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Conventional and omics approaches shed light on Halitzia cheese, a long-forgotten white-brined cheese from Cyprus

Photis Papademas [a, *], Maria Aspri [a], Maria Mariou [a, b], Scot E. Dowd [c], Maria Kazou [b], Effie Tsakalidou [b]

[a] *Department of Agricultural Sciences, Biotechnology and Food Science, Cyprus University of Technology, Limassol, Cyprus*
[b] *Laboratory of Dairy Research, Department of Food Science and Human Nutrition, Agricultural University of Athens, Athens, Greece*
[c] *MRDNA Molecular Research, Shallowater, TX, USA*

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ABSTRACT

Production and ripening of Halitzia cheese was examined by conventional physicochemical and microbiological analyses along with state-of-the art metagenomics. Cheese was made from (A) raw goat milk without the addition of starters; (B) pasteurised goat milk without the addition of starters; (C) pasteurised milk with the addition of starters. The type and counts of microorganisms were mainly influenced by ripening time; microbial counts for lactic acid bacteria were predominant and remained stable with little or no variation throughout ripening. Coliforms and coagulase positive staphylococci declined during ripening and at the end of ripening the staphylococci were not detected. Yeasts were detected at low counts but in great diversity throughout ripening. Metagenomics analysis confirmed the results obtained by the classical microbiological analysis. The physicochemical parameters during ripening were also determined; at 60 days the pH value and moisture, fat, protein, ash, and salt contents did not significantly differ amongst cheese types.

1. Introduction

White-brined cheeses are widely produced in the Eastern Mediterranean, Northern Africa as well as the Balkans. It is a distinct category of soft to semi-hard cheeses that are usually dry-salted and then ripened submerged in varying concentrations of NaCl solutions for variable times according to the specific production protocol. This type of salting is the main difference from cheese varieties produced in Northern European countries.

Characteristic cheeses are the Protected Designation of Origin (PDO) Feta-Greece and Batzos-Greece, as well as the non-PDO Telemes-Greece, Halloumi-Cyprus, Beyaz Peynir-Turkey, Domiati-Egypt, and others maybe not so well-known, such as Urfa-Turkey and Sjenica-Serbia. It is likely that these cheeses share the same origin and have differentiated over time according to the specific cultural and climatic conditions of each country. Traditionally, they were produced from raw milk (sheep, goat or cow, or mixtures of them) at an artisanal scale, solely relying on the natural microbiota for development of flavour and texture. Because their popularity grew over time, pasteurisation and addition of commercial mesophilic (i.e., *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*) and/or thermophilic (i.e., *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*) starter cultures are now widely used to comply with strict EU food hygiene legislation as well as the need for product uniformity, as demanded by high-output dairies (Bintsis & Papademas, 2002, 2017).

Halitzia is a traditional white-brined cheese manufactured in the remote peninsula of Tilliria in Northwest Cyprus from goat milk in small quantities at farm-level. It has been reported that in the 16th century the first inhabitants of this area were soldiers of Greek descent (i.e., North Epirus or Albania), carrying with them their customs and gastronomic preferences, hence the origin for this Feta-like cheese (Nasa Patapiou, personal communication). The cheese's name is derived from its shape that reminds one of a small white stone or pebble. Halitzia is a rind-less cheese with a characteristic fresh, sour taste that is moderately salty. It is soft, slightly crumbly, with mechanical holes and it has a rather smooth texture. Recently, local interest to revive this long-forgotten cheese has occurred to boost the local economy by promoting cheese manufacture and prompted product certification and hence application

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* Corresponding author. Tel.: +35725002581.
E-mail address: photis.papademas@cut.ac.cy (P. Papademas).

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for EU quality schemes (PDO; Protected Geographical Indication, PGI; or Traditional Speciality Guaranteed, TSG). This has deemed standardisation of the cheese making procedure as well as the technological and organoleptic characterisation of the end-product imperative.

Taking the above into account, the aim of the present study was to combine conventional and omics approaches to shed light on Halitzia cheese. Halitzia cheese was manufactured at farm-level in the area of Tilliria in two separate cheese makes. Three different types of cheese were produced, i.e., raw goat milk (no starters), pasteurised goat milk (no starters) and pasteurised goat milk with added commercial starter culture, to assess the physicochemical profile, the organoleptic characteristics and the microbial ecology.

The study of the microbial ecology of fermented foods has dramatically changed during the last two decades. A major priority for food microbiologists is to develop and optimise molecular methods for the detection, reliable identification and monitoring of food-associated microorganisms. Culture-independent analyses arose to overcome the limitations of classical culture-based approaches and have been extensively used in food microbiology. Nowadays, the study of food microbial diversity can be accomplished by using high-throughput sequencing (HTS), with the most widely application in food microbiology being amplicon-based sequencing. This leads to an in-depth description of the ecosystem and helps to understand microbial dynamics and evolution during food production (De Filippis, Parente, & Ercolini, 2017; Ferrocino & Cocolin, 2017).

2. Materials and methods

2.1. Cheese production and sampling

Halitzia cheese was produced at farm-level located in Tilliria area in Cyprus. Cheeses were classified into three groups in accordance with their production process: (A) cheese made with raw goat milk and commercial rennet (CHY-MAX, Chr.Hansen, Hørsholm, Denmark), (B) cheese made with pasteurised goat milk and commercial rennet, and (C) cheese made with pasteurised goat milk with addition of a commercial rennet and a mesophilic homofermentative lactic acid culture (5 g kg^{-1} milk; R-703, Chr. Hansen). Halitzia cheese production is described in Fig. 1. Samples were taken from two batches of the same type of Halitzia cheese during ripening at 1, 7, 20, 40 and 60 days. The two batches were produced on different days using milk from the same farm. The cheeses after ripening period were transferred to the laboratory under refrigerate conditions (4 °C) and analysed immediately or frozen, depending on the analysis.

2.2. Microbiological analysis

Cheese samples (10 g) were transferred aseptically to sterile stomacher bags with 90 mL of sterile maximum recovery diluent (MRD) (Merck, Darmstadt, Germany) and homogenised in a stomacher (Lab Blender 400, Seward, London, UK) for 60 s at room temperature. Homogenate was serially diluted with MRD, and 1 mL or 0.1 mL of appropriate dilutions were poured or spread on selective agar plates. Unless otherwise stated, all media and supplements were purchased from Merck. Total viable counts (TVC) were enumerated on plate count agar (PCA), incubated at 37 °C for 72 h (ISO, 2013); total mesophilic and thermophilic lactic acid bacteria (LAB) were enumerated on de Man, Rogosa, Sharpe (MRS, Oxoid, Basingstoke, UK) agar, incubated at 22 °C for 72 h and at 42 °C for 48 h under anaerobic conditions, respectively; mesophilic cocci (presumptive lactococci) and thermophilic cocci (presumptive streptococci) were enumerated on M17 agar (Biolab, Budapest, Hungary), incubated at 30 °C for 48 h and at 42 °C for 48 h, respectively; non-starter LAB (NSLAB) were enumerated on Rogosa agar under anaerobic conditions at 37 °C for 5 days; micrococci on mannitol salt agar (MSA) at 30 °C for 48 h; enterococci on kanamycin aesculin azide (KAA) agar incubated at 37 °C for 48 h; total staphylococci on Baird–Parker (BP, Biolab) agar base supplemented with egg yolk tellurite, incubated at 37 °C for 48 h; lactose-fermenting enterobacteria (coliforms) on double-layered violet red bile agar (VRBA, Biolab) incubated at 37 °C for 24 h (ISO, 2006); yeasts and moulds on rose bengal chloramphenicol (RBC, Biolab) agar incubated at 25 °C for 5 days. Finally, the presence of *Listeria monocytogenes* in a 25 g sample was determined on Harlequin™ Listeria Chromogenic Agar (HAL, LABM Ltd., Lancashire, UK) according to ISO (2017). The same groups of microorganisms were also enumerated in raw and pasteurised milk used for cheese making. All analyses were performed in duplicate.

2.3. Metagenomics analysis

2.3.1. DNA extraction

Microbial DNA from raw goat milk and raw goat milk cheese on day 40 and day 60 (end of ripening process) was extracted according to the protocol of Pitcher, Saunders, and Owen (1989), with some modifications. In brief, 0.5 mL of milk or 0.5 g of cheese was added in an Eppendorf tube along with 1 mL water for injection and vortexed briefly. The fat layer was removed after centrifugation (10,000 ×g, 10 min, 4 °C), 1 mL of phosphate buffered saline (PBS), pH 7.4, was added and the samples were incubated at 65 °C for 10 min to decrease the content of PCR inhibitors. After centrifugation (10, 000 ×g, 10 min), 600 μL of freshly prepared lysozyme (Sigma-Aldrich Chemie Gmbh, Munich, Germany) (50 mg mL^{-1}) in Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8), 100 μL RNAse A (Sigma-Aldrich) (10 mg mL^{-1}) and 40 μL mutanolysin (Sigma-Aldrich) (5 U μL^{-1}) were added to the pellet and vortexed briefly until the pellet was completely dissolved. The suspension was incubated at 37 °C for 3 h, subsequently 20 μL proteinase K (Sigma-Aldrich) (25 mg mL^{-1}) were added and incubation at 55 °C for 1 h followed. Afterwards, cells were lysed with 0.5 mL GES reagent (5 mol L^{-1} guanidium thiocyanate, 100 mM L^{-1} EDTA and 0.5%, v/v, sarkosyl). After centrifugation (10,000 ×g, 10 min), 1 mL of the supernatant was transferred to a new Eppendorf tube and cooled on ice for 5 min. Subsequently, 250 μL cold ammonium acetate (7.5 mol L^{-1}) were added, the tube was held on ice for 10 min and then 0.5 mL chloroform was added. The resulting phases were mixed thoroughly, the sample was centrifuged (10,000 ×g, 10 min) and 1 mL of the supernatant was transferred to a new Eppendorf tube, where phenol and chloroform, at a ratio sample:phenol:chloroform 1:0.5:0.5, were added. The resulting phases were mixed, the sample was centrifuged (10,000 ×g, 10 min) and 900 μL of the supernatant were transferred to a new Eppendorf tube. A second round of phenol-chloroform wash was performed, 800 μL of the supernatant were transferred to a new Eppendorf tube and an equal volume of chloroform was added. After centrifugation (10,000 ×g, 10 min), 700 μL of the supernatant were transferred to a new Eppendorf tube and an equal volume of chloroform was added. After centrifugation (10,000 ×g, 10 min), 600 μL of the supernatant were transferred to a new Eppendorf tube and 0.1 volumes of sodium acetate (pH 5.2) and 0.54 volumes of cold isopropanol were added and the tube was kept overnight at −20 °C. The next day, the fibrous DNA was pelleted by centrifugation (10,000 ×g, 20 min, 4 °C), the supernatant was discarded and the pellet was washed twice with 700 μL cold 70% (v/v) ethanol. Ethanol was removed completely from the pellet and the tubes remained at 37 °C for 5–10 min with open lid for evaporation of the remaining ethanol. Finally, the pellet was resuspended in a

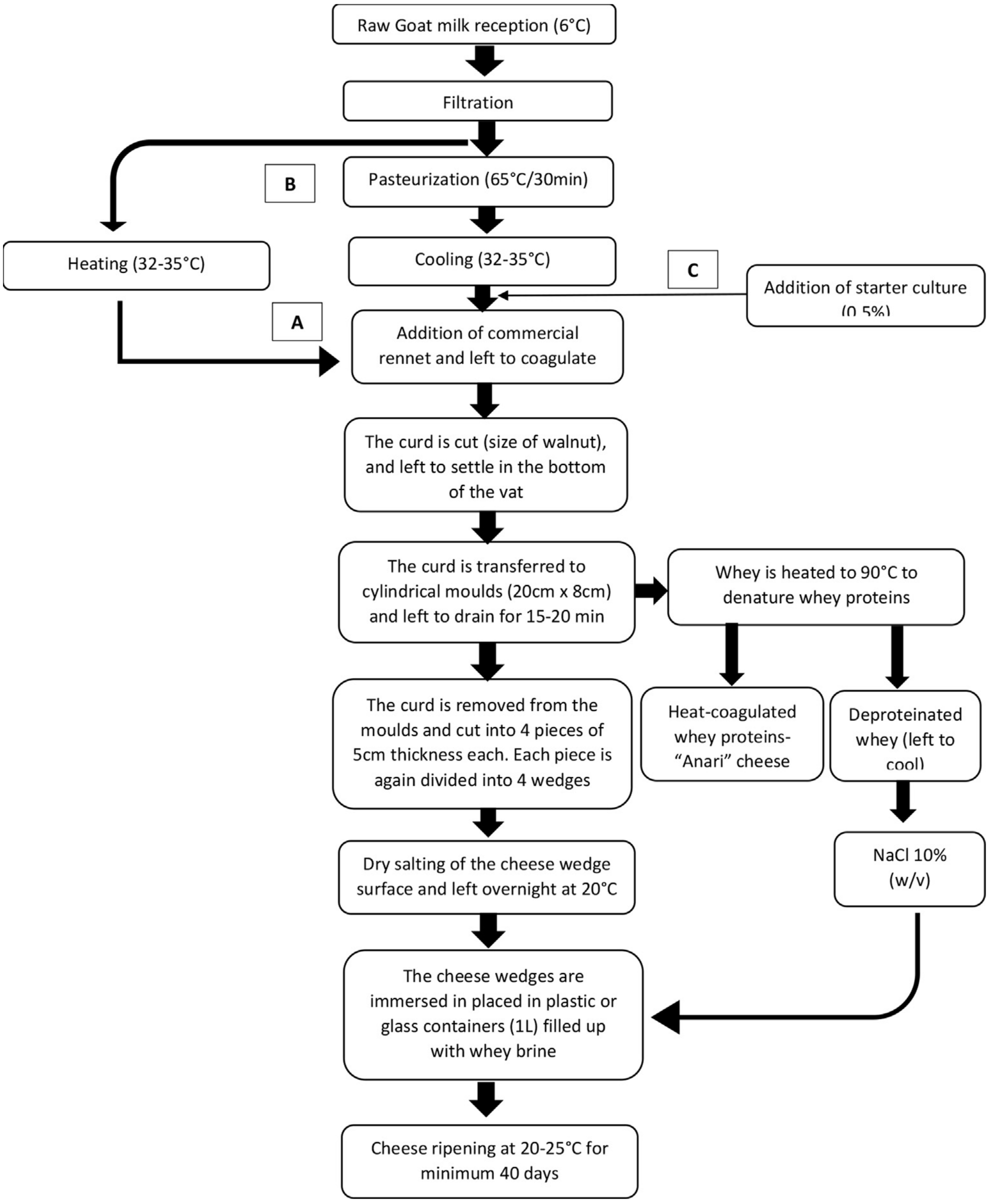

Fig. 1. Flow diagram for the production of Halitzia cheese. Cheese made with raw milk with commercial rennet (A), Cheese made with pasteurised milk with commercial rennet (B) and Cheese made with pasteurised milk and addition of commercial rennet and a mesophilic homofermentative lactic acid culture (C).

small volume of TE buffer (pH 8.0; 30–50 μL) and stored at −20 °C until use. DNA concentration was measured using a Quawell Q5000 Read First photometer (Quawell Technology Inc, San Jose, CA,USA), and DNA quality was determined in a 1% agarose gel 1 × Tris-acetate-EDTA (TAE) (1 × 40 mM Tris-acetate, 1 mM EDTA, pH 8.2), which was stained with ethidium bromide 10 mg mL^{-1} (Sigma-Aldrich).

2.3.2. *Sequencing*

Amplicon sequencing (bTEFAP®) was performed at Molecular Research (MR DNA, Shallowater, Texas, USA) and used for bacterial and fungal analysis. Primers 27F (5′-AGR GTT TGA TCM TGG CTC AG-3′) and 519R (5′-GTN TTA CNG CGG CKG CTG-3′), as well as ITS1F (5′-CTT GGT CAT TTA GAG GAA GTA A-3′) and ITS2R (5′-GCT GCG TTC TTC ATC GAT GC-3′), were used to evaluate the bacterial and fungal diversity, respectively. A single-step 30-cycle polymerase chain reaction (PCR) with the HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA, USA) was employed under the following conditions: 94 °C for 3 min, followed by 30 cycles at 94 °C for 30 s, 53 °C for 40 s, and 72 °C for 1 min. Amplification followed by a final elongation step at 72 °C for 5 min. Following PCR, all amplicon products from different samples were mixed in equal concentrations and purified using Agencourt Ampure Beads (Agencourt Bioscience Corporation, MA, USA). Samples were sequenced on the Illumina MiSeq with methods based upon the bTEFAP®. Sequence data deriving from the sequencing were processed using a standard analysis pipeline (MR DNA). Paired sequences were merged and depleted of barcodes and primers, then short sequences (<200 bp), sequences with ambiguous base calls, as well as sequences with homopolymer runs exceeding 6 bp, were removed. Sequences were then denoised and chimeras were removed. Operational taxonomic units (OTUs) were defined after removal of singleton sequences, clustering at 3% divergence (97% similarity). Final OTUs were taxonomically classified using the Nucleotide Basic Local Alignment

Search Tool (BLASTn) against a curated National Centre for Biotechnology Information (NCBI) deriving database (Dowd et al., 2008). Normalised and de-noised files were then rarefied and run through the Quantitative Insights into Microbial Ecology 2 (QIIME 2) pipeline for alpha- and beta-diversity analyses (Bolyen et al., 2018). Additional statistical analysis was performed using XLSTAT (Addinsoft, NY, USA) and NCSS (NCSS, UT, USA), and finally Chao1 and Shannon indices were used as diversity and richness measures to assess changes in microbiota composition. Raw sequencing data are deposited at the European Nucleotide Archive (ENA) under the study ID PRJEB31234.

2.4. Physicochemical analysis

Cheeses were analysed following the International Dairy Federation (IDF) and ISO Standards for moisture content (IDF, 2012), fat content (ISO, 2004), protein content (ISO, 2014), and ash content (IDF, 1964). Salt content was determined by the Volhard method (AOAC, 2012b). The pH was determined in a 20 g sample weighed in a beaker and suspended in 20 mL of distilled water previously heated at 40 °C. The mixture was homogenised by means of a laboratory peristaltic blender (Masticator, IUL, Barcelona, Spain) for 60 s, and the pH of the slurry was measured at room temperature using a digital pH-metre (pH 211, Hanna Instruments, Padova, Italy). Titratable acidity was determined by titration using lactic acid (expressed as g lactic acid per 100 g cheese) (AOAC, 2012a). All the analyses were performed in triplicate samples. Goat milk composition was determined by the Lactostar Dairy Analyser (Funke Gerber, Berlin, Germany).

2.5. Organoleptic evaluation

Cheese samples, cut in small cubes of ~2 cm side, were organoleptically assessed at 40 and 60 days by a panel, familiar with the product, consisting of 20 students and staff (men and women, 20–40 y) of the Department of Agricultural Sciences, Biotechnology and Food Science. After a brief training session, where terminology was discussed, the panel was asked to evaluate the appearance (exterior, interior), texture (body), flavour (odour, taste and aftertaste) and overall impression using a structured hedonic scale of nine points (1 = I disliked it very much, 5 = I neither liked it nor disliked it, 9 = I liked it a lot). Samples during evaluation were at ambient temperature (18 ± 2 °C).

2.6. Statistical analysis

Chemical and microbiological data obtained were subjected to analysis of variance (ANOVA) and where statistical differences were noted, differences among the distinct groups were determined by the Duncan's test. Differences were considered significant at $P < 0.05$. Statistical procedures were performed with the software package SPSS version 15.0 for Windows (SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1. Microbiological analysis

Results of the microbiological analysis of milk and cheeses are presented in Fig. 2. The aerobic mesophilic flora counts, obtained on PCA, for the raw goat milk used for cheese production in the present study, were high. The dominant populations were mesophilic cocci with 8.2 log cfu mL^{-1}, mesophilic lactobacilli with 7.8 log cfu mL^{-1}and staphylococci with 7.9 log cfu mL^{-1}. Coliforms and the foodborne pathogen *L. monocytogenes* were not detected in raw milk. The pasteurisation process eliminated the heat-sensitive raw milk flora, such as *Staphylococcus aureus* and, to a lesser extent, LAB. According to the Regulation 853/2004 (European Commission, 2004), the total microbial count for raw sheep and goat milk intended for production of heat-treated drinking milk or for the manufacture of heat-treated milk-based products should not exceed 1,500,000 cfu mL^{-1}, while for manufacture of products made from raw milk, whose manufacturing process does not involve any heat treatment, the microbial count limit is 500,000 cfu mL^{-1}.

Regarding cheeses, results showed that cheese made with raw milk (cheese A) had higher microbial population compared with the cheeses made from pasteurised milk (cheeses B and C). The high total bacteria counts on day 1, 7.8 (cheese C), 9.7 (cheese B) and 9.8 log cfu g^{-1} (cheese A) were rather stable until day 20, and subsequently started to drop, which is consistent with previous results for goat milk cheeses (Guizani, Al-Attabi, Kasapis, & Gaafar, 2006).

As expected, LAB comprised the main microbiota during ripening of Halitzia cheese. Mesophilic cocci (presumptive lactococci) counts on day 1 were high, 8.8 (cheese A), 9.4 (cheese B) and 7.9 log cfu g^{-1} (cheese C), but decreased until the end of ripening (day 60), reaching 5.3 (cheese B), 5.8 (cheese C) and 6.4 log cfu g^{-1} (cheese A), most probably due to competition with other microbial groups, but played a significant role in the early stages of cheese production (Manolopoulou et al., 2003; Psoni, Kotzamanidis, Yiangou, Tzanetakis, & Litopoulou-Tzanetaki, 2007; Quigley et al., 2011). The counts of thermophilic cocci on day 1 were high for all cheeses and at 7.0–8.0 log cfu g^{-1} and dropped to 5.0–6.0 log cfu g^{-1} at 60 days of ripening following very similar trends in all samples.

Mesophilic lactobacilli counts were higher than thermophilic lactobacilli throughout the ripening period for all cheeses, with 8.0 log cfu g^{-1} for all cheeses on day 1, compared with thermophilic lactobacilli counts of 7.8, 7.5, and 6.0 log cfu g^{-1} for cheese A, B and C, respectively. Counts of both groups slightly decreased during ripening, reaching at the end of ripening 6.0 (cheese A), 5.5 (cheese B) and 6.9 log cfu g^{-1} (cheese C) for mesophilic lactobacilli, and 4.2 (cheese A), 3.7 (cheese B) and 4.0 log cfu g^{-1} (cheese C) for thermophilic lactobacilli. The slow metabolism of lactobacilli and their capacity to better adapt to adverse conditions (acidity, low a_w, and high salt concentrations) than other LAB, facilitated their predominance in the last stages of ripening (Arenas, González, Bernardo, Fresno, & Tornadijo, 2004). Moreover, through their proteolytic and lipolytic activities, which can increase the concentration of small peptides, free amino acids and free fatty acids, they play an important role during cheese ripening (Bouton, Buchin, Duboz, Pochet, & Beuvier, 2009; Kongo, Gomes, Malcata, & McSweeney, 2009; Mannu, Comunian, & Francesca Scintu, 2000).

NSLAB were comprised of mesophilic lactobacilli and pediococci, which are an important part of the microbiota of most varieties of ripening cheeses (Beresford, Fitzsimons, Brennan, & Cogan, 2001). In all three groups of cheeses, the NSLAB population on day 1 was high at 8.5 (cheese B) to 8.7 log cfu g^{-1} (cheeses A and C). After a slight decrease on day 40, counts finally reached 7.7 (cheeses A and B), and 7.8 log cfu g^{-1} (cheese C) on day 60.

On day 1, enterococci were enumerated at 5.7 (cheese C), 7.3 (cheese B) and 7.5 log cfu g^{-1} (cheese A). After a slight increase on day 7, counts in all three cheeses started declining until day 60, reaching 2.8 (cheese C), 3.2 (cheese B) and 4.0 log cfu g^{-1} (cheese A), results that are in accordance with those found in other cheese varieties manufactured from raw or pasteurised milk (Manolopoulou et al., 2003). Enterococci can survive during ripening due of their tolerance to high salt concentrations, acidic conditions, high temperatures and low moisture environments (Fuka, Maksimovic, Tanuwidjaja, Hulak, & Schloter, 2017). Despite

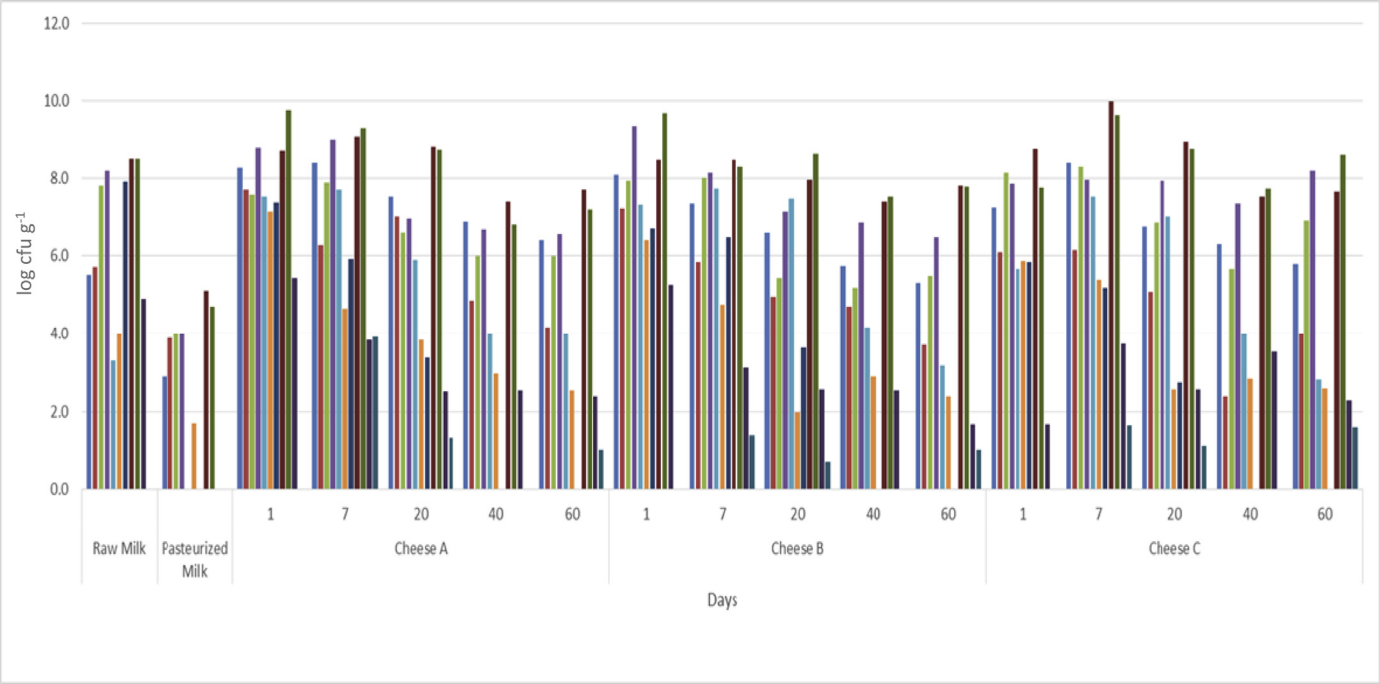

Fig. 2. Microbial population during cheese ripening: Cheese A, made with raw milk with commercial rennet; Cheese B, made with pasteurised milk with commercial rennet; Cheese C, made with pasteurised milk and addition of commercial rennet and mesophilic homofermentative lactic acid culture. Left to right for each set of data: 🟦, thermophilic cocci; 🟧, thermophilic lactobacilli; 🟩, mesophilic lactobacilli; 🟪, mesophilic cocci; 🟦, enterococci; 🟨, micrococci; ⬛, staphylococci; 🟫, non-starter lactic acid bacteria; 🟩, total plate count; 🟪, yeasts; 🟦, coliforms.

various concerns about enterococci safety, they are considered to play an important role in the ripening by shaping the sensorial profile of many cheeses (Foulquié Moreno, Sarantinopoulos, Tsakalidou & De Vuyst 2006).

Micrococci counts on day 1 were rather high, 5.9 (cheese C), 6.4 (cheese B) and 7.1 log cfu g^{-1} (cheese A), but significantly decreased by day 60, 2.4 (cheese B), 2.5 (cheese A) and 2.6 log cfu g^{-1} (cheese C), which is in agreement with other studies (Manolopoulou et al., 2003; Sarantinopoulos, Kalantzopoulos, & Tsakalidou, 2002). Micrococci are considered major components of the raw milk cheeses microbiota, occurring, also, in significant numbers in cheeses made from pasteurised milk as well (Manolopoulou et al., 2003; Sarantinopoulos et al., 2002). They survive throughout ripening, probably due to their resistance to salt and low a_w, and have a significant impact on the sensorial properties of cheese, due to their proteolytic and lipolytic activities (Bintsis & Papademas, 2002).

No coliforms were detected in either raw or pasteurised milk used. However, they appeared in all three cheeses on day 7, in cheese B 1.4, in cheese C 2.0 and in cheese A 3.9 log cfu g^{-1}, but decreased during ripening, reaching on day 60, counts of 1.0 (cheeses A and B) and 2.0 log cfu g^{-1} (cheese C). Coliforms are indicators of poor hygiene and possible faecal or environmental contamination during manufacturing, but pH drop during ripening results in their decrease/elimination (Alichanidis & Polychroniadou, 2008; Manolopoulou et al., 2003).

Yeast population was 1.7 in cheese C, 5.2 in cheese B, and 5.4 log cfu g^{-1} in cheese A, after the first day of ripening. During ripening, the population in cheeses A and B decreased, reaching on day 60 2.4 and 1.7 log cfu g^{-1}, respectively. On the other hand, in cheese C, yeast population initially increased up to day 7, but by day 60 declined to 2.6 log cfu g^{-1}. Relatively high counts of yeasts are frequently observed in many different types of cheese, especially in raw milk cheeses (Manolopoulou et al., 2003). Their occurrence is mainly due to their tolerance to low pH, reduced a_W and high salt concentrations (Nyberg, 2016). Yeasts can positively contribute to cheese ripening due to their proteolytic and lipolytic activities. However, they may also act as spoilage organisms causing defects, such as fruity, bitter or yeasty off-flavours and the appearance of a gassy, open texture, brown surface discolouration, and even increased acidity due to stimulant effects on LAB (Gardini et al., 2006; Pereira-Dias, Potes, Marinho, Malfeito-Ferreira, & Loureiro, 2000; Pintado et al., 2008).

The highest levels of staphylococci were noticed in all cheeses at the beginning of the ripening process, with populations of 7.4 (cheese A), 6.7 (cheese B) and 5.8 log cfu g^{-1} (cheese C). Like coliforms, staphylococci levels were drastically affected by the ripening time, and, after 40 days, no staphylococci were detected in any of the cheeses. Similar trends were also found in other studies on goat milk cheeses (Alonso-Calleja, Carballo, Capita, Bernardo, & García-López, 2002; Cabezas, Sánchez, Poveda, Seseña, & Palop, 2007; Psoni et al., 2007). Among staphylococci, the presence of *Staphylococus aureus* in food is of public health concern, due to the ability of some strains to produce heat-resistant enterotoxins, which can accumulate and cause food intoxication (Le Loir, Baron, & Gautier, 2003).

L. monocytogenes is considered to be a widespread environmental contaminant detected in cheese plants (Fox, Hunt, O'Brien, & Jordan, 2011). In our study, *L. monocytogenes* was used as an indicator of food safety according to EC Regulation 2073/2005 (European Commission, 2005), and it was not detected in any cheese sample throughout ripening.

3.2. *Metagenomics analysis*

Metagenomics analysis was performed only on cheese A, which was prepared from raw goat milk without the addition of starters. Analysis was performed on raw milk and cheese after 40 and 60 days of ripening. A total of 570,173 bacterial raw sequences were obtained from the three samples. After data quality screening, 361,157 sequences (an average of 47,272 sequences per sample) were used for metagenomic analysis. A total of 714 bacterial OTUs were assigned among samples, with an average of 521 OTUs per sample (Table 1). On the other hand, the number of fungal raw sequences obtained from the three samples was higher, i.e. 632,250. However, the 569,903 sequences (an average of 46,043 sequences per sample) used for metagenomic analysis after quality filtering, led to a total of 471 fungal OTUs in the three samples, with an average of 299 OTUs per sample (Table 1), i.e., far less than for bacteria. The rarefaction analysis for both 16S and ITS data, assigned to 97% of OTUs similarity, showed that the Shannon-Wiener Index curve plots reached a plateau at approximately 2000 sequences, indicating that sequencing depth was sufficient (Fig. 3).

According to the alpha-diversity metrics for the 16S data, it is evident that the goat milk is less diverse compared with cheese samples on days 40 and 60. However, alpha-diversity metrics for the ITS data revealed that diversities for all samples were relatively similar. However, it should be noted that although a decrease in ITS alpha-diversity was observed from raw milk to cheese on day 40, a subsequent increased diversity was found in cheese on day 60, higher than that observed in raw milk (Fig. 3). Furthermore, beta-diversity analysis based on principal coordinates analysis (PCoA) of the 16S data revealed a clear clustering of the two cheese samples, which were separated from the raw milk sample. In contrast, PCoA of the ITS data did not reveal a clear phylogenetic clustering among the three samples, indicating that the three fungal communities were quite diverse (Fig. 4).

A change in bacterial and fungal communities was observed throughout the ripening process of Halitzia cheese. Regarding bacteria, Streptococcaceae, Leuconostocaceae and Enterobacteriaceae families were predominant in all samples, reaching approximately 70% of the bacterial sequences (Fig. 5A; Supplementary material Table S1). This was in agreement with classical microbiological analysis, since mesophilic and thermophilic cocci as well as mesophilic lactobacilli and NSLAB were found in significant populations both in raw milk and in cheese on days 40 and 60 (Fig. 2). On the other hand, although Enterobacteriaceae was detected in all three samples in relatively high abundances (Supplementary material Table S1), coliforms counts were only obtained in cheese on day 60 (Fig. 2). Furthermore, 16S metagenomics analysis revealed a major difference between the raw milk sample and the two cheese samples regarding the abundance of Pseudomonadaceae and Lactobacillaceae families. Although Pseudomonadaceae was found among the dominant families (23.7%) in the milk sample, the abundance was sharply decreased in the cheese samples on days 40 and 60 (0.2% for both cheese samples). The opposite occurred with the Lactobacillaceae family, with only 0.5% abundance in the milk sample, which increased during cheese ripening reaching up to 25% on day 60.

At the genus level, *Lactococcus*, *Lactobacillus*, *Leuconostoc* and *Pseudomonas* dominated all samples with a total of 47 genera identified (Fig. 5B; Supplementary material Table S2). This finding confirmed the results of the microbiological analysis, since lactococci and lactobacilli were found in higher counts compared with the other microbial groups (Fig. 2). Moreover, both classical microbiological and 16S metagenomics analyses fingerprinted staphylococci the same way, since they were only detected in raw milk (Fig. 2; Supplementary material Table S2). In addition, the absence of *Listeria* in raw milk and cheese samples was confirmed by both classical microbiological and metagenomics analyses. It was interesting that even though micrococci were counted in all three samples, neither *Micrococcus* genus nor Micrococcaceae family were detected through the 16S metagenomics analysis. This could be probably due to the limited selectivity of the culture medium used for micrococci enumeration. Furthermore, according to the Venn diagram constructed on the basis of the 16S metagenomics results, only three genera were unique in raw milk, namely *Burkholderia*, *Janthinobacterium* and *Staphylococcus*, four in cheese on day 40, namely *Acidomonas*, *Hyphomicrobiumand*, *Cethylobacterium* and *Curvibacterone* and only one in cheese on day 60, namely *Alkalibacterium*, all below 0.01% (Fig. 6). Among the core bacterial communities, all samples shared 28 genera, while, as it was expected, the two cheese samples shared the majority of identified genera in similar abundances (Supplementary material Table S2).

Concerning the fungal families, Saccharomycetaceae, Saccharomycodaceae and Debaryomycetaceae were the dominant ones among the 70 families identified in all samples (Fig. 7A; Supplementary material Table S3). Saccharomycetaceae was found in similar abundance in all three samples ranging from 33 to 49%. Saccharomycodaceae was found in raw milk at relatively high abundance (25.5%), which gradually decreased in cheese on day 40 (9.4%) and 60 (6.6%). The same occurred with other families, such as Helotiales, Plectosphaerellaceae, Microbotryomycetes and Pleosporaceae. Interestingly, Debaryomycetaceae which was identified in low abundances in raw milk (3.6%), sharply increased in cheese on day 40 (39.7%) and then decreased in cheese on day 60 (10.5%). Moreover, Pichiaceae was detected in abundance below 1.5% in raw milk and cheese on day 40, while in cheese on day 60 reached up to 12%.

At genus level, *Kluyveromyces* (45.2%) and *Debaryomyces* (25.7%) were predominant in milk, *Verticillium* (39.4%), *Hanseniaspora* (17.4%) and *Kluyveromyces* (15.8%) in cheese on day 40, and *Kluyveromyces* (34.9%), *Hanseniaspora* (16.5%) and *Cadophora* (12.7%) in cheese on day 60 (Fig. 7B; Supplementary material Table S4). Overall, the fungal communities in the three samples were more diverse compared with the bacterial ones, which is also shown in the fungal Venn diagram (Fig. 8). From a total of 87 fungal genera identified, approximately 34.5% were shared among the three samples, compared with the 55.3% of the bacterial core genera. It is also interesting to note that the two cheese samples shared only 30 genera, while this number was higher between raw milk and cheese on day 40 (37 core genera) and milk and cheese on day 60 (38 core genera). Furthermore, raw milk had the majority of unique genera, i.e., 28, followed by cheese on day 60 (8 genera) and day 40 (5 genera). According to the results of the microbiological analysis, fungal counts in raw milk were higher (4.9 log cfu g^{-1}) compared with fungal counts in cheese on days 40 and 60 (both

Table 1
Bacterial and fungal number of sequences and operational taxonomic units (OTUs) assigned after filtering.

Sample	Bacteria		Fungi	
	Number of sequences	Number of OTUs	Number of sequences	Number of OTUs
Raw milk	58,271	475	81,674	340
Raw milk cheese at 40 days	43,801	550	35,244	258
Raw milk cheese at 60 days	39,744	539	21,213	298

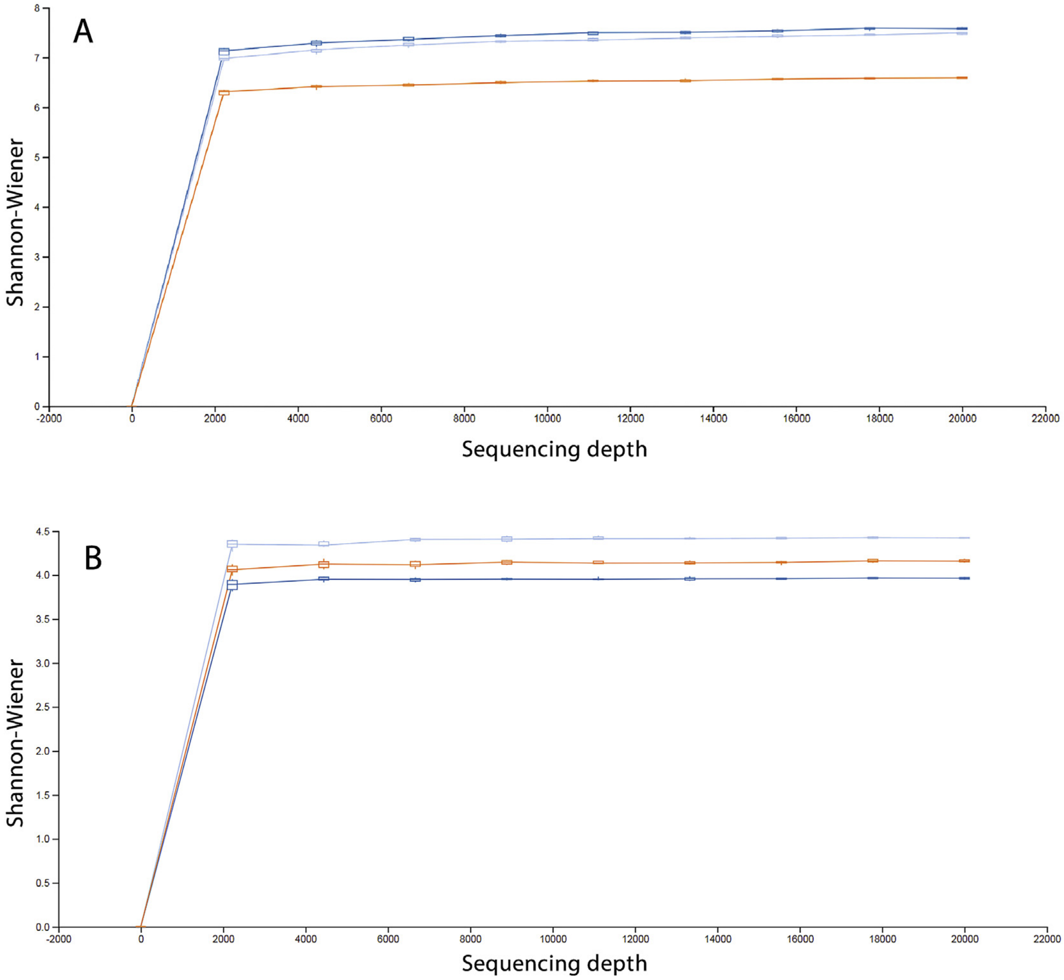

Fig. 3. Shannon-Wiener curves of bacterial (A) and fungal (B) communities in raw milk (orange line), and raw milk cheese at 40 and 60 days (dark and light blue lines, respectively). Rarefaction curves were calculated based upon 97% similarity. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

approximately 2.5 log cfu g^{-1}). However, since colonies were counted in RBC medium, no correlation between classical microbiological and metagenomics analyses is feasible.

Among fermented foods and beverages, dairy products, especially cheeses, are the ones, which have been explored the most using amplicon-based HTS (De Filippis et al., 2017). However, amplicon-based metagenomics studies of fungal communities are scarce compared with bacteria, despite the fact that fungi are also important during cheese ripening. The two main drawbacks are (i) the uneven ITS length among fungal species that may lead to an incorrect estimation of OTUs abundance and (ii) the significant part of deposited ITS sequences that are not updated or curated (De Filippis et al., 2017). Nevertheless, despite these limitations, the ITS region is generally accepted as the official fungal DNA barcode marker. In our study, both 16S and ITS amplicons were analysed with QIIME 2 using a high quality filtering so as to minimise the impact of sequencing errors, to achieve a reliable identification of bacterial and fungal populations.

Interestingly, the majority of amplicon-based metagenomics studies refer to cow milk cheeses, although there are plenty of cheeses made from sheep and/or goat milk. Among the few studies on goat milk cheeses, Quigley et al. (2012) analysed bacterial populations in 62 Irish artisanal cheeses prepared from unpasteurised or pasteurised cow, goat, or sheep milk using a 454 genome sequencer FLX platform (Quigley et al., 2012). According to the authors, milk origin and pasteurisation could influence the level of bacterial diversity. Based on their results, cow milk cheese appeared to be more diverse compared with goat and sheep milk cheeses, containing 21, eight and two bacterial genera, respectively (Quigley et al., 2012). Furthermore, Zhang et al. (2017) investigated bacterial diversity in raw milk of two goat breeds from the Guanzhong area of China, using an Illumina HiSeq2500 PE250 platform. The results of the 16S metagenomics analysis revealed that in both breeds, the main bacterial genera in raw milk were *Enterobacter*, *Acinetobacter*, *Pseudomonas*, *Staphylococcus* and *Stenotrophomonas*, all present in the raw goat milk we investigated as well. To the best of our knowledge, our analysis is the first report on both bacterial and fungal communities in a goat milk cheese, starting from the raw milk, until the final cheese product at the end of the ripening process.

3.3. *Physicochemical analysis*

The heat treatment of milk for cheese production is not only an effective way of preventing harmful effects of microorganisms, but it also causes changes in the physicochemical properties of milk components. At the same time, the changes in the physicochemical parameters occurring during cheese preparation and ripening are

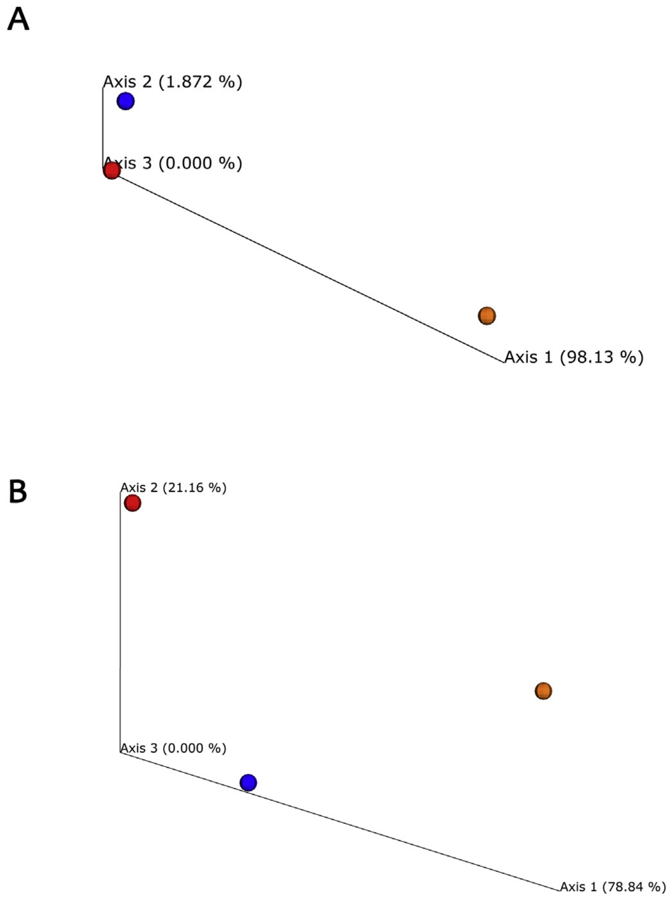

Fig. 4. Principal coordinate plot of weighted Unifrac 16S (A) and ITS (B) data: raw milk (orange circle), and raw milk cheese at 40 and 60 days (red and blue circles, respectively). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

crucial, as they will determine the structure, texture, flavour and aroma of cheese. The mean composition of the goat milk used for the manufacture of Halitzia cheese was: fat 4.49% ± 0.01, protein 3.93% ± 0.01, solids non-fat (SNF) 10.42% ± 0.02, and its pH was 6.7 ± 0.0.

Results of the physicochemical analysis of the three cheeses (A, B and C) during ripening are shown in Table 2. By the end of the ripening period the composition of cheeses manufactured using different treatments were quite similar.

On day 1, no significant differences were found in pH values among the different manufacturing processes, i.e., 5.7 in cheese C, 5.9 in cheese A and 6.0 in cheese B. On day 7, there was a pH decrease by more than one unit in all cheeses (Table 2). Afterwards, pH values for all cheeses remained stable without any major differences among the three cheeses until the end of ripening (day 60), where pH values reached 3.8 (cheese A), 3.6 (cheese B) and 3.7 (cheese C). These pH values are lower than those found in Feta cheese (4.25–4.50) (Sarantinopoulos et al., 2002), but maybe the fact that Halitzia cheese is ripened in a nutrient denser medium (i.e., whey-brine) rather than in brine as is Feta, could account for the higher metabolic activity and a greater pH-drop.

The average value of titratable acidity, expressed as percentage of lactic acid was the same (0.1%, w/w) for all cheeses. As expected, titratable acidity increased over ripening in all three cheeses until day 40 and then remained stable until the end of ripening, reaching 0.41, 0.44 and 0.42% for cheeses A, B and C, respectively.

On day 1, the fat content did not differ significantly among cheeses (cheese A: 14%, cheese B: 15%, cheese C: 16%). Fat percentage increased until day 20 for all cheeses and then remained stable until the end of ripening except for cheese A, where fat content was significantly lower (18%) than in cheeses B and C.

Protein content did not considerably differ among cheeses throughout the ripening period. During the first seven days of ripening, there was a small increase of protein content in all cheeses. Afterwards, the percentage of protein gradually declined reaching by day 60 values of 17.9, 14.5 and 16.4%, for

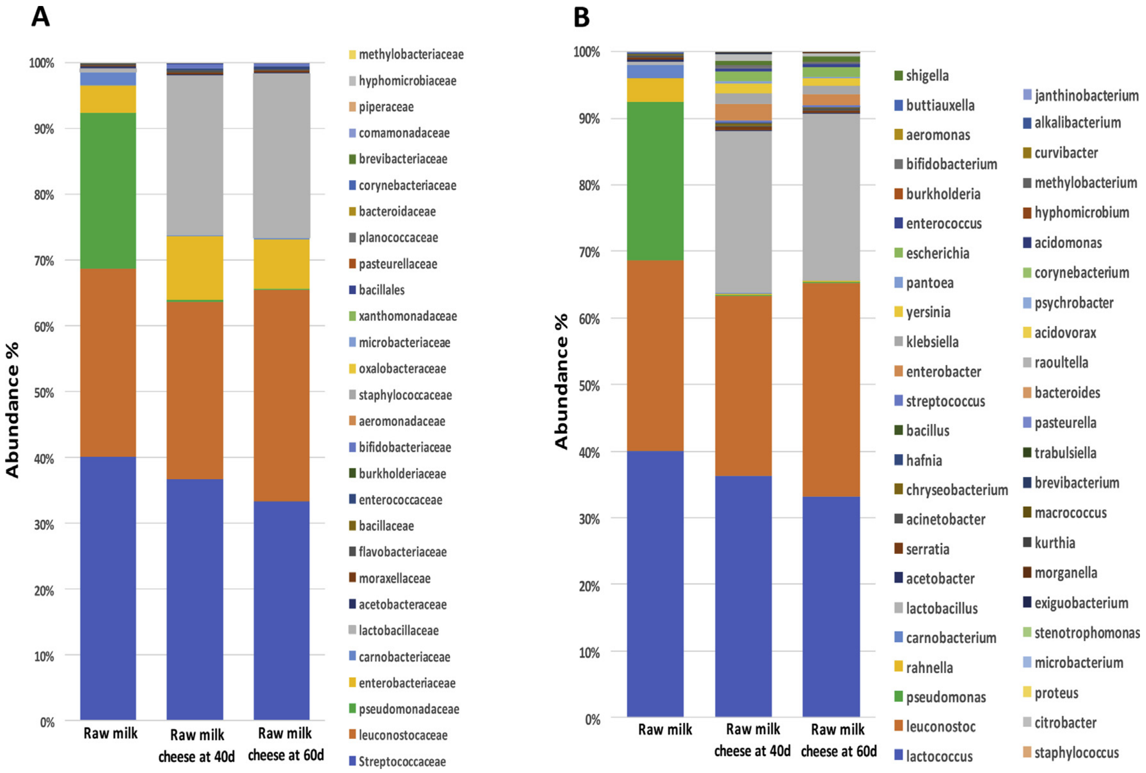

Fig. 5. Relative abundance (%) of bacterial families (A) and genera (B) obtained by 16S metagenomics analysis.

Fig. 6. Venn diagram showing the number of unique and shared bacterial genera in raw milk and raw milk cheese at 40 and 60 days.

cheeses A, B and C, respectively. The increase in the protein content at the beginning of the ripening was mainly due to loss of moisture.

Moisture content of cheeses steadily decreased until day 20 and then started to increase again until the end of ripening, which is typical for white brined cheeses (Barać et al., 2013; Tzanetakis, Vafopoulou-Mastrojiannaki, & Litopoulou-Tzanetaki, 1995), reaching values of 63.8, 57.5 and 54.5%, for cheeses A, B and C, respectively. This increase could be a result of proteolysis that helps to soften the cheese during ripening due to hydrolysis of peptide bonds and the release of ionic groups in the cheese, which then bind water from the whey, thereby increasing moisture content of the cheese (Fathollahi, Hesari, Azadmard, & Oustan, 2010). The effect of proteolysis on the cheese's moisture content could also explain the differences observed between cheeses A, B and C during maturation (Table 2). Cheese A is made with raw milk, hence a higher proteolysis index is expected (unpublished data) due to the diverse microflora present, when compared with cheeses B (pasteurised milk) and C (pasteurised milk and starter culture). Moreover, the fact that the salt content in cheese A remains practically constant after day 20 could also explain that concomitant changes occurring in cheese's moisture are not attributed to the migration of salt or water during brining.

Ash content of all cheeses showed a significant increase during ripening. On day 1, the ash percentages in cheeses A, B and C were 2.87, 2.77 and 2.85% respectively, and at the end of ripening reached 3.78, 3.58 and 3.87%, respectively.

The salt content on day 1 was 2.35, 2.75 and 2.94% for cheeses A, B and C, respectively. The salt content was fluctuating throughout ripening for all cheeses and at the end of ripening, was 3.12, 2.92 and 2.82% for cheeses A, B and C, respectively. Salting is an

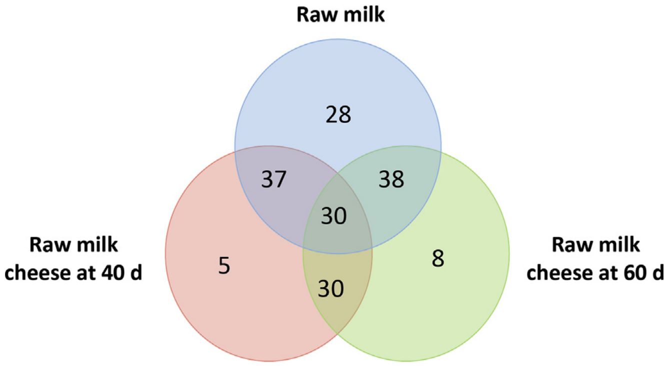

Fig. 8. Venn diagram showing the number of unique and shared fungal genera in raw milk and raw milk cheese at 40 and 60 days.

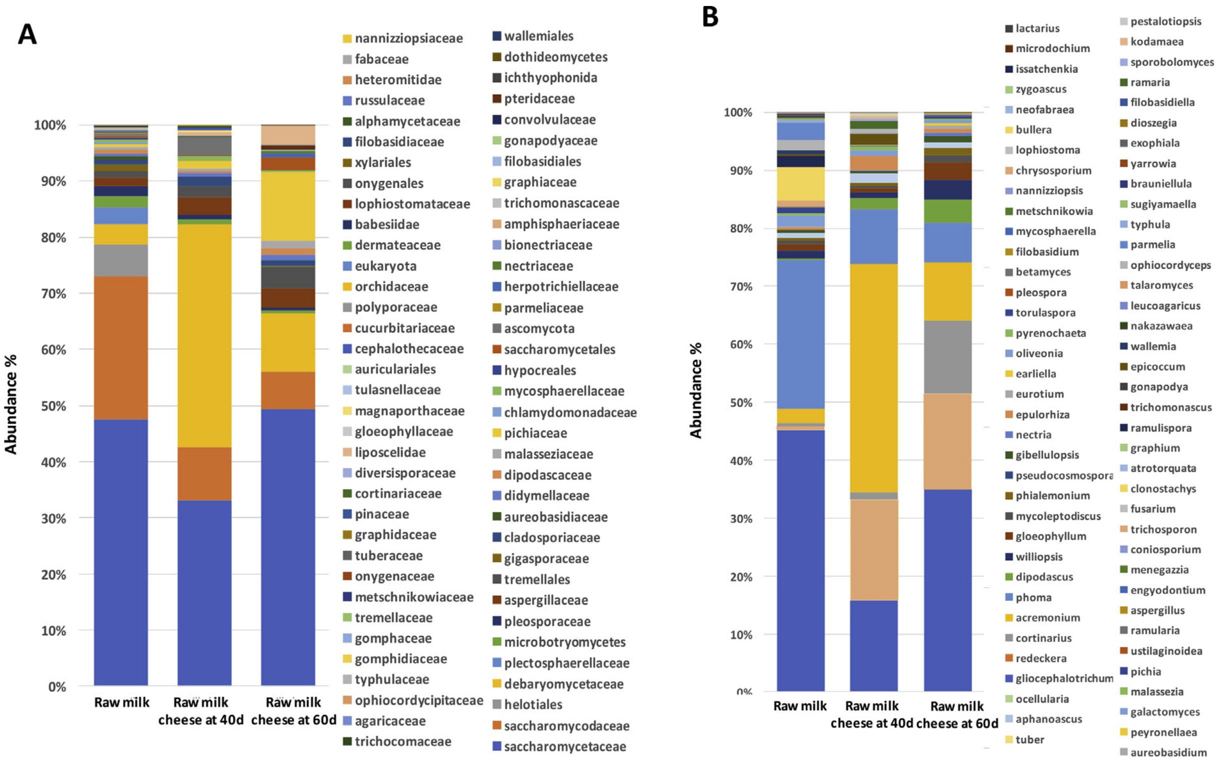

Fig. 7. Relative abundance (%) of fungal families (A) and genera (B) obtained by ITS metagenomics analysis.

Table 2
Physicochemical analysis of Halitzia cheese during ripening.[a]

Day	pH	Acidity	Moisture (%)	Fat (%)	Salt (%)	Protein (%)	Ash (%)	FDM	MFFS
Cheese A									
1	5.9 ± 0.03	0.1 ± 0.01	63.0 ± 0.50	14.0 ± 0.88	2.5 ± 0.26	15.8 ± 0.32	2.9 ± 0.03	37.8	73.3
7	4.4 ± 0.01	0.4 ± 0.02	53.8 ± 3.50	18.0 ± 1.77	3.2 ± 0.06	21.1 ± 0.80	4.3 ± 0.43	39.0	65.6
20	3.8 ± 0.04	0.4 ± 0.02	50.3 ± 2.40	23.0 ± 0.00	3.0 ± 0.16	18.9 ± 0.40	4.4 ± 0.13	46.3	65.3
40	3.9 ± 0.03	0.4 ± 0.02	57.5 ± 0.70	20.0 ± 0.71	3.0 ± 0.05	18.1 ± 0.40	3.4 ± 0.54	47.1	71.9
60	3.8 ± 0.02	0.45 ± 0.00	63.8 ± 1.20	18.0 ± 0.88	3.1 ± 0.06	17.9 ± 0.60	3.8 ± 0.23	49.7	77.8
Cheese B									
1	6.0 ± 0.11	0.1 ± 0.01	64.8 ± 0.70	15.0 ± 0.7	2.8 ± 0.03	13.3 ± 0.41	2.8 ± 0.26	42.6	76.2
7	4.1 ± 0.09	0.3 ± 0.07	54.2 ± 2.60	23.0 ± 0.53	3.1 ± 0.19	16.4 ± 0.60	4.0 ± 0.28	50.2	70.4
20	3.7 ± 0.08	0.4 ± 0.06	56.0 ± 0.90	23.0 ± 0.35	3.0 ± 0.13	15.1 ± 0.30	3.8 ± 0.15	52.3	72.7
40	3.7 ± 0.06	0.4 ± 0.00	58.7 ± 0.50	24.0 ± 0.18	2.6 ± 0.11	14.8 ± 0.40	3.8 ± 0.14	58.1	77.2
60	3.6 ± 0.08	0.4 ± 0.03	57.5 ± 2.10	24.0 ± 0.53	2.9 ± 0.02	14.5 ± 0.30	3.6 ± 0.24	56.5	75.7
Cheese C									
1	5.7 ± 0.96	0.12 ± 0.11	62.7 ± 0.00	16.0 ± 0.35	2.9 ± 0.03	14.1 ± 0.80	2.9 ± 0.01	42.9	74.6
7	3.9 ± 0.01	0.3 ± 0.01	53.5 ± 1.60	23.0 ± 0.71	3.0 ± 0.01	18.5 ± 0.10	3.8 ± 0.05	49.5	69.5
20	3.7 ± 0.01	0.3 ± 0.07	53.2 ± 1.20	24.0 ± 0.00	2.8 ± 0.09	16.9 ± 0.40	3.9 ± 0.63	51.3	70.0
40	3.7 ± 0.00	0.4 ± 0.01	53.8 ± 0.20	24.0 ± 0.71	2.6 ± 0.01	16.7 ± 0.40	3.3 ± 0.03	51.9	70.8
60	3.7 ± 0.01	0.4 ± 0.02	54.5 ± 0.70	24.0 ± 0.53	2.8 ± 0.18	16.4 ± 0.60	3.9 ± 0.42	52.7	71.7

[a] Cheese A, made with raw milk with commercial rennet; Cheese B, made with pasteurised milk with commercial rennet; Cheese C, made with pasteurised milk and addition of commercial rennet and mesophilic homofermentative lactic acid culture. FDM, fat in dry matter; MFFS, moisture in fat free solids.

important step in cheese making, since salt has a major effect on the control of microbial growth by acting as a preservative, promotes removal of whey from the cheese matrix, and contributes to the development of the characteristic cheese flavour, aroma and texture, through the control of biochemical pathways, such as proteolysis and lipolysis. The percentage of salt in the final products is consistent with that of Cypriot Halloumi cheese (Papademas & Robinson, 1998).

3.4. *Organoleptic evaluation*

The panel's scores for the different cheese samples on days 40 and 60 of ripening are presented in Table 3. Development of the characteristic appearance, taste, flavour and texture of each cheese results from a series of complicated biochemical transformations. One of the most important processes occurring during ripening of white-brined cheeses is proteolysis, which can affect the structure and flavour of the final product.

Cheese C, prepared with pasteurised goat milk with the addition of starters, obtained significantly higher scores in terms of appearance with values of 7.3, compared with the other two cheeses. Cheese C was characterised by intense white colour and few or no holes. Cheese A, prepared from raw goat milk without the addition of starters, received higher score for texture on day 40 compared with the other two cheeses. However, on day 60, the texture of the cheeses B (prepared with pasteurised goat milk without the addition of starters) and C seemed to be preferred by the panellists compared with cheese A. In terms of flavour, aroma and odour, cheese A received the highest score in both days 40 and 60 followed by cheese C and cheese B. This is probably due to the characteristic intense odour of raw goat milk (farm flavour). Finally, regarding the taste and aftertaste on day 40, cheese C had the highest average score, i.e., 6.7 and 6.2, respectively. Second in the consumers' preference, in terms of taste and aftertaste was cheese B with an average score of 6.69 and 6.56, followed by cheese A with

Table 3
Organoleptic evaluation of Halitzia cheese.[a]

Parameter	Cheese type	Ripening time	
		40 days	60 days
Appearance	A	6.8 ± 0.1^{Aa}	7.0 ± 0.1^{Aa}
	B	6.4 ± 0.3^{Aa}	7.2 ± 0.2^{Ba}
	C	$7.3b \pm 0.3^{Aa}$	7.3 ± 0.2^{Aa}
Texture	A	6.7 ± 0.3^{Aa}	6.7 ± 0.2^{Aa}
	B	6.4 ± 0.2^{Aa}	7.0 ± 0.1^{Ba}
	C	6.6 ± 0.2^{Aa}	6.9 ± 0.2^{Aa}
Flavour	A	6.8 ± 0.1^{Aa}	7.1 ± 0.2^{Aa}
	B	6.3 ± 0.2^{Aa}	6.5 ± 0.2^{Ab}
	C	5.9 ± 0.3^{Ab}	6.8 ± 0.1^{Ba}
Taste	A	4.7 ± 0.2^{Ab}	6.7 ± 0.3^{Ba}
	B	6.9 ± 0.2^{Aa}	6.8 ± 0.1^{Aa}
	C	6.8 ± 0.1^{Aa}	6.7 ± 0.2^{Aa}
After-taste	A	4.6 ± 0.2^{Ab}	6.7 ± 0.4^{Ba}
	B	6.8 ± 0.1^{Aa}	6.1 ± 0.2^{Ba}
	C	6.6 ± 0.3^{Aa}	6.2 ± 0.2^{Aa}

[a] Cheese A, made with raw milk with commercial rennet; Cheese B, made with pasteurised milk with commercial rennet; Cheese C, made with pasteurised milk and addition of commercial rennet and mesophilic homofermentative lactic acid culture. Means in the same column followed by different lowercase superscript letters and same row followed by different superscript uppercase letters are significantly different ($P < 0.05$).

Table 4
Quality parameters for Halitzia cheese.

Parameter	Specification
Geographical production area	Tilliria, Cyprus
Raw material	Pasteurised Goat Milk
Coagulant	Commercial rennet (fermentation produced chymosin)
Type	White-brined cheese
Fat in dry matter	Min. 46%
Moisture	Max. 60%
Salt	2.8–3.0%
pH	Max. 4.4
Colour	White
Taste	Fresh, sour/lemony taste, medium salty
Texture	Rindless, soft, crumbly cheese with small mechanical and bacterial holes
Shape	Small “pebbles”
Maturation	40 days at 25 °C
Packaging	Plastic or glass containers (1–4 L) in whey brine or in vacuum packed in individual pieces (100–200 g)

an average of 5.19 and 5.69, respectively. On day 60, cheese A was first followed by cheeses C and B. The taste and flavour of cheese is related to the action of LAB, as well as to the natural microbiota. LAB produce acetic acid, diacetyl, free fatty acids and peptides through their proteolytic, lipolytic or glycolytic activities that can affect the development of flavour, aroma and texture of cheeses during the ripening period (Nyberg, 2016). Overall acceptability and the wiliness to buy the product was equal between cheese A and C with an average percentage of 36.4% followed by cheese B with an average percentage of 27.3%.

4. Conclusions

Halitzia is a white-brined cheese, traditionally manufactured from raw goat milk. Overall, the microbiological, biochemical and sensory results of the present study will help to establish the specifications and the cheese-making technology for the production of Halitzia cheese. Results of both classical microbiological analysis and metagenomics analysis showed that Halitzia cheese produced with raw milk is as safe as the product from pasteurised milk. However, to (a) standardise production practises (see Table 4) and (b) adhere to strict hygiene rules as imposed by the local veterinary services, it is recommended to use pasteurised milk. Raw milk cheese was equally attractive to consumers as the cheese produced with pasteurised milk and the addition of cultures compared with the cheese produced only from pasteurised milk. Finally, 40 days of cheese ripening was adequate for the cheese to develop the expected sensorial characteristics.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2019.06.010.

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