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Research article

Major technological differences between an industrial-type and five artisan-type Greek PDO Galotyri market cheeses as revealed by great variations in their lactic acid microbiota

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Abstract: Galotyri is a Greek PDO acid-curd, traditionally ripened, soft spread cheese produced from boiled (>80–90 °C), naturally acidified ewes' or ewes'/goats' milk in the regions of Epirus and Thessaly. In this survey study, the microbiology of an industrial Galotyri cheese variety (11 batches) was compared with five artisan-type Galotyri cheese varieties (1–2 batches each), with emphasis on the species of lactic acid bacteria (LAB) prevailing in the final product. All market cheese samples distributed either in packaged or bulk form, were purchased from retail outlets at Ioannina, Epirus during two sampling periods, 2003–2006 and 2014–2019. A total of 366 (189 industrial and 177 artisan) cheese LAB isolates were identified by key biochemical criteria. Strains of *Streptococcus thermophilus* and/or *Lactobacillus delbrueckii* subsp. *bulgaricus* were isolated nearly as a pure symbiotic starter culture (97.4%) from all industrial batches, labeled as a fresh cheese combining the nutritional properties of yogurt. Conversely, the artisan-type cheeses were ripened and more diversified in terms of LAB ecology. They contained strains of *Lactococcus lactis* (19.8%), *Lb. plantarum* (16.9%), *S. thermophilus* (14.7%), *Leuconostoc mesenteroides* group (11.3%), *Pediococcus inopinatus* (7.3%), *Enterococcus 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 was cheese product batch-dependent. In conclusion: (i) the industrial Galotyri cheese technology results in a fresh yogurt-like cheese of limited (thermophilic) LAB diversity; (ii) the artisan-type Galotyri cheese technologies may vary significantly, but share a decisive ripening step that favors the predominance of mesophilic LAB species in the final market products; (iii) a regulatory distinction between fresh and ripened varieties of commercial PDO-certified Galotyri cheeses is required.

Keywords: Galotyri industrial vs. artisan cheese; LAB diversity; PDO certification

1. Introduction

Cheeses of Protected Denomination of Origin (PDO) are authentic traditional dairy products that are produced, processed and prepared in a specific geographical area, using recognized know how based on certified compositional and technological specifications [1]. They are economically and culturally important foods in Europe, particularly in Greece and other Mediterranean countries [2,3]. Generally, traditional (artisan) cheese types made of raw or thermized milk owe many desirable sensorial and nutritional characteristics to their microecological complexity and biodiversity, which are higher compared to similar industrial cheese types made of pasteurized milk [2–5]. Therefore, there is an increasing interest in investigating the microbial ecology of traditional cheeses, in priority of PDO cheese varieties, with emphasis on the autochthonous species and strains of lactic acid bacteria (LAB) and their biotechnological, antimicrobial and probiotic properties [6–11].

Galotyri is amongst the 21 traditional Greek PDO cheeses [12] recognized in 1994 [13]. It is a clean-white, acid-curd, soft, spreadable cheese without surface skin, eyes or cracks produced exclusively in the regions of Epirus and Thessaly. Its maximum permitted water content is 75% and its minimum permitted fat content in dry matter is 40%. The final cheese has a pleasant, slightly acidic, refreshing taste and aroma. The cheese curd should be made of ewes' or goats' milk, or their mixtures. The milk has to be collected from breeds raised in the aforementioned regions and it should be of good quality, full fat, raw or pasteurised. Addition of rennin, edible sea salt, and LAB starter cultures is allowed; addition of cow milk, condensed milk, milk powder, milk proteins, casein salts, colourings, and chemical preservatives is prohibited [12]. The traditional manufacturing method of Galotyri cheese and modifications to adopt modern cheese processing practices and satisfy economic needs in Greek dairies were first evaluated in our laboratory during challenge and validation studies with important dairy pathogens to assure compliance of commercial PDO Galotyri cheese with the microbiological safety criteria of the EC Regulation 2073/2005 [14–16].

Although Galotyri cheese remains very popular in Greece, no published studies exist to describe the most important LAB species and other microbial groups involved in its manufacturing technology. Most previous studies evaluated the microbiological safety of commercial Galotyri cheeses and the survival, growth and toxin production potential of *Listeria monocytogenes*, *Escherichia coli* O157:H7 and *Staphylococcus aureus* under the low pH conditions during acid-curd processing and storage [14–17]. Other studies have evaluated the effects of different processing procedures or different types of commercial starter cultures (thermophilic, mesophilic, or mixed, multi-strain) on the biochemical and sensory characteristics with the aim to help traditional Greek cheese makers to produce Galotyri-type products of more consistent and high quality [18–20]. Nisin and natamycin, singly or combined, have also been applied to improve shelf-life of Galotyri [21]; however, its microbial ecology has yet to be investigated.

Samelis and Kakouri [16] first published basic comparative data on the microbial characteristics of commercial Galotyri cheeses: An industrial-type product comprised thermophilic starter LAB only, whereas the artisan-type cheese products contained mesophilic starter and nonstarter LAB types plus numerous yeasts and enterococci and few enterobacteria [16]. This biotechnological difference and the observation that pilot, artisan-type cheeses produced with commercial mesophilic starter cultures received higher sensory panel scores than their industrial-type counterparts [18], prompted us to conduct further research with the aim to determine compositional differences in the LAB microbiota of Galotyri cheeses, as affected by their scale and year of production. Therefore, different PDO Galotyri cheese variety products from the Epirus market were analyzed before and after explosion of the financial crisis in Greece, which has resulted in major reformations in the national food sector and supply from 2010 onwards; i.e., the Greek regulatory authorities currently require all Small- & Medium-size Enterprises (SMEs) producing PDO (dairy) foodstuffs or specialties to be certified by AGROCERT, and their PDO products be packaged and labelled accordingly; statistical production data of each Greek PDO food should be kept also. According to the HAO DEMETER-General Directorate of Food Quality Control and Assurance records, the Greek dairy SMEs so far certified for PDO Galotyri cheese production are limited to six; two located in Thessaly and four in Epirus, including the dairy industry Dodoni S.A. at Ioannina which produces the popular 'PDO Galotyri Dodoni' brand. Therefore, in the period 2014–2017, the Ioannina prefecture contributed to the annual PDO Galotyri cheese production by 93.9 to 97.8% in Epirus and 82.2 to 84.6% all over in Greece. No other acid-curd cheese variety product without the aforementioned certification is permitted to be trademarked and distributed in the market as 'Galotyri cheese' and benefit by its PDO status.

In this survey, the microbiology of the industrial PDO 'Galotyri Dodoni', with emphasis on its LAB species composition, was studied comparatively with five PDO artisan-type Galotyri cheese varieties marketed in Epirus from 2003 to date in order to determine potential variability within their microbiota associated with differences in their manufacturing technology. In specific, individual batches of the market cheese 'Galotyri Dodoni' were collected before (2003–2006) and after (2014–2019) the onset of Greek crisis and compared to each other and with the respective market products of different three and two artisan Galotyri cheese processors during the two abovementioned survey periods (see section 2.1 for cheese sampling details). The observed variations in the micro-ecological characteristics of the six Galotyri products were correlated chronologically and discussed critically in association with the PDO description of this traditional-delicatessen acid-curd cheese.

2. Materials and methods

2.1. Commercial Galotyri cheeses—production scale and sampling information

All commercial Galotyri cheese samples (19 batches in total representing Dodoni S.A. and five traditional dairies) were purchased from retail outlets at Ioannina. During the first sampling period (2003–2006), the only packaged and fully-labelled PDO Galotyri cheese was the industrial product of Dodoni S.A., already distributed in 1 kg hermetically sealed, hard plastic containers; six individual batches (coded 1D3, 1D7, 2D3, 2D7, 3D3, 3D7) were sampled for analysis after 4 to 5 days of retail storage at 4 °C (i.e., samples had an in-package shelf-life of 30 days from the production date to the best-before date). Conversely, all the artisan-type PDO Galotyri cheeses (another six batches in total; coded 1M3, 1M7, 2M3, 2M7, 3M3, 3M7) were bulk retail products marketed unpackaged under the local names 'Galotyri Metsovou' or 'Metsovitiko', and were purchased in 500 g portions filled in flexible plastic containers. They were products of three small traditional dairy plants A (1M), B (2M) and C (3M) (two cheese batches each) although initially we were misinformed they were products of one plant in Metsovo [16]. Misinformation at the retail level was easy to happen because no labelling or other written information regarding the date and method of production of the bulk artisan-type Galotyri cheese products was available, except of two general trade statements: (i) their manufacture was according to the PDO Galotyri technology [13]; (ii) natural LAB starters were used. As an update, it should be noted that none of the above traditional dairy plants A, B and C has been certified for PDO Galotyri production so far. Instead plants A and C still produce unpackaged acid-curd cheese varieties that are no longer marketed as 'PDO Galotyri', while plant B has shifted to the production of another retail-packaged and fully-labeled, but not PDO, acid-curd cheese variety trademarked as 'Metsovitiko Kadotyri' (personal communications and local market information).

During the second sampling period (2014–2019), additional seven commercial Galotyri cheese batches were sampled. As it was discussed above, none of them was sold in bulk (unpackaged) form, as before. Instead, all of them were marketed in fully-labelled plastic containers of various sizes, from 180 g to 1 kg individual retail packs (Figure 1). Emphasis was given to study the microbiology and LAB ecology of the industrial PDO Galotyri, which was the only constant and most important cheese product of both sampling periods. Therefore, sampling included five individual batches of 'PDO Galotyri Dodoni' (coded D14, D15, D16, D18, D19; the number after the prefix D indicates the sampling year) plus two artisan-type PDO Galotyri cheese products of the certified traditional dairies 'Zagori' in Epirus and 'Roussas' in Thessaly (one representative cheese batch each coded Z16 and R18, respectively); details on the labeling information of each trademarked cheese variety, illustrated in Figure 1, are also tabulated and discussed comparatively in the Results.

Figure 1. The containers of the fresh industrial PDO 'Galotyri Dodoni' cheese (i.e., batch D16 is shown only as a representative sample) and of the ripened artisan PDO 'Galotyri Zagori' (batch Z16) and PDO 'Galotyri Roussas' (batch R18) cheese samples analyzed in the years 2016 and 2018, respectively. The containers' lids are raised to show the production, packaging and sell-by dates plus the cheese brand and other specifications.

2.2. Cheese analyses

All cheese samples were shipped to the microbiology laboratory of the Dairy Research Institute in insulated polystyrene ice boxes and stored at 4 °C promptly. Each sample was subjected to microbial quantification analyses and pH determination on the day of purchase, and within 2 h after transportation. The pH was measured with a Crison digital pH meter (model Micro-pH 2001, Alella, Spain) equipped with a glass electrode immersed in the soft cheese mass after the microbiological sampling. All samples of the first period (2003–2006) were also analyzed for moisture, protein, fat and salt content according to the AOAC procedures [22], whereas the samples of the second period (2014–2019) were analyzed for (%) moisture only because levels of total protein, fat and salt contents specified on the product labeling were not critical for affecting the cheese LAB composition.

For microbial quantifications during both sampling periods, the procedure was as follows: Each plastic container was opened near a Bunsen gas flame and the soft cheese mass was stirred well by a sterile spoon-spatula; 25 g samples taken from the upper central part of the cheese mass were mixed aseptically with 225 mL of 0.1% w/v buffered peptone water (BPW) in stomacher bags, and the mixture was stomached in a Lab Blender 400 (Seward, London, UK) for 60 sec 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 various agar plates. Unless otherwise stated, all enumeration agar media and supplements during the first and the second sampling period were purchased from Merck (Darmstadt, Germany) and Lab M Ltd. (Heywood, UK), respectively.

During the first sampling period (2003–2006) before the Greek crisis, no published research or governmental data on the Galotyri microbiology were available; therefore, we considered it as a typical, low-temperature ripened acid-curd cheese fermented with the aid of mesophilic starter LAB, in accordance with the artisanal manufacturing technology included in the PDO Galotyri description [13]. Accordingly, the enumeration agar media and incubation conditions selected for microbial (LAB) quantifications were as reported previously [16]; they are summarized in Table 1.

During the second sampling period, some modifications were made to improve quantification of total cheese microbiota and the discriminations between mesophilic and thermophilic LAB groups or species (Table 2), by combining suitable enumeration methods from previous dairy studies [23–25]: Briefly, total viable counts (TVCs) were determined on Milk Plate Count agar (MPCA), incubated at 37 °C for 48–72 h, instead of CASO agar at 30 °C (Table 1); total mesophilic and thermophilic LAB populations were separated on MRS agar incubated at 30 °C aerobically for 48 to 72 h and at 45 °C anaerobically (GasPack System, BBL, Becton Dickinson, Sparks, MD, USA) for 48 h, respectively. Anaerobic incubation of the MRS agar plates at 45 °C and the incubation of the skim milk-containing MPCA plates at 37 °C were performed to ensure optimal growth, and thus, selective enumeration of thermophilic starter lactobacilli, such as *Lactobacillus delbrueckii* (Table 2); this group might remain undetectable under aerobic mesophilic culturing conditions in Table 1. In accordance, the mesophilic and thermophilic dairy cocci, presumptively lactococci and streptococci, were enumerated as separate groups on M17 agar incubated aerobically at 22 °C and 42 °C, respectively (Table 2).

Common methods were used during both sampling periods for the enumeration of enterococci, enterobacteria and yeasts (Tables 1 and 2). Particularly for enterococci, kanamycin aesculin azide (KAA) agar applied first (Table 1) was later replaced by Slanetz & Bartley (SB) agar (Table 2), a medium of similar selectivity but of higher visual discriminative ability of the typical *Enterococcus* colonies (red-brown) compared to KAA (i.e., black enterococcal colonies may be confused with the also black colonies of certain species of mesophilic *Lactobacillus* which instead give white colonies on SB). Pseudomonad-like bacteria were enumerated on *Pseudomonas* agar base with cephalothin-fucidin-cetrimide (CFC; supplement X108, Lab M), incubated at 25 °C for 48 h, and total staphylococci on Baird-Parker agar base with egg yolk tellurite, incubated at 37 °C for 48 h [24,25].

2.3. Isolation of the cheese LAB microbiota

Following microbial enumeration, a total of 400 presumptive LAB colonies were collected from the 19 Galotyri cheese batches; in specific, 210 and 190 colonies were isolated during the first and second sampling period, respectively, as follows:

For the first (2003–2006) sampling period, 90 isolates were collected from the six industrial 'Dodoni' batches and another 90 isolates from the six artisan-type 'Metsovou' batch products by picking five colonies from one highest dilution plate (with 30–100 colonies) of each of the CASO/30 °C, MRS/30 °C and M17/37 °C agars in Table 1. Attention was paid to collect representative isolates of all macroscopically different colonies from each plate, which were considered as the predominant cheese LAB biota. Additionally, 30 subdominant presumptive *Enterococcus* colonies were picked from one highest dilution KAA agar plate of the artisan-type cheese batches only; no colonies grew on the KAA agar plates of the industrial cheese batches (Table 1).

In the years 2014 to date, the isolation procedure was modified in accordance to the improved LAB enumeration protocol: Hence, five presumptive LAB colonies were collected from one of the highest dilution plates of each of the MPCA/37 °C, MRS/30 °C, MRS/45 °C, M17/22 °C, M17/42 °C and KAA/37 °C agar media in Table 2. When no colonies had grown on the plates of any agar above, then additional five colonies were isolated from the rest agar media. Based on this isolation protocol, 130 presumptive LAB colonies were collected from the five industrial 'Galotyri Dodoni' cheese batches (D14, D15, D16, D18, D19) and additional 60 LAB isolates in total were collected from the two artisan 'Zagori' (Z16) and 'Roussas'(R18) PDO Galotyri cheese batches, respectively.

All 400 isolates were cultivated in 10 mL MRS or M17 broth and checked for purity by streaking on MRS or M17 agar plates followed by testing for gram staining by the rapid 3% KOH method (gram-negative colonies form a viscous slimy mixture with the alkali) and catalase reaction by direct dropping of 3% H_2O_2; all gram-positive and catalase-negative isolates were regarded as LAB and were kept as stock cultures in MRS broth with 20% (w/v) glycerol at −30 °C for biochemical characterization.

2.4. Biochemical characterization of the LAB isolates

The stock LAB isolates were resuscitated in 10 mL MRS or M17 broth at 30 °C (mesophilic LAB) or 37 °C (thermophilic LAB) for 24 to 72 h, and were subcultured overnight in the above media before testing them. The biochemical identification was conducted according to established phenotypic criteria reported in the most reliable and/or updated biochemical identification keys [26–30]; test methods were according to previous cheese LAB identification studies from our laboratory [24,31]. Briefly, all the isolates were tested for cell morphology by phase contrast microscopy, gas production from glucose, ammonia production from arginine, growth at 15 °C and 45 °C, in 2%, 4% and 6.5% salt, on KAA agar, and for the fermentation of 13 sugars in 96-well pre-sterilized miniplates: L-arabinose, cellobiose, galactose, lactose, maltose, mannitol, melibiose, raffinose, ribose, sorbitol, sucrose, trehalose, and xylose (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). All tests were conducted in duplicate and the isolates were grouped according to the aforementioned criteria. Representative isolates of each of the LAB groups or subgroups (to be discussed in the Results) were also subjected to additional biochemical tests as appropriate, i.e., slime formation from sucrose, acetoin production by the VP test, haemolysis testing for enterococci on 5% sheep blood agar [31,32]. The entire sugar fermentation profiles of representative LAB group isolates were obtained by the API 50 CHL kit (bioMérieux, Marcy l' Etoile, Lyon, France).

2.5. Statistical analysis

Duplicate samples of each Galotyri cheese product batch were analyzed for microbial quantification and pH determination. Then the microbiological counts were converted to log CFU/g and subjected to analysis of variance along with the pH values (Statistical Graphics Corp., Rockville, IL, USA). Means and standard deviations were calculated, and when F-values were significant at the $P < 0.05$ level, mean differences were separated by the Least Significant Difference procedure.

3. Results and discussion

3.1. Basic composition of Galotyri market cheeses

The industrial Galotyri market cheeses of the first sampling period (2003–2006) had 76.9 ± 0.2% moisture, 9.1 ± 0.7% fat, 8.3 ± 0.7% protein, and 1.8 ± 0.1% salt after their purchase [14–15]. The corresponding values of the artisan cheeses of the traditional dairy plants A, B and C (2 batches each; separately) were 76.8 ± 0.1, 75.2 ± 2.2, 77.8 ± 1.1% moisture, 9.8 ± 1.2, 9.5 ± 0.6, 8.0 ± 0.6% fat, 8.2 ± 0.7, 7.6 ± 0.5, 8.2 ± 0.3 % protein, and 1.9 ± 0.1, 1.5 ± 0.4 and 2.0 ± 0.4% salt, respectively. The moisture content of the five industrial market cheeses of the second sampling period (2014–2019) was 74.9 ± 0.9%; hence it was reduced by approximately 2% compared with the 2003–2006 Dodoni cheese batch samples, in compliance with the maximum level of 75% permitted by the current PDO specifications [12]. The moisture content of the two artisan PDO Galotyri cheese batches, Z16 and R18, was 73.6 ± 0.1% and 71.2 ± 0.2%, respectively. As stated in the Methods, fat, protein and salt contents of the 2014–2019 samples were not measured because all market cheese products were fully-labeled (see Results in section 3.3 below). Additionally, it has become apparent that the PDO Galotyri is a highly moist, acid-curd cheese, which in recent times is of significantly lowered salt content (≤2%) also; therefore, its microbial ecology, stability and safety are decisively affected by its acid pH rather than the other basic parameters above [14–16]. The pH values of cheese samples of this study are thus presented and discussed in association with their microbiological attributes below.

3.2. Microbiological attributes of Galotyri market cheeses of the period 2003–2006

Populations of LAB in commercial PDO Galotyri cheese batches analyzed from 2003 to 2006 ranged on average from 6.9–8.3 log CFU/g, while TVCs were at similar levels (Table 1). Mesophilic LAB on the MRS and CASO agars at 30 °C were lower ($P < 0.05$) in the artisan cheeses of plant A compared with those of plant B and were intermediate in plant C (Table 1). Overall, the observed variations in mesophilic LAB and cheese pH values, which were the highest in artisan-type A and C cheeses and the lowest in the industrial 'Dodoni' cheese (Table 1), were indicative of differences in the starter and probably the nonstarter LAB species prevailing in each PDO Galotyri cheese variety.

Additionally, while all the artisan cheeses displayed presumptive enterococcal populations on KAA agar at varying subdominant levels ($P < 0.05$) of 3–4 and 5–6 log CFU/g in plant B and plants A and C, respectively, all the industrial cheese batches were practically free (<100 CFU/g) of enterococcal contaminants (Table 1). A considerable contamination with enterobacteria was detected in one batch of plant A only (Table 1), while all batches had below 100 CFU/g of pseudomonads and total staphylococci (data not tabulated). All the unpackaged artisan cheeses contained ca. 2 to 3 log CFU /g higher ($P < 0.05$) yeast populations than the packaged industrial cheeses (Table 1).

The biochemical identification results of the Galotyri market cheeses analyzed in the years 2003–2006 (Table 3) confirmed our preliminary findings [16]; nearly all LAB colonies (94.5%) isolated from the industrial cheeses were homofermentative, arginine-negative, thermophilic cocci, unable for growth at 15 °C; all of them shared a restricted sugar fermentation profile, typical of *S. thermophilus* (biotype I in Table 3) that was positive with lactose and sucrose only. The remaining five industrial cheese colonies were sporadic mesophilic LAB isolates recovered from the MRS/30 °C plates of the batches 3D3 and 3D7 (data not tabulated); three of them were identified as *Lc. lactis* subsp. *lactis* [24] further differentiated into two strain biotypes based on their ability to hydrolyze arginine and ferment xylose (Table 3). The remaining two isolates were gas-forming, arginine-negative, slime-producing members of the diverse *Leuconostoc mesenteroides* group [29]. Conclusively, *S. thermophilus* occurred nearly as a monoculture in all industrial cheese batches, indicating its constant use as the primary commercial starter species in PDO 'Galotyri Dodoni' production. *S. thermophilus* colonies were recovered quite easily from all the high dilution plates, including the MRS/30 °C agar plates on which *S. thermophilus* shows weak or no growth, depending on the strain [25].

A different strain biotype of *S. thermophilus* was isolated from the artisan-type cheeses (biotype II in Table 3) at an overall isolation frequency of 28.9% amongst the predominant LAB groups (Table 4). Specifically, *S. thermophilus* was predominant in both cheese batches (2M3 and 2M7) of plant B (22 isolates in total), was subdominant in batch 1M7 (4 isolates), while it was not isolated from batch 1M3 of plant A or both batches 3M3 and 3M7 of plant C (Table 4). The biotype II isolates of *S. thermophilus* from the artisan-type cheeses differed from the respective industrial cheese biotype I isolates in growing in 2% salt and fermenting maltose and ribose weakly (Table 3).

In general, the artisan-type Galotyri cheeses of plants A, B and C were more diversified than the industrial 'Galotyri Dodoni' cheese (Tables 3 and 4): *Lc. lactis* (38.9%) and *Lactobacillus plantarum* (22.3%) were the most prevalent mesophilic LAB species isolated from the CASO, MRS and M17 plates followed by *Enterococcus* (6.6%) and *Leuc. mesenteroides* (3.3%) (Table 4). Specifically, *Lc. lactis* was present in all artisan cheeses, except of batch 3M3; it was subdominant of *S. thermophilus* and *Lb. plantarum* in the 2M and 3M batches, while it was dominant over *S. thermophilus* in the 1M batches (Table 4). Except of only two isolates from batch 3M7 (biotype II in Table 3), all *Lc. lactis* isolates displayed a strong arginine-positive reaction and a sugar fermentation pattern typical of the subspecies *Lc. lactis* subsp. *lactis* (biotype I in Table 3). Regarding *Lb. plantarum*, all 30 isolates of this major group were recovered solely from the 3M cheese batches of plant C; they occurred almost as monoculture in batch 3M3, but also prevailed in 3M7 which was the most diversified Galotyri cheese batch amongst all six batches tested (Table 4). Several of the *Lb. plantarum* isolates failed to ferment raffinose and/or sorbitol, while four of them could grow at 45 °C (Table 3); therefore, this 3M group was quite heterogeneous and probably some isolates belonged to *Lb. paraplantarum* or *Lb. pentosus* which share a higher than 99% 16S rRNA similarity with *Lb. plantarum* [26,27,33].

Indigenous *Enterococcus* strains were not isolated within the predominant LAB groups of the artisan Galotyri cheese isolates, except of the 1M batches of plant A (Table 4) which had the highest subdominant enterococcal populations in Table 1. Those six isolates plus another 20 subdominant *Enterococcus* isolates from the KAA agar plates of the 1M and 2M batches (Table 4) formed a heterogeneous group which included three distinct species, *E. faecium*, *E. faecalis* and *E. durans* (Table 3). None of the 26 isolates was β-hemolytic on 5% sheep blood agar plates at 37 °C. Their species discrimination was based on the most reliable biochemical key by Manero and Blanch [30]: Six L-arabinose-positive isolates were assigned to *E. faecium*, eleven sorbitol-positive but arabinose-negative isolates were identified as *E. faecalis*, whereas the remaining nine isolates (i.e., negative with the above two key sugars plus raffinose) were assigned to *E. durans* (Table 3). Seven *E. durans* isolates formed a typical group, which was unable to ferment mannitol, sucrose and xylose, while another two isolates were atypical *E. durans* in fermenting sucroce and mannitol or xylose (Table 3). Existence of high intra-species biochemical heterogeneity within *E. faecium* and *E. durans* biotypes is a very common feature [30,31,34]. Notably, none of the ten isolates from the KAA agar plates of the 3M batches of plant C were enterococci but instead all were members of the *Lb. plantarum* group (Tables 3 and 4) which proved to be kanamycin-resistant and esculin-positive. As it was emphasized in the Methods, the ability for colony growth with blackening on KAA agar of several strain biotypes of the *Lb. plantarum* genomic group of species may actually hamper the discrimination, and thus, the reliable selective enumeration of enterococci [25]; to avoid this confusion, we replaced KAA (Table 1) with SB agar (Table 2) in the years 2014 to date.

3.3. Microbiological attributes of Galotyri market cheeses of the period 2014–2019

Tables 5 and 6 summarize the labeling information of the certified PDO Galotyri cheese products analyzed from 2014 onwards, while Table 2 summarizes all microbial quantifications and cheese pH measurements.

Starting from the labeling information data, although all three cheese varieties had moisture and fat in dry matter contents according to the levels specified in PDO Galotyri description [12], their total fat, protein, salt and sugar contents showed moderate differences (Table 5), and their actual mean moisture values were 71 to 75%, they differed dramatically to each other with regard to their shelf-life (Table 6). Also, their microbial counts and pH values (Table 2), and consequently, their manufacturing technology were different: While all five industrial PDO 'Galotyri Dodoni' batches represented a fresh cheese product with an average in-package shelf-life (from the production date to sell-by date) of 31 $\pm$ 5 days, the corresponding time period of the two artisan cheese batch products Z16 and R18 was 298 and 325 days (Table 6). Thus, contrary to the fresh industrial cheese, both artisan Galotyri products were long-ripened. The ripening time of each artisan cheese on label was the period between the production and packaging dates, which were 239 days for the 'Zagori'-Z16 batch and 112 days for the 'Roussas'-R18 batch (Table 6). The in-package shelf-life of the artisan cheeses under refrigerated (4 °C) retail storage was the period from their packaging date to their sell-by date, which was 2 and 7 months for the Z-16 and R-18 batch products, respectively (Table 6).

The above major differences in cheese ripening times and shelf-life durations were reflected in the numbers of the different microbial groups (Table 2) and particularly in the types of LAB identified (Table 7) and the distribution of their isolates in the five industrial (Table 8) and the two artisan (Table 9) PDO Galotyri cheese batches. Based on the data in Table 6, the industrial cheeses were on average 16.4 day-old when they were analyzed microbiologically, while the ripened artisan cheeses were eight-month older (Z16, 253 day-old; R18, 264 day-old). So, as shown by the statistical comparison of quantitative data for the MPCA and the five LAB-selective media (in columns; Table 2), all fresh industrial cheeses contained high (>8 log CFU/g) viable thermophilic LAB populations, which consisted of *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* only (Table 7), while mesophilic LAB and enterococcal populations were by 4.5 to more than 6.0 log units lower ($P < 0.05$; Table 2). Therefore, a commercial symbiotic 'yogurt starter' was applied to acidify the milk for production of industrial Galotyri. As it was expected, the selective growth of *S. thermophilus* and *Lb. bulgaricus* was enhanced on M17/42 ℃ and MRS/45 ℃ agars, respectively, while MPCA/37 ℃ supported growth of both symbiotic species (Table 8). Surprisingly, almost a quarter (23.3%) of the *S. thermophilus* isolates fermented galactose (Table 7), suggesting existence of two strain biotypes in the industrial 'Galotyri Dodoni' starter, one dominant and typical (biotype I in Table 3) and the other subdominant and 'novel' in fermenting galactose. The recent emergence of galactose-positive *S. thermophilus* in Greek dairy plants and products is likely due to newly imported *S. thermophilus* starter strains that might have attained this property naturally or, most likely, by mutation following metabolic engineering in Northern Europe [35,36].

No mesophilic LAB populations were detectable in the industrial cheeses sampled in 2014 to date; all the subdominant presumptive 'mesophilic LAB colonies' ($P < 0.05$; Table 2) picked for testing and identification (Table 8) actually were either yeasts grown well on MRS/30 ℃ agar plates or yeasts mixed with invisible *S. thermophilus* cell lawns from M17/22 ℃ agar plates (Table 2).

Table 1. Populations (log CFU/g) of the main groups of the technological microbiota present in industrial and artisan-type Greek PDO Galotyri cheeses at the time of purchase from retail outlets of the Ioannina, Epirus, market in 2003–2006; data modified from [16].

Microbial group	Enumeration agar medium/incubation conditions	Type of Greek PDO Galotyri cheese			
		Industrial	Artisanal		
		Dodoni Milk Industry	Traditional dairy A	Traditional dairy B	Traditional dairy C
		(*n* = 6)	(*n* = 2)	(*n* = 2)	(*n* = 2)
Total mesophilic bacteria	CASO agar 30 °C for 72 h	8.03 ± 0.33	7.66 ± 0.42 a	8.17 ± 0.06 b	7.78 ± 0.26 a
Total mesophilic LAB	MRS agar 30 °C for 48–72 h	7.55 ± 0.88	6.89 ± 0.27 a	8.28 ± 0.36 b	7.58 ± 0.77 ab
Total dairy LAB	M17 agar 37 °C for 48 h	8.11 ± 0.49	7.76 ± 0.95 a	8.07 ± 0.66 a	8.22 ± 0.04 a
Enterococci	KAA agar 37 °C for 48 h	<2.00 *	5.40 ± 0.41 b	3.39 ± 0.44 a	5.06 ± 0.20 b
Yeasts	RBC agar 25 °C for 5 days	2.93 ± 0.78 *	5.33 ± 0.55 a	4.26 ± 1.78 a	4.77 ± 0.39 a
Total enterobacteria	VRBG agar (double-layered) 37 °C for 24 h	<1.00 *	2.97 ± 1.37 b	<1.00 a	<1.00 a
Cheese pH		3.75 ± 0.08 *	4.04 ± 0.04 b	3.93 ± 0.02 a	4.04 ± 0.08 ab

Note: Values are the means ± standard deviation of six individual industrial cheese batches (1D3, 1D7, 2D3, 2D7, 3D3, 3D7) and of two individual cheese batches each (1M3–1M7; 2M3–2M7; 3M3–3M7) of the traditional dairy plants A, B and C, respectively. Within the same row and for the artisan cheese products, values with different letters are significantly different ($P < 0.05$). The means of the six industrial cheese batches bearing an asterisk were significantly different ($P < 0.05$) from the respective means of all six artisan batches when their data were pooled and compared [16]. Abbreviations: CASO, casein-peptone, soymeal-peptone agar; MRS, de Man, Rogosa, Sharpe agar, KAA, Kanamycin Aesculin Azide agar; RBC, Rose Bengal Chloramphenicol agar; VRBG, Violet Red Bile Glucose agar. All agar media were purchased from Merck, Darmstadt., Germany.

Table 2. Populations (log CFU/g) of the main groups of the technological microbiota present in industrial and artisan-type Greek PDO Galotyri cheeses at the time of purchase from retail outlets of the Ioannina, Epirus, market in the years 2014–2019.

Microbial group	Enumeration agar medium/incubation conditions	Type of Greek PDO Galotyri cheese		
		Industrial Dodoni milk industry (*n* = 10)	Artisan Zagori (*n* = 2)	Artisan Roussas (*n* = 2)
Total viable count	MPCA/ 37 °C; 48–72 h; Aerobically	8.24 ±0.57 c C	7.61 ±0.07 b C	5.56 ±0.11 a C
Total mesophilic LAB	MRS agar/ 30 °C; 48–72 h; Aerobically	3.36 ±0.75 a B	7.77 ±0.03 c C	6.46 ±0.19 b D
Total thermophilic LAB	MRS agar/ 45 °C; 48 h; Anaerobically	7.84 ±0.44 b C	5.14 ±0.20 a B	5.19 ±0.41 a C
Mesophilic dairy cocci (presumptive lactococci)	M17 agar/ 22 °C;72 h, Aerobically	3.32 ±0.69 a B	7.82 ±0.01 c C	6.15 ±0.30 b D
Thermophilic dairy cocci (presumptive streptococci)	M17 agar/ 42 °C; 48 h; Aerobically	8.00 ±0.56 c C	5.04 ±0.06 b B	<3.00 a A
Enterococci	Slanetz & Bartley agar/ 37 °C; 48 h; Aerobically	<2.00 a A	3.36 ±0.11 b A	3.16 ±0.11 b B
Yeasts	RBC agar/25 °C; 5 d; Aerobically	2.40 ±0.55 a	5.92 ±0.02 b	5.70 ±0.02 b
Total enterobacteria	VRBG agar/ 37 °C; 24 h; Double-layered	<1.00	<1.00	<1.00
Cheese pH		3.69 ±0.11 a	3.85 ±0.01 b	4.43 ±0.04 c

Note: Values are the means ± standard deviation of five industrial cheese batches (D14, D15, D16, D18, D19) and two artisan cheese (Zagori-Z16 and Roussas-R18) batches, with two individual samples analyzed per batch. Within a row, values with different lowercase letters are significantly different ($P < 0.05$). Within a column, values with different capital letters are significantly different ($P < 0.05$). Abbreviations: MPCA, Milk Plate Count Agar; RBC, Rose Bengal Chloramphenicol agar; VRBG, Violet Red Bile Glucose agar. All agar media were purchased from Lab M (Heywood, UK).

Table 3. Biochemical identification of 210 LAB isolates from Greek PDO Galotyri cheeses produced at industrial (Ind-Ch) or artisanal (Art-Ch) scale and distributed packaged or in bulk form, respectively, at the market of Ioannina, Epirus, in the years 2003–2006.

Species	*Streptococcus thermophilus*		*Lactococcus lactis* subsp. *lactis* group		*Enterococcus faecium*	*Enterococcus durans*		*Enterococcus faecalis*	*Leuconostoc mesenteroides*	*Lactobacillus plantarum*
	Biotype I	Biotype II	Biotype I	Biotype II		Typical	Atypical		Group	Group
Ind-Ch isolates	85	0	2	1	0	0	0	0	2	0
Art-Ch isolates	0	26	33	2	6	7	2	11	3	30
Total isolates	85	26	35	3	6	7	2	11	5	30
Cell shape	LC	LC	C	C	C	C	C	C	BC	R/SR
CO_2/glucose	−	−	−	−	−	−	−	−	+	−
NH_3/arginine	−	−	+	−	+	+	+	+	−	−
Growth at: 15 °C	−	−	+	+	+	+	+	+	+	+
45 °C	+	+	−	−	+	+	+	+	4/5	4/30
2% salt	−	(+)/+	+	+	+	+	+	+	+	+
4% salt	−	−	+	+	+	+	+	+	+	+
6.5% salt	−	−	−/(+)d	−	+	+	+	+	+	+
KAA agar	−	−	−	−	++	++	++	++	−	+/(+)
Fermentation of:										
L- Arabinose	−	−	−	−	+	−	−	−	−	−
Cellobiose	−	−	+	(+)	+	+	+	+	4/5	+
Galactose	−	−	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+
Maltose	−	(+)d	+	+	+	+	+	+	4/5	+
Mannitol	−	−	+	+	+	−	1/2	+	4/5	+
Melibiose	−	−	−	−	4/6	+	+	5/11	1/5	+
Raffinose	−	−	−	−	−	−	−	−	1/5	11/30
Ribose	−	(+)d	+	−	+	+	+	+	1/5	+

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Species	*Streptococcus thermophilus*		*Lactococcus lactis* subsp. *lactis* group		*Enterococcus faecium*	*Enterococcus durans*		*Enterococcus faecalis*	*Leuconostoc mesenteroides*	*Lactobacillus plantarum*
	Biotype I	Biotype II	Biotype I	Biotype II		Typical	Atypical		Group	Group
Sorbitol	−	−	−	−	1/6	−	−	+	−	6/30
Sucrose	+	+	+	+	+	−	+	+	+	+
Trehalose	−	−	+	+	4/6	+	+	+	+	+
Xylose	−	−	+	−	2/6	−	1/2	2/11	3/5	−

Note: Symbols: +, all isolates were positive; −, all negative; 4/30, 4 out of the 30 isolates were positive; (+) weakly positive; d, delayed. Growth type on KAA: ++, abundant growth, + moderate growth, (+) weak growth, − no growth. Cell shape: LC, large cocci; C, cocci; BC, bacillococci or elongated cocci; R, rods; SR, short rods.

Table 4. Distribution of 120 isolates of the predominant or subdominant LAB groups of species in six batches of artisanal Greek PDO Galotyri cheeses purchased in bulk retail form from the Ioannina, Epirus, market in the years 2003–2006[a].

Species	Artisanal Galotyri cheese batches—Plants A, B and C in Table 1						Total isolates	% of total isolates
	1M3	1M7	2M3	2M7	3M3	3M7		
	Predominant LAB groups of isolates according to data in Table 1							
Streptococcus thermophilus	0	4	12	10	−	−	26	28.9
Lactococcus lactis subsp. *lactis* (arginine-positive)	11	9	3	5	−	5	33	36.7
Lactococcus lactis subsp. *lactis* (arginine-negative)	−	−	−	−	−	2	2	2.2
Enterococcus faecium	2	−	−	−	−	−	2	2.2
Enterococcus durans	1	1	−	−	−	−	2	2.2
Enterococcus faecalis	1	1	−	−	−	−	2	2.2
Leuconostoc mesenteroides Group	−	−	−	−	1	2	3	3.3
Lactobacillus plantarum Group	−	−	−	−	14	6	20	22.3
Total isolates	15	15	15	15	15	15	90	100

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Species	Artisanal Galotyri cheese batches—Plants A, B and C in Table 1						Total isolates	% of total isolates
	1M3	1M7	2M3	2M7	3M3	3M7		
	Subdominant LAB groups of isolates according to data in Table 1							
Enterococcus faecium	3	1	–	–	–	–	4	13.3
Enterococcus durans	1	1	2	3	–	–	7	23.3
Enterococcus faecalis	1	3	3	2	–	–	9	30.0
Lactobacillus plantarum Group	–	–	–	–	5	5	10	33.4
Total isolates	5	5	5	5	5	5	30	100

Note: [a]The isolates of the predominant LAB groups were picked from one of the highest dilution plates of the CASO, MRS and M17 agar media incubated at 30, 30 and 37 °C, respectively, whereas the isolates of the subdominant LAB groups were picked from one of the highest dilution plates of the selective KAA agar incubated at 37 °C for 48 h.

Table 5. Chemical and nutritional composition reported in the on-package-labeling information of the PDO Galotyri cheeses purchased from the Ioannina, Epirus, market in the years 2014–2019[a].

Cheese type	Brand/Batch	Moisture (%)	Fat in dry matter (%)	Nutritional information (g/100 g of cheese)							Starter type	Packing size (g)
				Total fat	Saturated fat	Total protein	Salt	Carbohydrates	Sugars	Energy (kcal)		
Industrial PDO (Fresh)	Dodoni D14 to D19[b]	≤75	≥40	10.0	6.0	8.0	2.00	4.6	4.6	140	Commercial	1.000 or 375[d]
Artisan PDO (Ripened)	Zagori Z16	≤75	≥40	12.4	8.3	11.5	1.17	1.4	1.4	163	Natural[c]	400
Artisan PDO (Ripened)	Roussas R18	≤75	≥40	12.7	9.7	11.9	1.00	5.9	4.0	186	Commercial	180

Note: [a]The brand-named Galotyri Dodoni (industrial) and Roussas (artisan) cheeses were made of pasteurized sheep and goat milk. All cheese products shared two warnings to the consumer: 'Keep refrigerated 2–4 °C; Consume within 3 days (Roussas) or 4 days (Dodoni & Zagori) after opening'. [b]All five industrial cheese batches (D14–D19) shared their labeling information, except of the following two updates: after 2016, the word 'fresh' was added to the previous 'soft spread' term for PDO 'Galotyri Dodoni' cheese categorization. Also, for marketing purposes, the drawings onto the industrial cheese packages changed from a white yogurt-like container (Figure 1) to a colored cottage cheese-like container (not shown). [c]Because the term 'starter culture' was not reported on the label of the Zagori brand, we contacted this SME to confirm use of a natural undefined yogurt-like starter for producing this particular artisan-type PDO Galotyri cheese specialty. [d]All industrial cheese batch samples were packaged in 1 kg containers, except of the D19 batch sample which was of smaller pack size.

Table 6. Ripening duration and in-package shelf-lives of the PDO Galotyri cheeses purchased from the Ioannina, Epirus, market in the years 2014–2019. The data were determined based on the production, packaging and sell-by dates indicated on the label of each cheese product batch.

Cheese type	Brand/Batch	Production date	Packaging date	Sell-by date	Ripening duration (days)	Shelf-life (days)	Cheese age on sell-by date (days)	Analysis date	Cheese age at analysis (days)
Industrial PDO	Dodoni D14	12-11-14	Not reported	10-12-14	None	28	28	28-11-14	16
	Dodoni D15	02-06-15	Not reported	29-06-15	None	27	27	11-06-15	9
	Dodoni D16	11-02-16	Not reported	09-03-16	None	27	27	25-02-16	14
	Dodoni D18	30-01-18	Not reported	10-03-18	Fresh [a]	39	39	27-02-18	28
	Dodoni D19	25-02-19	Not reported	31-03-19	Fresh [a]	34	34	12-03-19	15
Artisan PDO	Zagori Z16	17-06-15	11-02-16	10-04-16	239	59	298	25-02-16	253
Atrisan PDO	Roussas R18	13-10-17	02-02-18	02-09-18	112	213	325	04-07-18	264

Note: [a]Refer to the legend of Table 5 for the updated industrial cheese categorization.

Table 7. Biochemical identification of 156 LAB isolates from Greek PDO Galotyri cheeses produced at industrial (Ind-Ch) or artisanal (Art-Ch) scale and distributed in fully-labelled packages at the market of Ioannina, Epirus, in the years 2014–2019.

Species	*Streptococcus thermophilus* Biotype I & Biotype III	*Lactobacillus delbrueckii* subsp. *bulgaricus*	*Lactobacillus gasseri*	*Lactobacillus rhamnosus*	*Lactobacillus acidipiscis*	*Pediococcus inopinatus*	*Leuconostoc mesenteroides* Group	*Enterococcus faecium*	*Enterococcus faecalis*
Ind-Ch isolates	43	56	0	0	0	0	0	0	0
Art-Ch isolates	0	5	6	6	1	13	17	5	4
Total isolates	43	61	6	6	1	13	17	5	4
Cell shape	LC	LR	FR	SR	R	LC-TR	BC	C	C
CO_2/glucose	−	−	−	−	−	−	+	−	−
NH_3/arginine	−	−	−	−	−	−	−	+	+
Growth at: 15 °C	−	−	−	+	+	+	+	+	+
45 °C	+	+	+	+	−	−	−	+	+
2% salt	20/43	+	−	+	+	+	+	+	+
4% salt	−	−	−	+	+	+	+	+	+
6.5% salt	−	−	−	2/6	+	10/13	+	+	+
KAA agar	−	−	−	+	−	−/(+)	−	++	++
Slime/sucrose	NT	NT	−	−	−	−	7/17	−	−
Fermentation of:									
L- Arabinose	−	−	−	−	−	−	14/17	+	−
Cellobiose	−	−	−	+	−	NT	NT	+	+
Galactose	10/43	−	+	+	+	+/(+)	+	+	+
Lactose	+	+	+	+	+	+	+	+	+
Maltose	−	−	+	+	+	+	+	+	+
Mannitol	−	−	+	+	−	−	+	+	+
Melibiose	−	−	−	−	+d	−	15/17	+	−
Raffinose	−	−	−	−	−	−	14/17	−	−

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Species	*Streptococcus thermophilus* Biotype I & Biotype III	*Lactobacillus delbrueckii* subsp. *bulgaricus*	*Lactobacillus gasseri*	*Lactobacillus rhamnosus*	*Lactobacillus acidipiscis*	*Pediococcus inopinatus*	*Leuconostoc mesenteroides* Group	*Enterococcus faecium*	*Enterococcus faecalis*
Ribose	−	−	−	+	(+)d	−	+	+	+
Sorbitol	−	−	−	+	−	−	NT	1/5	+
Sucrose	+	−	+	+	+	+/(+)d	+	3/5	+/+d
Trehalose	−	−	+	+	+	NT	+	+	NT
Xylose	−	−	−	−	−	−	14/17	−	−

Note: Symbols: +, all isolates were positive; −, all negative; 20/43, 20 out of the 43 isolates were positive; (+) weakly positive; d, delayed reaction. Growth type on KAA: ++, abundant growth; + moderate growth; (+) weak growth-very little blackening; − no growth. NT, not tested. Cell shape: LC, large cocci; LC-TR tetrads; C, cocci; BC, bacillococci/elongated cocci; R, rods; LR, large rods; SR, short rods; FR, filamentous rods.

Table 8. Distribution of 105 predominant LAB isolates of the commercial symbiotic starter species *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* and 25 subdominant ‘intruder’ yeast isolates in five batches of industrial PDO ‘Galotyri Dodoni’ cheese purchased from retail outlets of the Ioannina, Epirus, market in the years 2014–2019 [a].

Species	Predominant species on thermophilic LAB isolation agar media															Total isolates
	MPCA/37 ℃					M17/42 ℃					MRS/45 ℃					
Cheese batch	D14	D15	D16	D18	D19	D14	D15	D16	D18	D19	D14	D15	D16	D18	D19	
Streptococcus thermophilus																
Biotype I (typical)	−	−	−	−	3	−	10	10	8	2	−	−	−	−	−	33 (31.4%)
Biotype III (Gal+)	−	−	−	−	−	−	−	−	2	8	−	−	−	−	−	10 (9.5%)
Lactobacillus delbrueckii																
subsp. *bulgaricus*	10	5	10	5	1	−	−	−	−	−	10	5	5	1	4	56 (53.4%)
Non-culturable isolates	−	−	−	−	1	−	−	−	−	−	−	−	−	4	1	6 (5.7%)
Total isolates/medium	35					40					30					105

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Species	Subdominant groups on mesophilic LAB plus *Enterococcus* isolation agar media															Total isolates
	MRS/30 ℃					M17/22 ℃					KAA/37 ℃					
Yeast isolates	–	–	–	5	5	–	–	5*	5*	5*	–	–	–	–	–	
Total isolates/medium	10					15					No colonies existed on KAA					25

Note: [a]The complete labeling information written on the container of the industrial cheese batches is given in Tables 5 and 6. *The asterisk indicates yeast colonies that were cross-contaminated with high-density underlying lawns of *S. thermophilus* unable to promote visible growth at 22 ℃.

Table 9. Species distribution of 57 lactic acid bacteria isolated from two individual artisan-type PDO Galotyri cheese product batches purchased from retail outlets of the Ioannina, Epirus in the years 2016 and 2018[a].

Species	Artisan PDO Galotyri cheese product												Total isolates
	Zagori (Batch Z16)						Roussas (Batch R18)						
Isolation agar	MPCA 37 ℃	M17 22 ℃	M17 42 ℃	MRS 30 ℃	MRS 45 ℃	KAA 37 ℃	MPCA 37 ℃	M17 22 ℃	M17 42 ℃	MRS 30 ℃	MRS 45 ℃	KAA 37 ℃	
Lactobacillus delbrueckii subsp. *bulgaricus*	–	–	–	–	5	–	–	–	–	–	–	–	5 (8.3%)
Lactobacillus gasseri	1	–	5	–	–	–	–	–	–	–	–	–	6 (10.0%)
Lactobacillus rhamnosus	–	–	–	–	–	–	–	–	–	–	5	1	6 (10.0%)
Lactobacillus acidipiscis	1	–	–	–	–	–	–	–	–	–	–	–	1 (1.7%)
Pediococcus inopinatus	–	–	–	–	–	–	3	5	–	5	–	–	13 (21.7%)
Leuconostoc mesenteroides	8	4	–	5	–	–	–	–	–	–	–	–	17 (28.3%)
Enterococcus faecium	–	–	–	–	–	5	–	–	–	–	–	–	5 (8.3%)
Enterococcus faecalis	–	–	–	–	–	–	–	–	–	–	–	4	4 (6.7%)
Non-LAB/Yeasts	–	1	–	–	–	–	2	–	–	–	–	–	3 (5.0%)
Total isolates/batch	35						25						60

Note: [a]The complete labeling information written on the container of each individual artisan cheese batch are given in Tables 5 and 6.

In full contrast with the industrial Galotyri, mesophilic LAB types dominated over thermophilic LAB types ($P < 0.05$; viz. Table 2), enterococci were subdominant, and yeasts were numerically important (approx. 6 log CFU/g) in the artisan cheeses (Table 2), as before (Table 1). Particularly the much higher ($P < 0.05$) populations of yeasts in the artisan cheeses as compared to the industrial cheeses could be associated with the higher ($P < 0.05$) pH values of the former (Z16 and R18) samples (Table 2). Yeasts generally increase the pH during cheese ripening by consuming part of the organic acids, mainly lactate, formed by LAB, while their inherent resistance to low pH conditions particularly enhances their prevalence and thus biotechnological role in the production, sensorial qualities and eventually the terminal spoilage defects of acid-curd cheeses [2,4,16].

Overall, the artisan cheese Z16 had higher ($P < 0.05$) TVC and mesophilic LAB populations than the artisan cheese R18; no major differences existed in their levels of yeasts and enterococci (Table 2). However, major compositional differences existed between the dominant and subdominant LAB species isolated from each artisan cheese specialty (Tables 7 and 9). In Z16 cheese, a predominant heterogeneous *Leuconostoc mesenteroides* group (42% formed slime) and a single mesophilic rod resembling *Lactobacillus acidipiscis* were isolated from MPCA/37 °C, MRS/30 °C and M17/22 °C agar plates, whereas *Lb. delbrueckii* subsp. *bulgaricus* and a homogeneous thermophilic group of filamentous, starch-fermenting isolates of *Lactobacillus gasseri* [27] were recovered from MRS/45 °C and M17/42 °C agar plates, respectively. Conversely, the mesophilic LAB biota of R18 cheese was dominated by *Pediococcus inopinatus* [28], while an acetoin-producing biotype of *Lb. rhamnosus* [27] was subdominant on MRS/45°C, also occurring on KAA agar plates (Tables 7 and 9). Isolation of aromatic, antifungal and/or probiotic *Lb. rhamnosus* [7,37–41], *Lb. gasseri* [7, 42–44] and *Lb. acidipiscis* [45] strains as primary or secondary LAB from natural whey starters, yogurt, acidified milks and artisan (Greek) cheeses [45,46], is frequent. On the contrary, few studies exist on the occurrence and usage of native *P. inopinatus* as adjuncts in traditional non-European cheeses [47,48]. Because most traditional Greek cheese makers refuse to provide information on the origin, type and handling of starter culture preparations they utilize, we can not assure whether *Lb. rhamnosus*, *Lb. gasseri*, *Lb. acidipiscis* and *P. inopinatus* isolated from the above two ripened PDO Galotyri cheeses were indigenous; or, they originated from craft-made natural whey or yogurt starters or from any of the numerous commercial mixed starter culture preparations currently available in Greece. However, neither *S. thermophilus* nor *Lc. lactis* were isolated from the ripened Z16 and R18 cheeses.

Another noteworthy difference regarded the isolates from KAA agar; *E. faecium* was exclusively isolated from cheese batch Z16 whereas *E. faecalis* from cheese batch R18 (Tables 7 and 9). Additional market cheese batches of the certified PDO Galotyri producers 'Zagori' and 'Roussas' should be analyzed in order to conclude whether the above differences in the LAB species composition of their products are preserved.

3.4. Biotechnological aspects of the Galotyri market cheeses in regard to their PDO certification – Research-based concerns and perspectives

According to the artisanal manufacturing technology reported in Galotyri PDO description, article 83D, page 46, of the Hellenic Code of Food and Beverages [12], this traditional Greek cheese product should be made of boiled milk that is left to cool in clay jars for 24 h, then is salted with 3–4% edible salt and left to acidify and curdle naturally, with or without rennet addition. On this basis, the authentic artisan Galotyri is a very distinct cheese specialty that differs from the majority of all other similar acid-curd cheese varieties, like the Anevato PDO made of raw or thermized milk [49,50], because it is traditionally made with boiled milk [13,46]. Boiling (>80–90 °C) of milk would inactivate all indigenous LAB and other bacteria present in raw milk at a much greater extent than milk pasteurization declared on the label of the commercial PDO Galotyri cheese products (Table 5). In the past, the natural acidification of boiled milk required for curd formation during the artisanal way of Galotyri cheese-making was insured by a diverse adventitious LAB biota from the surrounding farm or plant environment or by the application of empirical back-slope techniques for milk inoculation [14,46]. To date, in the Greek dairy industries and in the modern well-equipped and 'disinfected' traditional dairies applying HACCP, an enriched 'house' LAB biota is very unlikely to occur and be preserved; hence, it has to be replaced by industrial or natural whey or yogurt starter cultures in the industrially pasteurized (72 °C; 15 sec) or traditionally boiled milk. The simpler the starter composition is the lower the LAB diversity of the fresh acid-curd cheese made from the above heat-treated milks. This has been the main reason for the diachronic, extremely poor LAB species diversity (100% prevalence of the symbiotic starter *S. thermophilus* and *Lb. bulgaricus*) of the PDO 'Galotyri Dodoni' labeled as '*Soft, fresh creamy cheese from Greek pasteurized sheep and goat milk with pleasant, refreshing taste. It combines the nutritional properties of yogurt with the rich, savory flavor of cheese'*. Based on our findings, this popular industrial product is a tasteful, homogeneous, fresh yogurt-like curd from pasteurized milk with maximum 2% salt added, which is characterized by high microbial stability and safety and has an approximate one-month shelf-life in retail. Does it represent an authentic PDO Galotyri cheese variety though? According to the data in Table 5, it is a fresh cheese of reduced salt, compared to 3-4% salt of the authentic PDO Galotyri that additionally is ripened [12]. Low (≤2%) salt levels are absolutely essential to support optimal growth of *S. thermophilus*, the primary acidifier but also the most salt-sensitive dairy starter LAB (Tables 3 and 7); thus, in terms of acidification, 'Galotyri Dodoni' was the best-optimized cheese (pH 3.6–3.8) during both sampling periods (Tables 1 and 2).

The bulk (unpackaged) artisan-type Galotyri cheese products analyzed during the first sampling period (2003–2006) resembled the industrial product in terms of curd acidification (pH 3.9–4.1) and salt levels below 2%; also, their LAB diversity was lower than anticipated (Table 3). Probably the artisan cheese plants A and B used mixed commercial or 'natural' starter cultures consisting of *S. thermophilus* and *Lc. lactis* strains, whereas the plant C cheese was a different specialty that lacked detectable *S. thermophilus* while it was enriched in *Lb. plantarum* strains (Table 4). Notably, under the conditions of this study, members of the *Lb. delbrueckii* group were not recovered from any of the Galotyri cheese samples of the first period (Table 3), possibly because the aerobic and mesophilic incubation conditions suppressed growth of thermophilic *Lactobacillus* (Table 1). Also, because the date of production of bulk artisan Galotyri samples was neither labeled nor declared, it remains unclear whether they were fresh or shortly ripened [16]. However, all of them had a granular appearance and contained small cheese particles dispersed in the curd. This characteristic and the high isolation frequency of *Lc. lactis* and *Lb. plantarum* from the artisan cheese products A and C, respectively, suggested an 'arbitrary' dispersion in the basal Galotyri milk curd of Feta cheese granules as an empirical 'external aromatization' process. To validate this, 'Feta-like' particles were picked directly from the acid-curd mass of the artisan Galotyri A and C samples, washed with sterile saline, mixed to obtain a new soft cheese mass under aseptic conditions and analyzed for their LAB numbers and types in comparison with the corresponding whole-cheese data in Tables 1 and 3; *Lc. lactis*, *Lb. plantarum* and enterococci were isolated from those granulated cheese particles at levels 1–3 log units higher than their corresponding counts in Table 1, while no *S. thermophilus* strains were isolated (data not shown). More recently, the dispersion of Feta cheese in the basal curd of the PDO 'Galotyri Zagori' was confirmed also, whereas there was no indication of dispersed Feta granules in the PDO 'Galotyri Roussas' (batch R18); notably, the latter traditional dairy distributes in the market another non-PDO soft, acid-curd, granular cheese variety labeled as 'an artisan spread with Feta and yogurt' (LOT 200836, production date 10-4-2018 was analyzed; results not shown). In accordance, a recent review on the microfloras of traditional Greek cheeses describes the potential addition of fresh Feta cheese at 1:5 proportion in basal yogurt curds as a quite common empirical practice for Galotyri cheese makers [46]. In our opinion, the above practice should be neither permitted nor encouraged as an optional operation because it is not specified in the Galotyri PDO technology. Moreover, a clear distinction between artisanal (ripened) and fresh industrial or artisan-like Galotyri cheeses is required; this would certainly benefit to artisanal producers and allow the consumers to distinguish between the two cheese varieties and avoid confusion. At present, the fresh industrial PDO 'Galotyri Dodoni' brand and the ripened artisanal PDO Galotyri cheese brands 'Zagori' and 'Roussas' studied herein, are marketed side-by-side on the shelve, without any distinctive labeling trademark between them. Therefore, all three brands gain equal benefits from the PDO Galotyri certification, which is contradictive in terms of science, i.e., particularly in consideration of the major process-dependent differences in their microbial (LAB) ecology, diversity and biotechnology, and irrespective of their production cost and retail price. In our opinion, the Greek food regulatory authorities should revise the original Galotyri PDO description in article 83 in order to recognize fresh and ripened cheese varieties as totally different products or otherwise the PDO certification of all fresh Galotyri-type cheese brands, with or without addition of 'external' Feta cheese, should be recalled from the market to enhance protection of the authentic Galotyri.

In parallel, traditional Galotyri cheese manufacturers in Epirus and Thessaly must be assisted by dairy researchers in developing, preserving and applying successfully more diversified consortia of novel indigenous starter or adjunct LAB strains in boiled milk. An alternate approach is to combine indigenous strains of high biotechnological, protective and/or probiotic properties with industrial starter strains of *S. thermophilus*, *Lb. bulgaricus* or *Lc. lactis*, of proven efficiency which can be derived from selected commercial starter culture preparations. For this reason, we have preserved numerous representatives of all LAB species isolated from all different Galotyri cheese products for further biotechnological evaluations. In specific, the identity of 31 representative isolates from the LAB groups shown in Table 3 has been confirmed by genotyping at the species/subspecies [51,52], according to the procedures described by Vandera et al. [31]: they include three industrial (biotype I) isolates of *S. thermophilus* (GLD1, GLD3, GLD 14) and 28 isolates from the artisan A, B and C cheeses in Table 1, genotyped with *S. thermophilus* biotype II (isolates GL7, GL10, GL12), *Lc. lactis* subsp. *lactis* (GL36, GL38, GL43, GL45, GL48, GL50, GL61), *E. faecium* (GL31, GL35), *E. durans* (GL34, GL60, GL70), *E. faecalis* (GL66, GL68, GL69), *Lb. plantarum* (GL16, GL21, GL27, GL51, GL64), *Lb. paraplantarum* (GL17, GL25, G28) and *Leuc. mesenteroides* (GL52, GL57). Additional molecular identification studies are in progress in collaboration with the DEMETER Institute of Agricultural Products, Athens, Greece, for (i) in-depth investigations of the LAB and yeast ecology of additional artisan-type PDO-certified Galotyri cheese brands by culture-dependent and culture-independent methods; (ii) clear research-based distinctions between the artisanal and industrial PDO Galotyri cheese brands with emphasis on the biochemistry and particular sensorial characteristics of each brand as, for instance, it was recently surveyed for Maroilles cheese [5]; and (iii) development and *in situ* optimization of novel combinations of natural or commercial (industrial) starter LAB strains with various indigenous nonstarter (adjunct) LAB strains under commercial Galotyri cheese processing and ripening conditions in selected artisan plants. In this manner, the biodiversity of the heated milk will be enhanced, and the authenticity, the sensorial qualities and potential functional properties of the PDO Galotyri cheese will be preserved and improved, as recommended for all different types of European traditional cheese technologies [3,5,8,11,53–55]. This is our primary research task in ProMedFoods, an ongoing collaborative research project of Mediterranean partner countries (ARIMNet II), which targets on the ripened Galotyri cheese technology.

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Conflict of interest

The authors declare no conflicts of interest in this study.

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