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Research Article

Changes in physicochemical, microbiological and fatty acid composition during the ripening period of artisan sheep cheese from Bulgaria

Stanko Stankov[1]✉*, Hafize Fidan[1], Ivayla Dincheva[2], Aleksandar Balabanov[3], Ivanka Petrova[1]

[1] *Department of Tourism and Gastronomy Management, Faculty of Economics, University of Food Technologies, Plovdiv, Bulgaria*
[2] *Agrobioinstitute, Agricultural Academy, University of Chemical Technology and Metallurgy, Sofia, Bulgaria*
[3] *Department of Milk and milk products, Technological Faculty, University of Food Technologies, Plovdiv, Bulgaria*

Abstract
The composition of artisan sheep's milk cheese during the ripening period was studied. The raw material used for cheese production, was obtained from an indigenous Karakachan sheep from Bulgaria. It was found that the main physicochemical parameters of the cheese have changed during the study period. During the ripening process, chaaracteristics such as dry matter, total protein and fat content increased by approximately 7.0%, 3.0% and 4.0% respectively, while the water content of the samples decreased by up to 7.0%. A smooth acidification process was observed, which correlated with the high number of lactic acid bacteria. The fatty acid analysis showed that the saturated fatty acid composition was 66.0% and unsaturated fatty acids were 34.0%. During the biochemical ripening at the 60th day of the test period, the presence of linoleic polyunsaturated fatty acid was found to be 0.12%.

Keywords: artisanal cheese, traditional sheep's cheese, fatty acids, cheese quality

Abbreviations
MUFAs – monounsaturated fatty acids; PUFAs – polyunsaturated fatty acids; SFAs–saturated fatty acids; UFAs – unsaturated fatty acids.

✉*Corresponding author: Stanko Stankov, Department of Tourism and Culinary Management, Faculty of Economics, University of Food Technologies, 26 Maritza Blvd, 4002 Plovdiv, Bulgaria, tel.: +359 32 603 784; E-mail: docstankov@gmail.com*

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Introduction

Traditional Bulgarian cheeses are products obtained from processing of raw milk from sheep, goats, and cattle. The uniqueness of these products is due to the conventional processing methods, the environment's climatic conditions, and the milk microflora's activity (Beev et al. 2019; Enikova 2010). Typical Bulgarian dairy products such as ayran, "tarator," "prokish," and buttermilk give seasonality and additional traditional identification to local population's eating habits. Each of the food products mentioned above is part of the daily diet of Bulgarians and is characterized by a specific nutritional and biological composition. The milk fat in the composition of sheep's milk has a pronounced positive effect on human health. However, it contains more significant amounts of saturated fatty acids than cow's milk (Palombo et al. 2020).

The physical and chemical parameters of artisan sheep's cheese changed during the ripening period. The increase in the dry matter (between 7 to 11%) could be due to diffusion processes during ripening (Mezo-Solís et al. 2020). The increasing trends of the protein content in the period up to 60th day of ripening were reported between 5 and 15%, as the reason for the change could be the ripening conditions and the composition of the sheep's milk used (Dimitrova et al. 2021) for fermenation. The formation of free fatty acids, as well as the presence of fatty acid esters (Dimitrova et al. 2021), aldehydes, ketones, and sulfur-containing compounds in the composition of artisanal cheese was mainly determined by lipolysis processes (Palombo et al. 2020). Earlier studies (Calvo et al. 2004; Beev et al. 2019) reported the presence of oleic fatty acid (C18:1) ranged from 30-35 mg/100g, palmitic (C16: 0) fatty acid in the range of 22-25 mg/100g and lauric fatty acid (5-12 mg/100g).

Special attention has been paid to the conjugated fatty acids in the composition of dairy products, a geometric isomer of linoleic fatty acid. Conjugated fatty acids (CFAs) are a group of positional and geometric isomers of polyunsaturated fatty acids (PUFAs) with conjugated double bonds. There are several subgroups of CFAs.

Conjugated linoleic acids (CLAs) are polyunsaturated fatty acids that are found naturally in ruminant food products. There are at least 28 known isomers of CLA, defined by two conjugated double bonds in different geometric (i.e., cis or trans) and positional locations. According to Gortzi et al. (2022) and Kawęcka and Pasternak (2022), the participation of CLAs in the composition of lactic acid products from sheep's milk may classify these food products with antioxidant, anticarcinogenic effects, and immunomodulatory properties.

Changes in the chemical composition of artisan sheep's milk cheeses are related to the differences in the composition of the raw milk used, (Enikova 2010) the fermentation and climatic conditions (Kalit et al. 2009; Mezo-Solís et al. 2020), and the technological features for cheese production in different regions (Enikova 2010). The use of rennet enzymes extracted from the rumen of mammals in particular geographical areas forms a typical aroma-flavor profile of sheep's cheese (Beev et al., 2019). In some earlier studies, representatives of pathogenic microflora were identified (Mezo-Solís et al. 2020; Atanasova et al. 2020). According to the reported results, some of the physical and chemical parameters of the obtained product were defined as uncharacteristic (Trmčić et al. 2016; Jackson et al. 2012).

The presence of representatives of different bacterial strains, which get into the products mainly from the external environment, form the typical taste characteristics. Thus, in order to ensure the quality and safety of artisanal products, several studies on their microbiological status have been carried out. However, using unpasteurized milk from small ruminants carries the risk of contamination from the udder. In addition, some researchers (Mezo-Solís et al. 2020; Atanasova et al. 2020; Studenica et al. 2022) studied the potential risks during artisanal cheese production especially those ripened in lamb skin or under unfavorable climatic conditions.

Due to the increased interest in traditional dairy products obtained from autochthonous breeds of sheep in Bulgaria, the aim of the present study was to focus on determining the physicochemical, microbiological and fatty acid composition of artisan sheep cheese during the ripening period.

Materials and Methods

Materials. The study is part of previous analyses of traditional artisanal products from Bulgaria (Stankov et al. 2022). In the present study, the fatty acid composition of the cheese was determined on the 60th day of its ripening. Traditional technology using unpasteurized sheep's milk was used for cheese production. Milk was obtained by manual milking of 50 ewes of autochthonous Karakachan breed in area of the Topolchane village (East Stara Planina) in April 2021. Coagulation of milk was done by using rennet obtained from the foreskin of a mammalian lamb, pre-treated with sea salt. After coagulation and separation of the whey, the cheese was salted by dry salting and stored in polyethylene packages with additional wet salting with salt solution (8.0%). Ripening was occurred at an ambient temperature of 12-15°C for period of 60 days.

Methods. All the analyses and measurements were done in triplicate. The physicochemical analysis was carried out during 1st, 30th and 60th day of the ripening period. The dry matter of artisanal cheese was determined according to ISO 5534:2004. The amount of sodium chloride was obtained according to ISO 5943:2006. The amount of fat in the cheese composition was determined according to ISO 3433:2008. Acidity was determined potentiometrically using a pH meter and by Thorner`s method (BNS 1111-80). The content of total protein, nitrogen, and its fractional amounts were determined according to Vakaleris and Price (1959), with the adaptation of product characteristics (Ivanova et al. 2020).

The microbiological analysis was carried out during the ripening period.

Detection of total Lactobacilli (ISO 7889:2003) – 90 cm^3 of sterile diluent (buffered peptone water, saline, or sterile distilled water) was added to 10 g of the sample, followed by two more successive dilutions. In two sterile Petri dishes, 1 cm^3 of the final dilution was transferred, in each of the dishes separately. The Petri dishes were then filled with 15 cm^3 of MRS nutrient medium (pH 5.7), melted, and cooled to 45°C. The cultivation was carried out anaerobically at 37°C for 3 days.

Detection of total Lactococci (ISO 4831:2006) – 90 cm^3 of sterile diluent (buffered peptone water, saline, or sterile distilled water) was added to 10 g of the sample, followed by two further successive dilutions. In two sterile Petri dishes, 1 cm^3 of the final dilution was transferred, in each of the dishes separately. Then, petri dishes were filled with 15 cm^3 of melted and cooled to 45°C nutrient medium M 17 and incubated at 28°C for 2 days.

Detection of psychrophilic microorganisms (ISO 17410:2019) – 90 cm^3 of sterile diluent (buffered peptone water, saline, or sterile distilled water was added to 10g of the sample, followed by two further successive dilutions. In two sterile Petri dishes, 1 cm3 of the final dilution was transferred, in each of the dishes separately. Then, the Petri dishes were filled with 15 cm^3 of melted and cooled to 45°C standard plate count agar medium (Oxoid CM 463, Basingstoke, UK). The cultivation was carried out aerobically at 6.5°C for 10d.

The determination of presence of molds and yeasts (BS ISO 6611:2006) – 90 cm^3 of sterile diluent (buffered peptone water, saline, or sterile distilled water) was added to 10 g of the sample, followed by two more successive dilutions. In two sterile Petri dishes, 1 cm^3 of the final dilution was transferred, in each of the dishes separately. Then, the Petri dishes were filled with 15 cm^3 of CGY nutrient medium, melted, and cooled to 45°C. The samples were incubated at 25°C for five days.

Detection of Coliforms (ISO 4831:2006) - 90cm^3 of sterile diluent (buffered peptone water, saline, or sterile distilled water) was added to.10 g of the sample, after which another tenfold dilution was made. Both dilutions were plated in sterile petri dishes and inoculated with 15 cm^3 of cooled 47°C VRBL medium. The Petri dishes were placed in a thermostat and incubated at 30 or 37°C for 24 ± 2h.

Detection of *L. monocytogenes* (BS EN ISO 11290-1:2017) - *Listeria monocytogenes* was detected on a highly selective medium after two pre-enrichments.

For primary enrichment, 25 g of the analysed sample was added to 225 cm^3 of a selective enrichment nutrient medium with a reduced concentration of inhibitors - ½ Fraser's broth. The cultures were incubated at 30°C for 25±1h. The pre-enrichment flask was homogenized, and 0.1 cm^3 was inoculated into 10 cm^3 of Fraser broth. The studied cultures were cultivated at 37°C for 24±2h. The primary and secondary enriched cultures were

carried out on the surface of two different selective media - ALOA-agar and PALCAM-agar. Inoculated Petri dishes were incubated at 35°C or 37°C for 24 to 48h.

Staphylococcus aureus was detected according to BSS EN ISO 6888-1:1999/A2:2018. 90 cm^3 of sterile diluent (buffered peptone water, saline, or sterile distilled water) was added to 10 g of the sample. From this initial dilution, 0.1cm^3 was transferred onto the Baird-Parker selective agar medium.. The samples were incubated at 35±1°C or 37±1°C for 24 to 48h.

Salmonella spp. (BS EN ISO 6579-1:2017/A1: 2020) - the detection of *Salmonella* ssp was performed on selective media after two pre-enrichments. For primary enrichment, 25 g of the test sample was inoculated into 225 cm^3 of a non-selective nutrient medium (buffered peptone water). The sample was incubated at 34 to 38°C for 18±2h. From the primary enriched sample, 0.1 and 1cm^3 were placed respectively in a 10cm^3 RVS-broth and a 10 cm^3 MKTTn-broth tube. The samples than were enriched in - RVS-broth and MSRV-agar and incubated at 41.5±1°C for 24±3h. Cultures were examined for colonies with typical *Salmonella* culture characteristics, and biochemical and serological confirmation was carried out when such were detected.

Extraction of Lipids. Methanol (500µl) was added to 100.0 mg of lyophilized and powdered sample. The following internal standards were used: 50 µl ribitol and 50 µl nonadecanoic acid (each in 1.0 mg/ml concentration); then, vortex was used for 10s and incubated for 30min/70°C/300rpm; the mixture was cooled to room temperature and 300 µl distilled water + 500 µl chloroform were added. After that, the mixture was vortexed again for 10s, and put to centrifuge (10 min/22°C/13000 rpm).

Transmethylation. The resulting lower phase (apolar phase – 300 µl) was evaporated to dryness under vacuum. To the dry residue 1.0 ml (1M) solution of sulfuric acid in methanol was added, followed by incubation at 96°C for 90min. The cooled solution was extracted with hexane (3x500ml). The combined organic layers were evaporated to dryness under vacuum, and 50µl BSTFA (silating reagent) and 50 µl pyridine were added followed by incubation at 70°C for 30 min.

Chromatographic conditions for determination of fatty acids. For the determination of the fatty acids, 1.0 µl of the sample was used, which was injected into a system consisting of an Agilent GC 7890A gas chromatograph and an Agilent MD 5975C mass spectral detector. An HP-5MS column with parameters: length of 30m, diameter 0.32mm, and film coating thickness of 0.25 µm was used under the following conditions for the determination of fatty acids: initial temperature was 70°C, then hold 1min, and increase to 300°C at 5°C/min, hold 10min; injector and detector temperatures were 250°C; carrier gas was helium with a flow rate of 1ml/min; mass detector scan range was m/z=50-550; injected sample volume was 1 µl in 20:1 flow split mode (Radkova et al. 2019).

Statistical analysis. The statistical analysis was performed with Microsoft Excel 2010, and the results are presented as mean value ± SD.

Results and Discussion

The physicochemical parameters of the raw material for the production of artisanal cheese were determined in previous studies (Stankov et al. 2022). Dry matter (21.36±0.22%), solid-not-fat (SNF) (12.76±0.03%), fats (8.61±0.01%), protein content (6.61±0.07%), carbohydrates (4.66 ±0.07%), salt content (1.25±0.15%), density (1.033g/cm^3); titratable acidity (20.02±0.03°T); pH (6.82±0.33) were determined.

The cheese ripening process was completed and several changes in its physicochemical and biochemical properties were observed. Also, development of a characteristic flavor, aroma and texture was determined. The results on the physicochemical parameters of the artisanal sheep's cheese obtained by traditional technology from unpasteurized milk are presented in Table 1. It was observed that the main phisicochemical parameters of artisanal sheep`s cheese has been changed as some of them increase and others decreased. As a result of the increase in the dry matter during ripening, the water content values decreased. The level of proteins in the initial ripening stage was 11.08%, and during ripening, reaching the 60th day, it increased by more than 2.0%. Other authors have also reported similar processes (Dimitrova et al. 2021; Mezo-Solís et al. 2020).

Table 1. Physicochemical parameters of artisan sheep's cheese

Parameters	Ripening period		
	1 day	30 day	60 day
Dry matter, %	41.94±1.05	43.51±1.00	48,73±1.01
Total protein, %	11.08±0.12	11.76±1.24	13.51±1.55
Total nitrogen, %	21.55±0.62	22.79±1.20	26.04±1.87
Water content,%	58.06±1.07	56.79±1.25	51.27±1.51
Sodium chloride, %	3.47±0.29	3.55±0.08	3.74±0.03
Total fat content, %	26.00±1.88	27.00±1.35	30.50±1.26
Fat from dry matter, %	61.99±1.04	62.05±1.05	62.33±0.02
Titratable acidity,°T	147.00±1.98	244.00±1.50	222.00±1.25
pH values	5.29±0.55	4.61±0.65	4.74±0.09

According to them, the diffusion processes taking place in the curd directly depend on the formation of organic acids. In the initial stages of ripening, the amount of salt was 3.47%, while on the 60th day of ripening, it increased to 3.74%. The amount of milk fat in the studied samples was analysed as a total amount of fat, which on the 1st day of ripening was 26.00%, and at the end of ripening (60th day), increased by nearly 4.0%. The proportion of fat in the dry matter was at the relatively constant value, which on the 60th day of ripening decreases by about 0.5%. The changes in the active and titratable acidity values were a consequence of the active development of fermentation processes. The high acidity determined at the beginning of the fermentation process was a prerequisite for active multiplication of the native microflora, resulting in the transformation of lactose into lactic acid. At the end of the process, a slight decrease in titratable acidity values was recorded. The changes observed are related to the change in the redox properties and buffering capacity of the cheese. Over a period of 60 days, the pH of the cheese decreased from 5.29 to 4.74. These results correspond with those obtained by Ivanov et al. (2015) for white brine cheese produced from cow and buffalo milk.

The microbiological analysis of artisanal sheep's cheese during ripening is presented in Table 2. The microbiological evaluation of artisanal cheese constituted a significant part of the product quality assessment. The presence of pathogenic microorganisms and coliforms was not detected during the ripening period. In contrast with our results in a previous study, Beev et al. (2019) investigated traditional dairy products and identified higher number of staphylococci and enterobacteria. Other studies have reported the presence of *Salmonella spp.*, *Listeria monocytogenes*, and the absence of *Escherichia coli* (Trmčić et al. 2016; Jackson et al. 2012) In traditional cheeses, *Staphylococcus xylosus* has been found in lamb skins.

Table 2. Microbiological analysis of artisanal sheep's cheese during ripening

Microbiological parameter, log cfu/g	Ripening period		
	1 day	30 day	60 day
Total number of lactobacilli	4.3	6.3	4.1
Total number of lactococci	7.2	6.2	5.1
Psychrotrophic microorganisms	2.1	2.2	2.0
Molds and yeasts	<100	<100	nd
Coliforms	nd	nd	nd
L. monocytogenes	nd	nd	nd
Staphylococcus spp.	nd	nd	nd
Salmonella spp.	nd	nd	nd

Note: nd – not detected

The presence of *Leuconostoc mesenteroides*, *Lactobacillus plantarum*, *Leuconostoc mesenteroides*, and *Lactobacillus plantarum* was mainly reported (Frece et al. 2019; Vrdoljak 2016). The data (Table 2) from the microbiological analysis showed that, representatives of the lactococci and lactobacilli, remain at the high level at the end of the ripening period. The initial rapid development of lactic acid bacteria created favorable conditions for product formation and inhibited the development of mold, yeasts and other undesirable microorganisms (Enikova 2010). The high acidity of the medium as a result of the vital activity of lactic acid bacteria was actively involved in the fermentation processes that formed the flavour of the final product. On the 60th day of ripening period, a predominance of lactococci was observed, the development of which created conditions for maintaining the acidity of the medium and preserving the product from the development of undesirable bacteria (Beev et al. 2019; Bintsis and Papademas 2002). From the representatives of lactic acid bacteria in traditional sheep's milk cheeses the presence of *Leuconostoc mesenteroides* and *Lactobacillus plantarum* was mainly reported (Beev et al. 2019).

The fatty acid composition of the analysed artisanal sheep's cheese samples is presented in Table 3.

Our results showed that during the 60 days of ripening, no changes occurred in the fatty acid composition. Probable reasons for this could be the low lipolytic activity of lactic acid microflora found in the samples (Table 2). Similar conclusions have been reported by a number of authors, according to whom the specific parameters of the medium and the endoenzymes released by the lactic acid microflora have negligible influence on the lipolytic transformations of milk fat in the cheese ripening process (Gortzi et al. 2022; Atanasova et al. 2020; Beev et al. 2019). According to Kalit et al. (2009) during the ripening process of cheese obtained from raw milk, significant amounts of free fatty acids (FFAs) were formed, which were considered as an indicator of the development of the lipolysis process.

As can be seen, the concentration of four dominant FAs in cheese samples were C14:0, C16:0, C18:0 and C18:1,with C16:0 (palmitic acid) being present in higher levels. Butyric (C4:0) and capric acid (C10:0) ranged between an average content of ~1.05 % and 6.12% respectively, but there not found caproic acids (C6:0), Both FAs had a slight increase during ripening period. In early previewe study (Atanasova et al. 2020) also reported that not found caproic acids (C6:0) during ripening in the Bulgarian goat, sheep and cow white brined cheeses.

Table 3.
Fatty acid composition of artisan sheep's cheese

Fatty acids, (%)	**Ripening period**		
	1 day	**30 day**	**60 day**
C4:0	nd	1.02±0.01	1.09±0.03
C8:0	1.04±0.10	1.08±0.03	nd
C10:0	6,15±0.13	6.05±0.33	6.18±0.02
C12:0	5.27±0.18	5.33±0.60	5.02±0.00
C13:0	0.18±0.02	0.09±0.02	nd
C14:0	12.95±0.21	12.75±1.04	12.97±0.01
C14:19c	0.57 ±0.05	0.61±0.44	0.56±0.00
C15:0	1.02 ±003	1.04±0.01	1.04±0.01
C16:0	31.95±0.21	31.91±0.06	31.97±0.00
C16:19c	0.66 ±0.01	0.64±0.05	0.67±0.03
C17:0	0.75 ±0.10	0.81±0.04	0.78±0.05
C17:1	0.25 ±0.00	0.15±0.05	0.11±0.11
C18:0	7.03 ±0.16	7.11±0.02	7.05±0.29
C18:19c	32.00 ±0.30	32.15±0.15	32.10±0.00
C18:2 n6c	nd	0.13±0.00	0.12±0.01
C18:3 cis-6	nd	0.07±0.02	0.11±0.00
C20:0	nd	0.04±0.00	0.13±0.00
C20:1	0.18 ±0.00	0.16±0.21	0.10±0.04

Note: nd – not detected

It was found that a concentrations of fatty acids with carbon chains from C8 to C12 were considerably higher than their flavour thresholds found in references Palombo et al. (2020) and therefore they can affect the taste of artisanal sheep's cheese.

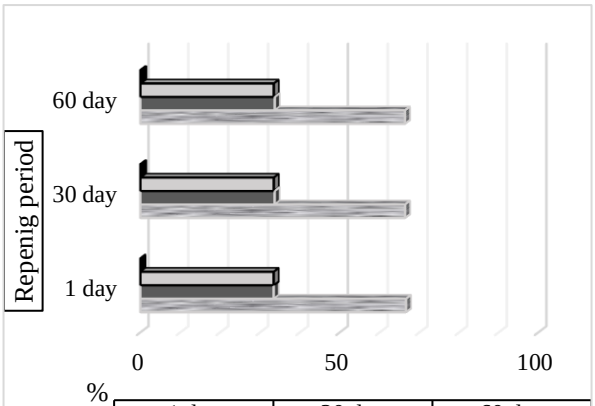
Fatty acids C15:0 (~1.2-1.4%) and C17:0 (~0.75-0.81%), typical of the composition of dairy products from sheep's milk.

From the perspective of human health, related to complete nutrition, short-chain fatty acids (C4:0; C8:0; C10:0; C12:0) represented about 10-13% of the fatty acid profile of the chesse. Other authors determined their antiviral and antibacterial action (Knutsen et al. 2018).

The values of myristic acid (C14:0) in the composition of the studied artisanal cheese (about 12-13%) remained relatively constant during the ripening and storage period. Its involvement in dietary intake had an important role in carcinogenesis processes and immune function (Thinon et al. 2014) and human colon health (Pakiet et al. 2019).

The predominant fatty acids in the composition of artisan sheep cheese belong to the group of saturated fatty acids (~66 %), compared to the unsaturated fatty acids (~33 %) (Fig. 1).

It is obvious that during the rippening process of artisan sheep cheese the amount of lauric saturated fatty acid acid decreased by 0,25 %. The other fatty acids showed similar changes, as described by other authors (Dimitrova et al. 2021; Palombo et al. 2020; Beev et al. 2019; Calvo et al. 2004). In this study the production of artisanal sheep`s cheese made from non-pasteurized milk describe the effect of of raw milk on the fatty acids profile and lipid oxidation of cheese throughout a rippening period of 60th days.



	1 day	30 day	60 day
PUFAs	0	0.20	0.23
MUFAs	33.41	33.44	33.44
UFAs	33.41	33.64	33.67
SFAs	66.59	66.36	66.33

Figure 1.
The percentage of total fatty acids composition

The conditions such as milking, various technological approaches, the local microflora, created the profile of the final product.

Conclusions

Microbiological indicators showed the absence of coliforms, *L. monocytogenes*, *Staphyloc*occus spp., and *Salmonella* spp between first and 60th day of the ripening. This indicates that astian sheep cheese is safe for consumption after 60 d ripening period. The fatty acid composition of cheese changed during ripening, and unsaturated fatty acids were also identified at the end of the storage period. The findings from this study confirmed that the application of domestic technologies for obtaining sheep's cheese did not create undesirable aspects to human health. However, the observance and control of technological processes were crucial for the quality of the final product. The present study could be extended by isolating and identifying local microorganisms (used as starters to improve the functional or quality characteristics of dairy products). Consequently, future research could be focused on microbiological analysis, including isolation and identification of the bacterial flora that influenced the formation of the organoleptic characteristics of the cheese.

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