Microbiota “Fingerprint” of Greek Feta Cheese through Ripening

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Abstract: Feta is a Greek protected designation of origin (PDO) brined curd white cheese made from small ruminants’ milk. In the present research, Greek Feta cheese bacterial diversity was evaluated via matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). Analysis of 23 cheese samples, produced in different regions of the country, was performed in two ripening times (three or six months post-production). The identified microbiota were primarily constituted of lactic acid bacteria. A total of 13 different genera were obtained. The dominant species in both ripening times were Lactobacillus plantarum (100.0% and 87.0%, at three or six months post-production, respectively), Lactobacillus brevis (56.5% and 73.9%), Lactobacillus paracasei (56.5% and 39.1%), Lactobacillus rhamnosus (13.0% and 17.4%), Lactobacillus paraplantarum (4.3% and 26.1%), Lactobacillus curvatus (8.7% and 8.7%). Other species included Enterococcus faecalis (47.8% and 43.5%), Enterococcus faecium (34.8% and 17.4%), Enterococcus durans (13.0% and 17.4%), Enterococcus malodoratus (4.3% and 4.3%), and Streptococcus salivarius subsp. thermophilus (21.7% and 30.4%). The increased ripening time was found to be correlated to decreased total solids (r = 0.616; p = 0.002), protein (r = 0.683; p < 0.001), and PH (r = 0.780; p < 0.001). The results of this study contribute to a better understanding of the core microbiota of Feta cheese.

Keywords: Feta cheese; microbiota; safety; ripening; MALDI-TOF MS

1. Introduction

Feta cheese is a white brined cheese produced traditionally in Greece, made of either 100% sheep milk or a mixture of 0–30% goat milk and 70–100% sheep milk. Its flavor is distinguished by its natural taste and texture as well as its saltiness and slight acidity. Since 2008, Feta cheese belongs to the Protected Designation of Origin Products (PDO) group [1], ensuring the strict quality and safety specifications imposed by the European Union. It is by far the most consumed Greek cheese, representing 90% of the total national cheese production. Four basic ingredients are needed to produce Feta cheese: milk, rennet, microorganisms, and salt. These ingredients follow the specific process of gel formation, whey expulsion, acid production, and salt addition. Then, a period of ripening takes place [2].

Matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI-TOF MS) has been recently utilized as a tool to characterize microorganisms fast and accurately, while it has been used as a tool to identify bacteria, yeasts, mycobacteria, and molds [3]. The microbial identification through mass spectrometry is achieved by analyzing the expression of their intrinsic proteins. This mass spectral pattern of protein expression is compared with reference patterns in a database and can be distinguished by a large spectrum of proteins in order to classify closely related organisms at the species level [4].
This has as a result to generate a characteristic mass and intensity distribution of the mainly ribosomal proteins, representing a “molecular fingerprint”. Some advantages stemming from this technology include the increase in the reporting of some relatively rare species over traditional methods, the easy pretreatment of samples, the quick identification results, and the lower costs. At the moment, MALDI-TOF MS profiling is accepted as a universally valid identification method and is used in food microbial ecology studies, including those on dairy products [5]. A recent study used MALDI-TOF MS to identify non-starter lactic acid bacteria (NSLAB) isolates from four artisanal kinds of cheeses, namely Kefalotyri, Anthotyro, Xynotyri, and Touloumotyri, synthesized from raw goat and sheep milk. The identified lactic acid bacteria (LAB) species included *Lactobacillus brevis*, *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Lactobacillus paracasei*, *Leuconostoc mesenteroides*, *Enterococcus faecium*, *Lactococcus lactis*, and *Pediococcus pentosaceus* [6]. Albesharat et al. assessed the LAB strains in breast milk, feces of mothers and babies, and local foods (fermented) using genotypic tools such as 16S RNA gene sequencing, random amplified polymorphic DNA (RAPD) and MALDI-TOF [7].

Feta PDO is defined by its distinctive organoleptic features. Various microbial populations coexist, operate, and interact, providing through their metabolism, the development of taste, texture, aroma, safety, and shelf-life of the cheese [8]. These microbiota dynamics and changes control both organoleptic and biosafety characteristics, thus defining the quality of the product [9]. The microflora of cheeses differ through time and are based on the cheese type, as well as the environmental, processing, and aging conditions. Feta cheese microbiota are originated from the milk microbiota of indigenous sheep and goat breeds, the starter lactic acid bacteria and the secondary microflora [6]. The starter lactic acid bacteria (SLAB), are inoculated starter cultures, involved in acid production during manufacture and contribute to the ripening process. Secondary microorganisms are not responsible for acid production during manufacture, but generally play an important role during ripening as these bacteria contribute to the lipolysis and proteolysis of cheese [10]. The secondary microflora consist of non-starter lactic acid bacteria (NSLAB) which grow internally and are often unique to specific cheese varieties. Specifically, the microbial ecology of cheese is based on the complex interaction among SLAB and NSLAB [11].

The screening of microbiota which affects Feta’s unique organoleptic properties and reflects product quality, authenticity, and safety is critical, as this is the Greek cheese with the higher consumption worldwide. Cheese microbiota diversity and overall safety is affected by production regions, traditional techniques, and ripening time. This diversity can also determine the sensorial appeal and nutritional value for the consumer. The microbial profile of Feta PDO has been reported in a very limited number of scientific papers. Rantsiou et al. studied the microbiota in four Feta cheese samples from pasteurized milk obtained from retail shops by polymerase chain reaction–denaturing gradient gel electrophoresis (PCR-DGGE) and identified LAB and yeast species [12]. Additionally, Bozoudi et al. studied the NSLAB, by molecular methods such as random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) and pulsed-field gel electrophoresis (PFGE) on fresh and mature artisanal Feta cheese samples at three mountainous areas of Greece [13]. Furthermore, Spyrelli et al. used 16s rRNA gene sequencing to examine the effect of container material on fresh and mature Feta cheese bacterial diversity [14].

The main goal of our work was to define the standard core microbiota of Feta PDO cheeses through the identification of the bacteria by the culture-dependent technique MALDI-TOF MS Biotyper. Moreover, this study aimed to identify and report the effect of the ripening period before consumption, which is often a period of three to six months (i.e., 90 to 180 days) post-production, on the microbial diversity and chemical composition of Feta PDO cheese.
2. Materials and Methods

2.1. Sample Collection

A total of 23 Feta cheese samples were collected from various production sites (dairy establishments) in different geographical regions in Greece. All Feta cheese samples were prepared in accordance with the requirements of a Protected Destination of Origin (PDO) cheese [15]. All the samples were placed in sterile sample containers and transported to the laboratory for microbiological analysis. The cheeses were analyzed in two different ripening periods, three and six months post-production. Subsamples of each sample were kept separately in brine. Half of the samples were examined after three months (90 days) of ripening and the rest after six months (180 days) under refrigerated storage conditions (2 °C).

2.2. Chemical Analysis

The cheese samples were analyzed for their chemical parameters affecting the microbial stability and safety such as pH, moisture, and salt, but also for fat, protein, and total solids with a FoodScan™ Lab (FOSS, Hilleroed, Denmark). Sampling was prepared by removing 250 g portions from each cheese sample and grinding it. Each cheese sample was measured in triplicate.

The pH of the cheese samples was measured with a digital pH meter, (Hanna pH meter, Koper, Slovenia), equipped with a glass electrode, which was immersed in 10 g Feta cheese portions.

2.3. Microbiological Analysis

Cheese sampling was carried out by cutting sectors from the core of the blocked shaped cheeses, at a depth of approximately 5 cm, under sterile conditions. From the core of each cheese block, two samples (of 20 g each) were collected. The samples were transferred to stomacher bags, mixed with 180 mL sterilized buffered peptone water solution (LAB M, Bury, Lancashire, UK) and homogenized, used a stomacher (Bag Mixer R 400, Interscience, ST Nom, Saint-Nom-la-Bretèche, France) for 60 s at room temperature. For each sample, appropriate decimal in buffer peptone water dilutions (10⁻¹ to 10⁻⁶) were prepared and 0.1 mL of each dilution was spread, in duplicate on agar plates. For total mesophilic bacteria, surface coating was performed on PCA agar and incubated at 30 °C for 72 h. Samples were analyzed also for mesophilic and thermophilic lactococci on M-17 agar (Merck, Darmstadt, Germany) anaerobically at 30 and 37 °C for 48 h, respectively, and lactobacilli on de Man, Rogosa and Sharp (MRS) (Merck, Darmstadt, Germany) agar, at 30, 37, and 42 °C anaerobically. Kanamycin aesculin azide (KAA) agar (Merck, Darmstadt, Germany) for enterococci at 37 °C for 24 h, incubated both in aerobic and anaerobic environments [16]. The samples were also tested for microorganisms which affect the safety of the cheeses according to relevant legislation [17–21]. For this purpose, analysis performed on blood agar base (Merck, Darmstadt, Germany) and MacConkey agar plates (Merck, Darmstadt, Germany) for the detection of foodborne pathogens while cheese samples were also tested for the presence of the pathogenic bacteria Salmonella spp. and L. monocytogenes according to ISO 6579:2002 and ISO 11290-1, 2017, respectively [22,23]. For the bacterial enumeration, Miles and Misra plate method (surface drop) was performed and 10 µL of each dilution was also inoculated to the appropriate culture media and conditions [24].

Identification of Bacteria with MALDI-TOF MS Biotyper

Pure cultures were identified using Microflex LT MALDI-TOF MS (Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry/Bruker Daltonics, Germany). Five single colonies were taken directly from the nutrient agar plate and the sample preparation was performed according to the instructions of Bruker Daltonics and analyzed running Flexcontrol 3.4 software. Each sample was spotted in triplicate. For each sample, the MS signals were acquired in positive linear, according to the manufacture’s instruction. The obtained experimental MALDI-TOF mass spectrum profiles were then compared to
the reference spectra and the matching between them was expressed by a Biotype Log score. The Log (score) considers the number of matching peaks, the total number of peaks, the peak weight representing species specificity, and a correlation factor related to the matching peak intensity. All samples were measured on the mass spectrometer (Bruker Daltonics, Germany) using the method “MBT_AutoX” and the peaks were evaluated with the processing method “MBT_Process” using flexControl (version 3.4, Bruker Daltonics, Germany). A calibration took place by using the Bacterial Test Standard (BTS) from Bruker. All spectra were compared with reference spectra of the BDAL database (version 8.0) and main spectrum profiles (MSPs) created [25].

Dendrograms of identified strains were plotted with the BioTyper MSP Dendrogram Creation Standard Method (version 1.4) using the default settings: the distance measure set as correlation; the linkage set as average; the score oriented dendrogram was selected, the score threshold value for single organism was 300 and the score threshold value for related organisms was zero (0).

2.4. Statistical Analysis

Experimental data were analyzed by the “Paired samples T test”, of the SPSS v. 21 statistical package (IBM Corp. Armonk, NY, USA) at a significance level of 5%. For each examined sample, the values from the chemical analysis and the microbiological analysis were paired between the two ripening time periods (three and six months post-production). Pearson’s correlations coefficient (r) and significance (p of the two-tailed test of significance) were also determined for the parameters that showed significant differences (p < 0.05). Results were expressed as “Means ± Standard deviation”. Microbiology data were log-transformed (log10) prior to analysis.

3. Results

3.1. Chemical Characteristics of Feta Cheese

Table 1 presents the chemical characteristics of the Feta cheese samples. The increase of ripening time (three vs. six months) significantly affected the total solids (p = 0.043), proteins (p = 0.008), and PH values (p < 0.001) of the Feta cheese samples. The increased ripening period correlated to decreased total solids (r = 0.617; p = 0.001), protein (r = 0.683; p < 0.001), and PH (r = 0.780; p < 0.001). The other examined parameters (moisture, fat, salt) were not significantly affected (p > 0.05) by the different ripening periods. All Feta cheese samples had composition values within expected limits for commercial production.

| Parameters | 3 Months       | 6 Months       | P (2-Tailed) | Pearson Correlation r | Pearson Correl. Signif. P |
|------------|----------------|----------------|--------------|------------------------|--------------------------|
| Moisture (%) | 51.62 ± 2.41    | 51.86 ± 2.90    | 0.684        | -                      | -                        |
| Total solids (%) | 48.55 ± 2.20    | 47.57 ± 2.69    | 0.043        | 0.617                  | 0.002                    |
| Protein (%)  | 18.30 ± 1.96    | 17.30 ± 2.19    | 0.008        | 0.683                  | <0.001                   |
| Fat (%)      | 25.42 ± 2.15    | 24.60 ± 2.40    | 0.086        | -                      | -                        |
| Salt (%)     | 1.87 ± 0.06     | 1.87 ± 0.08     | 0.927        | -                      | -                        |
| pH           | 4.60 ± 0.09     | 4.50 ± 0.09     | <0.001       | 0.780                  | <0.001                   |

Values are expressed as “Means ± Standard deviations”.a,b Different superscript letters in rows indicate statistically significant differences (p < 0.05).

3.2. Microbiological Analysis of Feta Cheese

The results of the microbiological analysis are presented in Tables 2 and 3, and Figures 1–4. Total viable counts (TVC) did not differ significantly between the two ripening times (p = 0.193). The most abundant bacterial genus was the Lactobacillus which was detected in all samples (100%) of both ripening periods. Total Lactobacilli counts did not differ
significantly \((p = 0.497)\) between the two ripening periods. However, differences were identified in the detection of specific \textit{Lactobacilli} species, with the predominant being \textit{Lb. plantarum} (identified in 100.0% and 87.0% of samples from the three and six months of ripening periods, respectively), followed by \textit{Lb. brevis} (56.5% and 73.9%), \textit{Lb. paracasei} (56.5% and 39.1%), \textit{Lb. rhamnosus} (13.0% and 17.4%), \textit{Lb. paraplantarum} (4.3% and 26.1%), and \textit{Lb. curvatus} (8.7% and 8.7%). \textit{Lb. pentosus} (4.3%) and \textit{Lb. delbrueckii} (4.3%) and \textit{Lb. fermentum} (8.7%) were only identified in samples from the three months ripening period. Furthermore, \textit{Lb. coryniformis} (4.3%), \textit{Pediococcus acidilactici} (4.3%), and \textit{Pediococcus parvulus} (4.3%) were only identified in samples from the six-month ripening period. The second major bacterial genus was \textit{Enterococcus}, while the most abundant species were \textit{E. faecalis} (identified in 47.8% and 43.5% of samples from the three and six months of ripening periods, respectively), followed by \textit{E. faecium} (34.8% and 17.4%), \textit{E. durans} (13.0% and 17.4%), and \textit{E. malodoratus} (4.3% and 4.3%). The third major bacterial genus was \textit{Streptococcus}, with prominent species \textit{Streptococcus sal.} \textit{spp. thermophilus} (identified in 21.7% and 30.4% of samples from the three and six months of ripening periods, respectively).

### Table 2. Total viable counts and total lactic acid bacteria of Feta cheese samples according to their ripening time (three vs. six months).

| Ripening Period | Paired Samples \(t\) Test |
|-----------------|--------------------------|
|                 | 3 Months | 6 Months | \(P\) (2-Tailed) | Pearson Correlation \(r\) | Pearson Correl. Signif. \(P\) |
| Samples N\#     | 23       | 23       |                |                         |                           |
| Total Viable Counts (TVC) \((\text{Log}10\text{ cfu/g})\) | 7.316 ± 0.882 | 7.044 ± 0.656 | 0.193 | - | - |
| Total Lactic Acid Bacteria \((\text{Log}10\text{ cfu/g})\) | 7.408 ± 0.906 | 7.554 ± 1.142 | 0.497 | - | - |

Values are expressed as “Means ± Standard deviations”.

### Table 3. Occurrence (% appearance in samples and number of isolates) of strains isolated from Feta cheese samples according to their ripening time (three vs. six months).

| Appearance in Samples (%) | Number of Isolates |
|---------------------------|---------------------|
|                           | 3 Months | 6 Months | 3 Months | 6 Months |
| \textit{Lactococcus lactis} | 13.0     | 8.7      | 2        | 2        |
| \textit{Lactobacillus rhamnosus} | 13.0     | 17.4     | 4        | 5        |
| \textit{Lactobacillus curvatus} | 8.7      | 8.7      | 3        | 3        |
| \textit{Lactobacillus plantarum} | 100.0    | 87.0     | 31       | 47       |
| \textit{Lactobacillus paraplantarum} | 4.3      | 26.1     | 2        | 7        |
| \textit{Lactobacillus brevis} | 56.5     | 73.9     | 14       | 29       |
| \textit{Lactobacillus pentosus} | 4.3      | 0.0      | 2        | ND       |
| \textit{Lactobacillus fermentum} | 8.7      | 0.0      | 3        | ND       |
| \textit{Lactobacillus paracasei} | 56.5     | 39.1     | 13       | 14       |
| \textit{Lactobacillus coryniformis} | 0.0      | 4.3      | ND       | 2        |
| \textit{Lactobacillus delbrueckii} | 4.3      | 0.0      | 2        | ND       |
| \textit{Lactobacillus kefiri} | 4.3      | 0.0      | 1        | ND       |
| \textit{Enterococcus durans} | 13.0     | 17.4     | 6        | 4        |
| \textit{Enterococcus faecium} | 34.8     | 17.4     | 9        | 21       |
| \textit{Enterococcus faecalis} | 47.8     | 43.5     | 14       | 13       |
| \textit{Enterococcus malodoratus} | 4.3      | 4.3      | 1        | 1        |
| \textit{Leuconostoc fallax} | 0.0      | 4.3      | ND       | 1        |
| \textit{Staphylococcus saprophyticus} | 4.3      | 0.0      | 1        | ND       |
| \textit{Staphylococcus equorum} | 0.0      | 4.3      | ND       | 1        |
| \textit{Staphylococcus haemolyticus} | 0.0      | 4.3      | ND       | 1        |
| \textit{Staphylococcus epidermidis} | 0.0      | 13.0     | ND       | 2        |
| \textit{Streptococcus salivarius subsp. thermophilus} | 21.7     | 30.4     | 6        | 6        |
| \textit{Pediococcus pentosaceus} | 8.7      | 0.0      | 3        | ND       |
| \textit{Pediococcus acidilactici} | 0.0      | 4.3      | ND       | 2        |
Table 3. Cont.

| Appearance in Samples (%) | Number of Isolates |
|---------------------------|--------------------|
|                          | 3 Months | 6 Months | 3 Months | 6 Months |
| **Pediococcus parvulus**  | 0.0      | 4.3      | ND       | 2        |
| **Candida lusitaniae**    | 4.3      | 0.0      | 2        | ND       |
| **Kocuria kristinae**     | 0.0      | 4.3      | ND       | 1        |
| **Bacillus cereus**       | 0.0      | 4.3      | ND       | 1        |
| **Corynebacterium flavescens** | 0.0      | 4.3      | ND       | 2        |
| **Kluyveromyces lactis**  | 13.0     | 13.0     | 1        | 2        |
| **Micrococcus luteus**    | 0.0      | 4.3      | ND       | 1        |
| **TOTAL**                 | -        | -        | 117      | 173      |

1 Results of bacterial identification in appropriate culture media. 2 Results of mass spectra analysis (MALDI-TOF, MS). ND: Not Detected.

Figure 1. Krona chart presenting all isolates of microorganisms from Feta cheese samples after three months-ripening time.

In total, 253 out of 290 isolates (87.20%) were classified to five LAB genera as: for three months of ripening, two isolates of *Lactococcus lactis*, 75 isolates of Lactobacillus genus (10 species), 30 isolates of Enterococcus genus (4 species), and three isolates of *Pediococcus pentosaceus*. For six months of ripening, the LAB genera were classified as: two isolates of *Lactococcus lactis*, 107 isolates of Lactobacillus genus (7 species), 39 isolates of Enterococcus genus (4 species), one isolate for Leuconostoc genus (*Leuconostoc fallax*), and four isolates of *Pediococcus* genus (three species). Most of these LAB species belong to the Lactobacillus genus (75 isolates for three months of ripening and 107 isolates for six months of ripening out of 290 strains, 25.9% and 36.9% respectively).

Some other identified microbial species were found in small number of isolates and included *Kluyveromyces lactis* (identified in 13.0% and 13.0% of samples from the three and six months of ripening periods, respectively), *Lactococcus lactis* (13.0% and 8.7%), *Leuconostoc fallax* (0.0% and 4.3%), *Pediococcus pentosaceus* (8.7% and 0.0%), *Candida lusitaniae* (4.3% and 0.0%), *Kocuria kristinae* (4.3% and 0.0%), *Bacillus cereus* (0.0% and 4.3%), *Corynebacterium flavescens* (0.0% and 4.3%), and *Micrococcus luteus* (4.3% and 0.0%). Major pathogenic
bacteria (*Listeria monocytogenes*, *Salmonella spp*, *Enterobacteriaceae*, and *Coagulase-Positive Staphylococci*) were absent in all tested samples.

Figure 2. Dendrogram derived illustrating the hierarchical clustering of the isolates after a period of three months ripening, identified by MALDI-TOF-MS.

Figure 3. Krona chart presenting all isolates of microorganisms from Feta cheese samples after six months-ripening time.

Figures 2 and 4 represent the main spectra profiles (MSP) dendrograms at the ripening periods (three and six months post-production). These MSP-derived dendrograms illustrate
the hierarchical clustering and provide information concerning the relationship status between the isolates at a genus but also at a species level. The core microbiota (common microbial species) of Feta cheese during the two ripening periods (three and six months) are represented in Figure 5.

**Figure 4.** Dendrogram derived illustrating the hierarchical clustering of the isolates after a period of six months ripening, identified by MALDI-TOF-MS.

**Figure 5.** The core microbiota of Feta cheese.
4. Discussion

The aim of the study was to characterize the microbiota ‘fingerprint’ of Feta cheese using culture-dependent methods such as MALDI-TOF MS during the ripening process. In Greece, one of the most emblematic cheese is Feta cheese as it is a highly consumed PDO product, by far the most popular and therefore it is crucial to understand and reveal the whole microbiota present in the cheese. There is a growing interest in the identification of microbiota, present in the Feta cheese core, as this part of bacterial communities can enhance the shelf-life of the cheese, determine its safety, its functionality and quality, as well as its nutritional properties. The number of live microorganisms in cheeses can vary significantly, depending on product manufacturing process, conditions of storage, and ripening time. According to the CODEX standards for fermented milk products, the minimum number of starter culture bacteria in yogurt is 7.0 log cfu/g [26]. In our results, the total viable microorganisms ranged from 7.79 log cfu/g (for three months ripening) to 7.80 log cfu/g (for six months ripening).

Various bacterial communities participating in the microbiota of Feta cheese have been studied by researchers in the past [6,8,12,27], yet only one study, as it is mentioned before, provided some data regarding the dominant lactic acid bacteria of traditional Feta cheese. Cheese is a living ecosystem whose bacterial elements originate from the milk and the dairy establishment (equipment, procedures, facilities, etc.) [28,29]. These microorganisms are responsible for the course of the fermentations and it is their mutual interaction resulting to the sensorial characteristics of the cheese. Besides their technological properties mainly important to the industry, most of these bacteria have beneficial impact to human health, also known as probiotics. Hence, there are often a lot of reasons based on quality and health aspects that dairy microorganisms, and particularly the ones from the most popular products, should be identified and thoroughly studied.

MALDI-TOF MS is considered as a method of choice for analysis of complex mixtures of bacterial proteins and represents a simple, reliable, and cost-saving tool for the rapid taxonomic characterization of microflora in dairy products not only at the genus level, but also at the level of species and subspecies [30–32]. The enrichment of the available data bases with reference material could be the key point to increase the discriminatory ability of the method, in cases where there is the need to distinguish close-related phylogenetic strains. The technique of MALDI-TOF MS has been applied to identify lactic acid bacteria from French Maroilles cheese and achieved the identification of LAB strains from Maroilles cheese, made from raw and pasteurized milk. In another study, were analyzed the derivatives from fruits with a genotyping method such as 16S rRNA gene sequencing and the culture dependent method such as MALDI-TOF MS to identify LAB species. The identification match for both techniques was 86% [33,34].

Feta cheese, according to its standards, must ripen for at least two months before consumption. However, Feta cheese continues to ripen after that period. Accordingly, the present research studied and verified the microbiota of the cheese and their alterations during a prolonged ripening time, ranging from the three to six months. The Lactobacillus genus being the most abundant of LABs, plays a crucial role during fermentations due to their diverse fermentative properties (heterofermentative, facultative heterofermentative) as well as their lipolytic and proteolytic activity. Our results were in accordance with the study in French Maroilles cheese in which, among the three identified genera, namely Lactobacillus, Leuconostoc, and Enterococcus, Lactobacillus was the most prominent genus with the Lb. brevis, Lb. plantarum, Lb. curvatus, Lb. paracasei, Lb. rhamnosus, Lb. parabuchneri, and Lb. fructivorans species to be dominant in the Feta cheese microbiota [35]. Although LABs are the main group of bacteria in all samples, there were differences noted between the third and the sixth month of ripening. Lactobacillus pentosus, Lactobacillus delbrueckii, Lactobacillus fermentum, and Pediococcus pentosaceus have been detected only in samples of three months of ripening, while Lactobacillus corynformis, Leuconostoc fallax, and Pediococci (acidilactici and parvulus) were only detected in samples at six months of ripening. To determine the relationship between the bacterial species, a score-oriented MSP
dendrogram was generated. Based on a distance level of 300, seven groups were present in both ripening periods (Figures 2 and 4). It is noteworthy that three bacteria in species level, such as Lactobacillus brevis, Lactobacillus rhamnosus, and Pediococcus pentosaceus belong to different clusters while only Lactobacillus coryniformis belong to different clusters at the six-month period of ripening.

L. plantarum, the most dominant species in the present study, were also isolated from Feta cheese by previous research [36], found to exert superior technological properties and contribute to the sensorial characteristics of the cheese. According to our results, L. plantarum continue to be the predominant species after three and six months of ripening. Technological properties, including fast acidification and resistance to high salt concentration were observed also in the case of Lactobacillus paracasei, a dominant species in long-ripened cheeses like PDO Grana Padano [37], semi hard cheeses [38], and in brine-ripened cheeses like Teleme, Briza, Haloumi, and Domiati [39]. L. brevis are believed to thrive as the ripening evolves and contributes to the quality of the final product by producing biogenic amines [40]. Lc. lactis were the only species of this genus isolated from the Feta cheese samples. They are considered a major contributor to the formation of the curd by fast acidification, while affecting the quality of the end product and particularly the moisture content and the cheese softness by their caseinolytic, aminopeptidase, and esterase lipase activities [41]. Finally, but equally important, Lc. lactis prevent the growing of pathogens and spoilage bacteria [42]. These results were in accordance with the findings of other researchers like Uymaz et al. in the Turkish cheese Ezine, who studied selected LAB strains for their technological and antimicrobial properties against pathogenic or spoilage microorganisms [43].

Pediococci were not isolated from cheese samples as frequently as other LABs and the number of isolates of this genus confirm this. In our results, it should be noted that these bacteria (Pediococcus pentosaceus) were isolated from samples of three months ripening, while Pediococcus acidilactici and Pediococcus parvulus have been detected in samples of six months ripening. Obviously, their importance is due to the fact that they contribute to the organoleptic profile (flavor) of the cheese by the production of diacetyl and acetaldehyde. They also exert a proteolytic activity against peptides containing a proline group [44]. The only Leuconostoc species was L. fallax (1 strain), isolated from six-month Feta cheese samples. (Table 3). This species is heterofermentive, resistant to 5% salt concentration and can grow in pH values as low as 3.9 [45]. Leuconostoc species produce polysaccharides contributing to the texture and stabilization of the cheese [46], while they are aroma producers [47].

Enterococci, as a group are very often isolated from various types of cheese made of ovine or caprine milk, Feta among them [48] and particularly in brine-ripened cheeses. In our study, four species have been detected, such as Enterococcus durans, Enterococcus faecium, Enterococcus faecalis, and Enterococcus malodoratus. More than 30 species of the Enterococcus genus have been isolated from dairy products, yet there are not of the same technological capacity. E. faecalis, for example, exerts higher proteolytic and lipolytic activity than E. faecium and E. durans. According to Ambadoyanni et al. [49], enterococci from cheese can survive at low pH values and at exposure to high concentration of bile salts and some strains such as E. faecium, E. faecalis, and E. durans are the main producers of enterocins. Some of these bacteriocins can be grouped with typical bacteriocins produced by LABs, providing opportunities for more effective control of pathogenic bacteria [50]. Four staphylococcal species have been detected in Feta samples, all belonging to the coagulase negative staphylococci (CoNS) group. Three of them were present only in the six months ripened-cheese, while S. saprophyticus were detected only in three months ripened-cheese (Table 3). Usually, these bacteria are spoilage, originating from the ripening chamber or the packaging device and most probably could be a source of contamination of the produced cheese [51]. The Regulation does not refer to the occurrence of CoNS species in food. Food is often a reservoir of these bacteria, and their common occurrence results primarily from resistance to unfavorable environmental conditions during the production
processes, food storage, and high adaptation abilities of these micro-organisms [52]. It is worth mentioning that some CoNs strains such as *Staphylococcus equorum* are frequently involved in cheese ripening while specific strains of this microorganism could be used as a starter culture for smear ripened cheeses. Other staphylococci such as *S. saprophyticus*, *S. lentus*, *S. sciuri*, and *S. succinus subsp. casei* can also be involved in dairy fermentations [53], having an impact in the sensorial characteristics of the final products [54]. *Streptococcus salivarius* spp. *thermophilus* is an industrial strain used in starter cultures which participate in the formation of aroma (aroma compounds such as leucine, phenylalanine, and methionine-derived aroma compounds), flavor, and texture through the metabolic pathway of proteolysis, the biosynthesis of vitamins (B-group), as well as the production of extracellular polysaccharides [55]. Since the cheese sampling of the present study was performed in registered dairy establishments in which the production process is according to the EU legislation and the milk was pasteurized, it is possible that the detected *Streptococcus thermophilus* strains originated from starter cultures [56].

*Micrococcus luteus* (1 isolate) detected only in six months ripened cheese. Due to its antimicrobial compounds, was found antagonistic to a group of foodborne pathogens such as *Listeria monocytogenes* and *Salmonella spp.* It is part of the microflora of the surface of smear-ripened cheeses and can be found in the core part of the cheese [57].

Despite the presence of the beneficial microbiota in the core Feta cheese samples, which characterize their identity of the product and its quality properties, a small number of microbial isolates detected refer to possible spoilage commensals considered as markers of poor hygienic situations in the holistic process of Feta cheese production. *Bacillus cereus*, (one isolate) was detected in one of the six months ripening samples (Table 3). These bacteria are rare and sporulate to survive pasteurization temperatures. Their presence indicates poor sanitation [58]. *Candida lusitaniae* (2 isolates) and *Kluyveromyces lactis* (3 isolates) are opportunistic yeasts, considered as spoilage but mozzarella cheese, where *Candida lusitaniae* is part of the natural microbiota counting 4–6 log cfu/g [59,60]. *Corynebacterium* species (two isolates) are aerobic microorganisms usually growing on the surface of the cheese [61] and hence the presence of *C. flavescens* in the core of a cheese sample is probably due to cracks and breaches.

The microbiota of Feta cheese, include diverse genera and reflect similarities and differences among the two ripening periods (three vs. six months). The increase of ripening time significantly affected the total solids, proteins, and pH values of the cheese. The decrease of the protein content was contributed to the proteolytic system of LABs which takes place during the ripening process. The catabolism of amino acids through microorganisms (LAB), is producing aromatic compounds [41]. The rest examined chemical parameters (moisture, fat, salt) were not significantly affected by the ripening periods. In the microbiological status of Feta cheese, appear several differentiations in species level compared to the examined ripening periods, demonstrating its core microbiota, which plays the crucial role for the technological properties and the sensory characteristics of Feta cheese (Figure 5). Major pathogenic bacteria (*Listeria monocytogenes*, *Salmonella spp*, *Enterobacteriaceae*, and *Coagulase-Positive Staphylococci*) were absent in all tested samples, thus all batches were considered safe according to the European Union regulatory criteria. Therefore, the examined Feta cheese representative microbiota profile was not influenced by pathogenic factors.

5. Conclusions

The present study is, to our knowledge, the first attempt to characterize the core microbiota of Feta cheese. Microbiological analysis revealed a variety of microorganisms, most of them belonging to the Lactic Acid Bacteria group. MALDI-TOF MS is a powerful tool for microbiological identification with adequate accuracy and sensitivity to the level of species. It requires less preparation time than conventional or molecular methods, is faster and cost effective. Between the third and the sixth month of ripening, statistically significant changes were observed in chemical parameters (PH, protein, total solids), although minor
microbiological changes were detected in species level. The unique sensory properties, nutritional value, authenticity, quality, and safety of Feta cheeses are believed to be attributed to the microbial species present, their dynamics, and the functional interactions concerned. The results of this study contribute to a better understanding of the microbiota of Feta cheese and determine the core microbiota from which Feta cheese consists of. The study of Feta cheese microbiota is an important tool for development of its processing technology, leading in improvements not only in the quality characteristics, but mainly in unique identification markers.

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