Article Microbiological and Metagenomic Analysis to Assess the Effect of Container Material on the Microbiota of Feta Cheese during Ripening

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Abstract: The aim of the present study was to assess the influence of ripening container’s material on the bacterial diversity of Feta cheese PDO (Protected Designation of Origin). The microbiota of fresh and mature cheese produced in plastic and stainless steel container was monitored by microbial enumeration and 16s rRNA gene sequencing. According to the obtained results, lactic acid bacteria (LAB) was the dominant microbiota of fresh and mature cheese. Metagenomics data revealed that fresh cheese was dominated by Lactococcus followed by members of Enterobacteriaceae family and Pseudomonas. Similarly, Lactococcus was the most abundant genus detected in mature cheese (54 days and 120 days), regardless of the container’s material. In both fresh and mature cheese, species of Pseudomonas, Streptococcus, Acinetobacter, Lactobacillus, Flavobacterium, and Carnobacterium were detected. The abundance of Enterobacteriaceae, Moraxellaceae and Pseudomonadaceae in mature cheese ripened in stainless steel container seems to be numerically reduced after 120 days of storage compared to the cheese ripened in plastic container but not significant differences were observed (p > 0.05). In conclusion, metagenomic analysis suggests that ripening container’s material does not affect the microbial community responsible for the ripening of feta cheese PDO.

Keywords: feta cheese PDO; microbiota; diversity; next generation sequencing; Lactococcus; cheese ripening

1. Introduction

The production of foods marked with a European Union geographical indicator is designated according to specific and strict guidelines, which have been established and published to Database of Origin & Registration (DOOR) database. Geographical indicators of foods protect not only the producers but also the consumers to recognize high quality food products with specific characteristics. These guidelines are specific for each food product and related to its traditional production originating in a specific place, region, or country. In order to produce food with a geographical indicator, the manufacturer has to follow strictly all the steps described in the appropriate Regulation.
In the case of Greece, one of the most important products that could serve as a flagship of Greece is Feta cheese PDO. Feta cheese PDO is a brined cheese which is produced by ewe milk or a blend of ewe milk and goat milk (up to 30%) in the geographic areas of Macedonia, Thraki, Ipiros, Thessalia, Sterea Ellada, Peloponnissos, and Lesvos Island [1]. Moatsou and Govaris [2] presented schematically the steps have to be followed in order to produce Feta cheese PDO. Ripening which is taking place in wooden or metallic containers is a key step for the production of feta [3] with rich smell and pleasant taste. Beyond the importance of applied techniques for the unique characteristics of feta, the effect of the qualified raw material on the final product is important. Ripening of feta cheese is driven by the used starter cultures and the natural microbiota of raw milk [4]. Streptococcus thermophilus and Lactobacillus delbrueckii subsp. bulgaricus are the commercial starter cultures used from the industries to produce feta cheese, while non-starter lactic acid bacteria (NSLAB) could be found during ripening. The microbiota of feta cheese during ripening has been monitored in previous studies [4–9]. Lactobacillus (especially plantarum), Lactococcus lactis, enterococci, pediococci are the commonly detected NSLAB during ripening [5–7]. The effect of alternative starter cultures e.g., Enterococcus, Lactobacillus plantarum on the microbiota of feta cheese was also studied before [10,11]. However, no data are available to our knowledge regarding the influence of an alternation of a production step on the microbiota of feta cheese. Thus, the present work aimed to assess whether there is an influence on microbial diversity of matured feta cheese PDO if the cheese is ripened in stainless steel container or in plastic container, to highlight the importance of the raw materials microbiota on the microbial quality of the final product. To assess this influence, microbiota of feta cheese was monitor by microbiological and metagenomics analysis during ripening of the cheese.

2. Materials and Methods

2.1. Cheese Sampling

Feta cheese PDO was produced in a local industry according to the requirement described at EL/PDO/0017/0427 [1] with a slight modification. In brief, fresh cheese was left to mature in plastic container (P) and stainless steel container (M). Samples were collected in duplicate for microbiological analysis during ripening at different time intervals i.e., 0, 7, 28, 54, 82, 104 and 120 days; 0 day represents the day of production of fresh cheese and prior its transfer in the ripening container. For microbiota estimation by next generation sequencing samples after 0, 54 and 120 days were collected.

2.2. Microbiological Analysis

Twenty-five grams of each sample were subjected in classical microbiological analysis. Total Viable Counts (TVC), LAB, streptococci, enterococci, yeasts and molds, Pseudomonas sp., Enterobacteriaceae, staphylococci and enterobacteria that utilize glucose were enumerated on the appropriate growth medium i.e., Tryptic Glucose Yeast Agar (TGYA), de Man, Rogosa and Sharpe agar (MRS), M17 Agar, Kanamycin Aesculin Azide Agar (KAA), Rose Bengal Agar (RBC) Pseudomonas Agar Base (PAB) supplemented with Cetrimide, Fusidate, & Cephaloridine (CFC), Violet Red Bile Glucose Agar (VRBGA), Baird- Parker Medium (BP) supplemented with Egg Yolk and Simmons Citrate Agar, respectively. The results were expressed as log CFU/g.

2.3. pH Value Measurement

pH value of each sample was measured by RL150 pH meter (Russel). Each presented value represents the mean value of three measurements.

2.4. Estimation of Microbiota by Next Generation Sequencing

DNA was extracted from cheese following the recommendation of Nucleospin Food (Macherey-Nagel, Düren, Germany). Ion 16S Metagenomics kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to amplify the V2-4-8 and V3-7-9 hypervariable regions of 16S rRNA gene and the resulted
amplicons (400 bp) were sequenced using Ion Torrent PGM by CeMIA SA (https://cemia.eu/) (Larissa, Greece). The analysis of sequences was performed using Ion Reporter software (Thermo Fisher Scientific, Waltham, MA, USA), which offers rapid and semi-quantitative evaluation of complex microbial samples. Chimeras and noise were removed from the sequences. Operational taxonomic units (OTUs) were taxonomically classified (at >97% similarity) using Nucleotide Basic Local Alignment Search Tool (BLASTn) against NCBI database (www.ncbi.nlm.nih.gov) (Bethesda MD, 20894 USA).

2.5. Statistical Analysis

Analysis of variance (ANOVA) was applied to analyze all microbiological data and abundance of OTUs were analyzed for statistical significance. The significant differences among results at a 95% confidence level was determined by Duncan’s multiple range test.

3. Results and Discussion

This study aimed to map the potential differences in microbial communities of feta cheese PDO associated with the ripening container’s material. Microbiota of feta cheese PDO ripened in plastic and stainless steel container was monitored by microbial enumeration and next generation sequencing. In parallel, the pH values were measured during the ripening process. According to the obtained results, similar microbial populations and pH values were observed during ripening in both containers ($p > 0.05$) (Table 1). Initial total viable counts found to be above 9 log CFU/g and slightly decreased during ripening, where lactic acid bacteria (enumerated on MRS agar) was the main microbial group detected on cheese samples. Similar results have been observed previously for feta cheese produced from ewe/goat milk [10] and ewe milk [12]. In these studies, they also found that streptococci population (enumerated on M17 agar) was detected in higher level at time 0 and decreased during ripening. Pseudomonads, yeasts, and molds were enumerated in lower levels, while Enterobacteriaceae was below the detection limit of the method. It was reviewed that in feta cheese sold in packages, Pseudomonas and Enterobacteriaceae populations were 3.7 log CFU/g and below the detection limit, respectively [13]. Microbial contamination of raw milk from the environment through direct contamination or indirect contamination e.g., teat, feed, air, water tanks, milking parlour has been associated with the microbial communities found in traditional cheeses [14]. The growth of undesirable microorganisms i.e., coliforms and yeasts could be controlled with a rapid decrease of pH during production of feta cheese, while pH value lower than 4.6 is desirable to avoid the softening of cheese [2]. In this study, pH values were lower than 4.6 during ripening. Rapid decrease of pH below the level of 4.6 was previously detected during ripening of feta cheese [6]. In another study, pH value below 4.6 was measured after 60 days of ripening of feta cheese produced with the use of Enterococcus faecium as starter culture [11]. On the other hand, the addition of a Lactobacillus plantarum strain decreased the pH value of feta cheese to 4.1 at the end of the storage period [10].
Table 1. The effect of ripening container’s material (plastic, stainless steel) on the final population of microorganisms and pH value of feta cheese Protected Designation of Origin (PDO) during ripening (0, 7, 28, 54, 82, 104, and 120 days).

| Microorganism                        | Plastics Container | Stainless Steel Container |
|--------------------------------------|--------------------|---------------------------|
|                                      | 0  | 7  | 28 | 54 | 82 | 120 | 0  | 7  | 28 | 54 | 82 | 104 | 120 |
| Total Viable Counts (TVC)            | 9.05 | 7.85 | 7.93 | 8.25 | 8.65 | 7.94 | 7.79 | 9.05 | 7.74 | 9.01 | 8.25 | 9.65 | 8.94 | 7.94 |
| Lactic acid bacteria (LAB)\(^1\)     | 8.26 | 7.89 | 7.89 | 8.18 | 8.48 | 7.80 | 7.84 | 8.26 | 7.76 | 8.99 | 8.13 | 9.70 | 9.08 | 7.87 |
| Streptococci \(^2\)                  | 9.22 | 8.20 | 8.98 | 5.00 | 2.80 | 4.18 | 5.12 | 9.22 | 7.89 | 9.00 | 5.57 | 4.45 | 5.45 | 4.95 |
| Enterococci                          | 6.25 | 6.67 | 6.77 | 7.08 | 6.71 | 6.86 | 6.67 | 6.25 | 6.86 | 7.15 | 7.01 | 6.72 | 6.66 | 6.73 |
| Yeasts and molds                     | 2.89 | 3.73 | 4.01 | 4.10 | 4.27 | 3.21 | 3.96 | 2.89 | 3.29 | 4.34 | 4.00 | 4.27 | 2.95 | 3.46 |
| Pseudomonas sp. \((\text{Enterobacteriaceae})\) | <2 | 3.49 | 3.20 | 2.35 | 2.00 | 2.00 | 3.55 | <2 | 2.00 | 3.09 | 3.25 | 2.00 | 2.00 | <2 |
| Staphylococci                        | <1 | 2.15 | <2 | <2 | <2 | <2 | <2 | <2 | <2 | <2 | 4.06 | <2 | <2 | <2 |
| Enterobacteria that utilize glucose  | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 |
| pH value                             | 4.19 | 4.20 | 4.16 | 4.30 | 4.14 | 4.06 | 4.12 | 4.19 | 4.21 | 4.34 | 4.28 | 4.21 | 4.05 |

\(^1\) enumerated on MRS agar. \(^2\) enumerated on M17 agar.
The investigation of main microbial culturable counts is not enough neither connect perfectly the source of microbial contamination nor highlight the microbial diversity [14,15]. This drawback could be overcome by collecting data from microbial characterization and metagenomics analysis. In previous studies, the microbiota of feta cheese during ripening has been monitored with culture dependent or independent methods (i.e., DGGE) [4–9]. In this study, a metagenomic approach was followed to investigate the microbial diversity of feta cheese. In brief, the metagenomic analysis revealed a complex microbiota that consisted of eleven bacterial families (Figure 1). The complexity of microbial community of feta cheese has been also described before [4,5]. The abundance of feta cheese PDO bacterial microbiota during ripening (0, 54 and 120 days) estimated by next generation sequencing are reported in Table S1. According to the obtained results, the cheese microbiota was not affected by the material of ripening container ($p > 0.05$).

![Figure 1: Abundance of the main families found in the feta cheese PDO samples during ripening (0,54, 120 days) in plastic (P) and stainless steel (M) container.](image)

Streptococcaceae was the most abundant family, where Lactococcus found to be the dominant bacterial genus (Figure 1,2). At a genus level, in both fresh and mature cheese, species of Pseudomonas, Streptococcus, Acinetobacter, Lactobacillus, Flavobacterium, and Carnobacterium were additionally detected. Lactococcus including Lactococcus lactis was increased during ripening reaching the level of 80.54 and 90.00 % after 120 days in plastic and stainless steel container, respectively. In previous studies, the dominance of Lactococcus in feta cheese and its increase during ripening have been reported [4–6,8,12,16]. The ripening process of feta cheese seems to favour the growth of Lactococcus lactis, which represents a member of starter culture used for the production of this cheese [4,10–11]. More specifically, it was reported previously that Lactococcus lactis could be used in combination with traditional starter culture consisted of Streptococcus thermophilus and Lactobacillus bulgaricus as starter cultures for the production of feta cheese PDO [2]. The rest portion of starter culture i.e Streptococcus thermophilus and Lactobacillus was not detected in high numbers in this study. More specifically, Streptococcus thermophilus decreased during ripening from 5.7% to 1.28 and 0.63% after 120 days ripening in plastic and stainless steel container, respectively. It has been reported previously that Streptococcus thermophilus and Lactobacillus were decreased during ripening [6,7]. It has to be noted that, the undesirable families like Enterobacteriaceae, Pseudomonadaceae and Moraxellaceae were detected in higher percentage at time 0, and their abundance was decreased during ripening in both cases. In an earlier study, Pseudomonas and Acinetobacter were also isolated from fresh and mature feta cheese [5]. On the other hand, the presence of the commonly detected genera i.e., Enterococcus and Pediococcus in earlier studies [5–7,12] were not confirmed in this study.
4. Conclusions

In conclusion, this study monitored the microbiota of fresh and mature cheese produced in plastic and stainless steel container by microbial enumeration and 16s rRNA gene sequencing. In our knowledge, this is a first study of the influence of an alternative ripening container’s material on the microbial diversity of feta cheese. It was shown that the used material i.e., plastic, stainless steel did not affect the microbial counts. Furthermore, metagenomic analysis highlighted that the microbial community responsible for the ripening of feta cheese did not alter by the ripening container’s material. Moreover, a better insight into the microbial diversity of feta cheese during ripening was offered by the application of next generation sequencing.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: Abundance of Feta cheese PDO bacterial microbiota during ripening (0, 54 and 120 days) estimated by next generation sequencing

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