Microbiological and Metagenomic Characterization of a Retail Delicatessen Galotyri-Like Fresh Acid-Curd Cheese Product

John Samelis [1,*] **, Agapi I. Doulgeraki** [2,*] **, Vasiliki Bikouli** [2] **, Dimitrios Pappas** [3] **and Athanasia Kakouri** [1]

[1] Dairy Research Department, Hellenic Agricultural Organization 'DIMITRA', Katsikas, 45221 Ioannina, Greece; kakouriathanasia@yahoo.gr

[2] Hellenic Agricultural Organization 'DIMITRA', Institute of Technology of Agricultural Products, 14123 Lycovrissi, Greece; mpvicky@otenet.gr

[3] Skarfi EPE—Pappas Bros Traditional Dairy, 48200 Filippiada, Greece; pappasbr@otenet.gr

[*] Correspondence: jsam@otenet.gr (J.S.); adoulgeraki@aua.gr (A.I.D.);
Tel.: +30-2651094789 (J.S.); +30-2102845940 (A.I.D.)

Abstract: This study evaluated the microbial quality, safety, and ecology of a retail delicatessen Galotyri-like fresh acid-curd cheese traditionally produced by mixing fresh natural Greek yogurt with 'Myzithrenio', a naturally fermented and ripened whey cheese variety. Five retail cheese batches (mean pH 4.1) were analyzed for total and selective microbial counts, and 150 presumptive isolates of lactic acid bacteria (LAB) were characterized biochemically. Additionally, the most and the least diversified batches were subjected to a culture-independent 16S rRNA gene sequencing analysis. LAB prevailed in all cheeses followed by yeasts. Enterobacteria, pseudomonads, and staphylococci were present as <100 viable cells/g of cheese. The yogurt starters *Streptococcus thermophilus* and *Lactobacillus delbrueckii* were the most abundant LAB isolates, followed by nonstarter strains of *Lactiplantibacillus, Lacticaseibacillus, Enterococcus faecium, E. faecalis*, and *Leuconostoc mesenteroides*, whose isolation frequency was batch-dependent. *Lactococcus lactis* isolates were sporadic, except for one cheese batch. However, *Lactococcus lactis*, Enterobacteriaceae, Vibrionaceae, *Salinivibrio*, and Shewanellaceae were detected at fairly high relative abundances culture-independently, despite the fact that their viable counts in the cheeses were low or undetectable. Metagenomics confirmed the prevalence of *S. thermophilus* and *Lb. delbrueckii*. Overall, this delicatessen Galotyri-like cheese product was shown to be a rich pool of indigenous nonstarter LAB strains, which deserve further biotechnological investigation.

Keywords: acid-curd cheese; Galotyri-like cheese; cheese microbiota; biochemical characterization; metagenomic analysis

1. Introduction

Acid-curd cheeses form a distinct group of soft, spreadable, low-pH (pH 3.5 to 5.0), but high-moisture (60–75%; water activity (aw) >0.98 to 0.99) dairy foods produced at industrial or artisan scale and consumed fresh or ripened [1,2]. Cottage cheese is the most popular and most often investigated acid-curd cheese variety worldwide [3,4]. Several traditional acid-curd cheese varieties are also produced in Greece from raw, thermized, pasteurized, or boiled ewe's and goat's milks, including five Protected Designation of Origin (PDO) cheese varieties: Anevato, Galotyri, Katiki, Kopanisti, and Pichtogalo Chanion [5–9]. Galotyri is considered the oldest and most popular Greek acid-curd cheese traditionally made using 'boiled' (>80 to 90 °C), naturally acidified milk in the regions of Epirus and Thessaly [5,10]. Samelis and Kakouri [11] pointed out major technological and microecological differences between an industrial-type and five artisanal Galotyri PDO cheese products marketed before or after the explosion of the Greek financial crisis, which has caused major reformations in the national food sector from 2010 onward. Specifically, the Greek regulatory authorities currently require all small and medium-size enterprises

producing PDO (dairy) foodstuffs or specialties to be certified by AGROCERT and their products to be packaged and labeled accordingly. Statistical production data of each Greek PDO food product should also be kept [11].

Particularly with regard to traditional PDO cheeses, the General Directorate of Food Quality Control and Assurance of the Hellenic Agricultural Organization 'DIMITRA' has increased inspections of products and plants in an attempt to preserve and protect PDO cheese authenticity. It has become pivotal to ensure that all certified brand-named PDO cheese products comply with the respective specifications described in article 83D of the Hellenic Code of Food and Beverages regarding the origin of the milk and the compositional and technological attributes of the final cheese [5,11]. Thus, according to its PDO description, the technology of authentic Galotyri cheese aims at the production of a uniform fresh curd following acidification of the 'boiled' milk with lactic acid bacteria (LAB) [5,12]. LAB can be added to ferment the 'boiled' or industrially pasteurized Galotyri cheese milk in the form of natural undefined starter cultures, including back slope techniques [10,13,14], or commercially defined starter cultures [11,15,16]. Rennet is often added to enhance curdling of the milk and to stabilize the curd [5,12], while draining, salting, and mainly ripening [17,18] further alter the microbial ecology, sensory characteristics, and total quality attributes of the artisanal Galotyri PDO cheese products [11,12].

It must be clarified that the manufacturing technology of Galotyri PDO cheese does not include any mixing steps of the fresh curd with other dairy products for 'external aromatization' purposes [5,12]. However, addition of fresh feta cheese trimmings at a 1:5 proportion in basic yogurt curds has been reviewed as a common empirical practice for traditional Galotyri cheese processors [19]. Samelis and Kakouri [11] opinioned that the 'arbitrary' dispersion in fresh Galotyri curds of feta trimmings should be neither permitted nor encouraged because it is in contradiction of the PDO certification of this authentic cheese [5]. In fact, following the recent increases in national food inspections, several new delicatessen Galotyri-like acid-curd cheese products have appeared on the Greek market with new trade names. Most of them are fresh yogurt spreads with feta cheese [11]. This more simplified and profitable acid-curd cheese making process also relies on traditional practices. In most Greek rural areas, excess ewe's/goat's milks, mainly in the summer, are preserved by making yogurt at home, which is strained and often mixed with trimmed white brined cheeses to consume as an appetizer. In addition to naturally fermented yogurt, fresh acid-curd cheeses and white brine cheeses produced in Greece, Bulgaria, and other Balkan or East Mediterranean countries since antiquity have been associated with beneficial effects on human health, attributed to the probiotic properties of their indigenous LAB [6,19,20]. Therefore, it is important to characterize the microbiota of Greek yogurt or of traditional Greek acid-curd cheese products prepared with yogurt in order to detect novel LAB strains with high biotechnological or probiotic potential for the development of novel functional foods.

An alternate traditional practice is to mix fresh naturally fermented Greek yogurt with naturally fermented and ripened whey cheese trimmings to prepare fresh acid-curd spread homogenates with pleasant, refreshing taste and aroma. The above type of fresh Galotyri-like cheese specialty, which may also have human health-promoting properties, has been commercialized locally, but its microbial quality, safety, and ecology have yet to be investigated. Therefore, the aim of this study was to characterize the natural microbiota of this retail delicatessen cheese product using classical microbiological methods and a culture-independent 16S rRNA sequencing metagenomic approach. The isolation and characterization of the natural LAB biota of this traditional acid-curd cheese of Epirus represent the first research tasks of BIO TRUST, an ongoing collaborative research project, which targets the development of novel indigenous starter, protective, or probiotic cultures for application in different traditional Greek cheese technologies [21].

2. Materials and Methods

2.1. Preparation and Sampling of Retail Galotyri-Like Cheese Products

Five sample portions (500 g each) of five individual batches of bulk ready-to-eat Galotyri-like acid-curd cheeses, distributed as "Galatyri" by the producer, were obtained from the Ioannina retail outlet shop of the Pappas Bros. traditional dairy (Skarfi E.P.E., Filippiada, Epirus, Greece). Samples were placed in plastic flexible containers with a lid and were transported to the microbiology laboratory of the Dairy Research Department in insulated ice boxes. Each 500 g sample represented one retail cheese batch. The five batches analyzed during this study were produced on independent and chronologically distant days, from December to March, corresponding to two annual ewe/goat milking periods. Each cheese batch was prepared by mixing 50 kg of a fresh uniform 'basal' curd of traditional Greek yogurt with 25 kg of a naturally fermented and ripened granular whey cheese, named 'Myzithrenio', traditionally produced as described below.

Briefly, the whey collected after processing of Graviera cooked hard cheese in the Pappas dairy plant, according to the standard manufacturing protocol described by Noutsopoulos et al. [22], was enriched with 5% raw ewe's milk and heated at 85–90 °C in a stainless-steel vessel over a gas burner to obtain a fresh whey cheese mass via the traditional manufacturing method. The whey–protein coagulum was transferred into perforated plastic cheese molds placed in a cooling room at 12 °C, drained for 48 h, dry-salted with ca. 3% edible sea salt, and filled into empty wooden cheese barrels. The barrels were closed firmly and turned upside down, while the cheese mass was left to acidify and ripen naturally at 18 °C for 30 days. Afterward, the mature 'Myzithrenio' cheese was distributed for bulk retail selling, directly from the barrel. Otherwise, relative to this study, the barrels were opened in the plant, and the ripened whey cheese mass was distributed in 5 kg portions into cryovac bags, which were vacuum-packaged (VP) to prevent growth of aerobic spoilage yeasts and molds and stored at 4 °C for 3–4 months. Periodically, the desired VP quantities of naturally acidified (pH 4.0–4.5) and ripened 'Myzithrenio' cheese were mixed proportionally with fresh yogurt, as described above, to obtain the fresh Galotyri-like acid-curd cheese product illustrated in Figure 1.

Figure 1. Traditional ready-to-eat (RTE) fresh Galotyri-like acid-curd cheese produced and retail-distributed by the Skarfi EPE-Pappas Bros. traditional dairy.

The basic yogurt curd was fresh and nondrained. It was traditionally made by seeding full-fat ewe's milk with a natural yogurt starter routinely prepared by batch circulation in the Pappas plant. The milk was heated at 90–92 °C and cooled at 48 °C to add the yogurt starter [23] diluted with water. The yogurt fermentation process lasted 3 h upon transferring the inoculated milk in a controlled chamber at 42 °C. We previously analyzed this type of natural yogurt starter and confirmed it to contain typical strains of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* [24], in agreement with relevant studies by others on the microbiology of yogurt and yogurt starters [20,23].

After the fresh yogurt curd and the ripened 'Myzithrenio' cheese were mixed, no further ripening was applied to the final RTE delicatessen cheese product (Figure 1).

In general, this Galotyri-like acid-curd cheese specialty is distributed for selling after preparation, is stored aerobically under refrigeration during retail distribution, and has an approximate retail shelf-life of 2 weeks before the manifestation of spoilage yeasts on the soft cheese surface. All present retail cheese samples were less than 1 week old at the time of analysis.

2.2. Cheese Analyses

All cheese samples were analyzed microbiologically and for pH within 2 h of their transportation to our laboratory. The pH was measured using a digital pH meter (Jenway 3510, Dunmow, Essex, UK). The glass electrode was immersed in the soft cheese mass after the microbiological sampling done as described below.

Each plastic container was opened near a Bunsen burner, and the cheese mass was stirred thoroughly with a sterile spoon spatula. Afterward, 25 g of cheese was homogenized with 225 mL of 0.1% *w/v* buffered peptone water (BPW) in a stomacher (Lab Blender 400, Seward, London, UK) for 60 s at room temperature. Serial decimal dilutions in 0.1% BPW were prepared, and duplicate 1 mL or 0.1 mL samples of appropriate dilutions were poured or spread on the total or selective agar plates. Unless otherwise stated, all diluents, enumeration agar media, and supplements were purchased from Neogen Culture Media (Heywood, Bury, UK).

All samples were analyzed for total bacteria, total mesophilic and thermophilic LAB, total mesophilic and thermophilic dairy (lactose-fermenting) LAB, enterococci, coliform bacteria, *Pseudomonas*-like bacteria, total and pathogenic (coagulase-positive) staphylococci, and yeasts. The agar media and incubation conditions were according to the analytical procedures described in previous studies [11,24,25]; they are summarized in Table 1. The lowest detection limit was set at 100 CFU/g. For coliforms and RFP+ staphylococci, the lowest detection limit was set at 10 CFU/g of cheese. The presence of natural *Listeria* spp. and *Salmonella* spp. contaminants was assessed by one-step culture enrichment of 25 g cheese samples [13,24]. Presumptive *Listeria* spp. or *Salmonella* spp. colonies on selective Palcam or Rambach plates were respectively identified using the API *Listeria* or the API 20E identification kits (BioMerieux, Marcy l' Etoile, France).

Table 1. Microbial populations (log CFU/g) and pH values of five retail individual batches of RTE Galotyri-like acid-curd cheese.

Microbial Group	Enumeration Medium/ Incubation Conditions	Cheese Batch A	Cheese Batch B	Cheese Batch C	Cheese Batch D	Cheese Batch E	Mean ± SD Values
Total viable cheese biota counts	Milk Plate Count agar (MPCA)/37 °C; 48–72 h; aerobically	8.60	8.59	7.78	6.89	7.94	7.96 ± 0.70
Total mesophilic LAB	MRS agar/30 °C; 72 h; Aerobically	7.72	7.49	7.32	7.00	8.01	7.51 ± 0.38
Total thermophilic LAB	MRS agar/45 °C; 48 h; anaerobically (in Gas-Pack jars)	7.82	7.13	5.15	7.19	6.43	6.74 ± 1.02
Total mesophilic dairy LAB (presumptive lactococci)	M17 agar/22 °C; 72 h, aerobically	7.87	7.54	7.74	7.04	7.84	7.61 ± 0.34
Total thermophilic dairy LAB (presumptive streptococci)	M17 agar/42 °C; 48 h; aerobically	8.27	8.62	7.68	7.34	8.82	8.15 ± 0.62
Enterococci	Slanetz and Bartley (SB) agar/37 °C; 48 h; aerobically	6.60	6.78	6.02	6.34	6.58	6.46 ± 0.29
Total staphylococci	Baird–Parker agar with egg yolk tellurite/37 °C; 48 h; aerobically	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00
Coagulase-positive staphylococci	Baird–Parker agar with RFP/37 °C; 18–24 h; aerobically	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00
Coliforms	Violet Red Bile (VRB) agar/37 °C; 24 h; double-layered	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00
Pseudomonas-like bacteria	Cephalothin–Fucidin–Cetrimide (CFC) agar; 25 °C; 48 h; aerobically	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00
Yeasts	Rose Bengal Chloramphenicol (RBC) agar/25 °C; 5 d; aerobically	6.13	5.96	6.92	5.96	5.81	6.16 ± 0.44
Cheese pH		3.84	4.09	3.91	4.28	4.44	4.11 ± 0.25

All cheese batches were free of *Salmonella* and *Listeria* species in 25 g culture-enriched samples; SD: Standard Deviation.

2.3. Isolation and Biochemical Characterization of the Cheese LAB Biota

The LAB types prevailing in each of the five cheese batches were determined by isolating five colonies from the highest dilution plate of each of the first six LAB-selective enumeration agar media listed in Table 1, namely, Milk Plate Count agar (MPCA)/37 °C (total cheese bacteria), MRS/30 °C and M17/22 °C (total mesophilic LAB), MRS/45 °C and M17/42 °C (total thermophilic LAB), and Slanetz and Bartley (SB) agar 37 °C (enterococci). Attention was paid to collect representative isolates of all macroscopically different colonies on each plate. In this manner, 150 presumptive LAB isolates (30/cheese batch) were collected. Colonies isolated from MPCA and M17 agar plates were transferred for growth to 10 mL of M17 broth. Colonies isolated from MRS and SB agar plates were transferred for growth to 10 mL of MRS broth. The tubes were incubated at 30 °C or 37 °C, depending on whether the isolates were from agar media incubated at 22 to 30 °C or 37 to 45 °C, respectively (Table 1). Following growth, all isolates were checked for purity on streaked MRS or M17 agar plates, and the purified isolates were stored in 5 mL of MRS broth with 20% glycerol at −30 °C. The stock LAB isolates were resuscitated in 10 mL of MRS or M17 broth at 30 °C or 37 °C for 24 to 72 h, and they were subcultured twice before testing.

The isolates were characterized biochemically according to established phenotypic criteria [26–28]. Test methods were according to our previous studies [11,24]. Briefly, all isolates were first tested rapidly for Gram stain and catalase reactions. Gram-positive and catalase-negative isolates were tested further for cell morphology by phase contrast microscopy, gas (CO_2) production from glucose, ammonia production from arginine, growth at 15 °C and 45 °C, growth in 6.5% salt, ability for typical growth on Kanamycin Aesclulin Azide (KAA) agar, and the fermentation of 13 sugars (2% v/w) in 96-well presterilized miniplates: L-arabinose, cellobiose, galactose, lactose, maltose, mannitol, melibiose, raffinose, ribose, sorbitol, sucrose, trehalose, and xylose (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Filter-sterilized sugar solutions (10% w/v; 40 μL) were placed into the wells first. Sugar-free basal MRS broth (pH 6.9–7.0), prepared from its basic constituents with 0.17 g/L of bromocresol purple (Sigma) added as a pH indicator, was used to prepare washed cell suspensions of the LAB isolates for inoculation of the miniplates (160 μL/well). All tests were done in duplicate, and the isolates were grouped at the genus or species level, according to key differentiating criteria [26–28].

2.4. DNA Extraction from Cheese Samples and 16S rRNA Sequencing of Microbiota

The cheese batches A and B were selected and subjected to characterization of the total bacterial biota culture independently, with the aim of comparing the two methods and gaining more information on the acid-curd cheese biodiversity. The Nucleospin Food kit (Macherey-Nagel, Düren, Germany) was used to extract the DNA (20 ng/μL) directly from the cheese samples (one sample per batch). Following DNA extraction, the V2–4–8 and V3–7–9 hypervariable regions of 16S rRNA gene were amplified using an Ion 16S Metagenomics kit (Thermo Fisher, Waltham, Massachusetts, USA). The sequencing of 400 bp amplicons was performed using the Ion Torrent PGM by CeMIA SA (https://cemia.eu/) (Larissa, Greece) (accessed on 17 May 2019). After removing the chimeras and noises, the Ion Reporter software was used to analyze the sequences, and the Nucleotide Basic Local Alignment Search Tool (BLASTn) against the NCBI database (www.ncbi.nlm.nih.gov) (accessed on 9 August 2019) was used to taxonomically classify the operational taxonomic units (OTUs) at >97% similarity level. The GenBank Bioproject ID is PRJNA722121. A *t*-test was applied to estimate the significant differences (95% confidence level) among OTU abundances. In addition, Venn diagrams were prepared using the online platform Venn 2.1 [29] to estimate the bacterial relationships at the family, genus, and species level shared by the two cheese batches.

3. Results and Discussion

3.1. Microbiological Attributes and pH Variation of Retail Galotyri-Like Acid-Curd Cheeses

The results of the microbial quantification analyses along with the pH values of the five cheese batches are shown in Table 1. Results are presented separately for each batch because, in many cases, populations grown on the same media varied greatly (Table 1). This indicated a high microbiological variability between the cheese batches, which prevented their treatment as replicate samples for statistical analyses. Nevertheless, to specify general trends, the mean and standard deviation values are also presented in Table 1.

Starting from the pH data, mixing of fresh natural Greek yogurt curds (pH 3.7–4.0; according to the routine plant measurements) with ripened 'Myzithrenio' whey cheese granules (pH 4.0–4.5; as above) at an approximate 2:1 ratio resulted in acidic (pH 4.11 ± 0.25) RTE Galotyri-like acid-curd cheese products, as desired technologically. However, the high variation in the resultant pH between batches (Table 1) indicated that this acid-curd cheese is a nonstandardized delicatessen dairy product.

Microbiologically, LAB prevailed in all batches followed by yeasts (Table 1). This finding was in general accordance with previous quantification data of the total LAB and yeast population levels in Galotyri PDO [10–12] and other traditional (Greek) acid-curd cheese products [6–9]. Particularly acid-tolerant spoilage yeasts are favored for growth under the low-pH conditions prevailing in fresh acid-curd cheeses after completion of the LAB fermentation process, and they most often become the terminal spoilers of traditional, fresh or ripened, acid-curd cheeses stored under refrigeration [1,10,30].

Major batch-to-batch variations existed in the populations of total thermophilic and/or mesophilic LAB grown on MPCA, M17, and MRS agars at incubation temperatures from 22 to 45 °C. Specifically, batches A, B, and E had higher LAB populations on M17 agar at 42 °C than batches C and D. Furthermore, batches A and B had the highest total viable counts on MPCA at 37 °C, as opposed to batch D which had the lowest total viable counts.

Overall, the LAB quantification results suggested that the natural yogurt starter strain(s) of *S. thermophilus* prevailed in batches A, B, and E, since *S. thermophilus* and other streptococci are favored for growth on M17 and MPCA agars at 37 to 45 °C. Conversely, the populations of mesophilic dairy LAB, whose growth is favored on M17 agar at 22 °C and MRS agar at 30 °C, were more stable within 7 to 8 log CFU/g in all cheese batches (Table 1). Mesophilic LAB in Galotyri-like acid-curd cheese likely originated from the ripened 'Myzithrenio' whey cheese rather than from the fresh yogurt curd, which should be a synergic natural culture of *S. thermophilus* and thermophilic lactobacilli [20,23,24].

However, major and quite randomized fluctuations also existed in the level of thermophilic *Lactobacillus* selectively enumerated on MRS agar at 45 °C. Their levels ranged from 7 to 8 log CFU/g in batches B, D, and mainly A, whereas batches E and C showed equal to lower thermophilic LAB levels, respectively, than those of enterococci on SB agar at 37 °C (Table 1). In previous studies, enterococci were the only LAB group isolated from MRS agar at 45 °C when thermophilic dairy lactobacilli in milk or cheese were few or absent [31]. Compared to the other LAB genera, enterococcal populations were subdominant by 1–2 log units in all cheese batches (Table 1). However, their counts (6.0–6.8 log CFU/g) were still high for a Galotyri-like acid-curd (pH < 4.5) cheese [11].

Total staphylococci and aerobic Gram-negative, *Pseudomonas*-like bacteria were below 2 log CFU/g. Coliforms and pathogenic (RFP+) staphylococci were below 1 log CFU/g in all cheese batches. Moreover, neither *Salmonella* nor *Listeria* spp. were detected in 25 g samples of cheese after culture enrichment. Hence, the hygienic quality and safety of all RTE cheese products was fairly good. The potent presence of hemolytic and/or antibiotic-resistant strains within the *Enterococcus* biota was the only concern [32,33]. Indeed, several previous *E. faecalis* isolates from PDO Galotyri cheese harbored the cytolysin gene, while one *E. faecalis* strain genotype was strongly β-hemolytic [33]. Thus, regardless of their strong antilisterial activity, all previous *E. faecalis* isolates from Galotyri cheese were a priori excluded from dairy (food) applications.

3.2. Biochemical Characterization and Distribution of the LAB Biota in Galotyri-Like Cheeses

In total, 145 out of the 150 isolates recovered from the five cheese batches were Gram-positive, catalase-negative, nonsporogenic bacteria, presumably LAB (Table 2). The remaining five colonies were catalase-positive bacteria or yeasts isolated from the cheese batches C and D (Tables 2 and 3).

Table 2. Biochemical characterization and grouping of the 145 LAB isolates from RTE Galotyri-like acid-curd cheese at the genus/subgenus level in accordance to their numerical distribution in each of the five cheese batches analyzed, and their total percent isolation frequency.

LAB Genus/Subgenus	Basic Differentiating Characteristics							Cheese Batch					Total Isolates (% Isolation Frequency)
	MA	CO_2	NH_3	15 °C	45 °C	6.5%	KAA	A	B	C	D	E	
Mesophilic, facultative heterofermentative *Lactobacillus*	R	−	−	+	−/V	+/++	−/+	7	9	0	15	3	34 (23.4)
Thermophilic, obligatory homofermentative *Lactobacillus*	R	−	−	−	+	−	−	5	5	0	5	0	15 (10.3)
Lactococcus (arginine-negative)	C	−	−	+	−	−	−	1	0	10	0	0	11 (7.6)
Lactococcus (arginine-positive)	C	−	+	+	−/V	−/+	−	0	0	2	0	1	3 (2.1)
Thermophilic *Streptococcus*	LC	−	−	−	+	−	−/V	10	4	2	2	5	23 (15.9)
Enterococcus	C	−	+	+	+	++	++	4	10	14	5	11	44 (30.3)
Leuconostoc-like bacteria	CB	+	−	+	−/V	+	−	3	1	0	0	9	13 (9.0)
Obligatory heterofermentative *Lactobacillus* or *Weissella* (arginine-positive)	R	+	+	+	−/V	V	V	0	1	0	0	1	2 (1.4)
Total LAB isolates								30	30	28	27	30	145

MA, microscopic appearance as rods (R), cocci (C), large cocci (LC), or coccobacilli (CB); CO_2, gas production from glucose; NH_3, ammonia production from arginine; 15 °C/45 °C, growth at 15 °C or 45 °C; 6.5%, growth in 6.5% sodium chloride; KAA, growth on Kanamycin Aesculin Azide agar; +, positive reaction; −, negative reaction; ++, strong positive reaction; V, variable reaction.

Table 3. Numerical distribution of the 145 LAB isolates from RTE Galotyri-like acid-curd cheese biochemically characterized at the genus/subgenus level and the remaining five non-LAB or yeast isolates in association with the selectivity of their enumeration/isolation agar media.

LAB Genus/Subgenus	Growth/Isolation Agar Medium						
	MPCA/ 37 °C	M17/ 22 °C	M17/ 42 °C	MRS/ 30 °C	MRS/ 45 °C	SB/ 37 °C	Total Isolates
Mesophilic *Lactobacillus*	6	11	3	11	−	3	34
Thermophilic *Lactobacillus*	−	−	−	−	15	−	15
Lactococcus	4	5	2	3	−	−	14
Streptococcus	6	−	17	−	−	−	23
Enterococcus	4	3	3	2	10	22	44
Leuconostoc-like bacteria	2	4	−	7	−	−	13
Heterofermentative *Lactobacillus*	1	−	−	1	−	−	2
Total LAB isolates	23	23	25	24	25	25	145
Non-LAB (catalase +) bacteria	−	1	−	−	−	−	1
Yeasts	2	1	−	1	−	−	4
Total cheese isolates	25	25	25	25	25	25	150

Abbreviations for the growth/isolation agar media are given in Table 1; the LAB genera/subgenera in the right column are listed in accordance to their biochemical characterization in Table 2.

The 145 LAB isolates were assigned to eight distinct biochemical groups at the genus level on the basis of the microscopic appearance and ability of each isolate to produce CO_2 from glucose and NH_3 from arginine, as well as to grow at 15 and/or 45 °C, in 6.5% NaCl, and on KAA agar (Table 2). According to their reactions, most (30.3%) of the isolates were characterized as *Enterococcus* followed by mesophilic, facultative heterofermentative *Lactobacillus* (23.4%), thermophilic *Streptococcus* (15.9%), and thermophilic homofermentative *Lactobacillus* (10.3%). Fewer isolates belonged to the genera *Lactococcus*, *Leuconostoc*, or *Weissella* and/or the obligatory heterofermentative *Lactobacillus* group (Table 2). However, at this point, it needs to be emphasized that the percentage isolation frequency of each LAB group in Table 2, calculated on the basis of the net number of five isolates from each of the first six LAB-selective enumeration agar media in Table 1, does not represent the actual population density of each same LAB group in each of the five cheese batches. Indeed, enterococci had the highest isolation frequency overall (Table 2) despite the fact that their populations were subdominant of the populations of most other LAB genera by 1–2 log units in all batches (Table 1). However, half of the *Enterococcus* isolates were collected from their selective SB agar plates, while the other half were random isolates from the MPCA/37 °C, M17/22 °C, MRS/30 °C, and mainly the MRS/45 °C agar plates. It is well documented that dairy (lactose-fermenting) enterococci promote excellent growth on all the above agar media at any incubation temperature in the range from 15 to 45 °C [31].

Therefore, to realize the importance of the media selectivity effects, the isolates of each of the cheese LAB genera or groups in Table 2 were tabulated in accordance with their growth agar medium (Table 3). Clearly, *Enterococcus* isolates were collected from all six isolation agar media, in full contrast to thermophilic lactobacilli isolated from MRS/45 °C agar only. Thermophilic streptococci, inclusive of *S. thermophilus*, were isolated from M17/42 °C and less often from the MPCA/37 °C agar plates only, whereas lactococci were isolated from all media, except for MRS/45 °C and SB/37 °C (Table 3). Notably, three mesophilic *Lactobacillus* isolates were recovered from SB/37 °C agar. This was not surprising because several mesophilic nonstarter lactobacilli, particularly from the *Lb. plantarum* group, have been shown to grow with a 24 to 48 h delay on SB (as whitish colonies; discriminated from the red-brown *Enterococcus* colonies) and KAA (as black colonies, like enterococci) at 37 °C, thus compromising the selectivity of the enterococcal media [11,31].

Enterococci and mesophilic lactobacilli, i.e., *Lb. plantarum* and *Lb. casei* groups, are among the most resistant nonstarter LAB of cheese, particularly able to survive long ripening periods [24,34–36] and grow on all LAB-selective growth media (Table 3) at or below 45 °C [31]. *Leuconostoc*-like bacteria, gas-forming lactobacilli, and the non-LAB and yeast isolates were recovered from MPCA, M17, and MRS agar plates incubated at 37 °C or below. In summary, MRS/45 °C and MPCA/37 °C were the most and the least LAB-selective agar media, respectively, under the conditions of this study (Table 3).

Based on these considerations, the major batch-to-batch variations noted among the five cheeses with regard to the size of thermophilic and mesophilic LAB populations (Table 1) were reflected in the type and numerical distribution of the LAB genera isolated from each batch (Table 2). Specifically, thermophilic streptococci were isolated from all batches. However, they were dominant in batch A, followed by batches B and E. In contrast, mesophilic lactobacilli were dominant in batch D, whereas batch C was the only cheese without detectable *Lactobacillus* spp., instead dominated by lactococci and enterococci (Table 2). Enterococci were isolated from all cheese batches. However, their isolations were more numerous from batches C, E, and B for reasons explained above regarding the selectivity of the isolation agar media (Table 3). *Leuconostoc*-like bacteria prevailed in batch E only. Lastly, in full accordance with their population sizes grown on the selective MRS/45 °C agar plates (Tables 1 and 3), cultivable thermophilic dairy lactobacilli were only recovered from the batches A, B, and D (Table 2).

On the basis of their sugar fermentation profiles, the 145 cheese LAB isolates were further characterized to the species level and, when possible, they were discriminated

at the subspecies or the strain biotype level. The biochemical characterization results, accompanied by the numerical distribution of each LAB species, subspecies, or biotype in the cheese batches A to E, are summarized in Table 4 for *Enterococcus*, Table 5 for mesophilic LAB (i.e., mesophilic *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and gas-forming *Lactobacillus*) and Table 6 for thermophilic dairy *Streptococcus* and *Lactobacillus*. For table simplification, the biochemical LAB species characterization of each group is listed in the footnote of Tables 5 and 6.

Table 4. Biochemical characterization of 44 *Enterococcus* spp. isolates from five retail RTE Galotyri-like acid-curd cheese batches and their numerical distribution in each cheese batch.

Biochemical Test	Species Identification (Biotypes)								
	Enterococcus faecium				*Enterococcus faecalis*				
	A1	**A2**	**A3**	**A4**	**B1**	**B2**	**B3**	**B4**	**B5**
Fermentation of:									
Maltose	+	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+	−
Lactose	+	+	+	+	+	+	+	+	+
Ribose	+	+	+	+	+	+	+	+	+
L-Arabinose	+	+	+	+	−	−	−	−	−
Xylose	−	−	−	−	−	−	−	−	−
Raffinose	−	−	−	+	−	−	1/2	+	2/6
Melibiose	8/9	10/11	−	+	−	−	+	+	−
Sucrose	+	−	−	+	+	−	+	+	+
Cellobiose	+	+	+	+	+	+	+	+	+
Trehalose	+	+	−	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+
Sorbitol	−	−	−	+	+	+	+	−	5/6
Melezitose	NT	NT	−	−	NT	NT	NT	+	NT
Total No of Isolates	9	11	3	1	8	2	2	2	6
Batch A	3	0	0	0	0	0	0	0	1
Batch B	1	2	3	0	0	0	1	2	1
Batch C	0	3	0	0	7	2	1	0	1
Batch D	2	2	0	0	1	0	0	0	0
Batch E	3	4	0	1	0	0	0	0	3

+, positive reaction; −, negative reaction; 8/9, 8 out of 9 isolates in the group were positive; NT, not tested.

Characterization of the 44 *Enterococcus* isolates at the species level was based on the biochemical identification key of Manero and Blanch [28] and the methodology used by Vandera et al. [24]. Accordingly, the present isolates were assigned to two species only: *E. faecium* (24 isolates; biotypes A1–A4) and *E. faecalis* (20 isolates; biotypes B1–B5) (Table 4). The key differentiating characteristic between them was the ability of all *E. faecium* isolates to ferment L-arabinose, unlike all *E. faecalis* isolates [28]. Beyond this key differentiation, all typical *E. faecium* isolates were included in the biotypes A1 and A2, which differed in the ability of the former isolates to ferment sucrose. The most atypical phenotypic feature of the *E. faecium* biotype-A3 isolates was their inability to ferment trehalose, whereas the single biotype-A4 GLP-217 isolate showed the richest sugar fermentation profile (data not shown), including sorbitol and raffinose (Table 4). Although biochemically identical to *E. faecium* KE102 genotyped by Vandera et al. [24], this particular isolate from batch E might be an *E. gallinarum* or *E. raffinosus* strain; additional biochemical tests and genotyping are required to elucidate this. Regarding *E. faecalis*, typical mannitol-, sucrose-, and sorbitol-positive isolates of this species were included in biotypes B1 and B3, while biotype-B2 included atypical sucrose-negative isolates. The most atypical sorbitol-negative and/or mannitol-negative *E. faecalis* isolates were grouped in biotypes B4 and B5, respectively (Table 4). they also require additional biochemical and molecular characterization because they possibly represent very atypical strain biotypes of *E. durans* or closely related species of the *E. faecium/durans* genomic group [24,28]. Regarding the numerical distribution of the

isolates, *E. faecium* was more manifest in batch E and *E. faecalis* was more manifest in batch C. Batches A and B were the least and most diversified cheeses, respectively, in *Enterococcus* biotypes (Table 4).

Table 5. Biochemical characterization of 66 mesophilic LAB isolates from five retail RTE Galotyri-like acid-curd cheese batches and their numerical distribution in each cheese batch.

Biochemical Test	Group Identification (Subgroups/Biotypes)										
	Mesophilic *Lactobacillus*				*Lactococcus*			*Leuconostoc*		*Lactobacillus* (Gas-Forming)	
	C1	C2	C3	C4	D1	D2	D3	E1	E2	E3	E4
CO_2 from glucose	−	−	−	−	−	−	−	+	+	+	+
NH_3 from arginine	−	−	−	−	−	−	+	−	−	+	+
Fermentation of:											
Maltose	+	+	+	+	+	+	+	8/12	+	+	+
Mannitol	+	+	+	+	−	+	+	2/12	(+)d	−	+
Lactose	+	+	+	+	+	+	+	+	+	+	−
Ribose	+	+	+	+	4/5	2/6	+	−	+	+	+
L-Arabinose	−	−	−	+	−	−	−	4/12	+	+	+
Xylose	−	−	−	−	−	−	−	+	+	+	+
Raffinose	+	−	−	−	−	−	−	9/12d	−	−	−
Melibiose	+	−	+	+	−	−	−	+	+	−	+
Sucrose	+	+	+	+	−	+	1/3	+	−	−	−
Cellobiose	+	+	+	+	NT	NT	NT	NT	−	−	+
Trehalose	+	+	+	+	+	+	+	+	−	−	−
Galactose	+	+	+	+	+	+	+	+	+	+	+
Sorbitol	4/17	1/8	4/8	−	−	−	−	−	−	−	−
Melezitose	+	+	+	+	NT	NT	NT	NT	−	−	−
Total No of Isolates	17	8	8	1	5	6	3	12	1	1	1
Batch A	0	3	4	0	1	0	0	3	0	0	0
Batch B	3	3	2	1	0	0	0	1	0	1	0
Batch C	0	0	0	0	4	6	2	0	0	0	0
Batch D	13	0	2	0	0	0	0	0	0	0	0
Batch E	1	2	0	0	0	0	1	8	1	0	1

+, positive reaction; −, negative reaction; (+) weak reaction; d, delayed reaction; 4/17, four out of 17 isolates in the group were positive; NT, not tested. Biochemical LAB species identification of each subgroup or strain biotype: C1: *Lactobacillus plantarum/paraplantarum*; C2: *Lactobacillus paracasei*; C3: atypical *Lactobacillus plantarum*/paraplantarum; C4: atypical *Lactobacillus plantarum*; D1: *Lactococcus lactis* subsp. *cremoris* (commercial starter strain/s); D2: *Lactococcus lactis* subsp. *lactis* (commercial starter strain/s); D3: *Lactococcus lactis* subsp. *lactis* (NH3-positive; xylose-negative; possibly starter strain); E1: *Leuconostocmesenteroides* group; E2: unidentified *Leuconostoc* spp.; E3: atypical *Lactobacillus brevis* or possibly *Weissella* sp. (NH3-positive); E4: *Lactobacillus brevis*.

Ιn total, 26 of the 34 (76.5%) mesophilic *Lactobacillus* isolates were characterized as rather atypical nonstarter dairy strain biotypes (C1, C3, and C4) of the *Lb. plantarum* group (Table 5). All fermented melibiose, mannitol, and of course lactose. However, only the single biotype C4 isolate fermented L-arabinose, while it failed to ferment sorbitol. Similarly, several of the L-arabinose-negative isolates of C1 and C3 biotypes failed to ferment sorbitol. Moreover, only the most prevalent L-arabinose-negative C1 biotype fermented raffinose (Table 5). The observed high degree of heterogeneity in the sugar fermentation pattern is uncommon for typical *Lb. plantarum* strains, which ferment raffinose, sorbitol, and most often L-arabinose [24,26,31]. Hence, biotypes C1, C3, and C4 are presently classified as members of the *Lb. plantarum/paraplantarum* cluster in Table 5. None of them fermented D-xylose, as *Lb. pentosus* typically does [26]. Differentiation of *Lb. plantarum*, *Lb. paraplantarum*, and *Lb. pentosus* by phenotypic criteria is difficult; actually, it requires a multiplex PCR assay with specific primers targeting the heterogeneity of the *rec*A gene [24,37]. Lastly, the C2 biotype included eight melibiose-negative isolates which matched the *Lb. paracasei* strains previously found to prevail in mature Graviera cheeses produced in Pappas plant. Seven isolates did not ferment sorbitol (Table 5), like the indigenous, least prevalent biotype II of *Lb. paracasei* isolates from mature Graviera cheeses [24,31]. Batch B also was the most

diversified batch with regard to the number of mesophilic *Lactobacillus* biotypes isolated (Table 5).

Table 6. Biochemical characterization of 38 thermophilic LAB isolates from five retail RTE Galotyri-like acid-curd cheese batches and their numerical distribution in each cheese batch.

Biochemical Test	Group Identification (Subgroups/Biotypes)					
	Thermophilic *Lactobacillus* sp.		Thermophilic *Streptococcus* sp.			
	Z1	Z2	H1	H2	H3	H4
Fermentation of:						
Maltose	−	5/7	(2/20)	+	+	+
Mannitol	−	+	−	+	+	+
Lactose	+	+	+	+	+	+
Ribose	−	4/7	(4/20)	+	+	+
L-Arabinose	−	3/7	−	+	−	−
Xylose	−	−	−	−	−	−
Raffinose	−	−	−	+	−	−
Melibiose	−	1/7	−	+	+	−
Sucrose	1/8	4/7	+	+	+	+
Cellobiose	NT	NT	−	+	−	+
Trehalose	2/8	6/7	−	+	+	+
Galactose	−	+	(2/20)	+	−	+
Sorbitol	−	−	−	−	−	−
Melezitose	−	−	−	+	+	+
Total No of Isolates	8	7	20	1	1	1
Batch A	3	2	10	0	0	0
Batch B	3	2	1	1	1	1
Batch C	0	0	2	0	0	0
Batch D	2	3	2	0	0	0
Batch E	0	0	5	0	0	0

+, positive reaction; −, negative reaction; 2/8, two out of eight isolates in the group were positive; (2/20), two out of 20 isolates showed a weak positive reaction; NT, not tested. Biochemical LAB species identification of each subgroup or strain biotype: Z1: *Lactobacillus delbrueckii* subsp. *bulgaricus* (natural yogurt starter strain/s); Z2: *Lactobacillus delbrueckii* subsp. *lactis* (mixed starter strains); H1: *Streptococcus thermophilus* (natural yogurt starter strain/s); H2–H4: unidentified *Streptococcus* (possibly atypical multi-fermenting strains of the *Streptococcus salivarius/thermophilus* group occurring as natural contaminants in the basic yogurt curd).

As mentioned, lactococci were found to be predominant in the cheese batch C only, while they were sporadically isolated from the same growth culture media used in batches A and E. The majority (85.7%) of *Lactococcus* isolates were arginine-negative, divided in two distinct biotypes, D1 and D2 (Table 5), representing typical industrial CSC starter strains of *Lc. lactis* subsp. *cremoris* (D1) and *Lc. lactis* subsp. *lactis* (D2), respectively. Conversely, biotype D3 included typical arginine-positive and quite oligo-fermenting *Lc. lactis* subsp. *lactis*, which also matched industrial CSC starter strains imported in Greece. Specifically, none of them fermented D-xylose (Table 5), as most wild plant-derived *Lc. lactis* isolates do [38], including the nisin A-producing *Lc. lactis* subsp. *cremoris* genotype (strains M78 and M104) from Greek raw milk [39], currently applied in commercial Graviera cheese production in the Pappas plant [22].

The remaining 15 mesophilic heterofermentative LAB isolates, preliminarily characterized as *Leuconostoc*-like bacteria, arginine-positive *Weissella*, or gas-forming *Lactobacillus* (Table 2), fermented D-xylose strongly (Table 5). Twelve of them (80%) formed a very heterogeneous biochemical group E1 which was assignable to *Leuconostoc mesenteroides* despite the variability of the isolates in several sugar fermentation reactions [27]. The remaining three were single gas-forming isolates. GL34 (biotype E3) and GLP205 (biotype E4) seemed to be *Lb. brevis* strain variants. GLP212 (biotype E2) resembled strain GLP205 in most sugar fermentation reactions, including the key negative reactions with trehalose and sucrose; however, it did not show an arginine-positive reaction, which is required for assignment to the *Lb. brevis* group [26]. Therefore, at present, GLP212 should be considered

an unidentified *Leuconostoc* sp. (Table 5). Molecular taxonomic approaches are required to accurately identify the gas-forming E1–E4 LAB groups at the species level.

All Galotyri-like acid-curd cheese isolates of thermophilic dairy lactobacilli (Table 6) were assigned to *Lb. delbrueckii*, specifically to the subspecies *bulgaricus* (Z1) and *lactis* (Z2), respectively [26]. Probably all were strain constituents of the natural yogurt starter [23,24]. None of the isolates were lactose-negative (Table 6) and, thus, the isolates were unable to be assigned to *Lb. delbrueckii* subsp. *delbrueckii* [26]. Moreover, most (87%) streptococci from all batches were typical isolates of *S. thermophilus* starter strain/s [11] since all fermented lactose and sucrose strongly, while few gave a very weak, practically negative, fermentation reaction with ribose, maltose, and most importantly galactose (biotype H1 in Table 6). The latter finding indicated that the natural yogurt starter used to make the basic yogurt curd in the plant did not contain 'new-generation' galactose-positive *S. thermophilus* starter strains, which were increasingly detected in industrial and small-size traditional Greek dairy plants and products during the last decade [11]. Most probably, these are also imported CSC *S. thermophilus* strains that gained the ability to ferment galactose via genetic manipulation [40–42]. The remaining three *Streptococcus* (biotypes H2, H3 and H4), also isolated from the most diversified batch B, had more complex sugar fermentation profiles than *S. thermophilus* biotype H1 (Table 6). They were single isolates of either autochthonous streptococci from animals or indigenous strains of the *S. salivarius/thermophilus* group derived from the basal yogurt curd or, less likely, the ripened 'Myzithrenio' cheese. Additional polyphasic identification tests are in progress to elucidate the species identity and evaluate the hygienic safety of these sporadic *Streptococcus*-like isolates from the delicatessen Galotyri-like cheese.

3.3. Bacterial Communities Estimated by 16S rRNA Amplicon Sequencing

DNA was directly extracted from two cheese sample portions, the respective Galotyri-like acid-curd cheese batches A and B (Tables 1–6), to estimate the bacterial communities present in cheese and figure out potential differences between the two batches. Sequencing analysis revealed the presence of 19 different families. Specifically, 13 of the 19 families had a relative abundance >0.2% in at least one of the two batches (Figure 2), while 18 and 14 of them were detected in batches A and B, respectively (Figure 3a). However, statistical analysis based on the relative abundances of detected OTUs, showed that no significant differences were observed between the two batches (*p* > 0.05).
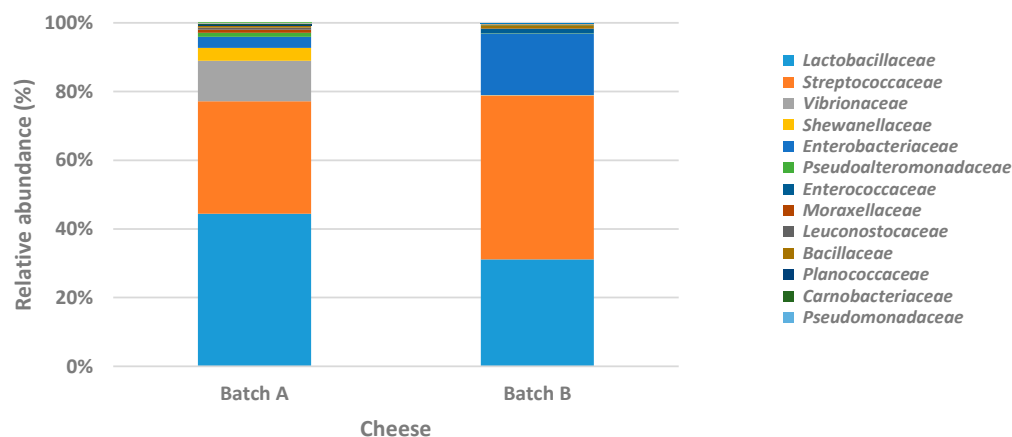

Figure 2. Abundances of bacterial families (relative abundances, %) detected on the two batches of Galotyri-like acid-curd cheese by next-generation sequencing.
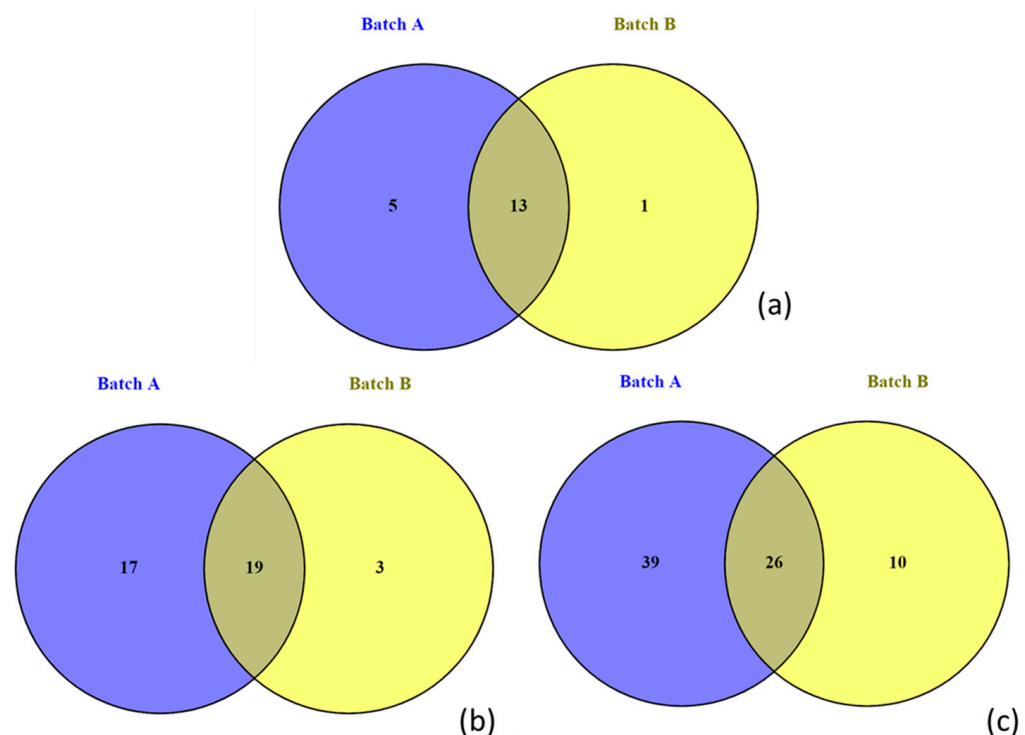
Figure 3. Venn diagrams showing the OTUs shared between batch A and batch B of cheese at the family (**a**)**,** genus (**b**), and species level (**c**).

Bacterial communities were dominated by Lactobacillaceae and Streptococcaceae in both batches. In brief, these families were detected at 77.21% and 78.76% total relative abundance in batch A and batch B, respectively. However, the relative abundance of Lactobacillaceae was higher in batch A (44.48%) compared to Streptococcaceae (32.73%), which in turn was detected in higher percentage (47.57%) in batch B compared to Lactobacillaceae (31.19%). Moreover, the Gram-negative bacterial families Vibrionaceae (11.77%), Shewanellaceae (3.73%), Enterobacteriaceae (3.29%), and Pseudoalteromonadaceae (1.08%) were also detected in batch A with relative abundance >1%. Conversely, in batch B, the only families apart from Lactobacillaceae and Streptococcaceae with a relative abundance >1% were Enterobacteriaceae (17.85%) and Enterococcaceae (1.31%). In Figure 3b,c, the number of shared OTUs between the two batches is illustrated at the genus and species level, respectively. The relative abundances of all detected genera and species are shown in Table S1 (Supplementary Materials). From the Venn diagrams, it seems that the bacterial diversity of batch A was higher than that of batch B; however, the percentage of the most of these genera or species was low (< 1%) (Figure 4). In brief, the number of OTUs shared between batch A and batch B marked on the overlapped area in Venn diagrams represents the number of the most common detected families, genera, or species on both batches of cheese. Here, it should be noted that the assigned family or genus names are mentioned throughout the text instead of the genus or species names in the case that sequencing analysis succeeded only up to family or genus level, respectively.

Considering the prevalence of Lactobacillaceae and Streptococcaceae families, the genera *Lactobacillus*, *Streptococcus*, *Lactococcus*, and marginally *Pediococcus* (0.03% in batch A only) were detected (Figure 4). In the case of the Lactobacillaceae family, *Lb. delbrueckii* was the most dominant species (43.62% and 29.4%) in both batches A and B, respectively (Table S1, Supplementary Materials). In addition, *Lb. helveticus* was detected marginally (0.07%) in batch B only. The remaining percentage of Lactobacillaceae failed to be identified at the genus (0.25% and 0.5%) or species (0.58% and 1.22%) level in batch A and B, respectively. Moreover, high abundances of *Streptococcus* (13.69% and 26.81%) and *Lactococcus* (17.81% and 18.72%) were observed in batches A and B, respectively (Figure 4). *S.*

thermophilus (11.03% and 17.75% in batches A and B, respectively) was the most abundant *Streptococcus* species detected in both batches (Table S1, Supplementary Materials). Beyond the unidentified percentages of 2.57% and 8.91% in batch A and B, respectively, assigned to the *Streptococcus* genus, the species *Streptococcus parauberis*, *Streptococcus uberis*, and *Streptococcus gallolyticus* were detected in low abundances (<0.1%). Regarding the genus *Lactococcus*, the primary species *Lactococcus lactis* was identified in batches A (5.05%) and B (7.82%). In addition, the species *Lactococcus chungangensis*, *Lactococcus raffinolactis*, and unidentified *Lactococcus* sp. were also detected in low abundances (<0.1%). However, a high percentage of the *Lactococcus* genus, ranging from 10.43% to 12.41%, failed to be identified at the species level.
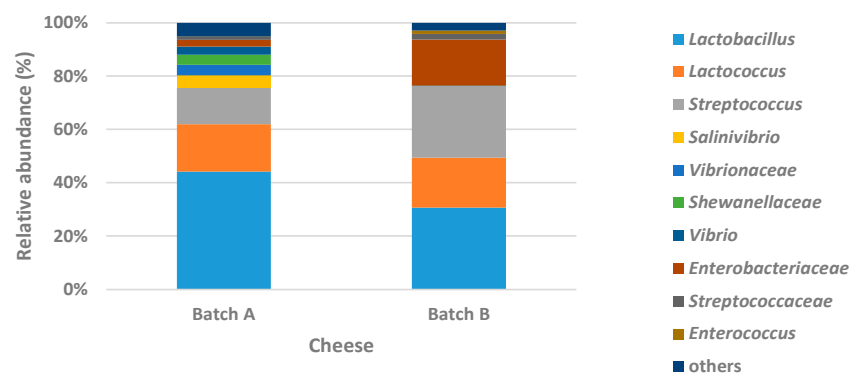
Figure 4. Abundances of bacterial genera detected on the two batches of cheese (relative abundance >1%) by next-generation sequencing. Family names are shown in the case that sequencing analysis succeeded only up to family level. The genera with relative abundances <1% are presented in the figure as "others".

Regarding the rest detected bacteria in relative high abundances (>1%) (Figure 4), *Salinivibrio*, unidentified members of Vibrionaceae, mainly *Vibrio* sp., Shewanellaceae, and Enterobacteriaceae constituted most of the bacterial community of batch A, together with Lactobacillaceae and Streptococcaceae. However, all former Gram-negative bacteria, except for Enterobacteriaceae, were detected in relatively low abundances (total <1%) in batch B. Additionally, the *Enterococcus* genus was marginally detected (0.03%) in batch A. In contrast, *Enterococcus* (1.28%) and mainly Enterobacteriaceae (17.30%), along with Lactobacillaceae and Streptococcaceae, constituted 96.85% of total bacterial community detected in batch B (Figure 4).

To compare the results of the 16S rRNA amplicon sequencing approach based on total bacterial DNA extraction directly from cheese, with the classical microbiological approach based on total and selective bacterial enumeration and characterization, the main findings of both approaches are summarized in Table 7. The relative abundances of the genera and species detected at >0.1% in at least one of the cheese batches (A or B) are compared with the respective relative population density (RPD, %) of each genus or main species found in the two cheese batches (Table 7); the RPD values were calculated as described previously [43]. Overall, the greatest difference between the two methods was the sole detection of LAB genera and species using a culture-dependent method, while additional non-LAB bacterial families/genera were detected by the culture-independent approach in both cheese batches (Table 7). This state could be attributed to (a) the lowest detection limit of 100 cells/g of the remaining bacterial populations being theoretically equal to an RPD < 0.000005% compared to the maximum total viable (LAB) population of 8.6 log CFU/g in both batches (Table 1), and (b) the primary observation that most of the above culture-independent detections of non-LAB (mainly of the Gram-negative families or genera) (Figures 2–4; Table S1, Supplementary Materials) were probably associated with bacterial DNA extracted or released from bacterial cells that had already died off. Otherwise, members of the Enterobacteriaceae family, found to be amongst the most

prevalent (17.30%) constituents of the bacterial community in batch B on the basis of OTUs (Table 7), should have been detected at levels above 6 log units on VRB/37 °C agar (Table 1). Their detection as viable counts should have been high even in the case that they were not coliforms but lactose-negative enterobacteria. However, the VRB/37 °C and CFC/25 °C agar plates of all batches were free (<100 cells/g) of Gram-negative bacterial growth, including batch A (pH 3.84) and batch B (pH 4.09) (Table 1) subjected to metagenomic analysis. Evidently, the fresh low-pH yogurt curds made using boiled ewe's milk with natural yogurt starter soon before Galotyri-like acid-curd cheese preparation did not contain Gram-negative bacteria. However, based on the whey cheese literature [44,45], natural contamination and growth levels of Enterobacteriaceae and other Gram-negative bacteria during natural fermentation and ripening of the traditional 'Myzithrenio' in wooden cheese barrels might have been quite high. In particular, the growth of salt-tolerant *Vibrio* and *Salinivibrio* might have been favored after the fresh whey cheese was dry-salted with 3% salt before transfer to the barrels. Furthermore, wooden cheese barrels have pores and are difficult to clean and disinfect; hence, in addition to various starter or nonstarter LAB, a great diversity of natural Gram-negative and Gram-positive non-LAB contaminants could be transferred to and grow early in fresh ripening cheese [6,19]. Acid-sensitive Gram-negative bacteria decline progressively during feta cheese ripening and may die off later [19,46], as they probably did in the acidified 'Myzithrenio' cheese after 1 month of ripening followed by 3–4 months of refrigerated VP storage. In general agreement, a recent microbiological and metagenomic study of feta cheese PDO matured in plastic or stainless-steel containers showed that the abundances of Enterobacteriaceae (which were high in the fresh cheese), Moraxellaceae, and Pseudomonadaceae were numerically reduced in the mature cheese after 120 days of storage [47]. *Lactococcus* was the most abundant genus in both fresh and mature industrial feta cheeses [47], produced with CSCs that probably contained *L. lactis* in addition to *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus*. In this study, CSC-like *L. lactis* strains were dominant in batch C only (Table 2), not selected for metagenomic analysis. Nonetheless, those *L. lactis* strains were probably acquired from the feta cheese barrels or other fresh whey cheese contamination sources in the plant. Moreover, the 'Myzithrenio' whey cheese used to make batch C might have been less ripened to harbor more *Lc. lactis* survivors compared to the other batches (Tables 2 and 7). During ripening of traditional Greek cheeses, the levels of *Lc. lactis* (starter) strains decline proportionally to the mesophilic nonstarter *Lb. casei/paracasei* and *Lb. plantarum/paraplantarum* groups [11,19,31], recently reclassified as two separate genera, *Lacticaseibacillus* and *Lactiplantibacillus*, following splitting of the large diverse genus *Lactobacillus* [48]. Additionally, many industrial CSC *Lc. lactis* strains have been genetically modified for autolysis in cheese to enhance flavor development via the release of intracellular enzymes during ripening [49,50]. The latter property might correlate with the low RPD (1.58%) or the absence (no isolates) of *Lc. lactis* in batches A and B, respectively, despite the relative abundance of *Lactococcus*, *Lc. lactis* in particular, being fairly high in both batches (Table 7). Probably most *Lactococcus* had also died off during 'Myzithrenio' ripening, as well as during the previous heating of the Pappas Graviera whey, obviously enriched in CSC *Lc. lactis* strains [22,31].

Regarding the dominant thermophilic yogurt-starter LAB in Galotyri-like acid-curd cheese, the metagenomic analysis and classical microbiological results followed similar trends, despite certain inconsistencies of the two methods between the two batches (Table 7). According to the classical method, *S. thermophilus* was predominantly isolated as the sole *Streptococcus* species from batch A (66.56%), while, in batch B, *S. thermophilus* occurred together with other thermophilic *Streptococcus* (total RPD 58.47%), which probably belong to the species listed in Table S1 (Supplementary Materials). Furthermore, *Lb. delbrueckii* was more prevalent in batch A (RPD 16.6%) than in batch B (RPD 3.4%) culture-dependently. The above differences between the two cheese batches were also detected culture-independently. However, particularly for *Lb. delbrueckii*, the related RPD percentages were always much lower than the relative abundances in both cheese batches,

probably because the populations of *Lb. delbrueckii* solely isolated from MRS/45 °C agar anaerobically (Tables 1–3) were lower than the actual prevalence of this species in batches A and B. *Lb. delbrueckii* may occur at highly viable, nonculturable levels on MPCA and MRS agars incubated at 37 °C or lower growth temperatures, under aerobic conditions. Conversely, detection of *Enterococcus* was higher in batch B than batch A, and vice versa for *Leuconostocs*, using both methods (Table 7). However, the percentage RPD values of the above two genera were higher than the corresponding percentage OTUs in both batches (Table 7). The death of the non-LAB groups discussed above and the fact that growth of leuconostocs and mainly enterococci was enhanced by the MRS and SB agar selectivity (Table 3) increased their RPD values. Likewise, the percentage RPD values of mesophilic *Lactobacillus* were always higher than the abundance of the remaining *Lactobacillus* species, excluding *Lb. delbrueckii*, in both batches. Surprisingly, neither *Lacticaseibacillus (Lb.) paracasei* nor *Lactiplantibacillus (Lb.) plantarum/paraplantarum* were identified culture-independently, despite their joint RPD values being 9.71% and 20.50% in batches A and B, respectively (Table 7).

Table 7. Prevalence of the main bacterial genera and species in the two retail RTE Galotyri-like acid-curd cheese batches based on their relative abundances of detected OTUs versus their relative population density (RPD) values calculated on the basis of microbial enumeration data.

Bacterial Genus or Species	Cheese Batch A		Cheese Batch B	
	RPD (%)	Relative Abundance (%)	RPD (%)	Relative Abundance (%)
Streptococcus	66.56	13.69	58.47	26.81
Streptococcus thermophilus	66.56	11.03	14.62	17.75
Lactobacillus	26.31	44.20	23.74	30.69
Lactobacillus delbrueckii	16.60	43.62	3.24	29.40
Other (mesophilic) *Lactobacillus*	9.71	0.58	20.50	1.29
Lactococcus	1.58	17.81	ND	18.72
Lactococcus lactis	1.58	5.05	ND	7.82
Leuconostoc	4.75	0.56	0.23	0.07
Enterococcus	0.80	0.03	17.56	1.28
Enterobacteriaceae	ND	2.48	ND	17.30
Vibrionaceae	ND	3.96	ND	0.13
Vibrio	ND	1.49	ND	0.00
Salinivibrio	ND	4.65	ND	0.00
Shewanellaceae	ND	3.73	ND	0.09
Other bacteria	ND	8.89	ND	4.91

ND, not detected.

In summary, the biochemical identification and metagenome analyses of the LAB species present in this retail delicatessen Galotyri-like cheese product based on yogurt corroborate the findings of Ivanov et al. [20], who reported different (batch-dependent) high abundances of *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* in Bulgarian home-made yogurts, accompanied by batch-dependent low abundances of several subdominant LAB species, including *Lc. lactis, Lc. garvieae, Pediococcus acidilactici, Lb. paracasei, Leuc. mesenteroides, Lb. helveticus, Lb. rhamsosus,* and wild *Streptococcus.* Commercial starter strains of *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* were isolated nearly as a pure synergic culture from an industrial Galotyri PDO product labeled as a fresh cheese combining the nutritional properties of yogurt [11]. Conversely, the artisan-type Galotyri PDO cheese varieties were far more diversified in terms of LAB ecology, containing (as percentage of total LAB isolates) strains of *Lc. lactis* (19.8%), *Lb. plantarum* (16.9%), *S. thermophilus* (14.7%), *Leuc. mesenteroides* group (11.3%), *Pediococcus inopinatus* (7.3%), *E. faecalis* (8.5%), *E. faecium* (6.2%), *E. durans* (5.1%), *Lb. rhamnosus* (3.4%), *Lb. gasseri* (3.4%), *Lb. bulgaricus* (2.8%), and *Lb. acidipiscis* (0.6%). The prevalence of each species in artisan Galotyri cheeses was also product- and batch-dependent, while it was difficult to discriminate which of

the strains were commercial starters or cultures in addition to the primary starter species *S. thermophilus* and *Lc. lactis* [11]. Limited identification data exist regarding the LAB species diversity in traditional Greek PDO acid-curd cheeses other than Galotyri [6,19]. Specifically, strains of *Lc. lactis* subsp. *lactis*, *Leuc. paramesenteroides*, and *Lb. plantarum* were most frequently isolated from Anevato, while *Lb. plantarum* and *Lb. casei* dominated in Kopanisti, followed by *Pediococcus pentosaceous* and enterococci [6]. Conversely, *E. durans* was the most frequently found species (27.5%) in Katiki, followed by *Lb. plantarum* (17.6%), *E. pseudoavium* (13.7%) and many other *Enterococcus, Leuconostoc, Weissella*, and *Lactobacillus* spp. at low isolation frequencies [19]. Altogether, our present and previous data and the preceding discussion justify that the microbial (LAB) ecology of the retail delicatessen acid-curd cheese analyzed during this study is very similar to that of PDO Galotyri produced with thermophilic natural or commercial yogurt starters.

4. Conclusions

In conclusion, the classical microbiological methods and the metagenomic analyses were in general agreement with regard to the dominant LAB families, genera, or species that remained viable during the quantification and isolation of the Galotyri-like acid-curd cheese biota. However, results were variable with regard to many other bacterial (LAB) genera or species that had died off in the cheeses; their DNA interfered with the DNA of the viable bacterial cells. Therefore, the total bacterial DNA-based metagenomic method used in this study seems a very powerful approach for mapping the microbial ecology and diversity of fresh foods, such as raw milk and raw milk cheese [51] and fresh meat [52], but it has obvious limitations when processed foods, which include preceding bacterial inactivation steps, are to be analyzed. In the case of Galotyri-like acid-curd cheese, the basal yogurt curd was fresh. However, the 'Myzithrenio' whey cheese had previously undergone several bacterial inactivation treatments, namely, salting, fermentation, and ripening, as well as boiling of the Graviera whey to obtain the fresh whey cheese mass, boiling of the 5% raw ewe's milk supplemented in the whey plus pasteurization or thermization of the original raw milk, and cooking of the whey during Graviera cheese production. Nevertheless, this delicatessen Galotyri-like acid-curd cheese product appears to be a rich pool of indigenous nonstarter strain biotypes of the new genera *Lactiplantibacillus* and *Lacticaseibacillus* and of the species *E. faecium*, *E. faecalis*, and *Leuconostoc mesenteroides* derived from the naturally ripened whey cheese used for its manufacture. Additional studies are in progress to evaluate the technological, safety, and antipathogenic and potential probiotic properties of selected Galotyri-like acid-curd cheese LAB isolates.

Data Availability Statement: The data presented in this study, as well as the bacterial strains identified, are available on request by from the corresponding authors. The GenBank 16S metagenomic Bioproject ID is PRJNA722121.

Conflicts of Interest: The authors declare no conflict of interest.

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