

Chemical, microbiological and sensory characteristics of ‘Tsalafouti’ traditional Greek dairy product

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Abstract

Tsalafouti is a traditional dairy product made from sheep's milk at the end of the lactation period. It is mainly manufactured as a farmhouse product, in high altitude mountainous areas of Southern Pindos. It has a high moisture content, creamy texture and mildly sour flavour. Its production process included the addition of salt to the milk, heating of milk to 90°C with continuous stirring, cooling and transfer to containers placed in caves under running water (around 10°C) and daily stirring for 10 to 20 days until thickening of the milk without the aid of starter cultures (i.e., natural acidification) occurred. The present work presents the physicochemical, microbiological and sensorial characteristics throughout the ageing of artisanal Tsalafouti. The acid-curd product had a pH of about 4.3 and an approximate moisture content of 79%, fat 10%, fat in dry matter 45-48%, salt 0.4% and protein 7%. Proteolysis increased during ripening at 10°C for 20 days and storage at 4°C for additional 70 days. Ethanol, 3-methyl-1-butanol, hexanal and heptanal were the most abundant volatile compounds. The indigenous microbiota was dominated by mesophilic lactic acid bacteria (LAB) whose numbers exceeded 8 Log CFU/g on day-10 of ripening. Enterococci also increased at an approximate level of 6.5 Log CFU/g, whereas thermophilic dairy lactobacilli were not found. Growth of aerobic spoilage yeasts and moulds was suppressed below 4.5 Log CFU/g for 45 to 60 days, but afterwards, yeasts and moulds outgrew on the product surface causing spoilage. Based on the microbiological results and primarily on sensory panel evaluations, Tsalafouti can have a shelf life of 45 days when stored aerobically under refrigeration.

1. Introduction

It is well known that Greece has many traditional dairy products which are manufactured locally, usually without any commercial ambition. These products have a strong linkage to the territory of origin and are the result of accumulated empirical knowledge passed from generation to generation. Each of their characteristics is tied to several factors, such as pedoclimatic conditions which dictate the composition of the natural pasture, the breed of the animals (usually autochthonous breeds), the use of raw milk and its natural microbiota, the manufacturing technology with the unique role of the producer and natural ageing conditions. Apart from providing cultural and social recognition, there is also an economic point of view in producing high-quality traditional products. It may give the opportunity to small size farms and rural farmers in less favoured environments, which are at the risk of disappearance, to continue to be on the market. For all those reasons, many

efforts have been made to protect traditional food products, at the European level, over the years (European Union, 2014).

Tsalafouti is a white, soft dairy product, with a creamy, paste-like texture, a smooth mouthfeel, mildly sour taste and pleasant aroma. It is similar to fresh, spreadable cheeses or yoghurt and traditionally it is made from sheep's milk at the end of the lactation period. It can be consumed fresh approximately after 10-20 days of production when its creamy texture is formed during maturation. Although it does not bear any certification or label, it is of high consumer acceptance as it is very versatile and suitable for the preparation of various dishes (e.g., sauces, desserts) and it can be consumed plain or in sweet or savoury dishes. During its traditional manufacture, there are no starter cultures or rennet used and it is left to acidify for several days, therefore, it resembles soft acid-curd cheeses. The composition of the grazing matter of the mountain Tzoumerka on the quality

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characteristics of Tsalafouti dairy product was studied previously by Koutsoukis *et al.* (2017). In the present work, traditional Tsalafouti which was manufactured using artisanal technology at the end of the lactation season, during summer, in the mountains of Tzoumerka in Northwestern Greece (Theodoriana village, height 1000 m) was studied. As Tsalafouti is gaining popularity nowadays in the local market and consumer demand is regular throughout the year, this study is necessary in order to establish its identity and enhance its production during the whole year. The composition, microbiological and biochemical characteristics were investigated in the present work. Therefore, the data of this study would be useful in order to standardize its manufacturing procedure, establish and improve its quality, and protect its identity and consumer's health.

2. Materials and methods

2.1 Artisanal production of Tsalafouti

Tsalafouti dairy product of this study was made in a farmhouse in the village of Theodoriana, Tzoumerka, following the traditional manufacturing protocol and using simple processing equipment. Briefly, raw sheep milk (15 kg) together with salt (30 g; 0.2%) was placed in a kettle and heated directly above a fireplace, under stirring at 90°C within 30 mins. At that 'boiling' temperature measured by a simple thermometer, the milk was withdrawn from the fire, transferred to another kettle and left for cooling under water. After cooling, the milk was transferred to clean plastic containers which were placed in caves under running water in nearby streams with low temperatures (approximately 10°C). The milk of the following day, treated as above, was also added to the same containers; this procedure was done up to three times. After its placement in the cave, the milk product was stirred twice daily and left to ripen for approximately 20 days, until its characteristic creamy texture occurred. On day 20, the naturally fermented and ripened acid-curd Tsalafouti product was transported to the pilot plant of the Dairy Research Department at Ioannina, placed in smaller containers (500g), and transferred to a cold room at 2-4°C for storage for up to 90 days. The above experiment was repeated twice by analyzing two samples per experimental trial. Tsalafouti samples were taken for analyses on days 0, 10, 20, 30, 45, 60 and 90 of ripening and storage.

2.2 Physicochemical analyses

The milk used for the manufacture of Tsalafouti cheese was analyzed for physicochemical parameters, i.e., fat, protein, lactose, and total solid by Milko-Scan, model 6000 (Foss Electric, Hillerød, Denmark). The pH and the content of moisture, fat, fat in dry matter (FDM),

salt and protein of Tsalafouti cheese were determined with established methods, as described by Pappa *et al.* (2006). The acidity (Dornic method; after mixing 10 g of Tsalafouti cheese with an equal mass of distilled water) is described by Ling (1963).

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

2.3 Sensory evaluation

Sensory quality was assessed by five trained panel members who were permanent staff of the Dairy Research Department and experienced tasters of dairy products. The panel was asked to evaluate the appearance, body and texture and especially the flavour of Tsalafouti and to notice any defects (such as defective colour, lack of texture uniformity, rancid or bitter flavour). For this purpose, a ten-point scale, with 1 being poor and 10 excellent, was used. The attribute of flavour was given dominating importance over the other two sensory attributes. Therefore, the score obtained for flavour was multiplied by five, for appearance by one and for texture by four (IDF,1987). The total score, for the overall acceptability, was obtained by adding the scores for the three attributes. Therefore, the excellent product received 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.* (2004).

2.5 Volatile compounds

The volatile compounds of mature Tsalafouti cheese (i.e., on day 30 of ripening and storage) were determined by Solid Phase Micro Extraction-Gas Chromatography-Mass Spectrometer (SPME-GC-MS) analysis (model GCMSQP2010, Shimadzu, Tokyo, Japan). The method used was described by Kondyli *et al.* (2016). Semi-quantification was performed by integrating the peak areas of total ion chromatograms (TIC) with the Shimadzu GCMS Solution software (Shimadzu, Tokyo, Japan).

2.6 Microbiological analyses

On each sampling day, two Tsalafouti samples (ca. 150 g each) from each product batch were aseptically poured into pre-sterilized 250 mL Duran flasks, which were placed in insulated ice boxes and transported to the microbiology laboratory of the Dairy Research Department for analyses within 1 hr after transportation. Each flask was opened near a Bunsen burner, and the cheese mass was stirred thoroughly with a sterile spoon spatula. Afterwards, 25 g of cheese was homogenized with 225 mL of 0.1% (w/v) buffered peptone water (BPW) in a stomacher (Lab Blender 400, Seward, London, UK) for 60 s at room temperature. Serial decimal dilutions in 0.1% BPW were prepared, and duplicate 1 mL or 0.1 mL samples of appropriate dilutions were poured or spread on the total or selective agar plates. All diluents, enumeration agar media and supplements were purchased from Neogen Culture Media (Heywood, Bury, UK).

Total viable bacteria (TVC) were counted on Milk Plate Count agar (MPCA) and incubated at 37°C for 48-72 hrs. Total mesophilic and thermophilic LAB populations were separated on MRS agar incubated at 30°C for 48-72 hrs and 45°C for 24-48 hrs, respectively. Mesophilic and thermophilic dairy (lactose-fermenting) LAB were enumerated on M17 agar incubated at 22°C for 48-72 hrs and 42°C for 48 hrs, respectively. Enterococci were selectively enumerated on Slanetz and Bartley (SB) agar and incubated at 37°C for 48 hrs. Based on previous acid-curd cheese studies from our laboratory (Samelis and Kakouri 2019), SB agar was preferred over the Kanamycin Aesculin Azide (KAA) agar to ensure accuracy in selective enumerations of enterococci at 37°C. We have repeatedly observed that apart from enterococci, KAA agar supports the growth of several kanamycin-resistant mesophilic lactobacilli, mainly of the *Lb. plantarum* group, and to a lesser extent the growth of several members of the *Leuconostoc mesenteroides* group (Samelis and Kakouri, 2019). All above non-enterococcal LAB species also catabolize esculin, and thus, form black (esculin-positive) colonies on KAA agar, which cannot be discriminated easily from the *Enterococcus* colonies which are black also. Conversely, enterococci are easily discriminated from mesophilic lactobacilli and other LAB on the SB agar because their colonies are red-brown and white, respectively.

Coliforms were counted by pouring 1 mL samples into melted (45 °C) Violet Red Bile (VRB) agar, overlaid with 5 mL of the same medium and incubated at 37°C for 24 hrs. Total staphylococci were enumerated on Baird-Parker (BP) agar base with egg yolk tellurite, incubated at 37°C for 48 hrs. Coagulase-positive staphylococci were selectively counted by spreading 1

mL of the first sample dilution on four plates of BP agar base with rabbit plasma fibrinogen (RFP; X086-Lab M supplement), incubated at 37°C for 18-24 hrs. Yeasts were selectively enumerated on Rose Bengal Chloramphenicol (RBC) agar (Merck, Darmstadt, Germany) plates, and incubated at 25°C for 5 days. When required, the selectivity of agar media was checked; representatives of macroscopically different colony types were confirmed at the LAB genus or phenotypic group, by rapid tests, phase contrast microscopy, gram staining by the KOH method, catalase and oxidase (Samelis and Kakouri, 2019; Samelis *et al.*, 2021).

2.7 Statistical analyses

The data were subjected to a 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 ($p < 0.05$).

3. Results and discussion

3.1 Physicochemical analyses

Because the milk used for the manufacture of Tsalafouti was collected at the end of the lactation period, i.e., by late summer, its composition was fat 8.83%, protein 7.90%, lactose 3.10%, total solids 20.50%.

Tsalafouti dairy product resembles acid curd cheeses, although it is not classified as cheese because its moisture content exceeds the 75% limit as defined by Greek legislation (Greek Codex Alimentarius, 2009).

It is known that slow quiescent acidification of milk as affected by the *in-situ* conversation of lactose to lactic acid is fundamental in the manufacture of fresh acid-curd milk products such as yoghurt and fresh cheeses. Acidification promotes two major physicochemical changes i.e., solubilization of micellar calcium phosphate and reduction of the negative charge on casein. These changes confer metastability on the casein system which through structural rearrangements reaches a new state in the form of a network (Guinee *et al.*, 1993). Tsalafouti is left to acidify for several days and its compact texture is normally induced through isoelectric precipitation of casein micelles by lowering the pH initialized by the native microorganisms of the milk and the microclimate of the environment of maturation.

The physicochemical characteristics of the Tsalafouti products of this study are shown in Table 1. The

moisture, fat, FDM, salt and protein content did not differ significantly throughout ageing, ranging from 78.97 to 79.14%, 9.75 to 10.25%, 45.09 to 48.18%, 0.34 to 0.54%, and 7.05 to 7.79%, respectively. Similar values were found by Koutsoukis *et al.* (2017) for Tsalafouti dairy products manufactured in a high altitudinal zone. The increased moisture content of Tsalafouti (78.97-79.14%) can be attributed to the fact that no drainage took place as well as to its short ripening time (20 days) under cool temperature conditions followed by refrigerated storage for 70 days. Galotyri spreadable acid-curd cheese made without rennet but with starter cultures only, had a moisture content of 76.4% because it was drained in a cloth bag during manufacture (Kondyli *et al.*, 2008). In the present work, the fat content was similar to that of Galotyri (Kondyli *et al.*, 2008); however, the protein and salt contents were lower than those found in other soft acid curd cheeses such as Galotyri, Pichtogalo Chanion or soft Xinotyri cheese (Papageorgiou *et al.*, 1998; Kondyli *et al.*, 2008; Pappa *et al.*, 2017). The differences in moisture content and in technological parameters, such as drainage and the application of increased milk fermentation temperatures during the manufacturing of the above cheeses, may explain the differences noted in Tsalafouti as regards its lower protein and salt content. Nevertheless, the less salted Tsalafouti meets the current consumers' demands regarding taste habits and health concerns.

Acidity did not differ significantly during ripening and storage (Table 1). The pH of Tsalafouti decreased ($P < 0.05$) during the first 10 days of ripening and storage

and then remained stable to 4.3; similar to other acid curd soft cheeses (Papageorgiou *et al.*, 1998; Kondyli *et al.*, 2008; Pappa *et al.*, 2017).

From Table 1 it was found that viscosity increased ($P < 0.05$) from day-10 to day-20 and then did not change ($P > 0.05$). Therefore, the characteristic texture of the Tsalafouti dairy product was obtained on day-20, which corresponded to the end of the natural ripening process in the cave environment. Tsalafouti showed lower viscosity values compared to yoghurt found by Pappa *et al.* (2018), probably because no added culture was used during its manufacture.

3.2 Proteolysis

Fractionation with water, with 12% TCA and with 5% PTA are commonly used as an index of the rate and extent of proteolysis (Christensen *et al.*, 1991).

The changes in the nitrogenous fractions at different sampling dates of traditional Tsalafouti dairy product are shown in Table 2 and to our knowledge, they are reported for the first time. The values of TN% did not change throughout ageing ($P > 0.05$) and were between 1.11-1.22%. The values of soluble nitrogen increased ($P < 0.05$) during ripening; WSN %TN increased ($P < 0.05$) from 8.37% to 12.37%, TCA content from 6.88% to 11.23% and PTA content from 3.09% to 4.7%TN, the 10th day and the 90th day, respectively (Table 2). This increase was due to the microorganisms of the milk used for its manufacture and from the environment (natural microflora of the region) since no starter culture or

Table 1. Physicochemical parameters of traditional Tsalafouti dairy product during ripening and storage

Age (days)	pH	acidity, %	moisture, %	fat, %	fat in dry matter %	salt, %	proteins, %	viscosity, cP (mPa*s) × 1000
0	6.88±0.15 ^b	---	----	---	---	---	----	---
10	4.38±0.01 ^a	1.47±0.03 ^a	78.97±0.16 ^a	10.13±0.88 ^a	48.11±3.81 ^a	0.44±0.25 ^a	7.05±0.48 ^a	12±0 ^a
20	4.27±0.07 ^a	1.51±0.07 ^a	78.5±0.45 ^a	10.0±0.0 ^a	46.54±0.98 ^a	0.49±0.1 ^a	7.79±0.13 ^a	44±2 ^b
30	4.36±0.10 ^a	1.49±0.09 ^a	78.49±0.69 ^a	10.25±0.25 ^a	47.67±0.37 ^a	0.34±0.24 ^a	7.63±0.16 ^a	46±10 ^b
45	4.28±0.01 ^a	1.52±0.08 ^a	78.38±0.47 ^a	9.75±0.15 ^a	45.09±0.19 ^a	0.34±0.05 ^a	7.31±0.10 ^a	48±8 ^b
60	4.26±0.01 ^a	1.53±0.09 ^a	78.77±0.79 ^a	9.75±0.24 ^a	48.18±0.73 ^a	0.54±0.15 ^a	7.31±0.04 ^a	48±1 ^b
90	4.23±0.10 ^a	1.57±0.04 ^a	79.14±0.35 ^a	9.75±0.25 ^a	46.76±1.97 ^a	0.43±0.02 ^a	7.09±0.39 ^a	47±1 ^b

Values are presented as mean±standard error of two manufacturing trials. Values with different superscript within the same column are statistically significant different ($P < 0.05$). ---, not measured.

Table 2. Proteolysis of traditional Tsalafouti dairy product during ripening and storage

Age (days)	TN, %	WSN %TN	TCA %TN	PTA %TN
0	---	---	---	---
10	1.11±0.08 ^a	8.37±0.44 ^a	6.88±0.46 ^a	3.09±0.58 ^a
20	1.22±0.02 ^a	8.74±0.14 ^a	7.5±1.08 ^{ab}	3.53±0.05 ^{ab}
30	1.20±0.03 ^a	10.46±0.48 ^b	9.0±0.98 ^{abc}	3.69±0.13 ^{ab}
45	1.15±0.02 ^a	10.59±0.57 ^b	10.51±0.09 ^{bc}	3.73±0.66 ^{ab}
60	1.15±0.01 ^a	10.87±0.57 ^{bc}	10.33±1.25 ^{bc}	4.45±0.1 ^b
90	1.11±0.06 ^a	12.37±0.36 ^c	11.23±1.3 ^c	4.7±0.03 ^b

Values are presented as mean±standard error of two manufacturing trials. Values with different superscript within the same column are statistically significant different ($P < 0.05$). ---, not measured, TN: Total Nitrogen, WSN: water-soluble nitrogen, PTA: nitrogen soluble in 5% phosphotungstic acid, TCA: nitrogen soluble in 12% trichloroacetic acid.

rennet was added during the preparation of Tsalafouti. An increase in the WSN values of other acid curd cheeses during storage was found (Mara and Kelly, 1998; Xanthopoulos *et al.*, 2000; Kondyli *et al.*, 2008, Pappa *et al.*, 2017).

3.3 Sensory evaluation

The organoleptic characteristics of the Tsalafouti dairy product are shown in Table 3. Statistical analyses showed that all characteristics scores remained stable during storage ($P > 0.05$) until the age of 45 days. On days 60 and 90, an unpleasant, over-acid and bitter flavour, as well as whey separation (syneresis), occurred in some samples reducing their quality. In general, panelists described Tsalafouti as thick and melting, with balanced sweet-acid, mildly sour and refreshing flavour, piquant aroma, smooth and creamy texture and they preferred it until the age of 45 days.

3.4 Microbiological analyses

The results of the microbiological analyses are shown in Table 4. At this point, it should be reported that after its transfer to the cave for ripening (day 0), the Tsalafouti milk (2-3 days old) had not curdled yet because neither it contained rennet nor the indigenous lactic acid bacteria (LAB) had grown at levels sufficient to reduce the $\text{pH} < 5.5$ to cause clotting of the milk due to lactic acid formation. The product pH was still high (6.8-6.9; Table 1) and its total viable counts (TVC) were still at an approximate mean level of 4.4 Log CFU/g only on day 0 (Table 4). That indigenous TVCs consisted of a few mesophilic LAB-like (gram-positive, catalase-negative) colonies plus numerous colonies of gram-positive, catalase-positive cocci grown as a mixed biota on M17 (mean level 3.76 Log CFU/g) and MRS (mean level 3.54 Log CFU/g) agar at 22°C and 30°C, respectively. Most catalase-positive cocci were typical staphylococci (mean level 3.64 Log CFU/g) grown selectively on Baird-Parker agar at 37°C (Table 4).

Of great importance relative to the hygienic quality and safety of the artisanal Tsalafouti was the fact that the spontaneous staphylococcal biota on day 0 included harmless, coagulase-negative *Staphylococcus* spp. This was proven because no characteristic coagulase-positive growth zones of *S. aureus* and closely-related pathogenic

Staphylococcus species were detected on the lowest dilution plates of the selective Baird-Parker+RFP agar tested in parallel to the typical Baird-Parker agar with egg yolk tellurite (data not tabulated). The elimination of RFP+ *Staphylococcus* colonies in the fresh (day-0) Tsalafouti samples was attributed to the preceding milk 'boiling' process that inactivated the coagulase-positive staphylococcal contaminants of the raw sheep milk used. In contrast, coagulase-positive staphylococci exceeded the 5-log safety threshold in fresh acid-curd Xinotyri soft cheeses made from raw goat's milk. Whereas they were suppressed (< 100 CFU/g) in all fresh Xinotyri cheeses made from pasteurized goat's milk (Pappa *et al.*, 2017), in agreement with the present findings.

Yeasts also had a remarkable presence in fresh (day 0) Tsalafouti products, whereas thermophilic LAB, enterococci, coliform bacteria and mould counts were below the corresponding lowest detection levels in Table 4. Particularly the absence of growth of typical coliform bacteria and other enterobacteria on VRB agar was a quite unexpected microbial characteristic for an artisanal cheese-making process; actually, this finding indicated that the hygienic quality of the salted Tsalafouti milk was retained overall high after its traditional 'boiling' discussed above. Based on the initial (day 0) microbial counts, it was evident that previous 'boiling' had inactivated the natural biota of raw sheep milk almost entirely. This technologically important result was confirmed by analyzing two milk samples per trial taken aseptically immediately after heating to 90°C. All had $\text{TVC} < 10$ CFU/mL (data not tabulated). Following that, excessive recontamination of the salted 'boiled' milk during subsequent cooling and handling operations before transfer for ripening in the cave was avoided, particularly with pathogenic staphylococci, coliforms and other gram-negative bacteria able to proliferate as small purple colonies on VRB agar.

After 10 days of Tsalafouti natural ripening, major (ca. 4.5 Log units; $P < 0.05$) increases of mesophilic LAB to population levels above 8 Log CFU/g occurred on M17/22°C and MRS/30°C agar plates. This dominant growth of mesophilic LAB, is also reflected as significant increases in TVCs grown on MPCA at 37°C (Table 4), indicating that the artisan Tsalafouti product underwent a fairly slow, low-temperature traditional

Table 3. Organoleptic characteristics of traditional Tsalafouti dairy product during ripening and storage

Age (days)	Appearance (10)	Texture (40)	Favour (50)	Total (100)
20	8.78±1.14 ^a	33.2±0.72 ^a	41.9±0.1 ^a	83.88±0.96 ^a
30	9.18±0.98 ^a	34.14±2.34 ^a	40.73±3.48 ^a	83.52±6.52 ^a
45	9.13±0.93 ^a	34.5±0.5 ^a	41.88±0.63 ^a	85.51±0.01 ^a

Values are presented as mean±standard error of two manufacturing trials. Values with different superscript within the same column are statistically significant different (LSD Test, $P < 0.05$). Values in brackets are the maximum scores.

Table 4. Microbial populations (Log CFU/g) of traditional Tsalamfouri dairy product during ripening and storage

Age (Days)	Total viable count (TVC) of cheese biota	Total mesophilic LAB		Total thermophilic LAB		Total mesophilic dairy LAB		Total thermophilic dairy LAB		Enterococci	Total Staphylococci	Yeast	Mould	Coliform bacteria
		LAB	LAB	LAB	LAB	dairy LAB	dairy LAB	dairy LAB	dairy LAB					
0	4.36±0.17 ^a	3.54±0.54 ^a	<2.00 ^a	3.76±0.76 ^a	<3.00 ^a	<2.00 ^a	3.64±0.12 ^c	2.54±1.54 ^a	<2.00 ^a	<1.00 ^a	3.64±0.12 ^c	2.54±1.54 ^a	<2.00 ^a	<1.00 ^a
10	7.88±0.28 ^b	8.03±0.15 ^b	5.49±0.17 ^b	8.04±0.03 ^b	6.92±0.89 ^b	6.07±0.23 ^b	2.96±0.22 ^b	3.34±1.04 ^{ab}	6.07±0.23 ^b	<1.00 ^a	2.96±0.22 ^b	3.34±1.04 ^{ab}	3.09±1.09 ^b	<1.00 ^a
20	8.09±0.09 ^{bc}	7.78±0.34 ^b	5.66±1.36 ^b	8.21±0.09 ^b	6.82±0.62 ^b	6.66±0.64 ^b	<2.00 ^a	3.53±0.23 ^{ab}	6.66±0.64 ^b	<1.00 ^a	<2.00 ^a	3.53±0.23 ^{ab}	3.42±0.16 ^b	<1.00 ^a
30	8.26±0.05 ^{bc}	8.20±0.04 ^b	5.71±1.23 ^b	8.21±0.06 ^b	6.86±0.59 ^b	6.68±0.54 ^b	<2.00 ^a	3.48±1.00 ^{ab}	6.68±0.54 ^b	<1.00 ^a	<2.00 ^a	3.48±1.00 ^{ab}	3.73±0.35 ^b	<1.00 ^a
45	8.17±0.05 ^{bc}	8.26±0.07 ^b	5.43±1.13 ^b	8.22±0.09 ^b	6.72±0.65 ^b	6.70±0.52 ^b	<2.00 ^a	3.28±0.58 ^{ab}	6.70±0.52 ^b	<1.00 ^a	<2.00 ^a	3.28±0.58 ^{ab}	4.14±0.22 ^b	<1.00 ^a
60	8.38±0.13 ^c	8.46±0.16 ^b	5.59±0.99 ^b	8.38±0.09 ^b	7.19±0.42 ^b	6.98±0.36 ^b	<2.00 ^a	4.15±0.26 ^{ab}	6.98±0.36 ^b	<1.00 ^a	<2.00 ^a	4.15±0.26 ^{ab}	4.10±0.29 ^b	<1.00 ^a
90	8.20±0.14 ^{bc}	8.20±0.16 ^b	5.94±1.68 ^b	8.32±0.15 ^b	7.03±0.37 ^b	6.84±0.43 ^b	<2.00 ^a	6.12±0.52 ^b	6.84±0.43 ^b	<1.00 ^a	<2.00 ^a	6.12±0.52 ^b	4.56 ^b	<1.00 ^a

Values are presented as mean±standard error of two manufacturing trials. Values with different superscript within the same column are statistically significant different (P<0.05).

fermentation process governed by mesophilic LAB (lactococci, leuconostocs, lactobacilli) types with optimum growth temperature around 30°C, but capable for major growth at 10-15°C. A significant part of this mesophilic LAB biota also was able to grow at levels of 7 Log CFU/g on M17 agar plates at 42°C. In parallel, although their initial (day-0) populations were below 100 CFU/g, autochthonous enterococci managed to increase at a level of 6 Log CFU/g on SB agar within the first 10 days of Tsalafouti ripening. Conversely, typical thermophilic (starter) LAB, such *Lb. delbrueckii* or *Lb. helveticus*, were not detected in any of the low-temperature fermented and ripened Tsalafouti samples. All LAB colonies that grew on MRS agar at 45°C at ca. 5.5 Log CFU/g were microscopically confirmed to be enterococci. Owing to their inherent heat resistance and broad growth temperature range (10-45°C), autochthonous dairy (lactose-fermenting) enterococci constitute a significant part of the natural LAB biota in traditional cheese fermentations. However, enterococci are neither strong acid-producers nor acid-resistant, and thus, generally, they have an approximate 2-log lower prevalence than other typical aciduric LAB species (i.e., *Streptococcus thermophilus*, *Lactococcus lactis*, *Lb. plantarum*) in traditional acid-curd (pH<4.5) Greek cheeses, like Galotyri PDO, Anevato PDO and Xinotyri (Xanthopoulos et al., 2000; Pappa et al., 2017; Samelis and Kakouri, 2019), also confirmed in Tsalafouti (Table 4).

Unlike the LAB groups, the initial (day 0) staphylococcal populations declined slightly on day 10, a result indicating failure of the acid-sensitive staphylococci to evolve under the low pH (<4.4) and low temperature (ca. 10°C) conditions prevailing in Tsalafouti by mid ripening. Also, there was no recovery/growth of coliform bacteria, while yeasts and mainly moulds increased remarkably (0.8-1.1 logs) during the first 10 days of ripening. No significant changes in the microbial populations occurred from day 10 to the end of ripening (day 20), only staphylococci declined below the detection level (100 CFU/g), while yeasts and moulds maintained an increasing tendency for growth (Table 4).

No significant changes in the predominant or subdominant LAB populations occurred during refrigerated (4°C) aerobic storage of Tsalafouti for up to 90 days. Neither staphylococcal nor coliform survivors, if any, recovered. Only yeasts and moulds increased progressively in all the acidic Tsalafouti samples, their aerobic growth at 4°C was controlled at levels below 4.5 Log CFU/g up to day-60. In general, no visible defects associated with the surface growth of spoilage yeasts and moulds were noted in Tsalafouti on days 30 and 45, while some samples started to spoil around day 60.

Afterwards, however, there was an outgrowth of surface spoilage yeasts and moulds on all cheese samples, which were terminally spoiled on day 90. Based on the microbiological results (Table 4) and primarily on the sensory evaluation data (Table 3), the shelf life of Tsalafouti was 30 days to a maximum of 45 days after manufacture, in case the product is stored aerobically at 4°C. The shelf life of Tsalafouti could possibly be extended for one or more months in case the aged RTE product on day 20 is thereafter stored at 4°C in a vacuum or other types of low-oxygen packaging conditions to suppress the growth of aerobic spoilage yeasts and moulds.

In summary, the artisanal Tsalafouti is an acid-curd dairy product made of 'boiled' milk which microbiologically resembles 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 (Samelis and Kakouri, 2019; Sameli et al., 2021; Samelis et al., 2021). Similarly, to Tsalafouti (Table 4), the technological LAB biota of ripened artisanal Galotyri PDO cheese products was dominated by mesophilic LAB (62.7% of total isolates, including the species *Lc. lactis*, *Lb. plantarum*, *Lb. rhamnosus*, other mesophilic lactobacilli, pediococci, and *Leuc. mesenteroides*), followed by enterococci (19.8%), whereas *S. thermophilus* (14.7%) and thermophilic *Lactobacillus* (2.8%) were subdominants or sporadic (Samelis and Kakouri, 2019). However, unlike fresh Galotyri and Galotyri-like cheese products frequently fermented at elevated temperatures (37-42°C) with the aid of natural thermophilic yoghurt-like starters added to the 'boiled' milk, the artisanal Tsalafouti products of this study were fermented and ripened slowly, at low (<15°C) temperature conditions without starters, according to the traditional manufacturer at Theodoriana. More advanced microbiological characterization studies are required to map the microbiota of Tsalafouti and to check whether the evolution and dominance of specific LAB species or strains during fermentation and ripening are enhanced by the possible use of any 'back-slope' techniques (Samelis et al., 2021).

3.5 Volatile compounds

In the Tsalafouti dairy products, twenty-four volatile compounds were identified, one ketone, ten alcohols, four esters, five aldehydes, two hydrocarbons and two free fatty acids (Table 5). Alcohols followed by aldehydes were the group of volatiles found in abundance in Tsalafouti dairy products (Table 5). Alcohols, together with ammonia, acids, ketoacids and carbonyls can originate from the deamination of amino acids during ripening (Fox et al., 1993). Aldehydes,

Table 5. Volatile compounds (peak area $\times 10^3$) of traditional Tsalafouti dairy product.

Volatile compound	Day-30
Ketones	
2- Nonanone	309.58 \pm 51.58
Total ketones	309.58 \pm 51.58
Alcohols	
Ethanol	102146 \pm 7875.85
Butanol - 3methyl	92197.3 \pm 3047.9
2 Heptanol	1372.65 \pm 646.65
2,3 Butanediol	911.38 \pm 161.03
Hexanol	5191.85 \pm 1357.15
Octen 3 ol	2009.85 \pm 150.95
Heptanol	2304.60 \pm 771.8
Menthol	1985.65 \pm 50.0
Octanol	1025.08 \pm 249.98
Phenylethyl alcohol	4296.3 \pm 1967.25
Total alcohols	213440.66 \pm 5158.95
Esters	
Butanoic acid ethyl ester	3599.75 \pm 1392.75
Hexanoic acid methyl ester	5138.9 \pm 1048.5
Acetic acid 2 phenylethyl ester	9130.2 \pm 1166.5
Octanoic acid ethyl ester	1350.8 \pm 463.9
Total esters	19219.7 \pm 4071.65
Aldehydes	
Hexanal	30414.5 \pm 7863.2
Heptanal	23802.5 \pm 2674.5
Octanal	3651.25 \pm 600.25
Nonanal	4323.25 \pm 303.55
Decanal	1078.45 \pm 99.95
Total aldehydes	63269.95 \pm 11542.5
Hydrocarbons	
Tetradecane	201.35 \pm 91.45
Undecane	10371.4 \pm 1435.2
Total hydrocarbons	10572.8 \pm 1343.75
Free Fatty Acid	
Hexanoic acid	2837.07 \pm 89.23
Octanoic acid	1960.85 \pm 550.35
Total FFA	4797.92 \pm 461.13
Total Volatile Compounds	311610.6\pm26353.3

Values are presented as mean \pm standard error of two manufacturing trials.

generally, are formed from amino acids or from lipid degradation and they are considered transitory compounds because they are further transformed into alcohols or acids (Molimard and Spinnler, 1996).

From Table 5, it can be seen that ethanol, 3methylbutanol, hexanal and heptanal were the most abundant compounds found in Tsalafouti dairy products. In general, these compounds were found in abundance in other acid curd soft cheeses (Kondyli *et al.*, 2013; Pappa *et al.*, 2017). Ethanol is derived from lactate via the

pentose phosphate pathway (Molimard and Spinnler, 1996) and has only a limited aromatic role despite being found in large quantities. However, it can be the precursor of several esters. 3-Methyl-1-butanol is produced from the transamination and decarboxylation of leucine (Leveau and Bouix, 1993) whereas straight-chain aldehydes, such as hexanal and heptanal are formed during β -oxidation of unsaturated fatty acids (Lee *et al.*, 1996).

4. Conclusion

In the context of preserving the heritage of artisanal Tsalafouti dairy product and protecting its characteristics, the present work was conducted to establish a general profile by studying its physicochemical and microbiological properties and investigating their changes during ageing. The obtained results could provide additional information regarding its characterization and establish a scientific basis for improvements in its quality. Achievement of this would yield not only health and technology improvements but also financial advantages as it would allow viable sale routes for small artisanal producers.

Conflict of interest

The authors declare no conflict of interest.

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