High-throughput sequencing of microbial communities in Poro cheese, an artisanal Mexican cheese

Alejandro Aldrete-Tapia, Meyli C. Escobar-Ramírez, Mark L. Tamplin, Montserrat Hernández-Iturriaga

ABSTRACT

The bacterial diversity and structure of Poro cheese, an artisanal food, was analysed by high-throughput sequencing (454 pyrosequencing) in order to gain insight about changes in bacterial communities associated with the cheese-making process. Dairy samples consisting of milk, fermented whey, curd and ripened cheese (during 7 and 60 d) were collected from three manufacturers located in the state of Tabasco, Mexico during dry (March–June) and rainy (August–November) seasons. Independently of producer and season, raw milk samples displayed the highest diversity in bacterial communities. In raw milk, genera found were Macrococcus, Staphylococcus, Enterococcus, Streptococcus, Lactobacillus and Enhydrobacter. Diversity in whey, curd and cheese was lower, principally containing Streptococcus and Lactobacillus; however, bacteria such as Staphylococcus, Acinetobacter, Chryseobacterium, Bacillus, Sediminibacter, Lactococcus and Enterococcus were occasionally present. After curdling step, the most dominant and abundant species were Streptococcus thermophilus and Lactobacillus delbrueckii.

1. Introduction

Poro cheese is a fresh or short-ripened, soft-pressed artisanal cheese, made with raw milk inoculated with fermented whey obtained from the previous production batch. It is regionally-made in the river zone of Tabasco, Mexico; usually an additional ripening is performed because of delays in distribution and sales (Cervantes and Villegas, 2006). The specific characteristics of typical products arise mainly from the raw materials, area of production, environmental conditions and traditional tools of manufacture (Ercolini et al., 2003). These factors influence the composition of microbial communities that are responsible for the unique and broad diversity of flavours, aromas and textures. Many bacteria from raw milk enhance the organoleptic qualities of cheeses, while others may have adverse effects or constitute a health risk. Milk pasteurization, which is a requirement for production of non-ripened cheese, destroys pathogenic but also spoilage bacteria that may affect the sensorial quality of the final products. Producers of Poro cheese need to meet the requirements of this regulation, however this change in artisan practices could affect the genuine characteristics of the cheese.

Cheese processing is largely based on fermentation by lactic acid bacteria, which are deliberately added as starter cultures or present in the raw materials and environment and selected by microbial interactions during fermentation (Fox et al., 2004). The changes in microbial population structure of cheese are influenced by environmental factors present during processing. Therefore microbial species and strains should be monitored, at least during the most critical phases, in order to achieve proper quality in the final product (Coppola et al., 2008). Community-level studies increasingly rely on culture-independent methods based on direct DNA analysis (Jany and Barbier, 2008). High-throughput sequencing has revolutionized the field of microbial ecology, allowing for more accurate identification of microbial taxa, including those which are difficult to culture and/or are present in low abundance, thus providing a more comprehensive analysis of microbial diversity (Ercolini et al., 2012; Quigley et al., 2012).

Producers from the state of Tabasco want to establish denomination of origin for Poro cheese. To accomplish this, standardization...
of cheese production process is needed which includes the use of pasteurized milk. However this change could affect the generation of flavours and aromas that are present in the artisanal cheese. A solution to this problem could be to use a native starter culture (instead of the fermented whey) to produce the desired sensorial characteristics (Candioti et al., 2002; Ayad et al., 2003; Goncu and Alpken, 2005). To accomplish this goal, knowing the microflora present during cheese production by using non-culture based studies could be very helpful to successfully isolate the microorganisms involved in the generation of flavours and aromas. In this study we attempt to provide an insight of the composition and dynamics of bacterial communities present during the production of Poro cheese, by three manufacturers during dry and rainy seasons using high-throughput sequencing.

2. Materials and methods

2.1. Cheese production

Manufacturers of Poro cheese located in the region of Tabasco, Mexico, follow the same production process but differ in milk supplier: some produce their milk while others purchase milk from external suppliers. Poro cheese production starts with addition of fermented whey (from the previous batch) to raw milk, and then a 40-min curdling step using calf rennet. After coagulation, the curd is cut and rested for a maximum of 4 h, during which the pH is reduced to 4.0. The curd is drained by self-pressing in wood containers, turned several times, and then mechanically pressed. The product is then salted by rubbing over a 3-d period, and then ripened on wooden shelves under environmental conditions. This process lasts seven days, after which the product is covered with paraffin wax. Ripening continues in this packaging during distribution and sales at ambient temperature.

2.2. Sample collection

Thirty samples consisting of milk, fermented whey, curd and ripened cheese (collect at 7- and 60-d) were obtained from three manufacturers in the state of Tabasco, Mexico. Milk is obtained from Zebu, brown Swiss, Holstein cow breeds and their crossbreeding. Milk, whey and curd samples were collected by personnel from Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP); the samples were frozen at −20 °C and shipped to Universidad Autónoma de Querétaro for processing. Manufacturers collected cheese samples (from the same batch as for the milk, curd, and whey samples) after ripening for 7 and 60 d, and shipped them to the University for analyses. Collections were done during dry (March–June) and rainy (August–November) seasons.

2.3. DNA extraction

Lipids, proteins and salts from dairy samples (5-g) were removed by emulsification in 45 ml of sterile trisodium citrate buffer (2%), followed by incubation at 37 °C for 5 min, homogenization in a Stomacher (milk, curd, whey during 1 min; cheese for 3 min) and centrifugation (12,000 g for 1 min); the process was repeated at least three times until a cell pellet was obtained. DNA extraction was done by lysis using heat and powder glass, followed by a purification step with phenol-chloroform. Briefly, the cell pellet was resuspended in 300 μl of buffer TE (1 mM TRIS, EDTA 1 mM; pH 8.0), heated at 100 °C for 10 min and cooled in ice. Then, 100 mg of powdered glass and 100 μl of equilibrated phenol (pH 8.0) were added, vortexed 30 s, and centrifuged (12,000 × g for 1 min). The supernatant was transferred to a new tube, 100 μl of chloroform-isooamyl alcohol (24:1) added, gently mixed, incubated on ice for 5 min, centrifuged 12,000 g for 1 min, and then the supernatant transferred to a new tube. DNA was precipitated with 1/10 volumes of sodium acetate 3 M (pH 5.0) and 2 volumes of ice cold absolute ethanol that was incubated at −20 °C for 30 min. DNA was pelleted by centrifugation at 12,000 g for 12 min, washed once with 200 μl of ethanol 70% (vol/vol), centrifuged, and the supernatant removed with a pipette and air dried for 5 min. The final pellet was resuspended in 50 μl of TE buffer.

2.4. Pyrosequencing (454 sequencing)

DNA sequencing was performed at MR DNA (www.mrdnlab.com, Shallowater, TX, USA). Briefly, the 16S rRNA gene was amplified using the primers 27Fmod (AGRTTTGATCMTGGCTCAG) and 530R (CCGNGCNGGTGGCAC); 454-adaptors were included in the forward primer including a barcode for each sample. The sequencing was performed utilizing a Roche 454 FLX titanium (Roche diagnostics Ltd, West Sussex, UK) instrument and reagents, following the manufacturer's instructions. The sequence data were processed using Mothur version 1.31.2 software (Schloss, 2009) with a modified pipeline. Briefly, sequences were subjected to quality controls and the 454-adaptors trimmed; unique sequences were aligned to the SILVA reference database. Chimera were removed from aligned sequences with the uchime algorithm and classified to obtain the taxonomic assignment using the Silva 16S rRNA gene database. The final sequences were subsampled to the minimum number of sequences in the sample. Good's coverage, Chao1 richness and Inverse Simpson diversity indices were calculated and rarefaction curves produced. Similarity, microbial populations assessed using the Bray–Curtis index were represented by principal coordinates analysis (PCoA).

3. Results

3.1. Characteristics of sample sequence reads

A total of 705,517 reads were obtained from 30 dairy samples with an average length of 469 bp (range of 39–871). After the quality control, 693,746 reads were obtained with an average length of 366 bp (range of 316–411). Filtering the sequences eliminated 16.7% of reads per sample, on average, and 3.6% of the quality sequences were not classified at the genus level. The total number of Operational Taxonomic Units (OTUs) was 273 with 97% similarity.

3.2. Diversity among bacterial communities in dairy samples

Diversity richness, diversity indices and coverage are presented in Table 1. In general, the highest number of OTUs observed was found in raw milk samples, and was higher in the rainy season (range = 62–358) compared to the dry season (range = 68–125). The number of OTUs in curd, fermented whey and cheese samples was lower; however, in some cases higher numbers were observed, e.g., whey and curd samples from dairy-3 (107 and 239 OTUs, respectively). The OTUs richness estimated by the Chao1 index was elevated compared with the OTUs observed. Bacterial diversity expressed by inverse Simpson index, was higher in raw milk than in the other dairy samples. The Good's coverage, an estimator of sampling completeness which calculates the probability that a randomly selected amplicon sequence from a sample has been already sequenced, indicate an adequate level of sequencing to identify the majority of diversity in the dairy samples. The rarefaction curves (Fig. 1) showed approximation to an asymptote, indicating that more sampling would not have significantly increased the number of OTUs.
3.3. Bacterial communities during manufacturing of Poro cheese

High-throughput sequencing determined the relative composition and bacterial species in artisanal production process of Poro cheese. At the phylum level, milk samples contained Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidetes. However, after curd fermentation the phyla Firmicutes dominated, with less abundance of Proteobacteria and Actinobacteria (data not shown). Diversity and composition of microbial communities in raw milk samples collected in two seasons and in three dairies were different (Fig. 2). The proportions of genera (range in parentheses) were: Macrococcus (0.17–47.8%) and Staphylococcus (1.50–44.09%). Lactobacillus (0.74–46.57%), Streptococcus (0.85–30.33%), and Enterococcus (0.21–15.25%) were detected (Fig. 2). Fermented whey displayed lower diversity than other samples taken during cheese production, principally containing Streptococcus (2.68–95.79%) and Lactobacillus (2.86–97.32%). The same genera were dominant in the curd and cheeses samples, but occasionally the presence of Staphylococcus, Acinetobacter, Chrystalobacter, Bacillus, Sediminibacterium, Enterococcus and Lactococcus were detected (Fig. 2).

A PCoA was performed to determine the effect of type of sample, producer, and season on microbial communities (Fig. 3). The principal coordinates 1 and 2 explained 56.10 and 30.20% of the variance, respectively. The type of sample showed relation with changes in the microbial population and two main clusters were observed: one grouping milk samples and the other curd, fermented whey, and cheeses samples. No clear grouping was observed among producers or seasons. Pooled sequences from all samples (milk, whey, curd, and cheese) were used to identify predominant species using BLAST (Table 2).

![Fig. 1. Normalized rarefaction curves from the V1–V3 region of the 16S rRNA sequences from dairy samples of Poro cheese production obtained from different producers in dry season (continuous line) and rainy season (discontinuous line) at 97% of similarity.](image)

### Table 1

OTUs identified at 97% similarity, species richness estimate (Chao1), diversity index (Inverse Simpson) and coverage for the 16S rRNA sequencing of dairy samples from Poro cheese production.

<table>
<thead>
<tr>
<th>Producer</th>
<th>Season</th>
<th>Sample</th>
<th>OTUs</th>
<th>Chao1</th>
<th>Inverse Simpson</th>
<th>Good's coverage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Dry</td>
<td>Milk</td>
<td>68</td>
<td>125.75 (86.63–247.01)</td>
<td>6.99 (6.99–6.68)</td>
<td>99.71</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Whey</td>
<td>14</td>
<td>28.00 (16.92–81.11)</td>
<td>1.65 (1.65–1.63)</td>
<td>99.94</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Curd</td>
<td>10</td>
<td>12.00 (10.25–26.01)</td>
<td>1.07 (1.07–1.06)</td>
<td>99.89</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cheese 7</td>
<td>10</td>
<td>13.33 (10.50–32.07)</td>
<td>2.00 (2.00–1.98)</td>
<td>99.83</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cheese 60</td>
<td>12</td>
<td>187.88 (154.72–259.29)</td>
<td>4.52 (4.52–4.45)</td>
<td>99.71</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Whey</td>
<td>92</td>
<td>175.15 (130.25–272.79)</td>
<td>1.27 (1.27–1.26)</td>
<td>99.81</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Curd</td>
<td>70</td>
<td>110.06 (85.89–173.75)</td>
<td>1.08 (1.08–1.07)</td>
<td>99.87</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cheese 7</td>
<td>9</td>
<td>10.50 (9.15–24.08)</td>
<td>1.25 (1.25–1.22)</td>
<td>99.85</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cheese 60</td>
<td>45</td>
<td>132.75 (74.88–302.73)</td>
<td>1.27 (1.27–1.27)</td>
<td>99.91</td>
</tr>
<tr>
<td>2</td>
<td>Dry</td>
<td>Milk</td>
<td>86</td>
<td>103.00 (91.34–140.16)</td>
<td>4.32 (4.32–4.08)</td>
<td>99.53</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Whey</td>
<td>8</td>
<td>8.33 (8.02–13.96)</td>
<td>2.04 (2.04–2.02)</td>
<td>99.95</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Curd</td>
<td>9</td>
<td>9.75 (9.07–17.45)</td>
<td>1.12 (1.12–1.1)</td>
<td>99.89</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cheese 7</td>
<td>4</td>
<td>4.00 (4.00–4.00)</td>
<td>1.85 (1.85–1.8)</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cheese 60</td>
<td>11</td>
<td>14.00 (11.36–36.23)</td>
<td>1.14 (1.14–1.11)</td>
<td>99.89</td>
</tr>
<tr>
<td></td>
<td>Rainy</td>
<td>Milk</td>
<td>62</td>
<td>71.75 (64.67–97.54)</td>
<td>3.59 (3.59–3.44)</td>
<td>99.72</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Whey</td>
<td>5</td>
<td>5.00 (5.00–0)</td>
<td>1.91 (1.91–1.87)</td>
<td>99.95</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Curd</td>
<td>16</td>
<td>61.00 (29.66–164.24)</td>
<td>1.05 (1.05–1.04)</td>
<td>99.74</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cheese 7</td>
<td>7</td>
<td>7.50 (7.03–15.26)</td>
<td>1.22 (1.22–1.20)</td>
<td>99.95</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cheese 60</td>
<td>10</td>
<td>10.60 (10.05–17.11)</td>
<td>1.08 (1.08–1.07)</td>
<td>99.91</td>
</tr>
<tr>
<td>3</td>
<td>Dry</td>
<td>Milk</td>
<td>125</td>
<td>188.59 (155.01–259.73)</td>
<td>5.12 (5.12–4.99)</td>
<td>99.40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Whey</td>
<td>107</td>
<td>269.75 (185.75–443.37)</td>
<td>1.89 (1.89–1.89)</td>
<td>99.93</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Curd</td>
<td>88</td>
<td>126.50 (101.06–201.47)</td>
<td>6.01 (6.01–5.87)</td>
<td>99.88</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cheese 7</td>
<td>22</td>
<td>26.20 (22.79–44.34)</td>
<td>1.58 (1.58–1.54)</td>
<td>99.81</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cheese 60</td>
<td>15</td>
<td>25.50 (17.03–69.19)</td>
<td>2.13 (2.13–2.11)</td>
<td>99.79</td>
</tr>
<tr>
<td></td>
<td>Rainy</td>
<td>Milk</td>
<td>358</td>
<td>520.98 (462.71–611.68)</td>
<td>2.60 (2.60–2.57)</td>
<td>99.78</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Whey</td>
<td>71</td>
<td>218.00 (134.72–410.14)</td>
<td>1.40 (1.40–1.39)</td>
<td>99.95</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Curd</td>
<td>239</td>
<td>375.88 (322.08–464.53)</td>
<td>1.50 (1.50–1.49)</td>
<td>99.87</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cheese 7</td>
<td>10</td>
<td>20.04 (11.92–62.16)</td>
<td>1.16 (1.16–1.15)</td>
<td>99.89</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cheese 60</td>
<td>15</td>
<td>18.33 (15.50–37.07)</td>
<td>1.67 (1.67–1.63)</td>
<td>99.88</td>
</tr>
</tbody>
</table>

---

**Notes:**

- Cheese ripened for 7-d.
- Cheese ripened for 60-d.
- Values in parentheses are 95% confidence intervals.
4. Discussion

The V1–V3 region of 16S rDNA was amplified from total DNA extracted from dairy samples obtained during a traditional production process of Poro cheese, allowing the identification of microbial communities and bacteria that could be potentially used in starter cultures.

In general, the diversity of microbial populations in dairy samples collected during dry season was less than in rainy season. This can be due to the influence of animal feeds and to the free-range production system, which affect the nutritional composition of milk and therefore, microbial communities in milk (Palmquist and Beaulieu, 1993; Chilliard et al., 2001). It has been reported that pasture plants and fodder types change composition seasonally and influence the physicochemical characteristics of milk (Galina et al., 2007). Eventually this can affect the nutritive properties, accented colours and flavours of food products (e.g. cheese) throughout the year (Lindmark-Mansson et al., 2003; Giannino et al., 2009).

The microflora of raw milk could be derived from the udder, the farm, milking barn environment and from workers and equipment in contact with milk (Oliver et al., 2005; Schmidt, 2008). For example, *Macrococcus caseolyticus* is part of the normal microbiota of cattle and other animals, and can hydrolyse casein (Randazzo et al., 2002). Environmental bacteria detected in this study, such as *Enhydrobacter*, *Acinetobacter*, *Chryseobacterium*, *Bacillus*, *Sediminibacterium*, *Diaphorobacter*, and *Elizabethkingia* have also been identified in other studies where culture-dependent and -independent techniques were used to define the microbiota of raw milk (Feurer et al., 2004; Delbes et al., 2007; Callon et al., 2007; Alegria et al., 2009; Quigley et al., 2012). The high proportion of
Staphylococcus aureus can provide information concerning the animal's health condition, handler practices in milking and in the cheese-making process. Depending on the strain and its ability to produce enterotoxins, the presence of S. aureus can be considered a health risk, especially if it can grow in Poro cheese (Akineden et al., 2008).

Among the three dairies, raw milk samples showed the highest species richness, however as the production process continued, diversity in dairy samples (fermented whey, curd, and cheese) was reduced. Several investigations using pyrosequencing to study the microbial composition of fermented foods, including dairy products, have found similar results (Humbiot and Guys, 2009; Roh et al., 2010; Kim et al., 2011; Masoud et al., 2011; Ercolini et al., 2012; Masoud et al., 2012; Nam et al., 2012; Quigley et al., 2012; Pangallo et al., 2013). It is well known that during fermentation step, there are shifts in the food environment causing selection of microbial populations present in raw milk. Also, environmental factors such as temperature and relative humidity impact the evolution of microbial diversity during the manufacturing and ripening of cheese (Barron et al., 2001; Caridi et al., 2002; Psomi et al., 2003; Bonetta et al., 2008).

In fermented whey, curd, and cheese, two genera were found in higher proportions: Streptococcus and Lactobacillus. Examining the predominant sequences in the NCBI for these genera, the most representative species were Streptococcus salivarius and Lactobacillus delbrueckii. Nevertheless, for the sequence of S. salivarius, the BLAST analysis of 16S rRNA matched two subspecies with similar DNA base composition: S. salivarius subsp. salivarius and S. salivarius subsp. thermophilus. S. salivarius subsp. salivarius is not suitable for milk fermentations, fails to grow symbiotically in the presence of Lactobacillus bulgaricus, does not produce sensory characteristics in products, and is an inhabitant of the buccal environment and not in dairy products (Marshall et al., 2008). In contrast, S. salivarius subsp. thermophilus along with L. delbrueckii, are well documented in starter cultures used to produce other types of cheeses (Randazzo et al., 2002; Ercolini et al., 2001). Thus, in our study the most suitable subspecies could be S. salivarius subsp. thermophilus.

Enterococci have been reported in natural starter of different cheeses (Giannino et al., 2009). However in Poro cheese it was found sporadically and in low levels after fermentation, indicating that its presence may not be significant for the development of sensory characteristics. The PCoA showed that independently of season and producer, microbial populations in Poro cheese are similar to those observed in fermented whey, suggesting that S. salivarius subsp. thermophilus and L. delbrueckii could be the bacteria involved in the production of characteristic flavours and aromas.

This work is the first study that reports the composition and dynamics of microbial communities during Poro cheese production using culture independent approach. Pyrosequencing showed the predominance of S. salivarius subsp. thermophilus and L. delbrueckii across dairies and seasons in fermented whey used as natural starter culture and in cheese. Additional studies to isolate these microorganisms and to characterize them using biochemical and molecular approaches are needed. Determination of technological characteristics of these isolates would be also necessary to establish which strain and their proportion could be included in the starter culture to generate typical flavours and aromas of Poro cheese using pasteurized milk.

The information generated in this work would be useful for producers of Poro cheese by helping them to standardize process improving quality and safety, as well as to preserve a traditional food that supports the economy of local producers and their families.

### References


