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Microbiological Characterization of Greek Galotyri Cheese PDO Products Relative to Whether They Are Marketed Fresh or Ripened

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Abstract: Galotyri is the most popular traditional Greek PDO soft acid-curd cheese. This study compared the microbial numbers and types and characterized the lactic acid bacteria (LAB) biota of two artisan-type Galotyri PDO cheese varieties, one marketed fresh (Brand-K) and the other ripened (Brand-Z). Two retail batches of each cheese variety were analyzed, and a total of 102 LAB isolates were biochemically identified. LAB (7.2–9.3 log CFU/g) prevailed in all cheeses, followed by yeasts (5.8–6.8 log CFU/g). Typical starter strains of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* were the most abundant species in all batches. However, the fresh Brand-K cheeses had 1–3 log units higher thermophilic starter LAB counts than the ripened Brand-Z cheeses, which contained a more diverse viable LAB biota comprising *Lacticaseibacillus paracasei, Leuconostoc mesenteroides, Lentilactobacillus (L. diolivorans, L. kefiri, L. hilgardii), Pediococcus inopinatus/parvulus,* few spontaneous nonstarter thermophilic streptococci and lactobacilli, and *Enterococcus faecium* and *E. faecalis* at higher subdominant levels. Conversely, the fresh Brand-K cheeses were enriched in members of the *Lactiplantibacillus plantarum* group; other LAB species were sporadically isolated, including *Lactococcus lactis.* All retail cheeses were safe (pH 3.9–4.0). No *Salmonella* spp. or *Listeria monocytogenes* were detected in 25-g samples by culture enrichment; however, *Listeria innocua* and coagulase-positive staphylococci (850 CFU/g) survived in one ripened batch. Gram-negative bacteria were <100 CFU/g in all cheeses. In conclusion, ripening reduced the starter LAB viability but increased the nonstarter LAB species diversity in the present Galotyri PDO market cheeses.

Keywords: acid-curd cheese; Galotyri PDO; cheese microbiota; biochemical characterization

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1. Introduction

Cheeses of protected designation of origin (PDO) are authentic traditional dairy products manufactured in a specific geographical area, using recognized know-how based on certified compositional and technological specifications [1]. PDO cheeses are economically and culturally important foods in Europe, particularly in Greece and other Mediterranean and Balkan countries [2–5]. Acid-curd cheeses form a distinct group of very soft, spreadable low-pH (pH 3.5–5.0) but high-moisture (60–75%; water activity (a_w) > 0.98 to 0.99) cheeses manufactured at industrial or artisan scale and consumed fresh or ripened [6,7]. Five traditional Greek acid-curd cheese types have PDO recognition [8]. Several artisan acid-curd cheese varieties are still produced without commercial starter cultures in many Greek mountainous and island areas and represent a diverse, rich pool for the isolation of indigenous (novel) lactic acid bacteria (LAB) strains with superior biotechnological, antimicrobial, and probiotic properties [9–12].

Galotyri PDO is the oldest and most popular traditional Greek acid-curd cheese, recognized in 1994 as an exclusive product in the regions of Epirus and Thessaly [13]. It is a clean-white, soft, spreadable cheese without surface skin, eyes, or cracks and with a pleasant refreshing acidic taste and aroma. According to the Galotyri cheese PDO specifications,

article 83D, pp. 45–47, of the Hellenic Code of Food and Beverages [8], the curd must be made of ewe's or goat's milk or their mixtures, collected from breeds raised and fed in the aforementioned regions; the use of cow or other types of milk is prohibited. The milk should be of suitable quality, full fat, raw, or pasteurized. The addition of rennin, edible sea salt, and starter LAB cultures is allowed, whereas the addition of milk concentrates, milk powder or proteins, casein salts, colorings, and chemical preservatives is prohibited. The authentic Galotyri PDO cheese is specified to be made of boiled milk left to cool in clay jars at ambient temperature for 24 h. Then the milk is salted with 3–4% edible sea salt and left to acidify naturally at ambient temperature for 2–3 days, followed by natural ripening of the fresh cheese into skin bags or wooden barrels at temperatures lower than 8 °C for at least two months in case the cheese is made of raw milk [8].

Samelis and Kakouri [14] first evaluated the microbial quality and safety of Galotyri PDO market cheeses manufactured at the industrial or artisan scale in Epirus and later described great variations in the microbial (LAB) ecology between an industrial-type and five artisan-type Galotyri PDO cheeses collected from the Epirus market in the years 2003–2006 and 2014–2019 [15]. In specific, all industrial cheeses were marketed fresh and contained viable commercial starter strains of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* only; whereas the artisan-type cheeses were more diverse and contained mesophilic starter and nonstarter LAB, mainly *Lactococcus lactis* and *Lactobacillus plantarum*, plus numerous yeasts, some enterococci, and few enterobacteria. The prevalence of *S. thermophilus* and *L. delbrueckii* in the retail artisan-type cheeses was product-dependent as the production method in each traditional dairy was different, particularly as regards the type of starter culture used for milk acidification and the duration of the ripening period, if any, before the cheese was packaged for selling [15].

Most recently, the microbial communities of retail pre-packaged samples of three artisan-type Galotyri PDO cheese varieties, all produced in SMEs located in Epirus and ripened for at least two months, were analyzed [16,17]. Based on their amplicon metabarcoding bacterial profiles, more *Lactobacillaceae* than *Streptococcaceae* occurred in all Galotyri cheese products, particularly *L. plantarum*, *L. acidipiscis*, *L. kefiri,* and exclusively *L. hilgardii* in great percentage (21.2%) in one cheese variety [16]. Conversely, based on the 16S rRNA identification of LAB isolates, *L. plantarum* was prevalent in all three Galotyri PDO cheese varieties [17].

Samelis and Kakouri [15] pointed out that the major variations in the microbial diversity between different Galotyri PDO cheese brands are due to diachronic critical modifications of the artisanal manufacturing method in order the commercial plants to adopt modern cheese processing practices and satisfy economic needs. Among others, modifications include (i) the constant use of pasteurized milk; (ii) the type of natural or commercial starters used for milk acidification in accordance with the fermentation temperature; (iii) whether the basal cheese curd is supplemented with fresh or ripened trimmings of white-brined (e.g., Feta) cheese [11,15] or ripened whey cheese (e.g., Myzithra) [18] for external aromatization purposes, an arbitrary operation in contradiction to the Galotyri PDO specifications [8,15]; and (iv) whether the fresh cheese from pasteurized milk is left to ripen, at what temperature, and for how long. Basically all artisan-type cheeses in the aforementioned studies shared a timely variant ripening step that generally favored the predominance of mesophilic LAB and enhanced the microbial diversity of the final product [15–18]. Artisanal and industrial cheeses of the same type are different in their microbiological, biochemical and sensory attributes [19], while ripening decisively affects all fresh cheese quality attributes by inducing complex microbial interactions and biochemical mechanisms [20,21].

This research represents a follow-up study of our aforementioned micro-ecological Galotyri studies [14,15] focusing on the comparison of the microbial numbers and types of two artisan-type Galotyri PDO cheese products of the Epirus region, one marketed fresh and the other after ripening. Special emphasis was given to the biochemical species characterization of the technological LAB biota prevailing in each product type relative

to whether it was marketed fresh or ripened. The practical aims of this study were (i) to microbiologically discriminate the above two artisan-type Galotyri PDO product brands, and (ii) to collect various autochthonous, potentially novel, LAB strains to be utilized in future biotechnological studies for the development of indigenous cheese starter strain combinations, other dairy applications and the production of novel functional foods.

2. Materials and Methods

2.1. Commercial Galotyri PDO Cheese Samples

Samples of four individual commercial batches of Galotyri PDO cheese produced by two traditional dairy SMEs (Brand-Z and Brand-K) located in Epirus, Greece, were purchased from a supermarket close to the Dairy Research Department at Ioannina. According to their labeling (Table 1), batches Z-A and Z-B represented the Galotyri PDO cheese product of Brand-Z subjected to ripening (for at least one month) before packaging for retail distribution, while batches K-A and K-B represented the Galotyri PDO cheese product of Brand-K distributed fresh after production. Both products were marketed hermetically sealed in brand-named plastic containers of various sizes, ranging from 250 g (Brand-K) to 400 g (Brand-Z) of cheese as regards the retail samples of this study. Two plastic containers from each cheese batch were purchased on the same day. All cheeses were analyzed before their sell-by date indicated on each batch sample's label. On the day of analysis, the two ripened batches Z-A and Z-B were already 58 and 70 days old, while the two fresh batches K-A and K-B were only 5 and 11 days old, respectively (Table 1). Despite their major technological difference with regard to ripening, both commercial cheese products were labeled to contain $\leq$75% moisture and $\geq$40% fat in dry matter, in compliance with the Galotyri cheese PDO specifications [8]. Conversely, the two products differed slightly in their nutritional information (g/100 g of cheese) labeling: the Galotyri-Z cheese contents in total fat, protein, carbohydrates, sodium chloride and energy were 12.4, 11.5, 1.4, 1.17 and 163 kcal, respectively; the corresponding values of Galotyri-K cheese were 11.8, 10.2, 1.7, 1.00 and 154 kcal, respectively. Additional technological issues relating to the labeled data presented in Table 1 will be reported and discussed in the Results section.

Table 1. Ripening duration and in-package commercial shelflife of two traditional Galotyri PDO cheese products (non-industrial; artisan-type; brand-named) purchased from the market of Ioannina, Epirus [a].

Cheese Brand	Cheese Batch	Production Date	Packaging Date	Sell-by Date	Ripening Duration (Days)	ShelfLife (Days) [b]	Cheese Age on Sell-by Date (Days)	Analysis Date	Cheese Age at Analysis (Days)
Brand-Z	Z-A	31 December 2017	26 January 2018	20 April 2018	26	84	110	27 February 2018	58
	Z-B	16 January 2018	15 March 2018	31 May 2018	58	77	135	27 March 2018	70
Brand-K	K-A	24 February 2018	ND	10 March 2018	None	14	14	1 March 2018	5
	K-B	16 March 2018	ND	8 April 2018	None	23	23	27 March 2018	11

[a] The data were based on the production, packaging and sell-by dates indicated on the label of each cheese product batch. [b] Both cheese product labels warn the consumer: 'Keep refrigerated 2–4 °C'; and 'Consume within 3 days (Brand K) or 4 days (Brand Z) after opening'. ND, Not declared.

2.2. Cheese Analyses

All cheese samples (n = 2 for each batch; n = 4 for each brand product) were analyzed microbiologically and for pH values within 2 h after purchase. The pH was measured with a digital pH meter (Jenway 3510, Dunmow, Essex, UK). The glass electrode was immersed in the soft cheese mass after the microbiological sampling performed as follows. Each plastic container was opened near a flamed Bunsen burner and the cheese mass was stirred thoroughly with a sterile spoon-spatula. Afterward, 25 g of cheese were 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, powdered agar media and supplements were purchased from Neogen Culture Media (formerly Lab M, Heywood, Bury, UK).

All samples were analyzed for total viable bacteria, total mesophilic and thermophilic LAB, total mesophilic and thermophilic dairy (lactose-fermenting) LAB, enterococci, coliforms, pseudomonad-like bacteria, total and pathogenic staphylococci and yeasts/molds. The agar media and incubation conditions were according to the analytical procedures described in a relevant previous study by Samelis et al. [18]; they are summarized in Table 2. Particularly for enterococci, two selective media, Slanetz and Bartley agar (as the best discriminative medium) and Kanamycin Aesculin Azide (KAA) agar, were used in parallel [15]. 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 [14,22]. Presumptive *Listeria* or *Salmonella* colonies on the selective Palcam agar or Rambach agar (Merck, Darmstadt, Germany) plates were identified using the API Listeria or the API 20E identification kits (BioMerieux, Marcy l' Etoile, Lion, France), respectively.

Table 2. Microbial populations (log CFU/g) and pH values of four retail individual batches of Galotyri PDO cheeses produced by two traditional dairy SMEs (Brand-Z and Brand-K) and distributed in the Epirus market with (Brand-Z; ripened cheese) or without (Brand-K; fresh cheese) a preceding ripening process.

Microbial Group	Enumeration Agar Medium/ Incubation Conditions	Brand-Z (Ripened Cheese)		Brand-K (Fresh Cheese)		Brand-Z (n = 4)	Brand-K (n = 4)
		Batch Z-A (n = 2)	Batch Z-B (n = 2)	Batch K-A (n = 2)	Batch K-B (n = 2)	Batch Z-A + Z-B	Batch K-A + K-B
Total viable cheese biota counts	Milk Plate Count agar (MPCA)/37 °C; 48–72 h; aerobically	8.27 ± 0.05	7.24 ± 0.12	9.29 ± 0.04	9.01 ± 0.07	7.75 ± 0.60 ^{a}	9.15 ± 0.17 ^{b}
Total mesophilic LAB	MRS agar/30 °C; 72 h; aerobically	6.36 ± 0.08	5.95 ± 0.13	6.36 ± 0.08	6.53 ± 0.06	6.15 ± 0.25 ^{a}	6.44 ± 0.11 ^{a}
Total thermophilic LAB	MRS agar/45 °C; 48 h;anaerobically (in Gas-Pack jars)	5.40 ± 0.05	4.90 ± 0.00	8.90 ± 0.25	8.50 ± 0.25	5.15 ± 0.35 ^{a}	8.70 ± 0.30 ^{b}
Total mesophilic dairy LAB (presumptive lactococci)	M17 agar/22 °C; 72 h; aerobically	6.07 ± 0.19	7.05 ± 0.04	7.21 ± 0.04	6.54 ± 0.08	6.56 ± 0.58 ^{a}	6.87 ± 0.39 ^{a}
Total thermophilic dairy LAB (presumptive streptococci)	M17 agar/42 °C; 48 h; aerobically	5.15 ± 0.11	6.74 ± 0.44	8.61 ± 0.02	8.30 ± 0.01	5.95 ± 1.12 ^{a}	8.45 ± 0.18 ^{b}
Enterococci	Slanetz and Bartley (SB) agar/37 °C; 48 h; aerobically	3.23 ± 0.16	5.11 ± 0.04	2.87 ± 0.12	<2.00	4.17 ± 1.09 ^{b}	2.48 ± 0.67 ^{a}
Enterococci plus kanamycin-resistant and aesculin-positive lactobacilli	Kanamycin Aesculin Azide (KAA) agar/37 °C; 48–72 h; aerobically	3.26 ± 0.04	5.00 ± 0.13	5.23 ± 0.05	5.58 ± 0.06	4.13 ± 1.01 ^{a}	5.40 ± 0.21 ^{b}
Total staphylococci	Baird-Parker agar with egg yolk tellurite/37 °C; 48 h; aerobically	<2.00	3.46 ± 0.14	2.30 ± 0.42	<2.00	2.73 ± 0.85 ^{a}	2.15 ± 0.30 ^{a}
Coagulase-positive staphylococci	Baird-Parker agar with RFP/ 37 °C; 18–24 h; aerobically	<2.00	2.93 ± 0.21	<2.00	<2.00	2.47 ± 0.55 ^{b}	<2.00 ^{a}
Coliforms	Violet Red Bile (VRB) agar/37 °C; 24 h; double-layered	<1.00	1.30 ± 0.42	<1.00	<1.00	1.15 ± 0.30 ^{b}	<1.00 ^{a}
Pseudomonad-like bacteria	Cephalothin-Fucidin-Cetrimide (CFC) agar; 25 °C; 48 h; aerobically	<2.00	<2.00	<2.00	<2.00	<2.00 ^{a}	<2.00 ^{a}
Yeasts	Rose Bengal Chloramphenicol (RBC) agar/25 °C; 5 d; aerobically	5.84 ± 0.16	6.27 ± 0.08	6.78 ± 0.11	6.18 ± 0.07	6.05 ± 0.27 ^{a}	6.48 ± 0.35 ^{a}
Cheese pH		3.80 ± 0.08	4.02 ± 0.15	3.90 ± 0.08	4.08 ± 0.09	3.91 ± 0.16 ^{a}	3.99 ± 0.12 ^{a}

All batches were free of *Salmonella* in 25 g culture-enriched samples; Cheese batches Z-A, K-A, and K-B were free of *Listeria* spp., whereas both samples of cheese batch Z-B harbored *Listeria innocua* in 25 g culture-enriched samples.Values in the last two columns to the right are the means of two retail cheese batches from each brand with two individual samples analyzed per batch (n = 4); within a row, means with different superscript letters ^{a,b} are significantly different ($p < 0.05$).

2.3. Isolation and Biochemical Characterization of the Cheese LAB Biota

The LAB types prevailing in each of the four Galotyri PDO cheese batches (Table 1) were determined by following the isolation protocol applied by Samelis et al. [18]. Briefly, five colonies were isolated from one highest dilution plate of each of the first six LAB-selective enumeration agar media listed in Table 2. One series of agar plates was used for the LAB isolation procedure from each cheese batch because the two replicate retail samples were homogeneous and gave very similar microbial (LAB) quantifications (Table 2). Colony selection was random; however, attention was paid to collect representative isolates of all macroscopically different colonies on each agar plate. In this manner, 120 presumptive LAB isolates (30 from each batch) were collected. Colonies isolated from Milk Plate Count and M17 agar plates were transferred in 10 mL M17 broth (Merck). Colonies isolated from MRS and Slanetz and Bartley agar plates were transferred in 10 mL 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, and 37 to 45 °C, respectively (Table 2). Following growth, all isolates were checked for purity on streaked MRS or M17 agar plates. The purified isolates were stored in 5 mL MRS broth with 20% glycerol (Merck) at −30 °C. The stock LAB isolates were resuscitated in 10 mL 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 [23–28]. Generally, the basic (key) differentiating criteria for each LAB group, the discrimination of the isolates at the genus or species level, and the test methods were according to Samelis et al. [18]. Briefly, all isolates were first tested rapidly for Gram reaction (with 3% KOH) and catalase reaction (with 3% H_2O_2). Gram-positive and catalase-negative LAB isolates were further tested 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 growth on KAA agar, and for the fermentation of 14 key sugars in miniplates: L-arabinose, cellobiose, galactose, lactose, maltose, mannitol, melezitose, melibiose, raffinose, ribose, sorbitol, sucrose, trehalose, and xylose (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). All tests were performed in duplicate. Next the isolates were grouped in phenotypes at the species level. Representative (or all) isolates from each group were tested for growth at 37 °C in MRS or M17 broth with 2% or 4% salt, or in MRS broth with 8% and 10% salt at 30 °C; for acetoin production from glucose and slime production from sucrose at the optimal growth temperature for each isolate; and, for the entire carbohydrate fermentation profiles using the API 50CHL identification kit (BioMerieux) to confirm or further elucidate the biochemical species identification of each LAB group. Recent dairy studies from our laboratory have described the effective use of the aforementioned phenotypic criteria to assign several *Streptococcus*, *Lactococcus*, *Enterococcus*, *Pediococcus*, *Leuconostoc* and *Lactobacillus* isolates to the species level; confirmed by 16S rRNA sequencing and additional genotypic tools in collaboration with molecular biology laboratories at the University of Ioannina [15,29–31]. Particularly for the lactobacilli isolates, their present assignment to the genus and species was based on the new nomenclature, following the recent splitting and reclassification of the old *Lactobacillus* genus in 23 new genera [32].

2.4. Statistical Analyses

Duplicate packaged samples of each individual Galotyri PDO cheese batch were analyzed for microbial quantification and pH determination. Microbial counts were converted to log CFU/g, pooled for the two batches of each brand (n = 4) and along with the corresponding cheese pH values were subjected to one-way analysis of variance (Statgraphics Plus for Windows v. 5.2, Manugistics, Rockville, MD, USA) to specify main differences between the Brand-Z (ripened) and Brand-K (fresh) cheese product. Means and standard deviations were calculated, and when F-values were significant at the $p < 0.05$ level, mean differences were separated by the LSD procedure.

3. Results

3.1. Differences in the Preservation Potential and Retail Shelf Life between the Two Galotyri PDO Cheese Brand Products Relating to Ripening

From the two Galotyri PDO cheese products' labeling data summarized in Table 1, it was evident that both Brand-Z cheese batches had been subjected to ripening before their retail distribution. Notably, the ripening duration, estimated as the time period from cheese production to packaging, was rather unstable as it was 26 and 58 days for batches Z-A and Z-B, respectively. Then, the two ripened cheese batches, Z-A and Z-B, were labeled to have a quite extended retail shelf life of 84 and 77 days, respectively, estimated as the time period from packaging to the sell-by date. In full contrast, based on their labeling information, the Brand-K cheese batches K-A and K-B were packaged fresh after production and, thereafter, had a quite short retail shelflife of 14 and 23 days, respectively. Accordingly, on the sell-by date (i.e., end of shelf life), the total age of the ripened cheese batches Z-A and Z-B was 110 and 135 days, as opposed to an age of 14 and 23 days only of the fresh cheese batches K-A and K-B, respectively (Table 1). These results clearly demonstrated the vital importance of a preceding ripening process for increasing the commercial shelf life of an artisan-type soft acid-curd cheese product, such as Galotyri PDO, manufactured from traditionally 'boiled' or industrially pasteurized milk. After cooling, the milk was acidified with the aid of natural undefined or commercially defined starter cultures under potentially varying fermentation temperature conditions. With regard to the latter critical technological issue, although the use of starter LAB cultures for enhancing and controlling Galotyri PDO cheese milk acidification is permitted, declaration of the type of 'starter culture', if any, is not required legislatively [8]; it was not declared on the label of the Brand-Z and Brand-K cheeses either. Therefore, a questionnaire was formally submitted to both SMEs to address the starter culture issue, plus other technical questions regarding their Galotyri PDO cheese processing. Only Brand-Z filled and returned the questionnaire, which clarified the use of a natural yogurt-like starter culture routinely applied for the production of the Galotyri-Z cheese specialty product.

3.2. Microbiological Attributes and pH Values of Retail Galotyri PDO Cheese Samples

The results of the microbial quantification analyses, along with the pH values of the four Galotyri PDO cheese batches, are shown correspondingly to the enumeration of agar media for each microbial group in Table 2. Results are presented separately for each cheese batch to facilitate comparisons between the LAB quantification and identification data within each batch and between all batches, presented in Sections 3.3 and 3.4 below. To specify significant differences, particularly relating to Galotyri cheese ripening, the mean and standard deviation values (n = 4) for Brand-Z (Z-A + Z-B) and Brand-K (K-A + K-B) products are statistically compared in Table 2.

Starting from the pH, no significant differences ($p > 0.05$) were found between the Brand-Z and Brand-K Galotyri PDO products, despite the former cheese was ripened whereas the latter cheese was fresh. In specific, the mean pH of all batches ranged from 3.8 to 4.1 (Table 2), despite the two Brand-Z batches were more than 8 weeks old, whereas the two Brand-K batches were less than 2 weeks old, on the day of pH measurement analysis (Table 1). Overall, the pH data indicated that (i) both Galotyri PDO brand products analyzed during this study were typical acid-curd cheeses, belonging to the a priori safe acidic (pH < 4.4) RTE foods [33]; and (ii) no reversal pH increases were prominent particularly in the ripened (aged) Brand-Z cheese product.

Microbiologically, LAB prevailed in all cheese batches, followed by yeasts (Table 2). However, the aforementioned major differences in the cheese ripening times and shelf-life durations (Table 1) were reflected in most of the microbial populations and particularly in the different types (groups) and numbers of LAB prevailing in each Galotyri PDO brand and/or batch. The most prominent difference was that the fresh Brand-K cheese batches contained significantly higher ($p < 0.05$) total viable microbial counts, grown abundantly on MPCA at the optimal growth temperature (37 °C) for all dairy microor-

ganisms, than the ripened Brand-Z cheese batches (Table 2). Further, it was evident that this very high (>9 log CFU/g) total viable microbiota of the fresh Brand-K cheese batches comprised mainly thermophilic LAB, presumptively dairy lactobacilli, and streptococci. Probably they were commercial symbiotic starter strains of *S. thermophilus* and *L. delbrueckii* because they also grew abundantly (8.3 to 8.9 log CFU/g) on the M17/42 °C and the MRS/45 °C agar plates, respectively (Table 2). In contrast, the corresponding thermophilic LAB populations recovered in the ripened Brand-Z cheese batches were lowered by 2.5 and 3.6 log CFU/g, respectively. Notably, all MRS/45 °C agar plates of batches Z-A and Z-B, as well as the M17/42 °C agar plates of batch Z-A, had a strong malty smell and seemed to contain thermophilic LAB and yeast colonies at approximate mixed population levels of 5.0 to 5.5 log CFU/g (Table 2). This observation was confirmed later by the phenotypic characterization of representative thermophilic MRS and M17 isolates from the Brand-Z cheese samples.

Meanwhile, neither the total mesophilic (dairy) LAB populations enumerated on the MRS/30 °C and M17/22 °C agar plates nor the total yeast populations selectively enumerated on the RBC/25 °C agar plates differed significantly between the Brand-Z and Brand-K cheese products ($p > 0.05$). Notably, none of the four cheese batches, even not the two ripened ones, displayed predominance (>8-log units) of mesophilic LAB. Instead the latter LAB populations were below 7.5 log CFU/g in all batches and clearly subdominant of the thermophilic LAB in the fresh (Brand-K) cheese batches. Overall, the populations of mesophilic LAB were similar with those of yeasts in both cheese brands, ranging around 6.0 to 7.0 log CFU/g (Table 2). The fact that levels of yeasts in the ripened (Brand-Z) cheese batches were around 6 log CFU/g was a reasonable finding. However, the presence of yeasts at population levels above 6 log CFU/g in the fresh (Brand-K) cheese batches was an unexpected finding to be discussed later.

Enterococci were the most subdominant LAB group in all Galotyri PDO cheese batches (Table 2). Based on their selective enumeration on the best discriminative SB agar medium, enterococci slightly exceeded 5 log CFU/g in batch Z-A only, whereas they were found to be below the detection limit of 100 CFU/g in batch K-B. Overall, populations of enterococci were significantly higher ($p < 0.05$) in the ripened (Brand-Z) than in the fresh (Brand-K) Galotyri cheese batches. Conversely, the fresh (Brand-K) batches contained significantly higher ($p < 0.05$) populations of kanamycin-resistant and aesculin-positive lactobacilli of the *Lactiplantibacillus* (*L. plantarum* group) genus [32], which also were subdominant and were selectively enumerated as black smaller colonies with a more delayed growth than enterococci on the KAA agar plates (Table 2).

Regarding the non-LAB bacterial groups, low levels of total staphylococci were detected in the batches Z-B and K-A only. Pseudomonad-like bacteria were below 100 CFU/g in all batches. Pathogenic staphylocooci and coliforms were below 100 CFU/g and 10 CFU/g, respectively, in batches Z-A, K-A, and K-B. Conversely, batch Z-B was of lowered hygienic quality because it contained an average of 850 CFU/g pathogenic staphylococci and 20 CFU/g coliform bacteria and, most importantly, it harbored *Listeria innocua* in 25 g of cheese after culture enrichment (Table 2).

3.3. Basic Phenotypic Characterization and Batch-Dependent Distribution of the Galotyri Cheese Isolates

All 120 presumptive LAB colonies (30/cheese batch) isolated from the first six LAB enumeration agar media in Table 2 were recovered in the form of original MRS or M17 broth cultures, as described in the Methods. All Galotyri isolates were coded with the prefix GL, followed by the letter Z or K to indicate the cheese brand product and an order number for each isolate, i.e., GL-Z1 or GL-K1; for text and tabular simplification, the prefix GL is omitted in all later sections. Following their purification, the number of isolated colonies for biochemical characterization was increased to 127. In specific, all five reddish-brown presumptive *Enterococcus* colonies isolated from the SB agar plates of batch K-A (mean level 2.87 log CFU/g in Table 2) were unavoidably contaminated by an underlying

dense lawn of kanamycin-resistant lactobacilli (mean level 5.23 log CFU/g on KAA agar in Table 2). These mesophilic lactobacilli were presumptive members of the *Lactiplantibacillus* (*L. plantarum*/*L. paraplantarum*/*L. pentosus* group) of species [32,34], also capable for growth as whitish (colorless) colonies on SB agar. In order to isolate representative strains of both LAB groups, the original five SB mixed cultures were purified to obtain two distinct colony isolates from each culture; the five rod-shaped isolates were coded K11-K15 while the five enterococcal isolates were coded K11B-K15B. Additional two original isolates, K6 (from MRS/45 °C/batch K-A) and K58 (from MPCA/37 °C/batch K-B) seemed to be either mixed or otherwise polymorphic isolates. Both of them were purified as above, increasing the final number of isolates subjected to macroscopic examination, Gram staining and catalase tests to 127, next differentiated into seven main phenotypic LAB groups plus two non-LAB groups (Table 3). The numerical distribution of the 127 isolates from each phenotypic group within each Galotyri PDO cheese batch (Table 3), and in association with the selectivity of their enumeration/isolation agar media (Table 4), were also evaluated comparatively.

Table 3. Biochemical characterization and main grouping of 102 LAB isolates (plus additional 25 non-LAB or yeast isolates) from Galotyri PDO cheese in accordance to their numerical distribution in each of the four batches analyzed, and their cheese brand or total percent isolation frequency [1].

LAB Group	Basic Differentiating Characteristics							Cheese Batch				Brand-Z Isolates	Brand-K Isolates	Total Isolates
	MA	CO_2	NH_3	15 °C	45 °C	6.5%	KAA	Z-A	Z-B	K-A	K-B	Z-A + Z-B	K-A + K-B	
Mesophilic lactobacilli	R	–	–	+	–	+	V	4	0	9	13	4 (6.7)	22 (32.8)	26 (20.5)
Thermophilic lactobacilli	R	–	–	–	+	–	–	6	1	6	5	7 (11.6)	11 (16.4)	18 (14.2)
Mesophilic cocci (lactococci, pediococci)	C/LC	–	V	+	–	(+)/+d	–	0	1	1	0	1 (1.7)	1 (1.5)	2 (1.6)
Thermophilic cocci (streptococci)	LC	–	–	–	+	–	–	1	11	10	11	12 (20.0)	21 (31.3)	33 (26.0)
Enterococci	C	–	+	+	+	++	++	5	5	5	0	10 (16.7)	5 (7.5)	15 (11.8)
Leuconostoc-like (gas-forming) bacteria	CB	+	–	+	-	+/(+)	–	0	2	1	0	2 (3.3)	1 (1.5)	3 (2.3)
Gas-forming lactobacilli	R	(+)/+d	V	+	–	–	–	4	1	0	0	5 (8.3)	0 (0.0)	5 (3.9)
Total LAB isolates								20	21	32	29	41 (68.3)	61 (91.0)	102 (80.3)
Non-LAB isolates (catalase-positive)	C	–	NT	NT	–	+	NT	0	1	0	1	1 (1.7)	1 (1.5)	2 (1.6)
Yeast isolates	Y	V	NT	+	+	NT	NT	10	8	4	1	18 (30.0)	5 (7.5)	23 (18.1)
Total isolates								30	30	36	31	60	67	127

[1] All 127 isolates, including the 25 non-LAB or yeast isolates, were recovered from the first six enumeration agar media in Table 2 after purification of 120 (30/batch) colony cultures. Mixed colonies recovered from the cheese Brand-K samples (7 in total) resulted in two different colonies each after purification.MA, Microscopic appearance as rods (R), cocci (C), large cocci (LC) or coccobacilli (CB); Y, yeast spherical or elongated cells; 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; (+), weak positive reaction; +d, delayed (>3 days) positive reaction V, variable reaction.

The first important observation was that 18.1% (23/127) of the presumptive LAB isolates recovered from the four Galotyri PDO cheese batches were actually yeasts (Table 3). Most of them (18/127) were recovered from the ripened Brand-Z cheeses, accounting for 30% of the 60 isolates in total from batches Z-A and Z-B. Conversely, the isolation frequency of yeast colonies from the fresh Brand-K cheeses was 7.5% only (Table 3). In addition, it was noteworthy that none of the 23 yeast isolates was recovered from the MPCA/37 °C or the SB/37 °C agar plates (Table 4), apparently because the total viable LAB counts at 37 °C were by 1–3 log units higher than the yeast counts, and thus, yeast colonies were absent on the highest dilution MPCA plates; SB simply inhibited yeast growth. Hence, yeast colonies were present only on the MRS and M17 agar plates of cheese samples with similar LAB and yeast counts, i.e., mainly the ripened Brand-Z cheese batches (Tables 2 and 3). Yeast isolates were recovered from MRS and M17 agar at all incubation temperatures, from 22 °C to 45 °C (Table 4). In particular, all five MRS/45 °C isolates from batch Z-B, and four out of

the five MRS/45 °C and M17/42 °C isolates from batch Z-A were not 'thermophilic LAB' as it was anticipated, but they were thermophilic yeasts (Tables 3 and 4).

Table 4. Numerical distribution of the 102 LAB isolates and the remaining 25 non-LAB or yeast isolates from the four Galotyri PDO cheese batches 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 lactobacilli	–	8	–	8	–	10	26
Thermophilic lactobacilli	5	–	–	1	12	–	18
Mesophilic cocci (lactococci, pediococci)	–	1	–	1	–	–	2
Thermophilic cocci (streptococci)	16	1	16	–	–	–	33
Enterococci	–	–	–	–	–	15	15
Leuconostoc-like (gas-forming) bacteria	–	1	–	2	–	–	3
Heterofermentative (gas-forming) lactobacilli	–	–	–	5	–	–	5
Total LAB isolates	21	11	16	17	12	25	102
Non-LAB isolates (catalase-positive cocci)	–	2	–	–	–	–	2
Yeast isolates	–	7	4	3	9	–	23
Total isolates	21	20	20	20	21	25	127

Abbreviations for the growth/isolation agar media are given in Table 2; the LAB groups in the first column on the left are listed in accordance withtheir biochemical characterization in Table 2.

Thus, additionally to the LAB biota, yeasts were of major micro-ecological and biotechnological importance in both brands of Galotyri PDO cheese products. Therefore, the present 23 yeast isolates, along with an additional 40 yeast isolates recovered from the RBC agar plates of the Brand-Z and Brand-K cheese batches, were characterized in the course of a parallel study focusing on the differences in the yeast ecology between ripened and fresh Galotyri PDO and Galotyri-like cheese products (unpublished data).

Only two non-LAB isolates, Z46 from batch Z-B and K49 from batch K-B, were recovered (Table 3). Both were Gram-positive, catalase-positive cocci isolated from M17/22 °C agar plates (Table 4). Both grew abundantly as typical, coagulase-negative *Staphylococcus* colonies when streaked on BP/BP+RFP agar; they were not characterized further. Since the levels of staphylococci in all batches were more than 4-log units lower compared to the mixed LAB and yeast populations enumerated on the M17/22 °C plates, the isolates Z46 and K49 most probably were environmental contaminants.

The results of the basic biochemical characterization and main grouping of the 102 LAB isolates (i.e., 80.3% of total isolates in Table 3) indicated that thermophilic cocci, presumptively (starter) streptococci, were isolated in numerical predominance (26%) from most retail Galotyri PDO cheese batches, followed by mesophilic (nonstarter) lactobacilli (20.5%), thermophilic (starter) lactobacilli (14.2%) and enterococci (11.8%). In contrast, only two sporadic isolates (1.6%) of mesophilic (starter) cocci, i.e., lactococci and/or pediococci, were recovered, one from each Galotyri brand (Table 3).

Altogether the above five phenotypic LAB groups comprised 74.1% of the total isolates or 92.2% of the total LAB isolates; they were obligatorily homofermentative or facultative heterofermentative LAB types, meaning that none of them formed gas from glucose. Conversely, only eight sporadic isolates of gas-forming LAB were detected; three (2.3%) were *Leuconostoc*-like bacteria. The remaining five (3.9%) isolates formed a quite strange group of obligatorily heterofermentative, mesophilic, salt-sensitive, slender lactobacilli showing a delayed (>24 to 48 h) and fairly weak growth with gas production and a negative or weak arginine hydrolysis reaction (Table 3).

Regarding the numerical distribution of each LAB group isolates within each batch, thermophilic streptococci were isolated in abundance from all Galotyri PDO batches, except of batch Z-A (Table 3). Mesophilic (nonstarter) lactobacilli were mainly isolated from the fresh Brand-K cheeses, including additional 10 presumptive isolates of the *L. plantarum* group picked from SB agar (Table 4). No mesophilic lactobacilli were isolated from batch Z-B. No enterococci were detected in the fresh batch K-B. Except of one *Leuconostoc* isolate

(K5), all gas-forming LAB, particularly the strange group of slow gas-forming lactobacilli, were isolated from the two ripened Brand-Z cheeses. Overall, Z-B and K-A were the most diversified cheese batches containing at least one isolate of all LAB groups except of mesophilic non-gas-forming and gas-forming lactobacilli, respectively. K-B was the least diversified batch containing thermophilic streptococci, thermophilic lactobacilli and mesophilic lactobacilli only (Table 3).

Regarding the selectivity of the LAB enumeration/isolation agar media, as it was expected, nearly all thermophilic streptococci were isolated from MPCA/37 °C and M17/42 °C agar plates, while nearly all thermophilic lactobacilli were isolated from MRS/45 °C agar (mainly) and MPCA/37 °C plates (Table 4). In contrast, all mesophilic LAB types (groups), including all sporadic isolates, were recovered from MRS/30 °C and M17/22 °C agar plates (Table 4). All 15 isolates of enterococci, which in terms of relative population density was the most subdominant LAB group (Table 2), were exclusively recovered from the selective SB agar plates, which also was the isolation medium of additional 10 presumptive isolates of the *L. plantarum* group (Table 4).

3.4. Biochemical Identification and Brand-Dependent or Batch-Dependent Distribution of the LAB Species Identified in the Retail Galotyri PDO Cheese Samples

The results of the biochemical characterization and differentiation at the species level of the main mesophilic (36 isolates in total) and thermophilic (66 isolates in total inclusive of enterococci) LAB groups are summarized in Tables 5 and 6, respectively. Based on the grouping data in the above two tables, Table 7 summarizes the LAB species identity of the 102 isolates, indicates the batch-dependent distribution of each of the LAB species identified in the fresh (Brand-K) or the ripened (Brand-Z) retail Galotyri PDO cheese samples, while it further specifies the GL-K or GL-Z order codes of the LAB isolates within each group. These isolate code numbers are required for validating the present biochemical LAB species identifications by molecular id-tools in future studies.

Table 5. Biochemical characterization of 36 mesophilic LAB isolates from four Galotyri PDO batches and their numerical distribution in each fresh (Brand-K) or ripened (Brand-Z) cheese batch.

Biochemical Test	LAB Group (Subgroups/Biotypes)									
	Mesophilic Lactobacilli			Gas-Forming Lactobacilli			*Leuconostoc*-Like Bacteria		*Lactococcus*	*Pediococcus*
	A1	A2	A3	B1	B2	B3	C1	C2	D1	E1
CO_2 from glucose	−	−	−	+	+	(+)d	+	+	−	−
NH_3 from arginine	−	−	−	(+)/−	−	−	−	−	+	−
Growth in:										
2% salt	+	+	+	+	+	+	+	+	+	+
4% salt	+	+	+	1/2	−	(+)d	+	+	+	+
6.5% salt	+	+	+	−	−	−	+	(+)	(+)	+d
8.0% salt	+	4/5	−	−	−	−	−	−	−	−
10.0% salt	5/18	−	−	−	−	−	−	−	−	−
Slime	−	−	−	−	−	−	−	+	−	−
Acetoin	−	4/5	−	−	−	−	−	−	−	−
Acid from:										
Maltose	+	+	+	+	+	+d	+	+	+	(+)
Mannitol	+	+	+	(+)	−	−	−	−	−	−
Lactose	+	+	+	+	+	+d	+	+	+	+
Ribose	+	+	+	+	+	+	1/2	+	+	−
L-arabinose	1/18	−	−	+	+	+	1/2	-	−	−
Xylose	−	−	−	+	+	−	+	+	−	−
Raffinose	1/18	−	−	+	−	−	−	+	−	−
Melibiose	+	−	−	+	−	−	1/2	+	−	−
Sucrose	+	+	−	−	−	−	+	+	+	−
Cellobiose	+	+	+	−	−	−	−	+	+	+
Trehalose	+	+	−	−	−	−	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	+
Sorbitol	17/18	+	−	(+)	−	−	−	+	−	−
Melezitose	+	+	−	−	−	−	−	−	−	−
Total isolates	18	5	3	2	1	2	2	1	1	1

+, positive reaction; −, negative reaction; (+) weak positive reaction; +d, delayed positive reaction; 5/18, 5 out of 18 isolates in the group were positive.

Table 6. Biochemical characterization of 66 thermophilic LAB isolates from four Galotyri PDO batches and their numerical distribution in each fresh (Brand-K) or ripened (Brand-Z) cheese batch.

Biochemical Test	LAB Group (Subgroups/Biotypes)						
	Thermophilic Lactobacilli			Thermophilic Cocci (Streptococci)		Enterococci	
	F1	F2	F3	G1	G2	H1	H2
CO_2 from glucose	−	−	−	−	−	−	−
NH_3 from arginine	−	−	−	−	−	+	+
Growth in:							
2% salt	+/+d	+d	−	+	+	+	+
4% salt	−	−	−	4/28	2/5	+	+
6.5% salt	−	−	−	−	−	+	+
8.0% salt	−	−	−	−	−	8/11	+
10.0% salt	−	−	−	−	−	−	−
Slime from sucrose	−	−	−	−	−	−	−
Acetoin from glucose	−/(+)	−	−	5/28	+/++	(+)	−
Acid from of:							
Maltose	−	+	+	1/28	4/5	+	+
Mannitol	−	+	+	−	4/5	+	+
Lactose	+	+	+	+	+	+	+
Ribose	−	+	−	−	3/5	+	+
L-arabinose	−	−	−	−	3/5	+	−
Xylose	−	−	−	−	−	−	−
Raffinose	−	−	+	−	−	−	−
Melibiose	−	−	+	−	NT	+	+
Sucrose	−	+	+	+	+	9/11	+
Cellobiose	−	+	-	−	NT	+	+
Trehalose	−	+	+	1/28	4/5	+	+
Galactose	−	+	+	8/28	+	+	+
Sorbitol	−	−	−	−	2/5	1/11	+
Melezitose	−	−	−	NT	NT	−	+
Total isolates	16	1	1	28	5	11	4

+, positive reaction; −, negative reaction; ++, strong positive reaction; (+) weak positive reaction; +d, delayed positive reaction; 4/28, 4 out of 28 isolates in the group were positive; NT, not tested.

The mesophilic, facultative heterofermentative group of lactobacilli was split in three biochemical subgroups, A1 to A3; none of them was arginine-positive (Table 5). The largest subgroup A1 (18 isolates; all recovered from the fresh Brand-K cheeses; Table 7) comprised all mesophilic lactobacilli isolates that were able to grow on KAA agar as small black (esculin-positive) colonies (Table 2). All promoted abundant growth in the presence of 8% salt, and five of them in 10% salt (Table 5). Their key sugar fermentation reactions confirmed that all were members of the former *L. plantarum-L. paraplantarum-L. pentosus* genomic group of species [23,34], currently reclassified within the new genus *Lactiplantibacillus* [32]. All fermented mannitol and melibiose, however, only one isolate (K16) fermented raffinose, a sugar typically fermented by *L. plantarum*, *L. pentosus* and most *L. paraplantarum* strains. Only one isolate (K4) fermented L-arabinose, while none of the 18 isolates fermented D-xylose, two sugars typically fermented by *L. pentosus* and several *L. plantarum* strains. Only *L. paraplantarum* does not ferment D-xylose [23]. Hence, all A1 isolates were biochemically assignable to the *L. plantarum* group (Table 7).

All eight mesophilic lactobacilli isolates in the two minor subgroups A2 and A3 fermented mannitol, but failed to ferment raffinose, xylose, L-arabinose and mainly melibiose (Table 5). Moreover, the three isolates in A3, recovered from the fresh cheese batch K-B (Table 7), had curved (horseshoe-like) cells and further failed to ferment sorbitol, sucrose, trehalose and melezitose (Table 5). Based on the above key differentiating criteria, the subgroup A2 was biochemically classified to *Lactobacillus paracasei* [23,30], currently reclassified as *Lacticaseibacillus paracasei* [32]. Whereas the subgroup A3 rather comprised atypical dairy

strains of *Lactobacillus curvatus* (i.e., the species typically fails to ferment mannitol) [23], currently reclassified as *Latilactobacillus curvatus* [32].

Table 7. Batch-dependent distribution of the 102 biochemically identified LAB isolates in the ripened (Brand-Z) or fresh (Brand-K) Greek Galotyri cheese PDO retail products.

LAB Species Identified	Total Isolates	Ripened Galotyri PDO (Brand-Z) Cheese Batches		Fresh Galotyri PDO (Brand-K) Cheese Batches		Biochemical Subgroup in Table 5 or Table 6: LAB Isolate Code [1]
		Batch Z-A	Batch Z-B	Batch K-A	Batch K-B	
Starter LAB isolates	46	7	6	17	16	
Streptococcus thermophilus	28	1	6	10	11	G1:Z25, Z49, Z54-Z56, Z59, Z60, K21-K30, K51-K60, K58B
Lactobacillus delbrueckii subsp. *bulgaricus*	16	6	–	5	5	F1:Z6, Z26-Z30, K6, K6B, K7, K8, K10, K36-K40
*Lb. delbrueckii*subsp. *lactis*	1	–	–	1	–	F2:K9
Lactococcus lactis	1	–	–	1	–	D1: K17
Nonstarter LAB isolates	56	13	15	15	13	
Unidentified thermophilic *Streptococcus* spp.	5	–	5	–	–	G2: Z51, Z52, Z53, Z57, Z58
Unidentified thermophilic *Lactobacillus* sp.	1	–	1	–	–	F3: Z35
Lactiplantiibacillus plantarum group	18	–	–	8	10	A1: K2, K4, K11-K15, K16, K31, K41-K48, K50
Lacticaseibacillus paracasei	5	4	–	1	–	A2:Z3, Z16, Z18, Z20, K3
Latilactobacillus curvatus (atypical biotype)	3	–	–	–	3	A3: K32, K33, K34
Lentilactobacillus diolivorans (or atypical *L. hilgardii*)	2	2	–	–	–	B1:Z2, Z5
Lentilactobacillus diolivorans	1	1	–	–	–	B2: Z1
Lentilactobacillus kefiri	2	1	1	–	–	B3: Z4, Z34
Leuconostoc mesenteroides	3	–	2	1	–	C1+C2: Z31, Z50, K5
Pediococcus inopinatus/parvulus	1	–	1	–	–	E1: Z32
Enterococcus faecium	11	4	2	5	–	H1: Z12-Z15, Z41, Z44, K11B-K15B
Enterococcus faecalis	4	1	3	–	–	H2: Z11, Z42, Z43, Z45
Total isolates	102	20	21	32	29	

[1] All Galotyri isolates bear the prefix GL before the cheese Brand-Z or K letter; for table simplification, GL is omitted in the last column specifying the isolate codes within each LAB species, according to the biochemical group identification data in Tables 5 and 6; isolates with GL code numbers Z1-Z30, Z31-Z60, K1-K30, and K31-K60 were recovered from the ripened batches Z-A, Z-B and the fresh batches K-A, K-B, respectively. Missing code numbers were yeast or non-LAB isolates in accordance to the data in Table 3. The GL-Z and GL-K isolates with numbers 1–5, 6–10, 11–15, 16–20, 21–25, and 26–30 were recovered from the agar media MRS/30 °C, MRS 45 °C, KAA/37 °C, M17/22 °C, M17/42 °C and MPCA/37 °C, respectively, in accordance to the data in Tables 2 and 4.

The minor mesophilic group B of slow-growing and slow-gas-forming lactobacilli, exclusively isolated from the ripened Brand-Z cheeses (Table 3), was biochemically assigned to the former *Lactobacillus buchneri* group of species [23], currently reclassified as a new genus *Lentilactobacillus* [32]. All five isolates fermented ribose and maltose and failed to ferment cellobiose and trehalose (Table 5), two of the key-negative sugars for most gas-forming lactobacilli [23]. Otherwise the five isolates were quite diverse, split in three subgroups, B1 to B3 (Tables 5 and 7). Because all failed to ferment melezitose, none was assignable to *L. buchneri* or *L. parabuchneri*, which both are melezitose-positive [23]. Because all fermented L-arabinose, none could be assigned to the L-arabinose-negative species *L. hilgardii*. Instead the two xylose-negative isolates, Z4 and Z34, in subgroup B3 were closer to *L. kefiri*, while the single xylose-positive but melibiose-negative isolate Z1 in B2 was closer to *L. diolivorans* (the species typically ferments melibiose). Accordingly, the Z2 and Z5 isolates in subgroup B1, which fermented xylose, raffinose and melibiose, were *L. diolivorans* or otherwise atypical (L-arabinose-positive) *L. hilgardii* strains [23].

The three sporadic *Leuconostoc*-like isolates were differentiated in two subgroups C1 and C2 (Table 5). Both C1 isolates, Z31 and Z50 from batch Z-B (Table 7), fermented xylose, but failed to form slime from sucrose and to ferment several other main (key) sugars, including mannitol, sorbitol, raffinose and cellobiose; Z50 further failed to ferment L-arabinose and ribose. Therefore, both isolates were atypical dairy isolates of *Leuc. mesenteroides*, or closer to *Leuc. pseudomesenteroides* [15,18,25]. C2 comprised K5, the only LAB isolate that

formed slime from sucrose (Tables 5 and 7). K5 failed to ferment mannitol and L-arabinose; probably it was a *Leuc. mesenteroides* subsp. *dextranicum* strain [25].

The only single *Lactococcus* (subgroup D1) isolate of this study, coded K17, from the fresh cheese batch K-A (Table 7), was a typical arginine-positive, xylose-negative *Lc. lactis* strain (Table 5). Thus, most likely, K17 belonged to the subspecies *lactis* [27,29] and represented a commercial *Lc. lactis* starter strain applied in the Brand-K dairy plant. The other single mesophilic coccoid isolate, Z32 from the ripened batch Z-B (Table 7), was an arginine-negative *Pediococcus* (subgroup E1; Table 5) based on the microscopic alignment of its large spherical cells in tetrads. Biochemically, Z32 was closer to the lactose-positive species *P. inopinatus* (Table 5) rather than to the lactose-negative *P. parvulus* [15,24].Both species are arginine-negative and closely related, sharing the same habitats [24].

The thermophilic obligatory homofermentative group of lactobacilli was split in three biochemical subgroups, F1 to F3; none of them was arginine-positive (Table 6). The largest subgroup F1 (16 isolates from all batches except of batch Z-B; Table 7) was highly homogeneous and displayed the very typical oligofermenting profile (i.e., positive for lactose only) of the dairy starter *L. delbrueckii* subsp. *bulgaricus* [15,23]. An additional single isolate, K9 from batch K-A (Table 7), in subgroup F2 was similar microscopically to the F1 isolates; however, it fermented several sugars additionally to lactose (Table 6). Biochemically, strain K9 resembled with *L. delbrueckii* subsp. *lactis,* although its positive reactions with mannitol and ribose were atypical for the species *L. delbrueckii* [23]. The single isolate Z35 (subgroup F3; Tables 6 and 7) was a thermophilic filamentous rod of the *Lactobacillus salivarius* group [23]; most probably a cheese contaminant of animal origin because it was the only β-hemolytic isolate found in this study (data not shown). Additional tests are required to elucidate the species identity of this strange isolate.

The large group of thermophilic streptococci was split further into two subgroups, G1 and G2 (Table 6). The largest subgroup, G1, was the most numerous LAB species, including 28 isolates. All fermented lactose and sucrose strongly (Table 6), displaying the typical oligofermenting profile of *S. thermophilus* starter strains [15,26]. Eight G1 isolates, four from batch Z-B and another four from batch K-B, fermented galactose. In contrast, two typical galactose-negative *S. thermophilus* isolates, K24 and K30, from batch K-A, fermented trehalose, and maltose, respectively. Most *S. thermophilus* (21 isolates) were recovered from the fresh Brand-K batches (Table 7). However, both brands used natural or commercial *S. thermophilus* starters in Galotyri cheese. The isolation from the ripened batch Z-B of additional three multi-fermenting thermophilic *Streptococcus* strain biotypes (a total of 5 isolates) was another interesting finding. These spontaneous isolates were included altogether in the heterogeneous subgroup G2 (Tables 6 and 7) because it was difficult to identify them at the species with biochemical criteria [26]. Additional investigations are required to validate the identity and the origin of the subgroup G2 isolates.

Finally, all 15 *Enterococcus* isolates were able to hydrolyze arginine strongly, with abundant ammonia formation, and to grow at 45 °C and in 6.5% salt; they were split further into two subgroups, H1 and H2 (Tables 6 and 7). All 11 isolates in subgroup H1 fermented L-arabinose, failed to ferment melezitose, and generally were typical strain biotypes of *E. faecium* (Table 6); whereas all 4 L-arabinose-negative but melezitose-positive isolates in subgroup H2 (Table 6) were typical *E. faecalis* [28,30,31].

Regarding the most abundant LAB species in each batch, it was confirmed that *L. delbrueckii* subsp. *bulgaricus* was dominant in the ripened batch Z-A, whereas *S. thermophilus* was most abundant, followed by a 10-fold lower mixed mesophilic LAB biota consisting mainly of *Leuc. mesenteroides*,*Pediococcus,* and *Lentilactobacillus* in the ripened batch Z-B (Table 2 in link with Table 7). Conversely, the viable LAB biota in both fresh cheese batches K-A and K-B was dominated by natural or industrial starter strains of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* at similarly high levels (>8.5 log CFU/g) followed by an approximate 3-log unit lower mixed mesophilic LAB biota primarily consisting of *Lactiplantibacillus* (Tables 2 and 7). *Leuc. mesenteroides* and *Lc. lactis* were sporadically

detected in batch K-A only, whereas batch K-B was the least diversified Galotyri PDO cheese of this study as regards its viable LAB biota (Table 7).

4. Discussion

The results of this study revealed that the manufacturing technology of both retail, brand-named artisan-type Galotyri PDO cheese products was based on an intensive fermentation-acidification step of the pasteurized ewes'/goats' milk with the aid of natural (Brand-Z) or, evidently, commercial (Brand-K) symbiotic starter strains of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* at temperatures ≥37 °C. In specific, based on the technical questionnaire filled by Brand-Z only, the earliest process step in the Galotyri-Z PDO variety was the transformation of the pasteurized ewes'/goats' milk into a basal yogurt curd by a natural thermophilic yogurt starter. Milk fermentation was initiated at ≥42–45 °C for about 3 h, followed by a gradual decrease in the curdling milk temperature at 20–22 °C within the first 24 h, and then cutting and salting of the fresh curd for draining and further handling. Although the natural yogurt starter used in the Galotyri-Z cheese variety was not provided to our laboratory for microbiological testing, the quantification (Table 2) combined with the LAB identification results (Tables 3 and 7) confirmed a high presence of *S. thermophilus* and/or *L. delbrueckii* subsp. *bulgaricus* in the ripened batches Z-A and Z-B, which were already about two months old on the day of analysis (Table 1). Fundamentally, the above two thermophilic LAB species are the primary symbiotic bacteria in naturally fermented (Greek) yogurt, which may contain additional species at subdominant population levels, like various other streptococci, probiotic lactobacilli, enterococci, lactococci, spontaneous heterofermentative LAB, yeasts, etc. [35–37]. Thus, the five unidentified thermophilic *Streptococcus* spp. isolated from batch Z-B (Table 7) might also originate from the natural yogurt starter [38] used to prepare the basal Galotyri-Z yogurt-like curd. The fact that the population density and isolation frequency of *S. thermophilus* was higher in batch Z-B (pH 4.0) than batch Z-A (pH 3.8), and vice versa for *L. delbrueckii* (Table 7), was probably due to the more acidic pH of the former cheese compared to the latter after ripening (Table 2). In general, *S. thermophilus* grows faster than *L. delbrueckii* in yogurt fermentations, with the latter species stimulating the former at an early exponential growth phase [35]. However, a progressive reversal prevalence of *L. delbrueckii* occurs as the yogurt 'ages' and becomes more acidic with storage, a phenomenon attributed to the increased lactate production and tolerance of *L. delbrueckii* to pH values below 4.0 down to 3.5, conditions that inhibit *S. thermophilus* [35]. A similar reversal trend might occur in Galotyri-Z yogurt-like basal curds during ripening: symbiosis of *S. thermophilus* with *L. delbrueckii* in yogurt has a successive pattern that eventually becomes antibiosis, and the latter species dominates and survives at the expense of catabolic products of the former species [35].

The preparation of a basal yogurt curd was most evident in the fresh Galotyri-K PDO cheese variety. The very high levels (>8.5 log CFU/g) of thermophilic LAB (Table 2), consisting of *S. thermophilus* and *L. delbrueckii* at an approximate 50:50 ratio, clearly indicated that the pasteurized Galotyri-K cheese milk was fermented/acidified by the thermophilic-K isolates of the above two symbiotic species (Table 7), most likely being the strain constituents of a commercial starter culture for yogurt production. Moreover, during microbiological sampling, it was easy to observe visually that the soft cheese body of the fresh retail batches K-A and K-B was not homogeneous, like a yogurt or the industrial Galotyri PDO cheese variety we studied previously [15], but rather it was a 'biphasic' cheese matrix consisting of a continuous, viscous, yogurt-like smooth curd phase in which small irregular particles of another granulated soft cheese curd were dispersed evenly. Based on our experience [15], it might be hypothesized that the above particles were Feta cheese trimmings, seemingly ripened before dispersion in the fresh basal yogurt-like Galotyri-K curd because they were salted, very tasty and aromatic after picking and rinsing them to be tasted separately. Litopoulou-Tzanetaki and Tzanetakis [11] first reported the addition of Feta cheese at a 1:5 proportion in basal yogurt curds as a quite common empirical practice for traditional Galotyri cheese making. Later, this empirical 'external aromatization' process

was confirmed for another two artisan-type Galotyri varieties by Samelis and Kakouri [15]; however, this operation is not specified in the Galotyri PDO technology [8,13]. Notably, those previous two shortly ripened artisan-type Galotyri varieties were characterized by a high isolation frequency of *Lc. lactis* or *L. plantarum*, both species particularly associated with the dispersed Feta cheese granules analyzed microbiologically as separate Galotyri cheese subsamples [15]. Hence, the high isolation frequency of *Lactiplantibacillus,* especially *L. plantarum*,as subdominant mesophilic LAB from both fresh Galotyri-K cheese batches might also associate with the dominance of this NSLAB group in the ripened Feta cheese trimmings being dispersed in the basal Brand-K yogurt curd. It is well documented that *L. plantarum* is the commonest heterogeneous species of the nonstarter mesophilic lactobacilli in Greek Feta cheese [39], generally increasing its prevalence during ripening due to its increased acid tolerance [2,40]. Particularly, the use of selected *L. plantarum* strains in industrial Greek Feta PDO cheese productions has recently attracted major attention due to their aromatic, antibacterial, antifungal and probiotic properties [41]. In accordance, *L. plantarum* had the highest occurrence (% appearance in samples and number of isolates) within a total of 20 different LAB species most recently identified by MALDI-TOF MS profiling of 23 Feta cheese samples collected from various dairy plants in Greece, which was 100% (31 isolates) and 87% (47 isolates) after 3 and 6 months of ripening, respectively [42].

The single *Lc. lactis* K17 isolate from batch K-A (Table 7) might also originate from the Feta cheese trimmings, although the direct addition of a mixed thermophilic (*S. thermophilus*, *L. delbrueckii*) and mesophilic (*Lc. lactis* strain variants) in the bulk milk during Galotyri-K manufacture cannot be excluded. *Lactococcus lactis* is the primary mesophilic starter LAB species in Greek Feta PDO cheese productions [2,11,40], usually applied as multi-strain mixtures of the subspecies *lactis* and *cremoris* [41]. A recent study by Papadimitriou et al. [43] based on amplicon sequencing and shortgun metagenomics found that *Lc. lactis* dominated two artisanal homemade samples while *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* dominated one industrial sample of Feta cheese [43]. Meanwhile, *L. plantarum* was more abundant in the two homemade Feta cheese samples dominated by *Lc. lactis* [43]. These findings are consistent with our early (2003–2006) findings and preceding discussions with regard to the high dominance of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* in an industrial Galotyri PDO variety, whereas *Lc. lactis* dominated in an artisanal Galotyri variety with dispersed Feta cheese trimmings [15]. However, the high initial *Lc. lactis* counts decline during the ripening of most Feta cheese productions [2,40,42,44], either naturally or due to controlled autolysis of the industrial starter strains [45]. Autolysis is desired for enhancing cheese ripening, reducing bitterness, and additional technological benefits, and it may be triggered shortly after fermentation, depending on the starter strain, the cheese type, and other factors [45,46]. Autolysis of commercial *Lc. lactis* starter strain/s might have also occurred in the aforementioned 'biphasic' fresh Galotyri-K cheese curds, probably enriched with mature Feta cheese trimmings after manufacture. In accordance, although *Lc. lactis* was detected at high relative abundances culture-independently, sporadic isolates of viable *Lc. lactis* were recovered during the microbiological and metagenomic characterization of a retail Galotyri-like acid-curd cheese traditionally manufactured by mixing fresh yogurt with 'Myzithrenio', a naturally fermented and ripened Greek whey cheese [18].

Additionally to *L. plantarum,* the fresh Brand-K cheese batches contained isolates of three of the commonest mesophilic LAB species in traditional Greek cheeses and other dairy products, namely *Lacticaseibacillus paracasei*, *Latilactobacillus curvatus,* and *Leuc. mesenteroides* (Table 7). In specific, numerous acid-tolerant strains of *L. paracasei* have also been reported to prevail in mature Feta PDO cheese [2,17,42,44], other low-pH white-brined cheeses [11], and traditional acid-curd cheeses such as Anevato [9]. However, no *L. paracasei* strains were isolated from the Galotyri PDO cheese varieties studied previously by Samelis and Kakouri [15] and Rhoades et al. [17]. On the other hand, *L. curvatus* is a meat-specific LAB used as a starter culture in meat fermentations [23,47], but also is a common NSLAB in dairy foods [23], including Feta [42] and traditional Greek acid-curd cheeses, such as Kopanisti [11,48]. *Leuconostoc mesenteroides* is another complex LAB species, consisting of

four subspecies [32], that still has limited dairy applications despite it's ubiquitous in milk and milk products [49]. In particular, it is one of the commonest indigenous NSLAB in artisan cheeses, including Galotyri PDO varieties [15] and nearly all traditional acid-curd and white-brined Greek cheeses [2,11]. Only *Leuc. mesenteroides* subsp. *cremoris* strains are ordinarily used as starters in the dairy industry [49,50].

The ripened Brand-Z cheeses also contained indigenous *L. paracasei* (batch Z-A) or *Leuc. mesenteroides* (batch Z-B) isolates. However, they were clearly discriminated from the fresh Brand-K cheeses technologically because of neither *Lactiplantibacillus*/*L. plantarum* nor *Lc. lactis* were isolated from them (Table 7). Based on the technical questionnaire, no *Lc. lactis* starters were used for the production of the ripened Brand-Z Galotyri PDO cheese samples. Instead, the pasteurized milk in Brand-Z was initially transformed into a basal yogurt curd with the sole use of natural thermophilic starters to assure an optimal and safe fermentation process [11,18,22,51], followed by natural aging (ripening) of the mildly (<2%) salted fresh cheese curd to enhance the diversity of the indigenous subdominant mesophilic LAB biota in the final Galotyri-Z cheese PDO variety.

On the above technological basis, the isolation of free-living *Lentilactobacillus* from both ripened Galotyri-Z batches, which were not identified as *L. buchneri* or *L. parabuchneri* as usual [2,11] but as *L. kefiri*, *L. diolivorans* and possibly *L. hilgardii* (Table 7), was the most interesting micro-ecological finding of this study. Although a recent metagenomic study by Michailidou et al. [16] detected *L. kefiri* and exclusively *L. hilgardii* at a relative abundance as high as 21.2% in one Galotyri PDO cheese variety from Epirus, to the best of our knowledge, this is the first time *L. kefiri* and *L. diolivorans* have been isolated from a traditional Greek acid-curd cheese. In particular, no Galotyri isolates of the above two species are described in the comparative culture-dependent LAB identification study [17] of the above amplicon metabarcoding cheese study [16]. To confirm this, we also searched the official Greek dairy (ACA-DC) collection of the Dairy Laboratory, Agricultural University of Athens, for possession of strains belonging to the above three rare *Lentilactobacillus* species. Only two strains of *L. diolivorans*, ACA-DC-0622 and ACA-DC-0624, both isolated from Feta cheese (Korinthos) by Asteri et al. [10], and only three Feta cheese (Evia) isolates of *L. kefiri* (ACA-DC-0900) or *L. kefiri*/mostly *otakiensis* (ACA-DC-0899 and ACA-DC-0902) are included in the collection, which lacks *L. hilgardii* strains. The isolation of non-deposited *L. hilgardii* from Greek Manura hard cheese has been reported [11], while most recently, *L. kefiri* was isolated, again from Feta cheese [42].

Overall, among the three *Lentilactobacillus* identified (Table 7), *L. kefiri,* originally isolated from kefir, is a quite common but always a subdominant species detected culture-dependently in naturally acidified cheeses and milk products from other countries, including the French RDO Camembert [52], the Italian Ricotta forte [53], the Serbian kajmak [54] and various traditionally fermented milks from Mongolia [55,56], Kazakhstan [57] and China [58]. Most recent NGS studies have detected *L. kefiri* in the microbial communities of industrially produced, ripened Edam cheeses [59] and of the Portuguese Queijo de Azeitao PDO [60]. *L. hilgardii* also is another subdominant heterofermentative LAB in low-pH dairy products, such as the Turkish Civil cheese [61] and various Himalayan ethnic fermented milk products where it coexisted with *L. kefiri* from the genus *Lentilactobacillus* and many other indigenous LAB genera and species [62]. More recently, *L. hilgardii* was found to be a member of diverse biofilms associated with the processing lines of the French Salers PDO [63] and the Italian Ragusano PDO, where it was particularly correlated with the formation of volatile ketones [64]. The 'free-living' style of *L. hilgardii* was also in accordance with its dominance in spontaneous cheese whey wastewater fermentations [65] and water kefir fermentations detected by shortgun metagenomic sequencing and linked with the production of mannitol from fructose [66]. Conversely, *L. diolivorans* is a rarely isolated species from dairy foods [67]. However, it was more frequent than *L. kefiri* in naturally fermented Mongolian milk products [56], while it was also involved in the natural fermentation of traditional Tibetan Qula cheese [68], an artisanal cheese from southern Brazil [69], and artisanal "Torta"-type Spanish cheeses [70]. In addition, *L. kefiri* and *L. diolivorans*

were among the LAB species isolated from traditional Iranian yogurts [71,72]. In summary, all three *Lentilactobacillus* species isolated from the ripened Galotyri-Z PDO cheeses share similar low-pH dairy habitats (i.e., yogurt, fermented milks, acid-curd cheeses, fermented whey cheeses, etc.) and may contribute to the probiotic properties and thereby the health benefits associated with these products. However, no *Lentilactobacillus* were isolated from the fresh Galotyri-K PDO cheeses (Table 7) or, previously, from the fresh Galotyri-like delicatessen cheese products [18].

Regarding the single *P. inopinatus/parvulus* isolate Z32 from the ripened batch Z-B (Table 7), Samelis and Kakouri [15] first isolated and reported the uncommon prevalence of lactose-fermenting strains of *P. inopinatus* in another artisan-ripened Galotyri PDO cheese variety produced by a traditional SME in Thessaly. In fact, *P. inopinatus* may be considered a natural co-starter in cheese: few previous studies exist on the occurrence and use of native *P. inopinatus* as adjuncts in traditional non-European cheeses, such as in white-brined Egyptian Domiati [73] and white-brined Iranian cheeses [74]. On the other hand, *P. parvulus* is a closely related but uncommon *Pediococcus* species in dairy niches and products, unable to ferment lactose [24]. Nevertheless, *P. parvulus* has been found in artisanal raw milk cheeses from Asian countries only, such as the Tulum acid-curd cheese from East Anatolian cities of Turkey [75] and the Iranian Siahmazgi cheese [76].

Owing to their inherent heat resistance and broad growth temperature range (10–45 °C), autochthonous dairy (lactose-fermenting) enterococci constitute a significant part of the indigenous LAB communities in traditional Greek cheese fermentations [2,11,30]. However, enterococci are neither strong acid-producers nor acid-resistant, and thus, generally, they have an approximate 2 to 4 log units lower prevalence than typical aciduric starter LAB and NSLAB species (i.e., *S. thermophilus*, *Lc. lactis*, *L. plantarum*) in traditional acid-curd (pH < 4.5) Greek cheeses, such as Galotyri PDO, Anevato PDO, Xinotyri [9,11,14,15,31], and the fresh or ripened Galotyri cheese products of this study.

Finally, all four cheese batches were safe (pH 3.8–4.1), according to the EC Regulation 2073/2005 criteria [33] and previous challenge and/or validation studies with regard to *L. monocytogenes* [22,51]. However, despite the inability of the pathogen to grow in RTE foods with pH < 4.4, the recovery of viable *L. innocua* from batch Z-B was a hygienic concern for reasons addressed after natural *L. monocytogenes* contaminants were previously isolated from one industrial and two artisan Galotyri PDO cheese batches [14]. Another concern, particularly regarding the fresh Brand-K cheese samples, was the quite high (>6.0 log CFU/g) levels of yeasts shortly after production. Native beneficial (aromatic) yeasts may progressively turn to become the primary surface spoilage microbiota in Galotyri [14] and other fresh acid-curd or ripened low-pH cheese types (e.g., Feta cheese) under aerobic storage conditions in retail. Correspondingly, the high yeast counts in the fresh Brand-K cheeses might also originate from the dispersed ripened Feta cheese trimmings, probably enriched in various yeast genera such as *Debaryomyces*,*Kluyveromyces*, *Cutaneotrichosporon*, *Pichia*, *Candida*, and *Rhodotorula* [43].

To this end, it should be noted that all 102 Galotyri PDO cheese LAB isolates that were biochemically identified during this study (Table 7) have officially been submitted for genotypic characterization in the molecular laboratory of the Institute of Agricultural Products (ITAP), ELGO-DIMITRA (Lykovrissi, Athens) along with the corresponding Galotyri-Z and Galotyri-K cheese samples for comparative metagenomic analysis (Scientific responsible: Dr. A. Doulgeraki). The culture-dependent molecular identifications primarily based on the 16S rRNA sequencing of representative LAB isolates have shown a high consistency with their biochemical identification (Table 7), with few taxonomic discrepancies existing only. Moreover, multiplex PCR amplification of the *rec*A gene has shown the presence of only one *L. paraplantarum* (strain K4) and one *L. pentosus* (strain K2) within the 18 subgroup A1 isolates (Table 7) of the *L. plantarum* group (unpublished data). This study serves as the basis for reporting on the molecular culture-dependent and metagenomic characterization of commercial fresh vs. ripened Galotyri PDO cheese products in future studies.

This study may also serve as a basis for future in-depth studies on the ecological and biotechnological discrimination of commercial Galotyri PDO cheese varieties sold fresh or ripened, as well as for their regulatory/trade discrimination for reasons previously addressed by Samelis and Kakouri [15]. The type and effects of ripening, as well as the benefits vs. the risks of ripening, should be evaluated in association with the type of milk (raw, pasteurized, or 'boiled') and the type and effects of starter LAB (i.e., solely thermophilic or mesophilic, or mixed thermophilic and mesophilic; with or without adjunct cultures) used in each PDO-certified dairy for Galotyri production. As mentioned, the authentic Galotyri is fermented slowly at ambient temperatures with the aid of a natural mesophilic 'starter' LAB biota, followed by cold ripening for at least two months. The current use of pasteurized milk inoculated with commercial thermophilic or mixed thermophilic with mesophilic starters in Galotyri processing and the shortening or even lack of ripening represent common industry practices, which are more convenient and profitable. Therefore, they are adopted by several traditional dairies also [15], including the Brand-Z and mainly the fresh Brand-K Galotyri products of this study. Because the Galotyri PDO specifications allow this cheese to be made of pasteurized milk with LAB starters, both products complied as regards the milk fermentation process. However, the particular technological Galotyri PDO specification "*the naturally acidified curd should be ripened at temperatures below 8 °C for at least 2 months in case the cheese is made of raw milk*" [8] is discrepant (i.e., the authentic Galotyri is made of 'boiled' milk). Unfortunately, this discrepancy allows several Greek cheese producers to market fresh (non-ripened) industrial or artisan-type Galotyri PDO cheese varieties from pasteurized milk, frequently supplemented with ripened Feta or whey cheese trimmings for external aromatization purposes [15], such as Brand-K. On the other hand, although the use of raw milk for making Galotyri, and generally cheese, is an uncommon practice in traditional Greek dairies to date, one of the three Galotyri PDO varieties from Epirus recently studied by Rhoades and co-workers was reported to be made of raw milk [16,17]. In summary, based on the literature and the findings of our present study, Galotyri probably represents the most variably manufactured and microbiologically diverse traditional Greek PDO cheese.

5. Conclusions

This study revealed significant differences in the microbiological characteristics and the NSLAB species diversity of two brand-named Galotyri cheese PDO varieties, as well as an important biotechnological similarity, which was the prevalence of thermophilic yogurt starter strains in both products regardless of whether they were marketed fresh (Brand-K) or ripened (Brand-Z). However, the fresh Galotyri-K market cheeses had ca. 2–3 log CFU/g higher viable populations of starter *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* than the ripened Galotyri-Z market cheeses. Generally, the two products were distinct from each other in terms of microbial and trade/labeling attributes. The secondary viable LAB biota of the ripened Brand-Z cheeses was quite diversified and comprised *L. paracasei*, free-living *Lentilactobacillus* (*L. diolivorans*, *L. kefiri*, *L. hilgardii*), *Leuc. mesenteroides*, *P. inopinatus*, plus *E. faecium* and *E. faecalis* at higher subdominant levels. Conversely, the secondary LAB biota of the fresh Brand-K cheeses was enriched in *Lactiplantibacillus*, primarily *L. plantarum*; sporadic isolates of *L. paracasei*, *Lc. lactis*, *Leuc. mesenteroides*, *L. curvatus*, and *E. faecium* at levels <1000 cells/g were also found. Further research is required to describe the biochemistry and potential health benefits associated with Galotyri ripening, including in-depth studies on the key technological and functional characteristics of indigenous NSLAB isolated from the ripened Brand-Z cheeses of this study. In general conclusion, ripening enhances the microbial ecology and LAB species diversity of Galotyri PDO cheese products, but concurrently, it reduces the total viable load of the beneficial LAB the cheese contains when it is sold and consumed fresh. This biotechnological issue also requires further investigation and verification.

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Data Availability Statement: The data presented in this study, as well as the bacterial strains identified, are available on request from the corresponding author. In addition, all LAB and yeast isolates reported herein were officially transferred from the Dairy Research Department (Katsikas, Ioannina, Greece) and deposited in the microbial culture collection of the Institute of Agricultural Products (ITAP) of ELGO-DIMITRA (Lykovrissi, Greece). This deposit was validated through a "Material Transfer Agreement" co-signed by the provider investigator (John Samelis), the recipient investigator (Agapi Doulgeraki), and the authorized representative (Efthymia Kondyli, Director of ITAP) on 25 May 2018, and it was a contractual obligation under the terms of the Nagoya protocol included in the Consortium Agreement of the funding project ProMedFoods.

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