

Bacterial diversity and production of volatile compounds during the ripening of Bola de Ocosingo cheese

Diversidad bacteriana y producción de compuestos volátiles durante la maduración del queso Bola de Ocosingo

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ABSTRACT

Bola de Ocosingo cheese is a genuine Mexican cheese produced in Ocosingo, Chiapas, Mexico, and processed through a 21-day light ripening process. In this study, microbial diversity was identified through metagenomic analysis, and volatile fatty acid production was assessed in Bola de Ocosingo cheese samples from three cheese factories using gas chromatography-mass spectrometry (GC-MS). The main bacterial genera identified were *Enterococcus*, *Lactobacillus*, *Streptococcus*, *Citrobacter*, *Acinetobacter*, *Lactococcus*, *Klebsiella*, *Escherichia*, *Shigella* and *Chryseobacterium* at 0, 7, 14, and 21 days of ripening. The production of various compounds such as acetic acid, propionic acid, butyric acid, isobutyric acid, pentanoic acid, isopentanoic acid, hexanoic acid, and heptanoic acid was also identified by gas chromatography-mass spectrometry (GC-MS). The presence of these bacterial genera is associated with the production of these compounds, which are related to the sensory characteristics of Bola de Ocosingo cheese.

Keywords: Metagenomics 1; Volatile compound 2; Bola de Ocosingo cheese 3

RESUMEN

El queso Bola de Ocosingo es un queso mexicano genuino producido en Ocosingo, Chiapas, México, cuyo proceso implica un paso de maduración ligera de 21 días. En este estudio, se identificó la diversidad microbiana mediante análisis metagenómico y la producción de ácidos grasos volátiles en muestras de queso Bola de tres queserías por cromatografía de gases acoplada a espectrometría de masas (GC-MS). Los principales géneros bacterianos identificados fueron: *Enterococcus*, *Lactobacillus*, *Streptococcus*, *Citrobacter*, *Acinetobacter*, *Lactococcus*, *Klebsiella*, *Escherichia*, *Shigella*, *Chryseobacterium* a los 0, 7, 14 y 21 días de maduración. También se

identificó la producción de diversos compuestos como ácido acético, ácido propiónico, ácido butírico, ácido isobutírico, ácido pentanoico, ácido isopentanoico, ácido hexanoico y ácido heptanoico mediante cromatografía de gases acoplada a espectrometría de masas (GC-MS). La presencia de estos géneros bacterianos está asociada a la producción de estos compuestos que están relacionados con las características sensoriales del queso Bola de Ocosingo.

Palabras clave: Metagenómica 1; Compuesto volátil 2; Queso Bola de Ocosingo 3.

INTRODUCTION

Artisanal cheeses are distributed regionally, however there is a growing demand among the population for quality, genuine and traditionally produced products (de Gantes and Cervantes, 2011). Artisanal cheeses are considered to be those produced using traditional production practices, from cow, sheep and/or goat milk (preferably raw), on farms or in small scale dairies interconnected with a geographical region and a culture (Montel *et al.*, 2014; Cirne *et al.*, 2019; Di Trana *et al.*, 2022). Bola de Ocosingo cheese is produced in Ocosingo, Chiapas, Mexico, and is one of the genuine Mexican cheeses, due to the particular characteristics of the area such as altitude, climate, soil types, and pastures, which generate particular conditions for the milk, and consequently, a special flavor for the cheeses. It is a double cream acid cheese that is matured for 21 days before being covered with a double, skimmed cheese coating (Cervantes *et al.*, 2008).

Nowadays, cheese is made by a few artisanal cheesemakers, who have received and continue to transmit, through generations, the know-how related to this product, which may be threatened by industrialization and by social,

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economic and political conflicts (de Gantes and Cervantes, 2011; Poméon, 2011). Therefore, the strategy for Mexican artisanal cheeses to continue to exist in the markets is through their revalorization and local recognition (Linck *et al.*, 2006; Cesin *et al.*, 2012; de Gantes and Cervantes, 2011).

Artisanal cheeses are characterized by a higher microbial diversity associated with flavor, aroma and texture, while standardized manufacturing of industrial cheeses leads to a less complex microbiota, in an attempt to improve product safety at the expense of sensory quality (Montel *et al.*, 2014). Lactic acid bacteria (LAB) are the main components of this microbial community, while pathogenic and spoilage bacteria such as *Escherichia coli*, *Staphylococcus* spp. and *Pseudomonas* spp., are absent or present in low amounts in cheese (Riquelme *et al.*, 2015; Bozoudi *et al.*, 2016; Tzora *et al.*, 2021; Kothe *et al.*, 2022).

However, the consumption of Mexican cheeses made with raw milk has been associated with outbreaks of food-borne illnesses due to pathogens such as *Salmonella* spp., *Staphylococcus aureus*, *Mycobacterium bovis*, and *Brucella* spp. (Parrilla-Cerrillo *et al.*, 1993; Laniado-Laborin *et al.*, 2014; Morales-García *et al.*, 2015; Ortiz *et al.*, 2021). Mexican artisanal cheeses should not be considered unsafe simply because they are made with raw milk; however, there are several practices that influence their quality and microbiological safety (Cuevas *et al.*, 2024).

A study of the microbiota of raw milk, curd, and Bola de Ocosingo cheese at 50 and 110 days of ripening during the dry (March-June) and rainy (August-November) seasons, detected the presence of *Streptococcus thermophilus*, *Lactococcus lactis*, *Lactobacillus helveticus*, *L. delbrueckii*, and *L. plantarum*. Low proportions of *Escherichia coli*/*Shigella flexnerii* were also found (Aldrete *et al.*, 2018).

The *Lactococcus*, *Streptococcus*, *Lactobacillus* and *Leuconostoc* genera are responsible for the rapid acidification of milk during the initial fermentation, while the *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Enterococcus* genera influence the development of sensory properties through their proteolytic and lipolytic activities during cheese ripening (Settanni and Moschetti, 2010; Blaya *et al.*, 2017).

The study of microbiota during cheese making and ripening is essential to understand its contribution to the sensory characteristics of the final product. Recent advances in high-throughput sequencing methodologies and high-resolution metabolomics have provided greater knowledge about the diversity and interactions of the cheese microbiota, which contribute through their metabolism to the production of numerous compounds, which provide aroma and flavor to cheese products (Ercolini, 2013; Afshari *et al.*, 2018; Sousa *et al.*, 2019; Van Leeuwen *et al.*, 2020; Zotta *et al.*, 2021). The substrates for biochemical processes are carbohydrates, lipids and proteins, the latter being considered the main ones for the formation of cheese flavor (Blaya *et al.*, 2017). The objective of this project was to identify the microbiota and the production of volatile fatty acids during the 21-day ripening process of Bola de Ocosingo cheese, using meta-

genomic analysis and gas chromatography coupled to mass spectrometry.

MATERIAL AND METHODS

Cheese sample collection

The Cheese samples were collected at the El Dorado, Queshil and San Martín cheese factories located in the municipality of Ocosingo, Chiapas, Mexico. For each day of sampling, three samples were collected at each cheese factory. The first sample was taken on the day of cheese production (day 0) and at 7, 14 and 21 days after its production. As they were collected, they were stored at 4 °C for later analysis.

DNA extraction from cheese samples

For DNA extraction, 500 mg of each cheese sample were taken and pretreated by the addition of 500 µL of the pre-lysis solution (20 mM Tris-HCl, 2 mM EDTA, 1% SDS, pH 7), then homogenate and incubated at 65 °C. DNA extraction was then continued with the omega BIO-TEK (E.Z.N.A.® Genomic DNA Isolation Kits, Omega Bio-Tek) according to the manufacturer's instructions. The DNA obtained was stored at -20 °C.

Library preparation and sequencing

DNA was quantified with a Qubit® 3.0 fluorometer using the Qubit dsDNA HS assay kit (Life Technologies, Carlsbad, CA, USA). For sequencing amplicons from cheeses samples, variable regions 3 and 4 of the 16S rRNA gene were amplified (Klindworth *et al.*, 2012). Amplicon libraries were constructed as described in the Illumina Sequencing Library Preparation Protocol for 16S (Illumina, Inc., San Diego, CA, USA) and sequenced on the Illumina MiSeq platform (Illumina, Inc., San Diego, CA, USA) with a 600-cycle paired-end read configuration. Reads passing QC filters (read quality ¼ Q20) were used to reconstruct the original amplicon region (450-490 bp).

Bioinformatic analysis

The Illumina paired end raw reads (2x300) were processed using the QIIME2 pipe-line. The amplicon sequence variants (ASVs) were resolved using the DADA2 plugin (Callahan *et al.*, 2016), with the following parameters for the 16S libraries: "truncation_length 280, truncation_length_reverse 200, trim_left 30, trim left reverse 30, min overlap 12, chimera method consensus". Representative ASVs were classified using the V-SEARCH plugin (Rognes *et al.*, 2016), utilizing the SILVA 138 database for 16S (Glöckner *et al.*, 2017). For each data type, a phylogeny of the representative ASVs was performed using the align to tree mafft fasttree plugin, which performs sequence alignment with the MAFFT program (Kato and Toh, 2008) and the phylogenetic tree with FastTree2 (Price *et al.*, 2010).

All data were imported into the R environment using the phyloseq library (McMurdie and Holmes, 2012). The samples were rarefied to a depth of 6,300 counts per sample. The observed ASV alpha diversity indices, Shannon, ACE, Simpson, and InvSimpson were calculated, and beta diversity was de-

terminated as a function of the unweighted UniFrac distance, representing it in a PCoA. Permutational multivariate analysis of variance (PERMANOVA) was performed for this study. The differential abundance of ASV between the sample groups was determined using the DESeq2 library (Love *et al.*, 2014).

Extraction and determination of volatile fatty acids in cheese

For the quantification of volatile fatty acids (VFAs) in cheese samples by headspace gas chromatography (Headspace-GC), a VFA standard from Sigma-Aldrich at concentrations of 0.01, 0.02, 0.04, 0.06 and 0.08 % (Volatile Fatty Acids Mixture, Cat. No. 32345) was utilized. Five grams of cheese were weighed into a 50 mL headspace vial. To acidify the sample and release VFAs, 1 mL of 25 % (v/v) phosphoric acid was added. The vial was sealed with a PTFE/silicone cap and equilibrated for 30 m at room temperature. The vial was placed in a TriPlus RSH autosampler with temperature control and agitation. The sample was incubated at 80 °C for 20 m with agitation cycles of 5 m followed by 2 m of rest.

One milliliter of headspace gas was injected into a Trace 1300 GC Ultra chromatograph in splitless mode. The programmable temperature vaporizer (PTV) injector was set to 40°C initially and heated to 250 °C at 10 °C/second, holding at this temperature for 2 m. Separation of VFAs was achieved using a DB-FFAP capillary column (Agilent Technologies, 30 m × 0.25 mm ID × 0.25 µm film thickness). The oven temperature program started at 40 °C for 2 m, ramped to 150 °C at 10 °C/min, and then to 250 °C at 5 °C/min, holding for 10 m. Helium was used as the carrier gas at 1.0 mL/min.

The flame ionization detector (FID) was set to 300 °C with hydrogen (35 mL/min), air (350 mL/min), and nitrogen makeup gas (30 mL/min). VFA quantification was performed using calibration curves from the Sigma-Aldrich standard. Compounds were confirmed using an ITQ 900 mass spectrometer in full scan mode (50-500 m/z). The ion source temperature was 230 °C, and the transfer line temperature was 280 °C. This methodology enabled precise identification and quantification of VFAs in the cheese samples.

Fatty acid analysis by GC-MS

Fatty acid profiles as methyl esters were derivatized by base- and acid-catalyzed methylation. Samples were injected into a Trace 1300 GC Ultra chromatograph in splitless mode. The programmable temperature vaporizer (PTV) injector was initially set to 40 °C and heated to 250 °C at 10 °C/second, held at this temperature for 2 m. Separation was performed using a DB-FFAP capillary column (Agilent Technologies, 30 m × 0.25 mm ID × 0.25 µm film thickness). The oven temperature program started at 40 °C for 2 m, gradually increased to 150 °C at 10 °C/min, then to 250 °C at 5 °C/min, and held for 10 m. Helium was used as the carrier gas at 1.0 mL/min. The flame ionization detector (FID) was set to 300 °C with hydrogen (35 mL/min), air (350 mL/min) and nitrogen as filler gas (30 mL/min). The compounds were confirmed with an ITQ 900 mass spectrometer in full scan mode (50-500 m/z).

Statistical analysis

The data from cheese samples from three cheese factories were analyzed with three replicas for each one. The data for volatile fatty acids were expressed in percentages. The percentages were compared between days of ripening for each fatty acid and were also compared between factories. An analysis of variance and a Tukey posteriori test were performed with a significance of $\alpha=0.05$. The R environment version 4.4.1 was used for data analysis.

RESULTS

Microbial diversity

In total, 138,887 readings were sequenced from the samples at 0, 7, 14 and 21 days of the cheese bola maturation process from the three cheese factories, normalizing to 6,300 readings per sample. The rarefaction curves of the samples at 0, 7, 14 and 21 days of maturation of the Bola de Ocosingo cheese from the three cheese factories had a similar species diversity, except for the samples from the Queshil cheese factory, which at 7 days had lower species richness compared to the other samples at the same time of maturation, and the sample from the Queshil cheese factory at 14 days also had greater species richness (Fig. 1).

We can also observe that the alpha diversity index of the microbial populations analyzed at 0, 7, 14, and 21 days of ripening of Bola de Ocosingo cheese behaves very similarly in the three cheese dairies. It can also be observed that the Queshil cheese dairy shows less diversity at 7 days of ripening compared to the other two dairies (Fig. 2).

In the Principal Coordinate Analysis (PCoA), we can observe the Beta diversity in the Bola de Ocosingo cheeses. In the San Martín cheese factory, the samples analyzed at 0, 7, 14, and 21 days show a very similar microbial diversity (Fig. 3). In contrast, in the cheeses from the El Dorado cheese factory, the sample from day zero shows a similar microbial diversity to that of the San Martín cheese factory. Finally, we can observe that the microbial diversity of the cheeses from the Queshil cheese factory shows similarity at 0, 14, and 21 days, while at 7 days it shows a difference in microbial diversity (Fig. 3).

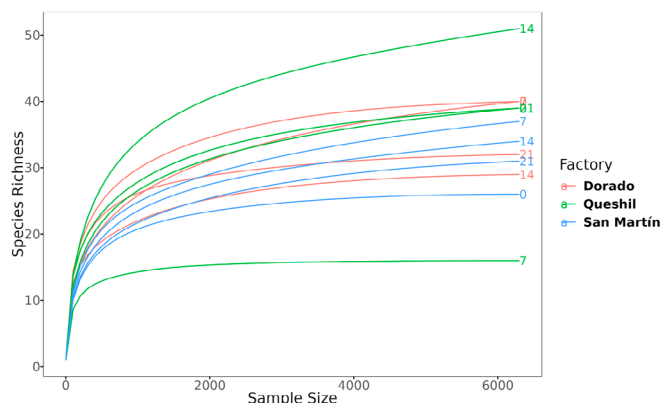


Figure 1. Rarefaction curves of Bola de Ocosingo cheese samples from three cheese factories at 0, 7, 14 and 21 days during the maturation process.

Figura 1. Curvas de rarefacción de las muestras de queso Bola de Ocosingo de tres queserías a los 0, 7, 14 y 21 días durante el proceso de maduración.

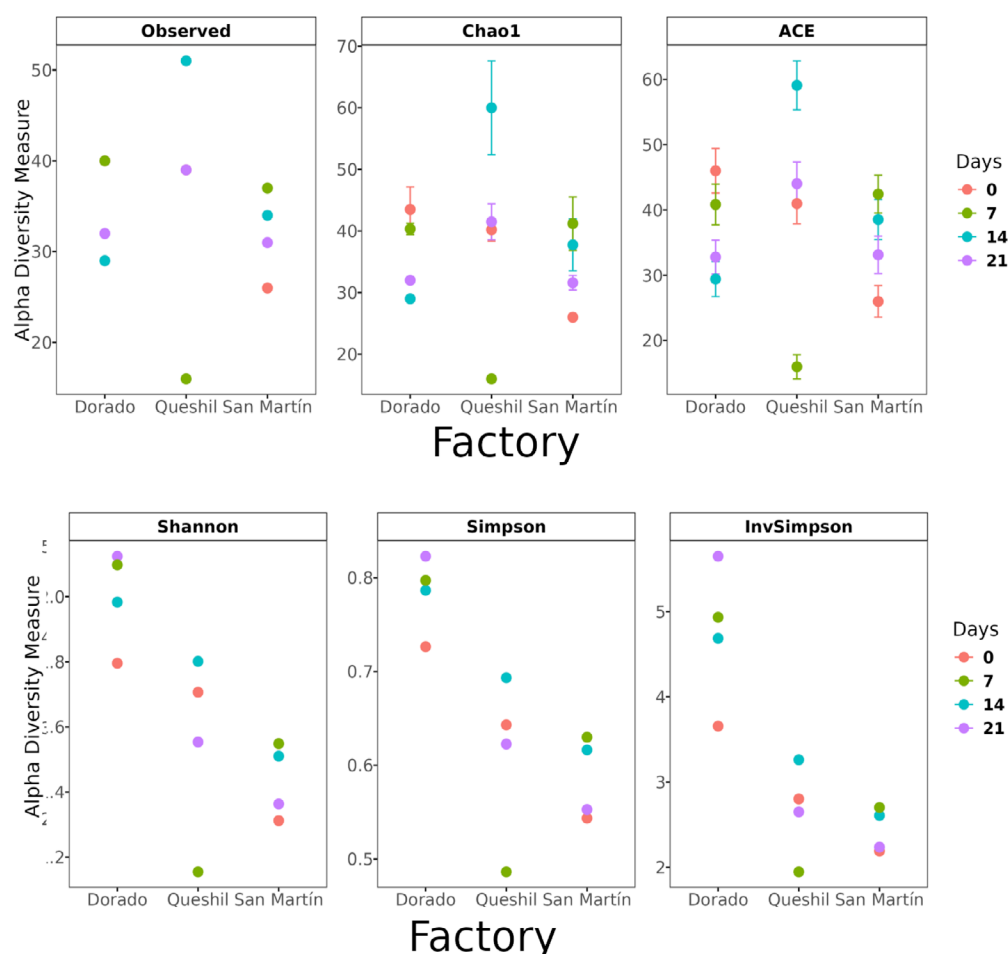


Figure 2. Alpha diversity of bacterial communities in Bola de Ocosingo cheeses from different cheese factories.
Figure 2. Diversidad alfa de las comunidades bacterianas de los quesos bolas de Ocosingo de las diferentes queserías.

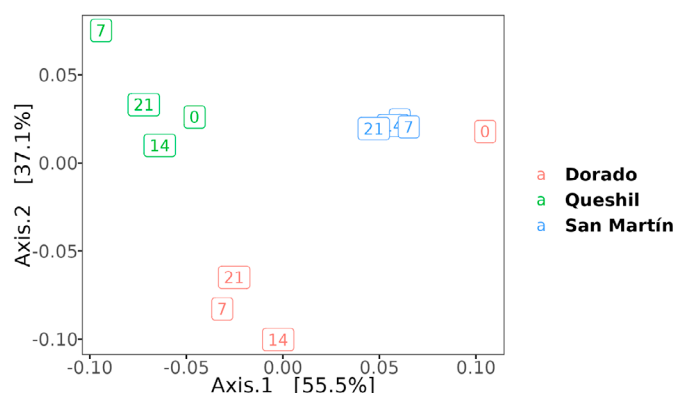


Figure 3. Principal Coordinate Analysis (PCoA) graph of the Bola de Ocosingo cheeses from the three cheese factories.

Figure 3. Gráfico del Análisis de Coordenadas Principales (PCoA) de los quesos Bola de Ocosingo de las tres queserías.

The microbial diversity of Bola de Ocosingo cheeses from the three dairies analyzed during the ripening process was taxonomically assigned to three phyla, Firmicutes, Proteobacteria, and Bacteroidota (Fig. 4), and 10 bacterial genera. The most abundant genera in the cheeses from the El Dorado dairy on day zero were *Lactococcus* (48.80%), *Citrobacter* (19.57%), *Enterobacter* (14.17%), and *Streptococcus* (6.11%). By day seven, the relative abundance was *Streptococcus* (38.47%), *Lactococcus* (19.26%), and *Lactobacillus* (15.49%). By day 14, the relative abundance was *Streptococcus* (38.55%), *Lactobacillus* (21.76%), and *Lactococcus* (12.96%). On day 21, the relative abundance was *Streptococcus* (28.92%), *Lactococcus* (27.12%), and *Lactobacillus* (16.90%) (Fig. 5). In contrast, in the cheeses from the Queshil dairy, the most abundant genera on day zero were *Lactococcus* (58.6%), *Streptococcus* (10.93%), *Klebsiella* (10.55%), and *Lactobacillus* (9.96%). On day seven, the relative abundance of the genera was 71.23% *Lactococcus*, 16.19% *Lactobacillus*, and 8.88% *Streptococcus*. On day 14, the relative abundance was 51.41% *Lactococcus*, 19.03% *Streptococcus*, and 14.20% *Lactobacillus*. On day 21, the relative abundance was 57.98% *Lactococcus*, 18.41%

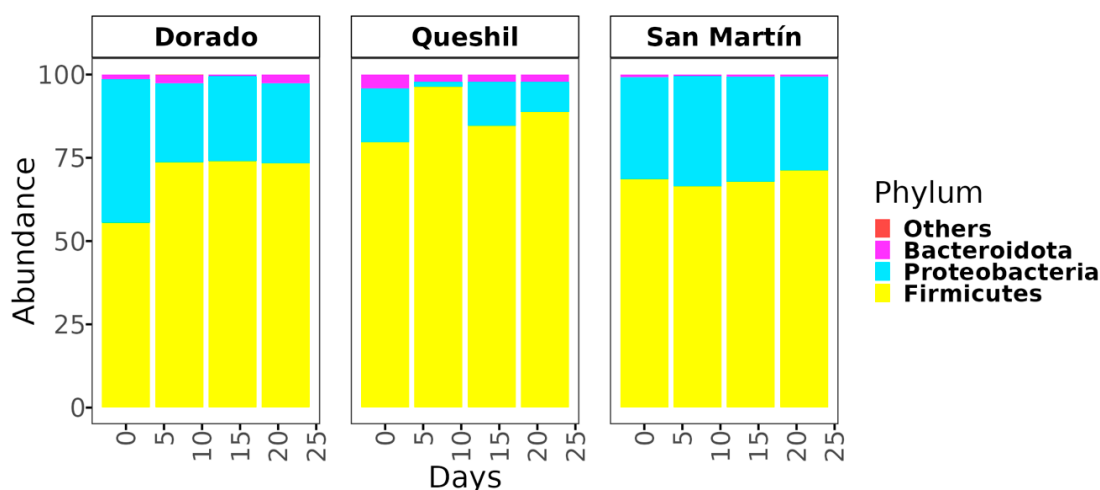


Figure 4. Relative taxonomic abundance of ASV from the bacterial phylum in the cheese bola ripening process. Bars designated as "Others" indicate ASV that had a relative abundance less than 1%.

Figura 4. Abundancia taxonómica relativa de ASV del phylum bacteriano durante la maduración del queso bola. Las barras designadas como "Otros" indican ASV con una abundancia relativa inferior al 1%.

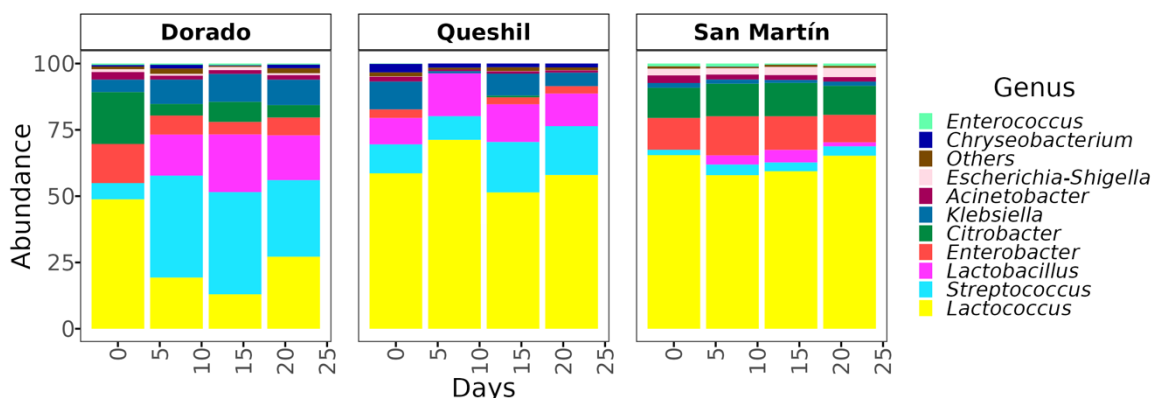


Figure 5. Relative taxonomic abundance of ASV from bacterial genera in the ripening process of cheese bola. Bars designated as "Others" indicate ASV that had a relative abundance of less than 1%.

Figura 5. Abundancia taxonómica relativa de ASV de géneros bacterianos en el proceso de maduración del queso bola. Las barras marcadas como "Otros" indican ASV con una abundancia relativa inferior al 1%.

Streptococcus, and 12.30% *Lactobacillus* (Fig. 5). Finally, at the San Martín cheese factory, the relative abundance on day zero was 65.47% *Lactococcus*, 12.01% *Enterobacter*, and 11.36% *Citrobacter*. After 7 days, it was 57.90% *Lactococcus*, 14.76% *Enterobacter*, and 12.28% *Citrobacter*. After 14 days, it was 59.39% *Lactococcus*, 12.69% *Enterobacter*, and 12.28% *Citrobacter*, and after 21 days, 65.25% *Lactococcus*, 10.96% *Citrobacter*, and 10.34% *Enterobacter* (Fig. 5).

Production of volatile fatty acids in the ripening process of Bola de Ocosingo cheese

Volatile fatty acids (VFAs) are commonly used as markers in the characterization of ripened cheeses due to their significant contribution to flavor and aroma; in this study, seven VFAs were detected. Between days 0 and 14, an increase was observed in most VFAs, except for pentanoic and butyric acids, which showed minimal changes between days 14 and 21. Acetic acid consistently presented the lowest levels among the identified compounds.

The results, summarized in Table 1, highlight that the highest levels of volatile fatty acids (VFAs) were found in the samples from the three dairies at 21 days of ripening. On day 0, the absence of statistical differences between the cheeses from the three dairies was likely due to their freshness and limited microbial activity. However, significant differences were observed at 7, 14, and 21 days, emphasizing the role of microbial and enzymatic activities in the increase of fatty acid levels during ripening.

Aromatic compound profile in the ripening process of Bola de Ocosingo cheese

The compounds identified by GC-MS analysis of Bola de Ocosingo cheeses from the El Dorado, Queshil and San Martín cheese factories at days 0, 7, 14 and 21 of ripening are shown in tables 2, 3 and 4. During the maturation process of the cheeses from the El Dorado cheese factory (table 2), 17 compounds were detected. It can be observed that on day 21 is where the greatest diversity of compounds was detected,

Table 1. Characterization of the volatile fatty acids profile during the ripening process of Bola de Ocosingo cheese from three cheese factories.
Tabla 1. Caracterización del perfil de ácidos grasos volátiles durante el proceso de maduración del queso Bola de Ocosingo de tres queserías.

% Volatile Fatty Acids					
Cheese factories	Compounds	Day 0	Day 7	Day 14	Day 21
El Dorado	Acetic acid	0.029dA	0.066cC	0.281aA	0.206bC
	Propionic acid	0.048dA	0.201cA	0.344aA	0.311bC
	Butyric acid	0.062dA	0.239bB	0.195cC	0.369aB
	Isobutyric Acid	0.057dA	0.208cB	0.387aA	0.363bC
	Pentanoic acid	0.059dA	0.207bA	0.2843aB	0.158cC
	Isopentanoic acid	0.060dA	0.174cA	0.426bA	0.606aA
	Hexanoic acid	0.062dA	0.246cA	0.3074bB	0.591aA
Queshíl	Acetic acid	0.029dA	0.086cB	0.242bB	0.449aB
	Propionic acid	0.048dA	0.188cB	0.287bB	0.552aA
	Butyric acid	0.062dA	0.260cA	0.483aA	0.404bA
	Isobutyric Acid	0.058dA	0.165cC	0.212bC	0.4715aB
	Pentanoic acid	0.059dA	0.175cB	0.375bA	0.567aA
	Isopentanoic acid	0.059dA	0.111cC	0.206bB	0.4753aB
	Hexanoic acid	0.062dA	0.265cA	0.438bA	0.508aB
San Martín	Acetic acid	0.029dA	0.151cA	0.181bC	0.520aA
	Propionic acid	0.048dA	0.185cB	0.276bB	0.371aB
	Butyric acid	0.063dA	0.139cC	0.354aB	0.333bC
	Isobutyric Acid	0.057dA	0.234cA	0.328bB	0.690aA
	Pentanoic acid	0.060cA	0.177bB	0.389aA	0.369aB
	Isopentanoic acid	0.061dA	0.259bA	0.225cB	0.414aC
	Hexanoic acid	0.063dA	0.182cB	0.244aC	0.206bC

Percentages of seven fatty acids in Bola cheeses from three different manufacturers during 21 days of ripening. Different uppercase letters indicate significant differences among manufacturers, and different lowercase letters indicate significant differences over time ($p < 0.05$).

Table 2. Compounds identified during the maturation process of Bola de Ocosingo cheese samples from the El Dorado cheese factory.
Tabla 2. Compuestos identificados durante el proceso de maduración de las muestras de queso Bola de Ocosingo de la quesería El Dorado.

RT	Compound	Days of maturation (SI/RSI)							
		0	SI/RSI	7	SI/RSI	14	SI/RSI	21	SI/RSI
17.16	Acetic acid, 2-phenylethyl ester			+	859/926				
27.88	Acetic acid, 3,7,11,15-tetramethyl-hexadecyl ester			+	811/831			+	768/809
26.20	Dodecanoic acid, ethyl ester					+	795/817		
27.63	Tetradecanoic acid, ethyl ester			+	721/770	+	822/858	+	821/842
28.57	Hexadecanoic acid, ethyl ester					+	893/902	+	
29.27	(E)-9-Octadecenoic acid ethyl ester					+	840/878	+	
21.21	n-Decanoic acid							+	790/844
26.0	Dodecanoic acid							+	911/932
27.48	Tetradecanoic acid					+	826/844	+	922/925
28.01	Pentadecanoic acid							+	845/867
28.47	n-Hexadecanoic acid							+	867/880
29.55	I-(+)-Ascorbic acid 2,6-dihexadecanoate							+	791/795

RT: Retention time; +: Presence; SI: Scan index; RSI: Reverse scan index

Table 3. Compounds identified during the maturation process of Bola de Ocosingo cheese samples from the Queshil cheese factory.
Tabla 3. Compuestos identificados durante el proceso de maduración de las muestras de queso Bola de Ocosingo de la quesería Queshil.

RT	Compound	Days of maturation (SI/RSI)							
		0	SI/RSI	7	SI/RSI	14	SI/RSI	21	SI/RSI
26.22	Dodecanoic acid, ethyl ester			+	845/860	+	847/849	+	864/865
27.61	Tetradecanoic acid, ethyl ester			+	908/912			+	881/885
28.13	Pentadecanoic acid, ethyl ester			+	783/834			+	795/811
28.5	Ethyl 9-hexadecenoate			+	797/806				
28.58	Hexadecanoic acid, ethyl ester			+	905/913	+	894/894	+	905/906
29.27	(E)-9-Octadecenoic acid ethyl ester					+	894/915	+	893/914
15.21	Octanoic acid					+	897/901	+	904/907
21.53	n-Decanoic acid					+	917/921	+	868/877
26.0	Dodecanoic acid					+	925/936	+	924/934
27.48	Tetradecanoic acid					+	846/859	+	922/924
28.01	Pentadecanoic acid					+	871/887	+	852/874
28.47	n-Hexadecanoic acid					+	867/880	+	862/875

RT: Retention time; +: Presence; SI: Scan index; RSI: Reverse scan index

including n-decanoic acid, dodecanoic acid, tetradecanoic acid, pentadecanoic acid n-hexadecanoic acid and l-(+)-ascorbic acid 2,6-dihexadecanoate. Likewise, it can be seen that the compound Tetradecanoic acid, ethyl ester remained present from day 7 onwards.

In the cheeses from the Queshil cheese factory (Table 3) a total of 17 compounds were detected. It can be observed that the highest presence of compounds is at 14 and 21 days of ripening, which is when the cheese can be marketed. The compounds detected were hexadecanoic acid, ethyl ester, (E)-9-octadecenoic acid ethyl ester, octanoic acid, n-decanoic acid, dodecanoic acid, tetradecanoic acid, pentadecanoic acid and n-hexadecanoic acid. It can be observed that the esters dodecanoic acid, ethyl ester and hexadecanoic acid, ethyl ester remained present from day 7.

In the San Martín cheese factory, a total of 5 compounds were detected: decamethylated cyclohexasiloxane, dodecamethylated cyclohexasiloxane, hexadecamethylated cyclooctasiloxane, octadecamethylated cyclononasiloxane, and eicosamethylated cyclodecasiloxane. These compounds may be residues from the column, so they cannot be considered compounds produced by the metabolism of the microbiota of the Bola de Ocosingo cheese.

DISCUSSION

Bola de Ocosingo cheese is an artisanal cheese produced in Ocosingo, Chiapas, Mexico, using raw cow's milk. These cheeses are prized for their sensory characteristics, such as their flavor and aroma. These characteristics are the result of the metabolism of the microbiota present in the milk. During the 21-day ripening process of Bola de Ocosingo cheese, three phyla were detected: Firmicutes, Proteobacteria, and Bacteroidota. These results coincide with those reported by Nelli *et al.* (2023), who reported these same microbial phyla in artisanal Gidotyri-type cheese.

We can observe that during the ripening process of Bola de Ocosingo cheese, there is a very similar microbial diversity at 0, 7, 14 and 21 days in the Bola de Ocosingo cheeses analyzed from the three cheese factories (Fig 3). The predominant genera were *Lactococcus*, *Streptococcus* and *Lactobacillus*. This agrees with what was reported by Aldrete *et al.* (2018) where they analyzed Bola de Ocosingo cheese ripened for 50 and 100 days, very different from the 21-day maturation period in which this cheese is consumed. This same relative abundance of the genera *Lactococcus*, *Streptococcus* and *Lactobacillus* has been observed in other artisanal cheeses such as Galotyri cheese, a cheese made with raw sheep's milk (Samelis and Kakouri, 2019). Species of the genus *Lactococcus* contribute to acidification by converting lactose into lactic acid, in addition to contributing to the conversion of amino acids into compounds that generate aroma in artisanal cheeses (Salazar *et al.*, 2018).

Furthermore, metagenomic analysis of Bola de Ocosingo cheese detected the presence of bacterial genera belonging to the Enterobacteriaceae family: *Enterobacter*, *Klebsiella*, *Escherichia*, and *Shigella*. Coliforms, belonging to the Enterobacteriaceae family, are microbiological indicators of hygiene during the cheese production process, and the presence of these genera was identified in samples from all three cheese dairies. This is consistent with the findings reported by Aldrete *et al.* (2018), who detected low proportions of the pathogens *Escherichia coli* and *Shigella flexnerii* in Bola de Ocosingo cheese matured for 50 and 100 days using metagenomic analysis. The presence of pathogens such as *E. coli* has also been reported in fresh cheese, cream cheese, and cream (Torres *et al.*, 2006; Cuevas *et al.*, 2024). This contamination could be due to the environment or originate from animal feces (Martín *et al.*, 2017). The presence of these pathogens has also been detected in cheeses made with pasteurized milk, which could be related to the resistance of some coli-forms to heat treatment (Selover *et al.*, 2021), as well as to the

lack of good hygiene practices during milking, processing and storage (Herrera *et al.*, 2014; Selover *et al.*, 2021). The *Enterobacter* and *Acinetobacter* genera were also detected in samples from the Dorado, Queshil, and San Martín cheese factories, as well as in Bola de Ocosingo cheese matured for 50 and 100 days (Aldrete *et al.*, 2018). This same pattern of microbial diversity was observed in artisanal cheeses made with goat's milk, where a relative abundance of the *Acinetobacter*, *Carnobacterium*, and *Enterobacter* genera was reported (Nelli *et al.*, 2023). Masiello *et al.* (2015) reported the presence of coliforms of the *Citrobacter*, *Enterobacter*, *Hafnia*, *Raoultella* and *Serratia* genera in pasteurized milk. The presence of these bacteria can affect the sensory characteristics of cheeses (Nörnberg *et al.*, 2010; Cai *et al.*, 2018; Tempini *et al.*, 2018). Another identified genus is *Chryseobacterium*, belonging to the phylum Bacteroidetes; its presence is common in dairy products and is associated with spoilage (Hantsis-Zacharov and Halpern, 2007).

The volatile fatty acid (VFA) analysis results presented in this study are consistent with previous research, such as that of Tavaría *et al.* (2004), who identified five volatile fatty acids (acetic acid, butyric acid, hexanoic acid, and isopentanoic acid) in Serra da Estrela artisanal cheese after 180 days of ripening. Although these compounds are present, their concentrations are higher in Serra da Estrela cheese compared to Bola de Ocosingo cheese. Similarly, Mureşan *et al.* (2021) confirmed the presence of volatile fatty acids in Alpuşeni cheese, many of which coincide with those observed in the present study. However, the concentrations of these compounds are also higher than those in Bola de Ocosingo cheese, which may be related to the ripening time. The presence of acetic and propionic acids in cheeses is related to bacterial control, in addition to contributing characteristic flavors to artisanal cheeses. Likewise, the other VFAs (butyric acid, isobutyric acid, pentanoic acid, isopentanoic acid and hexanoic acid) contribute to these flavors (Ziino *et al.*, 2005; Thierry *et al.*, 2015).

The formation of free fatty acids (FFA) in ripened cheeses is a complex process influenced by multiple factors. Previous studies have identified three main biochemical pathways: lipolysis, proteolysis and lactose fermentation (Bintsis, 2018). In the case of cheeses made with raw milk, such as those analyzed in this study, lipolysis seems to be the predominant mechanism, which can cause the release of linear chain fatty acids (butanoic, pentanoic, hexanoic, heptanoic, octanoic, decanoic and dodecanoic) while proteolytic enzymes are responsible for the breakdown of proteins into amino acids such as valine, leucine and isoleucine (Grossmann and McClements, 2021). Native milk lipases, not lactic acid bacteria, are primarily responsible for the release of straight-chain and branched-chain fatty acids (Sharma *et al.*, 2020). These enzymes hydrolyze triglycerides, releasing fatty acids such as butyric, caproic, caprylic, capric, and lauric. In addition, proteases may contribute to the formation of branched-chain fatty acids from the degradation of amino acids.

At 21 days of maturation, which is when the cheeses are marketed, the observed volatiles that predominate most in the El Dorado cheese factory are: a) Octanoic acid, also known as caprylic acid, a short-chain saturated fatty acid, with a strong, rancid odor, similar to old cheese or sweat (Enzenauer, 2021), b) Dodecanoic acid, also known as lauric acid, a medium-chain saturated fatty acid with a mild, slightly soapy odor (Grossmann and McClements, 2021), c) Tetradecanoic acid, also known as myristic acid, a medium-chain saturated fatty acid, with a very weak, almost imperceptible odor (Miroljub *et al.*, 2022), d) Hexadecanoic acid ethyl ester, an ester of a saturated fatty acid (palmitic acid), which has an odor that could be described as slightly waxy or soapy, and e) Tetradecanoic acid ethyl ester, an ester of a saturated fatty acid (myristic acid), with a mild odor and may have a slight fruity nuance (Eroglu *et al.*, 2016).

In the cheeses from the Queshil factory, the predominant fatty acids were: a) Octanoic acid, also known as caprylic acid, a short-chain saturated fatty acid that has a strong, rancid odor, similar to old cheese or sweat (Enzenauer, 2021), b) Dodecanoic acid, also called lauric acid, which has a mild, slightly soapy odor (Grossmann and McClements, 2021), c) Tetradecanoic acid, known as myristic acid, which has a very weak, almost imperceptible odor (Miroljub *et al.*, 2022), d) Decanoic acid, also called capric acid, which has a strong, rancid odor; these are medium-chain saturated fatty acids (Chen *et al.*, 2022); e) Ethyl ester of hexadecanoic acid, also known as palmitic acid and ethanol, which has a milder and less defined odor, which could be described as slightly waxy or soapy (Grossmann and McClements, 2021).

In the San Martín cheese factory, a high percentage of compounds such as hexadecamethyl cyclooctasiloxane, decamethyl cyclopentasiloxane, eicosamethyl cyclodecasiloxane and hexadecamethyl, were found. These compounds do not have characteristic or defined aromas but rather belong to the siloxane family, organosilicon compounds that form chains or rings of repetitive silicon and oxygen units. It is important to mention that the presence of siloxanes would affect the quality of dairy products (Zhang *et al.*, 2012). Despite the short ripening time, the three types of cheeses have the presence of the most representative VFAs of ripened cheeses, however, esters and long chain fatty acids were not detected in the San Martín cheese. This could be attributed to the absence or low abundance of microorganisms involved in the production of the compounds or by enzymatic inhibition due to heat treatment during production. Based on the above, we can determine that Queshil cheese preserves a greater variety and abundance of compounds characteristic of a commercially mature cheese. Secondly, we observed that the samples from the El Dorado cheese factory preserve a notable composition, although relatively lower than that of Queshil. Finally, the San Martín cheese showed less diversity and a much lower profile of compounds. These findings denote the importance of microbial and enzymatic processes in the development of the chemical profile of the Bola de Ocosingo cheese.

CONCLUSIONS

In this study, metagenomic analysis was used to identify the main genera of lactic acid bacteria present during the 21-day ripening period of Bola de Ocosingo cheese, an artisanal cheese. The bacterial genera identified were *Lactococcus*, *Streptococcus*, and *Lactobacillus*. Additionally, eight main aromatic compounds, acetic acid, propionic acid, butyric acid, isobutyric acid, pentanoic acid, isopentanoic acid, and hexanoic acid, were identified by GC-MS as being present during this ripening process.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest

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