0.01	7.84±0.01
Lactococcus	S	7.81±0.02	8.80±0.02	9.21±0.02	8.86±0.09	7.59±0.01	7.96±0.01	7.84±0.01	6.59±0.01	6.54±0.05	8.19±0.04	7.90±0.03
	W	8.01±0.03	8.92±0.09	9.48±0.07	9.56±0.03	7.74±0.01	8.75±0.05	7.53±0.04	6.71±0.01	7.69±0.02	8.56±0.04	8.47±0.08

Note: S[a]: Summer, W[b]: Winter. Results are the average and Std. Dev of two replicates.

3.2. Genetic Identification of microbiota

3.2.1. Microbiota Analysis in summer

Cheese samples were collected on the same day of the summer of 2014. The temperature for sampling places varied between 36-40 ºC. The DNA extracted from the 11 samples of fresh cheese collected underwent further processing. Fig. 1 shows the amplification performed by the PCR technique using the GoTaq® Green Master Mix (Promega), thus achieving an amplification of approximately 450 bp, observing non-specific fragments, using the oligos B=G+R and G17. PCR was performed with oligonucleotides F=G17-CG and B = G+R for DGGE.

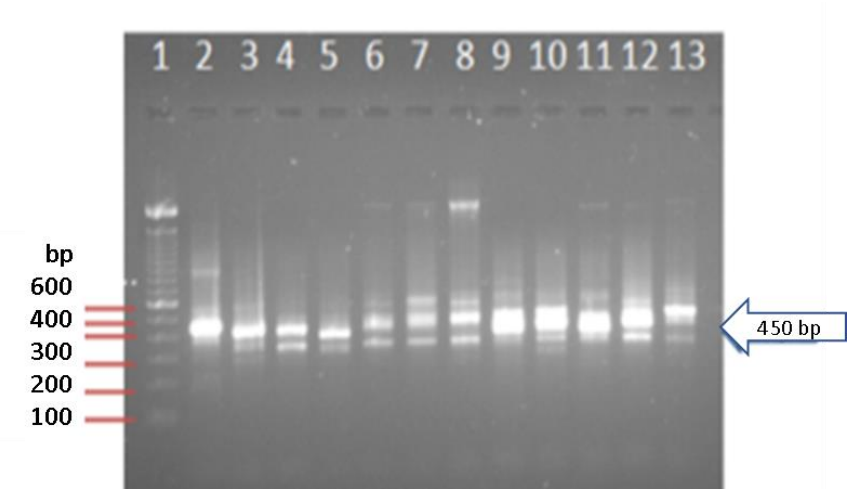

Fig. 1. 16S rRNA amplification gene. Gel electrophoresis (1.5% agarose) stained with ethidium bromide from amplicons of fresh cheese samples from the summer season. 1) Molecular weight marker 1000 bp 1.5 µl (100 ng / µL). 2) Positive control. 3) TUX1. 4) TUX2. 5) CHIL. 6) LB1. 7) LB2. 8) LB3. 9) LB4. 10) BJ1. 11) BJ2. 12) BJ3. 13) BJ4. A volume of 10 µL of each sample was added into different wells. Image analyzed with ImageJ.

Fig. 2 shows the banding profile of the bacterial populations. Subsequently, the PCR-DGGE gel was analyzed and bands of interest were chosen based on the intensity of the banding or the presence of some bands only in some samples. Each band represents a bacterial species. Subsequently, the profile of each community was associated with groups by calculating a matrix of Euclidean distances with the help of BioNumerics software (version 6.6; Applied Math, Austin Tx) and using the UPGMA technique. The DNA contained in the selected bands were re-amplified with the same nucleotide pair from the selected bands, the numbers 15, 17, 18, 21, 22, 23, and 26 were not sequenced as the concentration and purity were not adequate for their sequencing. For each sequence obtained, the forward and reverse products were assembled, obtaining the consensus sequences for a total of 19 samples managed.

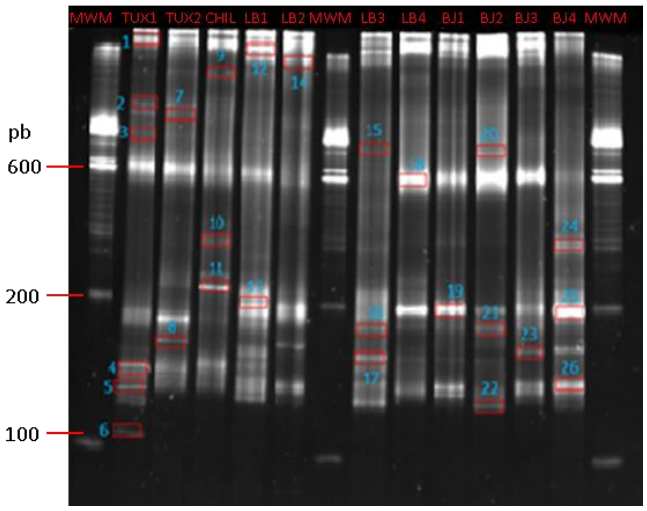

Fig. 2. Banding profile of bacterial populations. Selected bands are identified with numbers in blue color.

Fig. 3 shows the dendrogram obtained by calculating Euclidean distances. The clusters obtained are the results of the difference in sampling place and preparation mode. The percentage of similarities are shown in the formed nodes. The results showed that the profiles originating from the samples collected in Tuxtepec (TUX1 & TUX2) were the most similar, forming a group with 66.7% similarity. This result suggests that cell count is affected by the origin and the method of preparation of the cheeses. The other samples were more related to each other, groups of 50.9% similarity, where groups and subgroups were formed. Besides, they were different from the first group with 43.2% similarity. For the rest of the samples, the place of origin and mode of preparation did not influence their bacterial cell count.

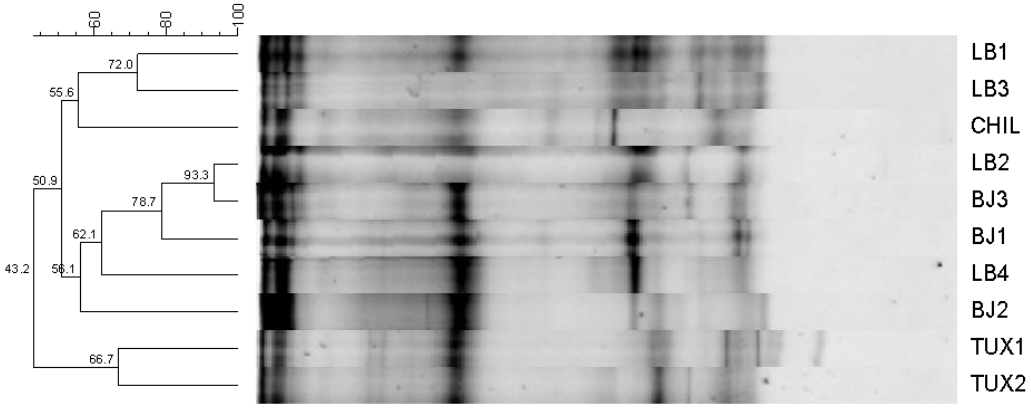

Fig. 3. UPGMA dendrogram showing the genetic relationships among the communities. PCR-DGGE of the bacterial communities present in fresh artisan cheeses collected in summer.

According to the database from GenBank, the most common microorganisms found in these fresh artisan cheeses were *Streptococcus*, *Lactococcus*, *Leuconostoc*, *Lactobacillus*, *Enterococcus, Pediococcus, Deinococcus,* and *Enterobacter*. Other microorganisms found were *Halobacteroides*, *Holoterrigena*, and *Salmonella* (Table 2). The original sequences, without making any changes, were analyzed using Sequencer 5.3 (Gene Codes Corp., Ann Arbor, Ml) and compared to the sequences available from GenBank using the BLASTN program at NCBI (National Center for Biotechnology Information). Fig. 4 shows the bacteria found in the samples and analyzed according to the results shown in the gel of the DGGE products. The analyses show the variety of microorganisms of the sample from different geographical areas in the Papaloapan region.

Table 2. Reference markers obtained from the GenBank database

DGGE band	Taxonomic identification*	Similarity (%)	Access Number
1	*Haloterrigena saccharevitans* strain JCM 12889	100	NR_113512.1
2	*Pediococcus lolii* strain NGRI 0510Q	99	NR_041640.1
3	*Halobacteroides halobius* strain DSM 5150	99	NR_102480.1
4	*Deinococcus peraridilitoris* strain DSM 19664	92	NR_102475.1
5	*Pediococcus lolii* strain NGRI 0510Q	99	NR_041640.1
6	---	--	--
7	*Streptococcus oligofermentans* strain AS 1.3089	100	NR_103943.1
8	*Enterobacter aerogenes* strain KCTC 2190	100	NR_102493.1
9	*Lactobacillus fermentum* strain CIP 102980	96	NR_104927.1
10	*Streptococcus oligofermentans* strain AS 1.3089	100	NR_194033.1
11	*Pediococcus lolii* strain NGRI 0510Q	100	NR_041640.1
12	*Staphylococcus saprophyticus* strain ATCC 15305	94	NR_074999.1
13	*Enterococcus faecalis* strain LMG 7937	91	NR_114782.1
14	*Streptococcus oligofermentans* strain AS 1.3089	100	NR_103943.1
16	*Enterococcus hirae* strain ATCC 9790	100	NR_075022.1
19	*Salmonella enterica* subsp. enterica serovar *Typhimurium* strain LT2.	100	NR_074910.1
20	*Streptococcus saliviloxodontae* strain NUM 6306	100	NR_126178.1
24	[A]*Leuconostoc rapi strain* LMG 27676	98	NR_136799.1
25	[A]*Lactococcus lactis* subsp. tructae strain L105	82	NR_116443.1

Note: All the identification was done by the 16S ribosomal RNA gene complete sequence, only bands 24 and 25 were obtained by partial sequence[A]

Lactococcus lactis was detected in band 13 in the sample LB1. LAB such as *Streptococcus* and *Pediococcus* were found in the samples, as previously reported by Stiles & Holzapfel, (1997). The results show the presence of other genera in a lower percentage as *Proteus*, *shimia*, *Rhizophydium*, *Pseudomonas*, *Halocella*, *Longilinea*, *Myxococcus*, *Nannocystis*, *Phormidium*, *Serratia*, and *Sorangium*. Most of these genera are present because of the cheese handling and from raw milk, to the final product.

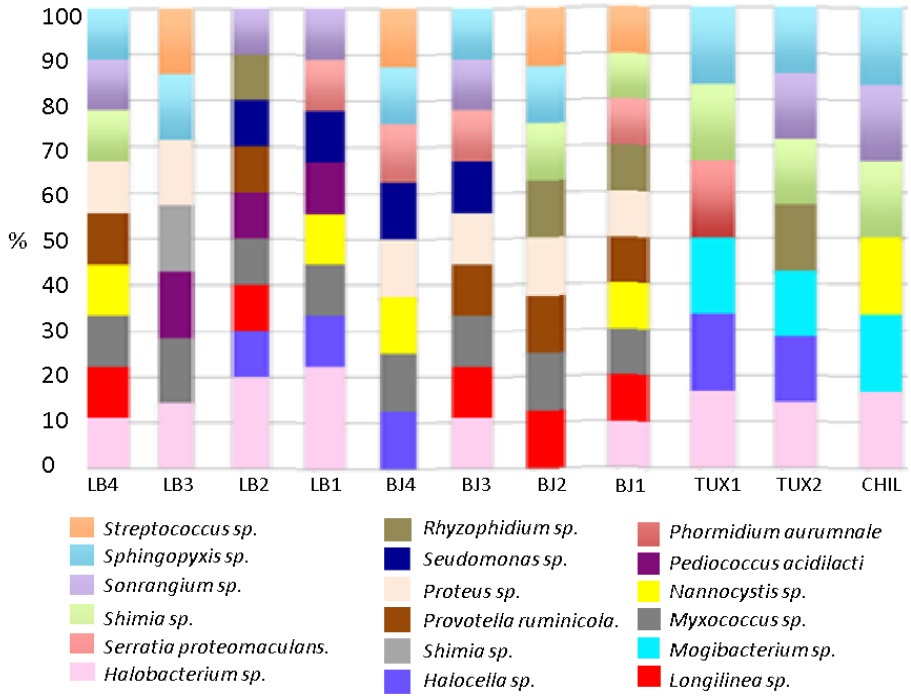

Fig. 4. Bacteria found in artisan fresh cheese samples in summer.

3.2.2. Microbiota Analysis in the winter season.

The samples were collected in January 2015 from the same places as the samples collected in summer, and treated with the same protocols. Thus, the aim was the same to identify the microbiota of the fresh artisan cheese in the winter season.

The banding profile of the bacterial populations from 11 artisan fresh cheeses, applying PCR technique as in summer samples and is illustrated in Supplemental Materials. The electrophoresis gel in 1.5% agarose, stained with ethidium bromide from amplicons of fresh cheese samples from the winter season is reported in Fig. 5. The selected bands of better resolution are reported in Fig. 6, each band corresponds to a bacterial species. These data were the key to identify the microorganisms present in the artisan fresh cheese in the winter season. Fig. 7 shows the dendrogram obtained by calculating

Euclidean distances. It is observed that the groups were the main source of variation, being the place of origin and method of preparation, indicated in the nodes formed, the percentage of similarities.

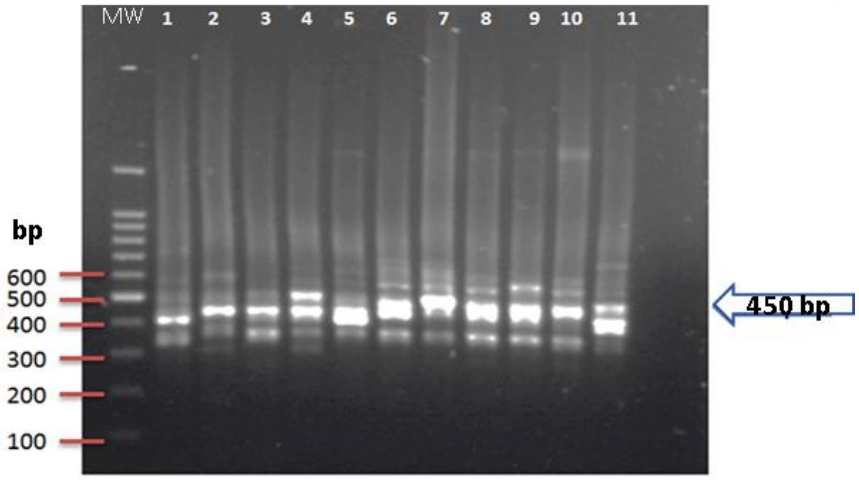

Fig. 5. Electrophoresis gel in agarose at 1.5% made with the amplicons of the samples of fresh cheese from the winter season and stained with ethidium bromide. WP) Molecular weight marker 1000 bp 1.5 µL (100 ng / µL). 1) CHI 10 µL. 2) Tux1 10 µL. 3) Tux2 10 µL. 4) BJ1 10 µL. 5) BJ2 10 µL. 6) BJ3 10 µL. 7) BJ4 10 µL. 8) LB1 10 µL. 9) LB2 10 µL. 10) LB3 10 µL. 11) LB4 10 µL. Image analyzed with ImageJ.

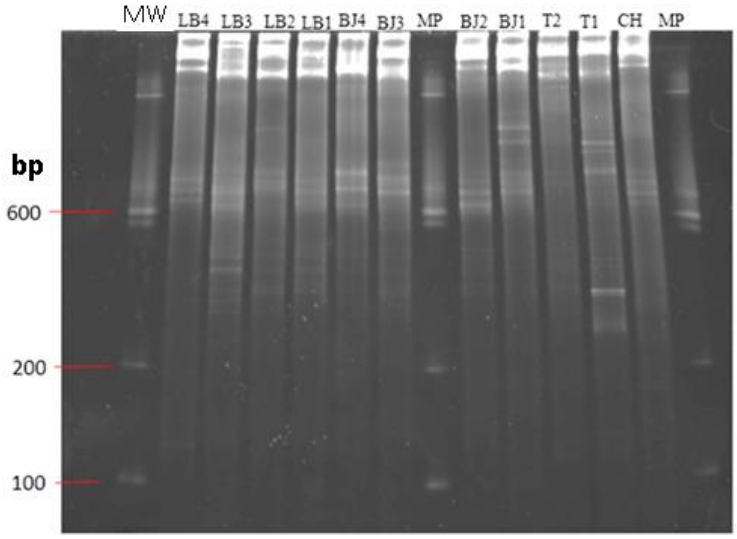

Fig. 6. Banding profile of bacterial populations. Each band represents a bacterial species. Running amplicons were obtained with DNA samples from fresh artisan cheeses collected in winter.

The results show two groups, the profile originated from the TUX1 sample with a 40.8% similarity to the rest of the samples. For the other samples, they were more related to each other and with 57.1% of similarity, where groups and subgroups were formed. These results show that the samples, the place of origin, and the method of cheese preparation influence their bacterial cell count.

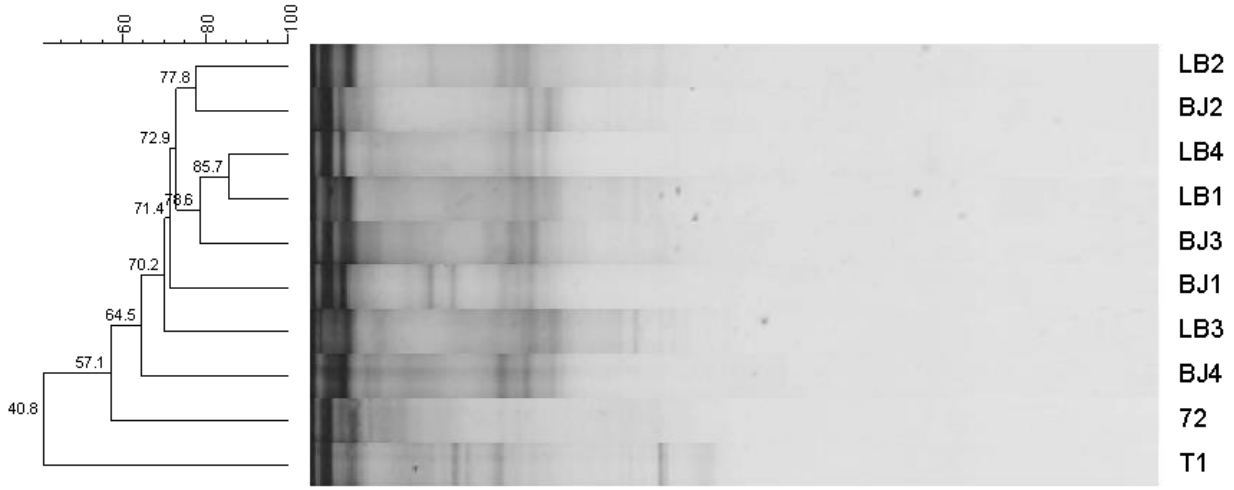

Fig. 7. UPGMA dendrogram showing the genetic relationships among the communities. PCR-DGGE of the bacterial communities present in fresh artisan cheeses collected in winter.

The PCR-DGGE gel was analyzed, and the bands of interest were chosen, based on the intensity of the banding or the presence of some bands only in some samples. The DNA contained in the selected bands is shown in Fig. 8. The products were re-amplified with the same nucleotide pairs. From the total selected bands, band numbers 1, 3, 8, 13, 16, and 18 were not sequenced because the concentration and purity were not reached for their sequencing. In total, 11 bands were sequenced.

From the results found in the GenBank database (Table 3), a variety of microorganisms were identified, as follows: *Lactococcus*, *Streptococcus*, *Lactococcus*, *Leuconostoc*, *Enterococcus*, *Pediococcus*, *Enterobacter*, *Klebsiella*, *Bacillus*, *Staphylococcus*, and *Salmonella*. *Lactococcus* and *Streptococcus* were the most representative in the DGGE profile of the cheese samples. *Lc. lactis* subsp. *Cremoris* was the bacteria found at two sampling points and was the most widespread. In the results obtained from the products of the DGGE gel, the analysis showed the variety of microorganisms for each sample collected at different geographical points in the Papaloapan region. The predominant bacteria in all samples were *Bacillus sp*., with 30-50% of its concentration (Fig. 9). The presence of *Bacillus sp*. in cheese samples is related to the presence of multicopy copies of the 16S rRNA gene for these microorganisms (Cocolin *et al*., 2004, 2007; Dahllöf *et al*., 2000).

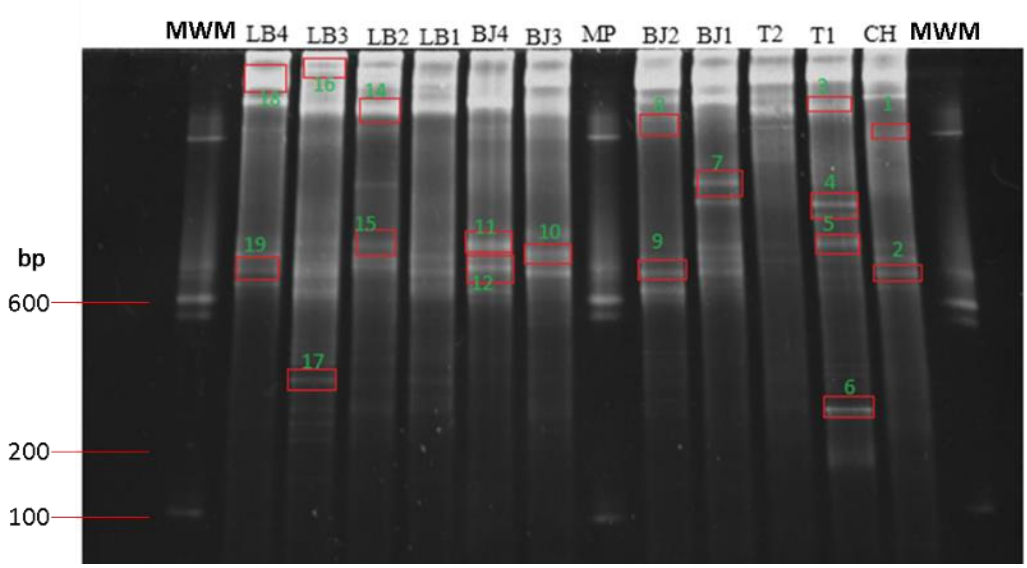

Fig. 8. Selected gel bands in DGGE seen with the aid of BioRad software. The running amplicons were obtained with samples of fresh artisan cheeses collected in winter.

Table 3. Reference markers obtained from the GenBank database for winter samples

DGGE band	Taxonomic identification	Similarity (%)	Access Number
2	*Klebsiella quasipneumoniae* subsp. quasipneumoniae strain 01A030.	82	NR_134062.1
4	*Pediococcus lolii* strain NGRI 0510Q	92	NR_041640.1
5	*Leuconostoc kimchii* strain IMSNU 11154	93	NR_075014.1
6	*Streptococcus oligofermentans* strain AS 1.3089	97	NR_103943.1
7	*Lactococcus lactis subsp. cremoris strain NBRC 100676*	82	NR_113925.1
9	*Enterobacter aerogenes* strain KCTC 2190	100	NR_102493.1
10	*Enterococcus hirae* strain ATCC 9790	100	NR_075022.1
11	*Salmonella enterica* subsp. enterica serovar *Typhimurium* strain LT2	100	NR_074910.1
12	*Lactococcus lactis* subsp. cremoris strain NBRC 100676	100	NR_113925.1
14	*Streptococcus oligofermentans* strain AS 1.3089	92	NR_103943.1
15	[A]*Staphylococcus pseudolugdunensis* strain B006	100	NR_115938.1
17	[A]*Lactococcus lactis* subsp. cremoris strain NBRC 100676	94	NR_113925.1
19	*Bacillus coagulans* strain NBRC 12583	99	NR_041523.1

Note: all the identification was done by the 16S ribosomal RNA gene complete sequence, only bands 15 and 17 were obtained by sequence[A]

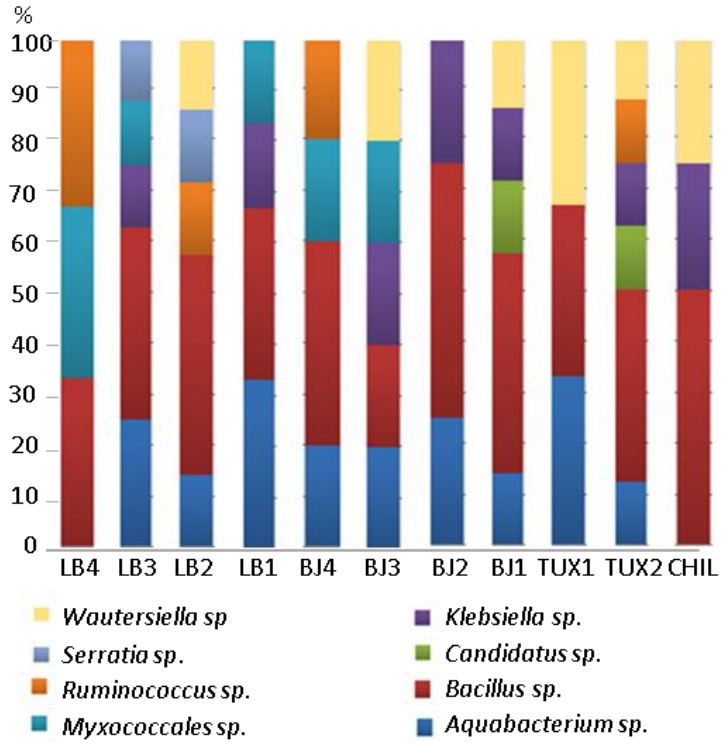

Fig. 9. Bacteria found in artisan fresh cheese samples in winter.

Aqueabacterium sp. was found in most of the samples, except LB4 and CHIL. This bacterium has been found in drinking water (Kalmbach, 1999). *Klebsiella*, *Wautersiella*, and *Serratia* were found in CHIL, TUX1, TUX2, BJ1, BJ2, BJ3, LB1, LB2, and LB3. These bacteria can infect the urinary or respiratory systems, including pneumonia (Podschun, and Ullmann, 1998). *Candidatus sp.* was found in the TUX2 and BJ1 samples at concentrations of 10%. This bacterium could be well characterized because it is not easily cultivable (Murray, 1995). *Ruminococcus sp.* was found in sample LB4 at a concentration of 30% and in a lower concentration in samples LB2, LB4, and TUX2. *Ruminococcus* is a genus of bacteria in the class *Clostridia*, present in significant quantities in the intestines of humans (Liu *et al*., 2008). *Myxococcales sp.* presented in LB4, LB3, LB1, BJ4, and BJ3. This bacterium lives in sites rich in organic matter such as soil, manure, animal excrement, and plant wastes. Furthermore, they are capable of degrading polysaccharides, such as cellulose and chitin (Kiskowski *et al*., 2004).

4. DISCUSSION

All products for human consumption must have control of the microbial load to be consumed safely. Fermented products, such as cheeses, yogurts, canned vegetables, and even meat products, are more at risk of being contaminated by manual handling of

the process and raw materials. In the case of fresh artisan cheeses, the situation is the same. According to Klaenhammer *et al*. (2002), the main genres of the BAL group present in cheese are *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Enterococcus*, and *Streptococcus*. Concordantly, our results also confirmed the presence of these kinds of microorganisms in samples of fresh artisan cheeses (Ruvalcaba-Gómez *et al*., 2020; Sánchez-Juanes *et al*., 2020; Yeluri Jonnala *et al*., 2018).

Some strains were found because of the processing of the raw milk, raw milk from the origin to produce the fresh cheeses. *Halobacteria sp*, predominance is because this strain grows in salty environments at temperatures of 42°C (DasSarma *et al*., 2006). *Provotella ruminicola* (TUX1, TUX2, and CHIL) is one of the bacteria that carry out the digestion of pectin through a fermentative process (Dehority, 1969). Pectin represents about 10 to 20% of the total carbohydrates that make up the forages used in ruminant nutrition. The presence of *Mogibacterium sp* (TUX1, TUX2, and CHIL) is associated with livestock feed and milk production. These bacteria are associated with moderate and severe oral diseases, found in oral cavities (including dental plaque), periodontal lesions, dental infections, and carious dentin (Holdeman *et al*., 1980; Nakazawa *et al*., 2000). *Sphingopyxis* (CHIL, TUX1, TUX2, BJ2, BJ3, BJ4, LB3, and LB4) is a ubiquitous bacterium in a variety of environments, such as salty water, compost, wastewater, and water contaminated with hexachlorocyclohexane. *Sphingopyxis sp*. is of industrial interest because this genus is capable of degrading mono and polycyclic aromatic hydrocarbon compounds (Kim *et al*., 2014).

Castro *et al*. (2016) isolated LAB from Minas, Brazil artisan cheese from the Campo das Vertentes region, regardless of the season. *Enterococcus faecalis* was isolated with higher frequency in samples of raw milk, endogenous starter culture, and fresh cheeses, followed by *Lc. lactis* and *Lb. plantarum*. *Lb. paracasei*, *Lb. brevis*, *Enterococcus pseudoavium*, and *Aerococcus viridans*.

In our study, *Lc. lactis* was present in the samples and it has an industrial interest since it produces the bacteriocin called Nisin. Nisin is currently the only bacteriocin approved by the FDA as a food preservative to inhibit the growth of pathogenic microorganisms (Fraga Cotelo *et al*., 2013; Klaenhammer, 1988). However, there are bacteriocins isolated from other genera of LAB such as *Pediococcus acidilactici*, *Lb*. *plantarum*, *Lb*. *sake*, *Lb*. *plantarum*, and *Lb. helveticus* (Castro-Castillo *et al*., 2013). Therefore, it is preferable to have a dominant LAB because the production of bacteriocins could help to maintain a low level of the count of pathogen microorganisms.

Moreover, it is a common practice to use sodium chloride (NaCl) during the production of artisan cheeses. The positive effect of NaCl is that it modifies the physical properties of the cheese curd and rind, controls the growth of the cheese-ripening microflora, and also limits the development of both pathogens and spoilage microbes. However, partial substitution of NaCl by other chloride salts such as potassium chloride represents an alternative option to limit the negative effects of reducing NaCl content in soft cheese. (Dugat-Bony *et al*., 2016). Synergistically, the production of bacteriocin contributes to

stopping the proliferation of pathogens. LAB increase the shelf life of fresh cheese due to the production of these antimicrobial compounds. Evaluating the microbiological quality of the final fresh cheese product as well as the intermediates throughout the production line is an important contribution to control the quality of the product, as suggested for the production of mozzarella cheese (Losito *et al*., 2014). In other studies, dynamic populations isolated from whey, from artisan string cheeses manufactured with raw milk, have been reported in the literature (Martínez-López *et al*., 2016; Moser *et al*., 2018). Isolates of whey from artisan string cheese showed that *Enterococcus* and *Lactobacillus* were the dominant genera (Luiz *et al*., 2016). In another characterization, Oaxaca cheese showed a similar microbiota pattern. The microbiota of Oaxaca cheese had 43 strains of LAB, 38 coliforms, 24 yeasts, and 16 *Staphylococcus*. This microbiota represents high health risks for its consumption (Castro-Castillo *et al*., 2013). The same type of cheese called Aro cheese, marketed in the municipality of Teotitlán de Flores Magón, Oaxaca, Mex., showed similar microbial characteristics. The most representative microorganisms were: Aerobic mesophilic bacteria, psychrophilic bacteria, total coliforms, *Escherichia coli*, *S. aureus*, *Salmonella*, *Enterococcus*, *Streptococcus*, LAB, molds, and yeasts (González-Montiel & Franco-Fernández, 2015) (González-Montiel & Franco-Fernández, 2015). In another study, genera analysis showed that among three brands of Hispanic fresh cheese from the USA, the microbial community showed more similarity within the brands than when compared among them. Thermus *Anoxybacillus*, and *Streptococcus* accounted for the dominant genera of cheese brands (Holle *et al*., 2018). The literature shows that artisan cheeses have similar microbiota.

On the other hand, the pathogenic bacterium *Salmonella enterica* was present in the samples and was detected by the sequence analysis. *Salmonella enterica* is responsible for infections in humans and domestic animals (Porwollik *et al*., 2004). *Salmonella* can adapt to organic acids, particularly at pH 6.0 or pH 5.0. However, when the pH is lower (pH 4.0), bacterial survival is not viable after 6 to 24 h (Burin *et al*., 2014).

Fresh cheese is a very nutritious food and there are no reports of outbreaks in the Papaloapan region and hence, it seems like a relatively safe food product. However, the most common microorganisms associated with food poisoning outbreaks caused by cheese are *Salmonella spp*., *S. aureus*, *Listeria monocytogenes*, and enteropathogenic strains of *Escherichia coli*. Most of the cheeses that use raw milk are contaminated with these types of microorganisms (Paswan & Park, 2020). Thus, their consumption must be under precaution. To keep the level of pathogens low, it could be possible to maintain a low pH or close to the pK_a (3.86) of the lactic acid and controlling the addition of NaCl.

Now that the microbiota has been characterized, it could be possible to suggest to the producer to prepare an inoculum with the correct concentrations of the beneficial strains of microorganisms and using pasteurized milk. Moreover, due to the nature of fresh cheeses to possess around 46% to 67% moisture (González-Córdova *et al*., 2016), it

must be consumed as soon as possible after production to reduce the reproduction of the pathogen microorganisms.

In conclusion, all samples of fresh artisan cheeses were under high microbial loads. Lactic acid bacteria were in a typical load, as established by the quality and safety standards in the food industry. Conversely, pathogenic bacteria exceeded this limit. Therefore, these cheeses were not recommendable for consumption. However, among the diversity, some microorganisms of industrial importance were identified, primarily LAB, such as *Streptococcus*, *Lactococcus*, *Leuconostoc*, *Lactobacillus*, and *Enterococcus*. Among these microorganisms, *L. lactis* is essential for fresh artisan cheeses. There was no significant difference between the summer and winter seasons in the count of microorganisms. Besides, total coliforms such as *Salmonella*, *Klebsiella*, and other groups of pathogenic origin were found. PCR-DGGE method showed specific bacterial profiles for each sample in the two seasons, and there was no variability among microorganisms. Seasons, production place, as well as the manufacturing method did not affect the microbiota or the typical characteristics of this product.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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