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Research article

Semi industrial production of Tsalafouti dairy product

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Abstract: Tsalafouti is a fermented dairy product produced from 'boiled', naturally acidified sheep milk during summer (end of lactation period) as a farmhouse product. As consumer's demand is regular throughout the year, a semi-industrial production method of Tsalafouti was investigated based on the artisanal processing method aided with the use of a commercial multi-strain *Lactococcus lactis* starter culture for optimal milk acidification. Main physicochemical and microbiological characteristics, level of proteolysis and volatile compounds of the new product were determined. The semi-industrial Tsalafouti had smooth firm texture, mild sour taste, pleasant aroma, and received high panel-sensory scores up to day 45 of storage; afterward, the product developed an unpleasant flavor. The ripened (day 30) product had pH 4.28, moisture 81.5%, fat 6%, fat-in-dry matter 32.53%, salt 0.33% and proteins 5.49%, and contained high levels of heptanal, acetone, hexanal and 3-methyl butanal. The *Lc. lactis* starter (4 strain biotypes) grew abundantly (> 9 log CFU/g) and acidified the milk within the first 10 days of ripening at 10 °C. Viable starter cell populations declined significantly during late ripening and storage, probably due to autolysis. No growth of thermophilic streptococci, mesophilic non-starter lactobacilli or leuconostoc-like bacteria, enterococci, staphylococci, coliform bacteria and yeasts occurred during ripening. Only spoilage molds grew on the product surface after 45 to 60 days of aerobic storage at 2−4 °C. The present data may contribute to the industrial production of Tsalafouti, giving an added value to this traditional Greek dairy product.

Keywords: Tsalafouti; dairy product; semi-industrial production; traditional; sheep

1. Introduction

Fermentation is a technique that has been used for centuries in order to preserve milk. Additionally fermentation can improve the organoleptic properties, while consumption of fermented milk products is associated with health benefits [1]. Traditional fermented milk products have been produced since antiquity by various civilizations [1–3]. In Greece, traditional fermented products are associated with the local culture and artisanal practices of small-scale manufacturers, while some of them are produced in many cases by back-slopping or spontaneous fermenting processes using naturally occurring microorganisms [4].

Artisanal Tsalafouti is a fermented milk product manufactured in many Greek areas, mainly in the regions of Agrafa and Tzoumerka in the mountain range of Pindos [5]. It is produced traditionally using ovine milk during summer (end of lactation period) and its manufacture relies primarily on the spontaneous milk fermentation by indigenous microbiota, mainly lactic acid bacteria (LAB). Pappa et al. [6] studied the artisanal production and the biochemical and microbiological characteristics of artisanal Tsalafouti. For its manufacture, the milk was initially heated at 90 ℃ and salt was added. The boiled and salted milk was then put into containers, which were placed in caves under running water in nearby streams for natural ripening at low temperature (approximately 10 ℃). The microbial community of the artisanal Tsalafouti consisted primarily of mesophilic LAB [5,6]. It is well known that heating of the milk over an open fire slightly increases the solid content and yields in a more viscous coagulum due to the modified properties of the casein [7]. Also, during artisanal Tsalafouti production, the heating of milk helped to eradicate any vegetative pathogenic microorganisms present in the raw milk. The addition of fresh product to the same vessel (i.e., to an ongoing batch fermentation procedure that was done up to three times) could ensure the build-up of a specific indigenous microbiota to sour the milk during ripening. However, milk fermentation and acidification by adventitious LAB and non-LAB mixtures sometimes results in a product with no standard composition and flavor [7].

As nowadays there is an increasing demand for Tsalafouti, it is necessary to obtain a dairy product of high quality and safety that can be sold outside its region of origin throughout the whole year. Therefore, it should be manufactured under controlled conditions with specified starter cultures. The use of selected (commercially defined) starter LAB cultures can act on milk to derive a controlled fermentation process as well as desirable and predictable flavor and texture [8], and thereby, to result in a product that meets current consumer demands and supplies the market regularly during the year. In the present study, Tsalafouti dairy product was produced by applying the traditional manufacturing method [6] under semi-industrial (pilot-plant) conditions, using a commercial starter culture, and its physicochemical, biochemical, microbiological and organoleptic characteristics during ripening and storage were determined. The present results add new information to the literature and may contribute to standardize the manufacture, identify the best ripening time and all this information can be used effectively for the final industrialization of Tsalafouti.

2. Materials and methods

2.1. Semi-industrial production

The traditional manufacture of artisanal Tsalafouti dairy product, at height 1000 m in the village

of Theodoriana, North West part of Greece, at the end of the lactation period using sheep milk, was described by Pappa et al. [6]. Briefly, raw milk (15 kg) together with salt (30 g) was heated by fire, under stirring at 90 ℃ within 30 min. Afterward the milk was cooled and transferred to plastic containers placed in caves under running water in nearby streams with low temperature (approximately 10 ℃). At that place, the Tsalafouti dairy product was stirred twice daily and left to ripen for approximately 20 days, until its characteristic creamy texture was obtained and it was ready for consumption.

For the semi-industrial production of Tsalafouti throughout the year, the following procedure took place in the pilot-plant laboratory of the Dairy Research Department at Ioannina (Epirus, Greece). The processing protocol was based on the basic milk heating, fermentation and ripening parameters of the artisanal Tsalafouti manufacture. Raw sheep milk from local breeds was used. The milk was concentrated (from 5 to 4.5 kg) by heating to 'boiling' at 90 ℃ under continuous stirring in order to increase the total solid content. This step lasted approximately 40 min. The milk was cooled at 30 ℃, 9 g of re-crystallized coarse-grained salt were added, followed by inoculation with the selected starter culture (FD-DVC MO-10, Hansen) consisting of *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*. The inoculated milk was poured into sterilized glass containers and incubated for 1 hour at 30 ℃. After, the glass containers were transferred to a ripening room (10 ℃) for 30 days and then to a cold room (2–4 ℃) for storage up to 90 days. The experiment was repeated three times. Samples were taken for analyses on days 0, 10, 20, 30, 45, 60 and 90 after manufacture.

2.2. Physicochemical analyses

The content of moisture, fat, fat in dry matter (FDM) and salt of Tsalafouti was determined with established methods, as described by Pappa et al. [9]. The acidity (Dornic method; after mixing 10 g of Tsalafouti with an equal mass of distilled water) as described by Ling [10]. The pH was measured with the micro-pH 2001 meter (Crison, Barcelona, Spain).

Viscosity was measured at 4˚C using a viscometer (Brookfield Engineering Laboratories Inc, Massachusetts, USA; model RVT) at a speed of 2.5 rpm using No 7 spindle and it was derived from the maximum deflection of the needle on the scale after 1 min of shearing.

All materials and reagents used for the analyses in this study were of analytical grade and all analyses were performed in duplicate.

2.3. Organoleptic evaluation

Organoleptic evaluation of Tsalafouti dairy product was performed by a panel consisting of five trained members familiar with fermented dairy products, which were permanent staff of the Dairy Research Department. They were asked to evaluate the appearance, texture and flavor of Tsalafouti and to notice any defects (such as defective color, lack of texture uniformity, rancid or bitter flavour etc.). For this purpose, a ten-point scale was used, with 1 being poor and 10 excellent. The attribute of flavor was given dominating importance over the other two; therefore, the score obtained for flavor was multiplied by five, for appearance by one and for texture by four [11]. The total score, that described the overall acceptability, was calculated by adding the scores for the three attributes. An excellent product would receive a total score of 100. Water was provided for mouth washing between samples.

2.4. Proteolysis

Proteolysis was assessed by measuring water-soluble nitrogen (WSN), nitrogen soluble in 5% phosphotungstic acid (PTA-N) and nitrogen soluble in 12% trichloroacetic acid (TCA-N). The above soluble nitrogen fractions were determined as described by Mallatou et al. [12] and expressed as % Total Nitrogen (TN). Total nitrogen content was estimated by the Kjeldahl method [13] using the Kjeldahltherm digestion and Vapodest 30 distillation systems (Gerhardt GmbH & Co KG, Bonn, Germany) equipped with an end-point titrator (Gerchard, Vap. 5). Protein values were determined by multiplying the TN values with the factor 6.38.

2.5. Volatile compounds

The volatile compounds of mature Tsalafouti (at 30 days of ripening and storage) were investigated by Solid Phase Micro Extraction-Gas Chromatography-Mass Spectrometer (SPME-GC-MS) analysis as described by Kondyli et al. [14]. Peak areas (arbitrary units, AU) were calculated from the total ion chromatograms by the Shimadzu GCMS Solution software (Shimadzu, Tokyo, Japan). Peak identification was done by comparing the mass spectra with NIST libraries and by comparing their retention times with authentic standards when available.

2.6. Microbiological analyses

On each sampling day, the glass container with the bulk Tsalafouti dairy product, which was either liquid/viscous or soft-curdled depending on the processing step, was first stirred with a sterile spatula to mix well. Then 25 g of sample were aseptically transferred in a sterile stomacher bag and 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. All diluents, microbiological enumeration agar media and supplements were purchased from Neogen Culture Media (formerly Lab M; Heywood, Bury, UK).

Microbial quantification analyses were conducted as reported for artisanal Tsalafouti by Pappa et al. [6]. Selection of appropriate enumeration agar media and incubation conditions were according to procedures justified during our previous microbiological ecology studies on traditional Greek soft, acid-curd cheeses [15,16]. Briefly, total viable bacteria (TVC) were counted on Milk Plate Count agar (MPCA), incubated at 37 ℃ for 48−72 h. Total mesophilic and thermophilic LAB populations were separated on MRS agar incubated at 30 ℃ for 48−72 h and at 45 ℃ for 24−48 h, respectively. Mesophilic and thermophilic dairy (lactose-fermenting) LAB were enumerated on M17 agar incubated at 22 ℃ for 48−72 h and 42 ℃ for 48 h, respectively. Enterococci were selectively enumerated on Slanetz and Bartley (SB) agar, incubated at 37 ℃ for 48 h. Coliforms were counted by pouring 1 mL samples into melted (45 ℃) Violet Red Bile (VRB) agar, overlayed with 5 mL of the same medium and incubated at 37 ℃ for 24 h. Total staphylococci were enumerated on Baird-Parker (BP) agar base with egg yolk tellurite, incubated at 37 ℃ for 48 h. Yeasts and molds were selectively enumerated on Rose Bengal Cloramphenicol (RBC) agar, incubated at 25 ℃ for 5 days. When required, the electivity of the SB, BP and RBC agar media was checked. In addition, to assess the expected predominance of the mixed *Lc. lactis* commercial starter strains during semi-industrial Tsalafouti

production and storage, random representatives of all macroscopically different colony types enumerated on M17 and MRS agar plates were confirmed at the LAB genus or phenotypic group, by rapid tests, i.e., phase contrast microscopy, Gram staining by the KOH method, catalase reaction, gas production from glucose, ammonia production from arginine and main sugar fermentation profiling in 96-well mini-plates [15,16].

2.7. Statistical analyses

The data were subjected to one-way analysis of variance to compare the values of each parameter of Tsalafouti dairy product, at different ages. The software Statgraphics Plus for Windows v. 5.2 (Manugistics, Rockville, Maryland, USA) was used and the means were separated by the LSD test, at the 95% confidence level ($P < 0.05$). The same software was used to find a correlation between parameters.

3. Results and discussion

3.1. Physicochemical analyses

The physicochemical characteristics of Tsalafouti during ripening and storage are presented in Table 1. In general, the values of pH, moisture and salt were similar to those of artisanal Tsalafouti [6]. On the contrary, the values of fat and fat-in-dry-matter (FDM) were higher compared to the traditional product; this was expected as the fat content of the milk used in this study was lower than that of the milk used for the artisanal manufacture. The acidity of the semi-industrial Tsalafouti was 1.31% at 30 days giving a mild acid flavor which was appreciated by the panelists during the organoleptic evaluation; acidity was lower than that of the artisanal product (1.49%).

Semi-industrial Tsalafouti obtained its creamy, soft, smooth and firm texture by lowering the pH values to the isoelectric precipitation of casein micelles, due to the action of the *Lc. lactis* starter culture. The pH values of Tsalafouti from 6.32 on day-0 decreased ($P < 0.05$) to 4.24 on day 10, and then remained stable ($P > 0.05$). As a consequence, the acidity increased ($P < 0.05$) from 0.26% at the beginning of ripening to 1.28% at the 10th day, which remained constant ($P > 0.05$) until the day-90.

As previously reported [5,6], the characteristics of artisanal Tsalafouti are similar to those of soft cheeses; however, Tsalafouti cannot be classified as cheese because its moisture exceeds the 75% upper limit [17]. This increased moisture is due to the fact that during manufacture there is no drainage of the product in combination with its short ripening time [5]. The moisture content of semi-industrial Tsalafouti was approximately 80% and the fat content was approximately 6% (Table 1).

In a recent study, samples obtained from the Greek market of Galotyri, Katiki and Xygalo Siteias spreadable cheeses showed moisture content 74%, 75% and 78.5%, and fat content 10.9%, 11.3% and 8.83%, respectively. In the present work, the protein content of Tsalafouti was approximately 5.5%, whereas the above cheeses had respective values of 8.24%, 8.54% and 9.02% [18].

Table 1. Physicochemical parameters of semi-industrial Tsalafouti dairy product during ripening and storage.

Age (days)	pH	moisture, %	fat, %	fat-in-dry-matter, %	salt, %	acidity, %	viscosity, cP (mPa*s) × 1000
0	6.32 ± 0.00^{a}	80.27 ± 0.15^{a}	$6.42 \pm 0.22_{a}$	32.53 ± 1.27^{a}	0.39 ± 0.01^{a}	0.26 ± 0.01^{a}	0 ± 0^{a}
10	4.24 ± 0.05^{b}	80.50 ± 0.24^{a}	6.00 ± 0.00^{a}	30.78 ± 0.36^{a}	0.47 ± 0.06^{a}	1.28 ± 0.03^{b}	140 ± 16^{b}
20	4.21 ± 0.01^{b}	81.19 ± 0.93^{a}	6.17 ± 0.17^{a}	33.37 ± 0.68^{a}	0.40 ± 0.02^{a}	1.13 ± 0.07^{b}	141 ± 12^{b}
30	4.28 ± 0.03^{b}	81.50 ± 0.42^{a}	6.00 ± 1.52^{a}	32.53 ± 0.83^{a}	0.33 ± 0.06^{a}	1.31 ± 0.01^{b}	133 ± 37^{b}
45	4.24 ± 0.03^{b}	80.30 ± 1.65^{a}	6.50 ± 0.25^{a}	33.22 ± 1.57^{a}	0.47 ± 0.05^{a}	1.31 ± 0.01^{b}	154 ± 50^{b}
60	4.21 ± 0.02^{b}	80.37 ± 0.15^{a}	6.66 ± 0.08^{a}	33.84 ± 0.56^{a}	0.43 ± 0.11^{a}	1.28 ± 0.02^{b}	133 ± 38^{b}
90	4.27 ± 0.03^{b}	80.12 ± 0.05^{a}	6.16 ± 0.16^{a}	31.02 ± 0.90^{a}	0.40 ± 0.28^{a}	1.29 ± 0.02^{b}	133 ± 37^{b}

Note: Means of three manufacturing trials ± standard error. a-b: Mean values of each parameter in the same column with different superscripts are statistically different ($P < 0.05$).

From Table 1 it was found that viscosity increased ($P < 0.05$) from day 10 to day 20 and then it did not change ($P > 0.05$); therefore, the characteristic texture of Tsalafouti dairy product was obtained on day 20. Viscosity of the semi-industrial Tsalafouti was higher than that of the artisanal product [6] and this could be due to the starter culture added. However, the present Tsalafouti showed lower viscosity values compared to yoghurt found by Pappa et al. [19] and this could be attributed to the different starter culture used and to the different manufacturing method.

3.2. Proteolysis

The biochemistry of ripening is very complex and involves three primary processes, glycolysis, lipolysis and proteolysis; the latter is the most complex [20]. The changes of the values of the principal soluble nitrogenous components of semi-industrial Tsalafouti are shown in Table 2.

The TN content remained constant during maturation since no significant differences were observed ($P > 0.05$). Similarly, the protein content remained stable ($P > 0.05$) at all sampling dates. The TN and protein contents were lower compared to the artisanal Tsalafouti product [6].

Soluble nitrogen fractions increased ($P < 0.05$) during ripening and storage. Specifically, from the day of manufacture to the end of storage (day 90), the WSN increased from 7.05% to 11.14%TN, the TCA from 5.97% to 8.51%TN and the PTA from 3.77% to 5.25%TN, respectively (Table 2). It is known that the fraction of WSN consists of the proteolysis products and of the whey proteins that have been extracted with water. The fraction of nitrogen soluble in 12% TCA includes free amino acids, medium-size and small-size peptides with 2–20 amino acid residues. Free amino acids and very small peptides up to 600 $g \cdot mol^{-1}$ consist the fraction soluble in 5% PTA. Although, proteolysis is a complex procedure, it can be considered that PTA-SN expresses its end-products [21].

At 30 days of ripening and storage, Galotyri cheese made only with starter culture [22] had lower WSN (6.76%TN) values than Tsalafouti in the present study (8.44%TN); the use of different starter culture and manufacture procedure could explain this difference.

Table 2. Proteolysis of semi-industrial Tsalafouti dairy product during ripening and storage.

Age (days)	TN, %	WSN %TN	TCA %TN	PTA %TN	Proteins, %
0	0.88 ± 0.02^{a}	7.05 ± 0.64^{a}	5.97 ± 0.37^{a}	3.77 ± 0.26^{a}	5.59 ± 0.13^{a}
10	0.92 ± 0.04^{a}	7.31 ± 0.59^{ab}	6.33 ± 0.41^{ab}	3.91 ± 0.43^{ab}	5.87 ± 0.26^{a}
20	0.87 ± 0.02^{a}	8.36 ± 0.38^{abc}	7.27 ± 0.14^{ab}	4.60 ± 0.18^{abc}	5.53 ± 0.15^{a}
30	0.86 ± 0.02^{a}	8.44 ± 0.28^{abc}	8.14 ± 0.39^{ab}	4.64 ± 0.16^{bc}	5.49 ± 0.11^{a}
45	0.90 ± 0.04^{a}	9.03 ± 0.57^{bc}	7.26 ± 0.33^{ab}	4.89 ± 0.10^{c}	5.72 ± 0.21^{a}
60	0.91 ± 0.01^{a}	9.32 ± 0.92^{c}	8.24 ± 1.32^{ab}	4.91 ± 0.35^{c}	5.83 ± 0.09^{a}
90	0.87 ± 0.02^{a}	11.14 ± 0.40^{d}	8.51 ± 1.32^{b}	5.25 ± 0.32^{c}	5.59 ± 0.11^{a}

Note: Means of three manufacturing trials ± standard error. a–d: Mean values of each parameter in the same column with different superscripts are statistically different ($P < 0.05$).

3.3. Organoleptic evaluation

The scores of the organoleptic evaluation are shown in Table 3. Panelists appreciated the semi-industrial Tsalafouti. Generally, the product was characterized with a mild, piquant aroma and a delicate, mildly acid and sour, pleasant and refreshing taste, which was intensified with increasing ripening time. It had a soft and smooth body and no whey off. Flavor was positively correlated with salt (correlation coefficient = 0.52) and negatively with moisture content (correlation coefficient = 0.70), while texture was highly correlated with viscosity (correlation coefficient = 0.88). Statistical analyses showed that all the characteristics scores remained stable ($P > 0.05$) until the day-45. After the 45th day an unpleasant odor and taste occurred resulting in the deterioration of the quality of the samples.

Table 3. Organoleptic characteristics of semi-industrial Tsalafouti dairy product during ripening and storage.

Age (Days)	Appearance (10)[(ii)]	Texture (40)	Flavor (50)	Total (100)
10	7.93 ± 0.07^{a}	30.7 ± 0.00^{a}	39.4 ± 0.74^{a}	78.33 ± 0.80^{a}
30	8.40 ± 0.4^{a}	31.5 ± 1.5^{a}	39.4 ± 0.6^{a}	79.25 ± 1.25^{a}
45	8.6 ± 0.31^{a}	32.67 ± 2.16^{a}	41.9 ± 1.22^{a}	83.2 ± 3.63^{a}

Note: Means of three manufacturing trials ± standard error. Values in brackets show the maximum scores. a: Means of each parameter within the same column without different superscripts do not differ significantly ($P > 0.05$).

3.4. Volatile compounds

Two ketones (acetone, 2-nonanone), six alcohols (ethanol, hexanol, octen-3ol, heptanol, hexanol-2ethyl, octanol), one ester (decanoic acid methyl ethyl ester), six aldehydes (butanal-3methyl, hexanal, heptanal, octanal, nonanal, decanal), four hydrocarbons (dodecane, tridecane, tetradecane, 2-undecanane) and three free fatty acids (butanoic acid-2ethyl, hexanoic acid, octanoic acid) consisted the volatile fraction of semi-industrial Tsalafouti at the end of ripening (day 30), as shown in Table 4. Although the evolution of different volatile groups was not studied

at different sampling dates, at day-30, aldehydes (40686.1 ± 7532.51 AU) followed by ketones (16349.8 ± 3887.98 AU) were the groups found in abundance. Aldehydes are unstable compounds that are reduced to alcohols or oxidized to acids, but can have an important impact on flavor due to their low perception thresholds [23]. It is known that the concentration of ketones depends on the amount of fat in the original milk [24]. The total alcohols, total esters, total hydrocarbons and the total free fatty acids were found to be 5463.71 ± 304.38 AU, 1251.4 ± 281.70 AU, 5062.01 ± 298.29 AU and 5082.6 ± 587.96 AU respectively.

Table 4. Volatile compounds (peak area × 10^3, AU) of semi-industrial Tsalafouti dairy product at 30th day of storage.

Volatile compounds	Peak area × 10^3, AU
Acetone (ketone)	13471.9 ± 3790
2-Nonanone (ketone)	2877.9 ± 114.95
Ethanol (alcohol)	1786.73 ± 233.73
Hexanol (alcohol)	964.43 ± 146.32
Octen-3ol (alcohol)	483.4 ± 36.07
Heptanol (alcohol)	600.1 ± 53.71
Hexanol-2ethyl (alcohol)	1259.98 ± 301.10
Octanol (alcohol)	369.07 ± 43.02
Decanoic acid methyl ethyl ester (ester)	1251.4 ± 281.70
Butanal- 3methyl (aldehyde)	5754.93 ± 1612.17
Hexanal (aldehyde)	11497.2 ± 2272.13
Heptanal (aldehyde)	19515.9 ± 3679.32
Octanal (aldehyde)	1156.7 ± 110.52
Nonanal (aldehyde)	2425.82 ± 141.45
Decanal (aldehyde)	335.47 ± 40.95
Dodecane (hydrocarbon)	3762.67 ± 114.97
Tridecane (hydrocarbon)	485.27 ± 123.13
Tetradecane (hydrocarbon)	427.82 ± 92.35
2-Undecanone (hydrocarbon)	386.25 ± 20.78
Butanoic acid- 2ethyl (free fatty acid)	435.2 ± 33.17
Hexanoic acid (free fatty acid)	813.93 ± 98.88
Octanoic acid (free fatty acid)	3833.5 ± 514.97

Note: Means of three manufacturing trials ± standard error.

From Table 4 it can be seen that heptanal, acetone, hexanal and 3-methyl butanal were the compounds found in high levels. Hexanal and heptanal originate from unsaturated fatty acids with the formation of an intermediate hydroperoxide [25]. Hexanal has been positively correlated with a caramel and creamy odor and a balanced flavor in a study carried out on ten European cheese varieties [26]. Acetone generally originates either from milk or is produced from the thermal degradation of β-ketoacids [27] while 3-methyl butanal is produced from the catabolism of the amino acid leucine [28].

Hexanal, acetone and 3 methyl butanal were found in high levels in Galotyri spreadable cheese [29].

3.5. Microbial evolution in semi-industrial Tsalafouti during processing and storage

Table 5 summarizes the microbial quantification results obtained for TVC and the populations of LAB enumerated on MRS, M17 and SB agar media during Tsalafouti processing and storage. Prior to discussing them, the following critical control points of the present processing protocol should be emphasized from a microbiological point of view: (i) the semi-industrial production of Tsalafouti was conducted in the pilot-plant laboratory of the Dairy Research Department under carefully monitored and inspected hygienic conditions; (ii) hence, microbial cross-contamination of the bulk milks from the pilot-plant environment was prevented to the greatest possible extent, particularly after raw milk heating at 90 ℃ for 40 min and cooling at 30 ℃; (iii) as a consequence, the commercial starter culture, consisting of an unknown to end-users number of mixed *Lc. lactis* strains of the subspecies *lactis* and *cremoris*, was expected to grow practically as a 'single LAB species' culture in the absence or low presence of native microbiota, following its addition to the 'boiled' milk after cooling; (iv) all Tsalafouti bulk milk batches were incubated at 30 ℃ for only 1 h after inoculation with the starter and then transferred for ripening at 10 ℃; thus neither the initial starter cell populations nor the populations of microbial post-thermal contaminants, if any, would have enough time for growth under optimal milk fermentation temperatures (> 20−30 ℃, or 37−42 ℃ progressively reduced to 20−25 ℃) applied in typical fermented cheeses [30,31], including typical Greek acid-curd cheeses [32,33], within the first 24 h after manufacture; (v) instead, semi-industrial Tsalafouti milk batches were fermented slowly, under 'cool' (10 ℃) conditions, in simulation of the artisanal fermentation [6], herein monitored by the mixed lactococcal starter, i.e., *Lc. lactis* is a typical mesophilic LAB species and thus most *Lc. lactis* strains can grow and acidify the milk adequately at temperatures 10−15 ℃ [34].

In general, the total microbial quantification results obtained, including the TVC and LAB counts shown in Table 5, were consistent with the above microbiological attributes expected for a pilot, semi-industrial Tsalafouti production. In specific, after inoculation with the commercial starter and incubation at 30 ℃ for 1 h (day 0), the initial contamination levels of the Tsalafouti milk batches with total staphylococci and yeasts were on average 1.88 ± 0.12 and 1.67 ± 0.33 log CFU/g, respectively. Also, post-thermal contamination of the 'boiled' bulk milks with pathogenic staphylococci, coliform bacteria and moulds was totally prevented (absence per g), or it was very low (< 10 CFU/g). Afterward the low Tsalafouti-specific fermentation temperature in association with the high initial (> 7.5 log CFU/g expressed as TVC in Table 5) cell population density and the subsequent major (> 8.5 log CFU/g) growth of the *Lc. lactis* starter mix (Table 5) suppressed the growth of total staphylococci, coliform bacteria, and yeasts/moulds below 100 CFU/g during ripening at 10 ℃ for 30 days. Similarly, during storage 4 ℃ for an additional 60 days, none of the above microbial groups managed to increase above 100 CFU/g, except of spoilage molds (2.03 ± 1.03 log CFU/g) that appeared on the Tsalafouti product surface after two months. In conclusion, no growth of gram-positive non-LAB or gram-negative bacteria and yeasts occurred; their counts remained undetectable (< 100 CFU/g) throughout Tsalafouti processing and storage. Therefore, the corresponding microbiological data are not tabulated in Table 5.

Regarding growth of the commercial multi-strain *Lc. lactis* starter culture during semi-industrial Tsalafouti processing and storage, variable results of prominent technological importance were

obtained (Table 5). Significantly different ($P < 0.05$) total *Lc. lactis* populations were enumerated on MPCA, M17 and MRS agar media from day 0 to day 90, which primarily were dependent on the incubation temperature of each medium (Table 5). According to the Methods, the incubation temperature of LAB enumeration agar media ranged from 22 to 45 ℃.

Starting from day 0, the mixed *Lc. lactis* starter populations, enumerated as total mesophilic dairy LAB on M17 agar at 22 ℃, were 7.54 ± 0.06 log CFU/g. Very similar also were the populations of total mesophilic LAB on MRS agar at 30 ℃ as well as the TVC on MPCA at 37 ℃ (Table 5). Hence, the initial (day-0) Tsalafouti LAB biota was dominated by at least two, or likely more, commercial *Lc. lactis* starter strains of the subspecies *lactis* and *cremoris*, able for prolific growth at a temperature range from 22 to 37 ℃. However, the incubation of a second series of M17 agar plates at 42 ℃ to selectively enumerate total thermophilic dairy LAB resulted in ca. 2 log CFU/g lower populations than those enumerated on M17/22 ℃ agar plates after milk inoculation with the starter (day 0; Table 5). This incubation temperature-dependent major difference in LAB growth on M17 agar indicated that at least one, or more, of the primary strain constituents of the mixed *Lc. lactis* starter could not grow at 42 ℃. This finding was logical because at least the typical *Lc. lactis* subsp. *cremoris* dairy starter strains are unable to grow at 40 ℃, as well as, unable to grow in 4% salt and breakdown arginine [34]. In contrast, most dairy (industrial) starter *Lc. lactis* subsp. *lactis* strains as well as most wild *Lc. lactis* strains of both *lactis* and *cremoris* genotypes grow at 40–42 ℃, but not at 45 ℃ [34–36]. In the present case, the observed growth temperature variability of *Lc. lactis* was a rather constant strain-dependent technological feature of the mixed starter culture FD-DVC MO-10 used for Tsalafouti production, as it is confirmed in later paragraphs.

Regardless of their ability to grow at 42 ℃, *Lc. lactis* starter strains promoted significant growth ($P < 0.05$) in all Tsalafouti batches from day 0 to day 10, which on average ranged from 1.6 to 2.1 log CFU/g on M17/22 ℃, M17/42 ℃ and MRS/30 ℃ agar plates. Starter growth on MPCA/37 ℃ agar plates, reflected as the TVC increases (Table 5), was restricted by 0.7 log CFU/g probably because the predominant *Lc. lactis* starter strain/s that could not grow at 42 ℃ also grew less profoundly at 37 ℃ on MPCA, a medium of poorer nutrient composition compared to M17 and MRS. Most dairy *Lc. lactis* strains grow optimally at around 30 ℃, but are not particularly well adapted to elevated temperatures (37–39 ℃) to which they are often exposed during cheese production [37]. Importantly, from day 10 to day 20, major ($P < 0.05$) reductions in the viability of all *Lc. lactis* starter strain fractions were observed, which were greater in the order M17/42 ℃ > MRS/30 ℃ > M17/22 ℃ > MPCA/37 ℃ (Table 5). However, from day 20 to the end of ripening at 10 ℃ (day 30), new, mostly significant, increases of the viable *Lc. lactis* populations occurred on the above agar media which were succeeded by new major ($P < 0.05$) progressive *Lc. lactis* population decreases during Tsalafouti storage at 4 ℃ for an additional 60 days (Table 5).

Table 5. Microorganisms of Tsalafouti dairy product during ripening and storage.

Age (Days)	Total viable count (TVC) (37 °C)**	Total mesophilic LAB (30 °C)	Total thermophilic LAB (45 °C)	Total mesophilic dairy LAB (22 °C)	Total thermophilic dairy LAB (42 °C)	Enterococci (37 °C)
0	7.54 ± 0.02^{bc}	7.60 ± 0.00^{e}	1.33 ± 0.33^{a}*	7.54 ± 0.06^{b}	5.31 ± 0.07^{d}	1.67 ± 0.33^{a}
10	8.51 ± 0.31^{c}	9.20 ± 0.04^{g}	$< 2.00^{a}$	9.19 ± 0.06^{e}	7.41 ± 0.06^{e}	$< 2.00^{a}$
20	7.29 ± 0.14^{bc}	6.46 ± 0.22^{d}	$< 2.00^{a}$	7.85 ± 0.59^{bc}	3.00 ± 0.00^{b}	$< 2.00^{a}$
30	8.42 ± 0.02^{c}	8.29 ± 0.02^{f}	$< 2.00^{a}$	8.57 ± 0.07^{d}	4.80 ± 0.17^{c}	$< 2.00^{a}$
45	6.71 ± 0.24^{b}	6.00 ± 0.00^{c}	$< 2.00^{a}$	8.06 ± 0.04^{c}	$< 2.00^{a}$	$< 2.00^{a}$
60	5.00 ± 0.00^{a}	5.10 ± 0.10^{b}	$< 2.00^{a}$	7.50 ± 0.05^{b}	2.93 ± 0.47^{b}	$< 2.00^{a}$
90	5.20 ± 1.11^{a}	$< 3.00^{a}$	$< 2.00^{a}$	4.99 ± 0.27^{a}	$< 2.00^{a}$	$< 2.00^{a}$

Note: Means of three manufacturing trials ± standard error. a–g: Mean values of each parameter in the same column with different superscripts are statistically different ($P < 0.05$); * For the lowest detection limit: 0.1 mL samples from all Tsalafouti liquid milk samples were spread directly on the agar plates on day 0; thus the lowest detection limit was 10 CFU/g on day 0 and increased to 100 CFU/g on days 10 to 90, after the samples became solid due to milk curdling; ** Temperature values in bracket indicate the incubation/growth temperature of each different LAB group enumerated on the corresponding agar media reported in section 2.6. of the Methods.

Table 6. Different strain biotypes of the commercial *Lactococcus lactis* starter culture isolated from Tsalafouti after ripening (day-30).

Lc. lactis	Acid production from (Key sugar fermentation reactions)												Tsalafouti batch/Isolation medium						Total isolates
biotype	Mal	Man	Lac	Rib	Lara	Xyl	Raf	Mel	Suc	Tre	Gal	Sor	A		B		C		
													M17/22 °C	M17/42 °C	M17/22 °C	M17/42 °C	M17/22 °C	M17/42 °C	
L1	+	+	+	+	−	−	−	−	−	+	+	−	5		4		3		12
L2	+	+	+	+	−	−	−	−	−	−	+	−			2		3		5
L3	+	−	+	+	−	+	−	−	−	+	+	−		6		6		6	18
L4	+	−	+	+	−	−	−	−	−	−	+	−	1						1
Total isolates													6	6	6	6	6	6	36

Note: Mal: maltose; Man: mannitol; Lac: lactose; Rib: ribose; Lara: L-arabinose; Xyl: xylose; Raf: raffinose; Mel: melibiose; Suc: sucrose; Tre: trehalose; Gal: galactose; Sor: sorbitol. All isolates were gram-positive, catalase-negative, homofermentative cocci. All produced ammonia from arginine. All grew at 15 °C; none grew at 45 °C. All failed to grow on selective agar media for enterococci (SB and KAA agar) and staphylococci (Baird-Parker).

The above major fluctuations in starter viability during semi-industrial Tsalafouti ripening and storage did not occur in the corresponding indigenous LAB populations of the artisanal product [6]. Because the present fluctuations showed a quite constant and repeatable pattern in all three product batches, they probably were due to variations in the autolysis rate of the mixed industrial *Lc. lactis* strain constituents of the FD-DVC MO-10 starter [37,38]. To validate this, first we ensured that no major growth of other starter (*Leuconostoc* spp., *Streptococcus thermophilus*) or non-starter (*Lactobacillus plantarum*, *Lb. casei/paracasei*, *Lb. rhamnosus*) [15,39,40] 'accidental' LAB species contaminants occurred in semi-industrial Tsalafouti, particularly as regards the subdominant LAB populations selected for growth on M17 agar at 42 ℃ (Table 5). To ensure this, 6 dominant and 6 subdominant LAB colonies were picked randomly after enumeration from one highest dilution M17/22 ℃ and one M17/42 ℃ agar plate, respectively, of each ripened (day-30) Tsalafouti batch for biochemical identification (Table 6). Results confirmed that all 36 isolates were homofermentative, arginine-positive, mesophilic cocci which possessed main sugar fermentation reactions that matched those of the species *Lc. lactis*: in specific, all failed to ferment L-arabinose, raffinose and sucrose and displayed variant subspecies lactis phenotypes [35,36]. The 36 isolates were split into four *Lc. lactis* strain biotypes, L1 to L4 (Table 6). Notably, all 18 representative isolates of the subdominant *Lc. lactis* populations grown selectively on M17/42 ℃ agar (4.8 ± 0.17 log CFU/g; Table 5) were identical to each other and grouped in L3, the only *Lc. lactis* strain biotype that fermented D-xylose but failed to ferment mannitol and sucrose (Table 6). Based on the above reactions, L3 was the closest starter biotype to the *Lc. lactis* subsp. *lactis* LMG6890T type strain, which also is arginine-positive and ferments D-xylose, while it does not ferment mannitol and sucrose [36]. Conversely, all three arginine-positive but xylose-negative biotypes L1, L2 and L4 (Table 6) represented the dominant multi-strain *Lc. lactis* populations in the ripened Tsalafouti, grown at high levels (> 8.5 log CFU/g) on M17/22 ℃ agar on day 30 (Table 5). None of them matched the typical dairy (starter) strains of *Lc. lactis* subsp. *cremoris*, including the type strain LMG6897T, which cannot hydrolyze arginine and possess very restricted sugar fermentation profile [35,36]. Biotype L1 appeared to be the most prevalent and fermented mannitol and trehalose. Biotype L2 failed to ferment trehalose, while biotype L4 failed to ferment both sugars (Table 6). Additional genotypic studies are thus required to identify the *Lc. lactis* L1 to L4 isolates at the subspecies *lactis* or *cremoris* [34,36]. For the aims of this study, altogether the results in Tables 5 and 6 suggested that all or certain *Lc. lactis* starter biotypes autolyzed at variable times and rates during Tsalafouti ripening. Autolysis is a desired strain-dependent feature of *Lc. lactis* starters in ripened cheeses that is affected by the cheese type and pH and the ripening temperature [38], as well as, by different environmental or analytical stressful growth conditions [37]. Therefore, further studies are also required to follow and monitor the autolysis of different *Lc. lactis* starter strains during Tsalafouti fermentation and ripening at low temperatures and the associated benefits.

From the results in Table 6, we concluded that LAB species other than *Lc. lactis* did not evolve during the traditional fermentation and ripening process of semi-industrial Tsalafouti at 10 ℃, contrary to the more diversified native LAB biota of the artisanal Tsalafouti [6]. Particularly all typical thermophilic dairy LAB species, including autochthonous enterococci, were practically absent (< 100 CFU/g) throughout processing and storage (Table 5). The mean initial (day 0) level of thermophilic LAB on MRS agar at 45 ℃ was 1.33 ± 0.33 log CFU/g only and solely comprised few spontaneous *Enterococcus* contaminants, also enumerated selectively on SB agar at an initial level of 1.67 ± 0.33 log CFU/g (Table 5); i.e., the commercial starter did not contain *S. thermophilus* or

thermophilic lactobacilli (*Lb. delbrueckii*, *Lb. helveticus*). Enterococci were confirmed by streaking the MRS/45 ℃ colonies on SB and kanamycin aesculin azide (KAA) agar plates to observe their typical enterococcal colony growth [6].

The low initial (< 100 CFU/g) contamination and absence of *Enterococcus* growth (< 100 CFU/g) in semi-industrial Tsalafouti during ripening at 10 ℃ was justified. All spontaneous heat-resistant enterococci at 3–6 log CFU/ml levels in raw Greek milk [41], were inactivated during 'boiling' of the milk at 90 ℃, as previously shown by Pappa et al. [6]. Following that, a major initial post-thermal contamination of Tsalafouti milk with enterococci was prevented due to our pilot-plant hygiene. Next the high inoculation level (> 7.5 log CFU/g) and the subsequent major competitive growth of the *Lc. lactis* starter fully suppressed the ability of few *Enterococcus* contaminants for growth within the first 10 days of semi-industrial Tsalafouti fermentation at 10 ℃. Conversely, during the artisanal Tsalafouti fermentation [6], the initial (day 0) population levels of indigenous mesophilic LAB were by 3-log units lower than the starter inoculation levels (Table 5); thus spontaneous enterococci managed to increase at an approximate level of 6 log CFU/g [6]. Enterococci grow optimally at 37 ℃ and despite most of them grow well in pure culture at 10 ℃ they are normally outnumbered by mesophilic starter or wild lactococci in most traditional raw milk cheese fermentations [42]. However, enterococci survive much better than wild lactococci and leuconostoc-like bacteria in thermized (mainly) or in open-batch pasteurized ewes'/goats' cheese milks and require an early cheese milk fermentation step at optimal temperatures (37–42 ℃) to grow competitively or even predominate [41]. On the other hand, enterococci are weak acidifiers, and generally have an approximate 2-log lower prevalence than typical aciduric starter or non-starter LAB species (i.e., *S. thermophilus*, *Lc. lactis*, *Lb. plantarum*) in traditional acid-curd (pH < 4.5) Greek cheeses, like Galotyri PDO, Anevato PDO and Xinotyri [15,32,43], as well as in traditional artisan-made Tsalafouti [6].

In summary, the microbiological results (Tables 5 and 6) supported the physicochemical and sensory results (Tables 1–4) regarding the technological feasibility to produce Tsalafouti of high quality by applying the traditional manufacturing method at semi-industrial production scale, and with the aid of a commercial mesophilic starter culture consisting of *Lc. lactis* strains only. The starter promoted abundant growth at 10 ℃ (Table 5), sufficiently acidified the milk by reducing the pH at 4.2–4.3 within 10 days (Table 1), and fully suppressed growth of spontaneous bacteria and yeast/molds during ripening for 30 days and at least another 15 days of aerobic refrigerated (4 ℃) storage of ripened Tsalafouti. Compared to the artisanal Tsalafouti studied by Pappa et al. [6], the semi-industrial product was free of enterococci, staphylococci, other gram-positive spoilage bacteria and yeasts, while both products were free of coliform and other gram-negative spoilage bacteria. The sole use of mesophilic starter cultures for the production of traditional Greek acid-curd cheese types has been justified [32,44,45]. In particular, Katsiari et al. [45] compared four commercial starter cultures [two mesophilic (MA01 1 and Probat 222), one thermophilic (CH-1) and one mixed thermophilic/mesophilic (CHOOZIT MT 1)] for a similar semi-industrial production of Galotyri-type cheeses. By analyzing compositional, lipolytic and sensory characteristics, the above authors concluded that high quality Galotyri could be produced by using any of the four commercial cultures, although the plain mesophilic MA01 1 culture, consisting of *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris*, gave cheese with the most consistent flavor and overall quality during storage [45]. Pappa et al. [6] emphasized that Tsalafouti is an acid-curd dairy product which microbiologically resembles with the artisanal Galotyri PDO or other Galotyri-like acid-curd cheese varieties also traditionally made from 'boiled' sheep or goat's milk or their mixtures [15,16]. However, compared to

the semi-industrial Tsalafouti (Tables 5 and 6), the indigenous (LAB) microbiota of artisanal Tsalafouti was more diversified; this ecological difference corroborates with an overall higher proteolysis index and total volatile compound content found in artisanal Tsalafouti [6]. The technological LAB biota of artisan-type Galotyri PDO cheeses was even more diversified; it was dominated by mesophilic LAB (62.7%) and included, additionally to *Lc. lactis*, *Lb. plantarum*, *Lb. rhamnosus*, other mesophilic lactobacilli, pediococci, *Leuc. mesenteroides*, plus enterococci (19.8%), *S. thermophilus* (14.7%) and few thermophilic *Lactobacillus* (2.8%) [15]. The higher microbial (LAB) diversity and the high sensory quality of traditional Galotyri PDO and Galotyri-like cheese products was partially attributed to the fermentation of the basal 'boiled' milk at elevated temperatures (37–42 ℃) with the aid of natural thermophilic yogurt-like starters, which however are not applicable in traditional Tsalafouti production.

4. Conclusions

Artisanal Tsalafouti is produced seasonally in mountainous rural areas as a farmhouse dairy product. As there is an increasing demand for this local product, its seasonal manufacture limits its expansion to the markets. Therefore, it is essential to produce high quality Tsalafouti which is palatable and marketable to consumers the whole year. In the present study, a semi-industrial production method of Tsalafouti was investigated resulting in a product of good quality, which received high sensory scores, had no gas holes or whey-off defects and remained fresh for 45 days at 2–4 ℃. The ripened (day 30) product had pH 4.28, moisture 81.5%, fat 6%, fat-in-dry matter 32.53%, salt 0.33% and proteins 5.49%, contained high levels of heptanal, acetone, hexanal and 3-methyl butanal. Its soluble nitrogen fractions increased ($P < 0.05$) during ripening and storage. The limited biodiversity and the sensory quality of semi-industrial Tsalafouti may be enhanced by a more enriched and diverse (LAB) biota. Therefore, the application of more complex commercial starter cultures or of craft-made cultures consisting of indigenous beneficial mesophilic non-starter LAB species and strains should be tested. An alternate option may be the application of defined (commercial or natural) starter cultures along with 'back-slope' techniques for preserving Tsalafouti biodiversity. Finally, the shelf life of semi-industrial Tsalafouti could possibly be extended by storing it at 4 ℃ in vacuum to suppress the growth of aerobic spoilage yeasts and molds. The data of this study may contribute to the industrial production of Tsalafouti, giving an added value to this traditional Greek dairy product.

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Conflict of interest

All authors declare no conflicts of interest in this paper.

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