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Evolution of the biochemical and microbiological characteristics of mountainous Kefalotyri cheese during ripening and storage

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Abstract

As artisanal cheeses are gaining recognition during the last years by consumers, efforts should be made to standardize their manufacture in order to produce safe products of high and constant quality, supporting the local economy and spreading them outside the region of origin. In this work, the biochemical and microbiological characteristics of Kefalotyri cheese produced during summer in the mountains of Pindos using an artisanal cheese-making procedure were studied. Sheep raw cheese milk was used without starter culture addition for Kefalotyri cheese manufacture. At 90 days of ripening and storage (the date that hard cheese can be sold in the market), its moisture was 40.4%, fat 28.8%, salt 4.1% and proteins 23.3%. Butyric acid and 3methyl butanoic acid were the most abundant volatile compounds found in this cheese. Mesophilic lactic acid bacteria and cocci were dominated; high numbers of thermophilic lactic acid bacteria and cocci, enterococci and Enterobacteriaceae were also present and the microbiological data revealed a rather satisfactory hygienic sanitary condition of the cheeses.

1. Introduction

Traditional products can be associated with broad concepts such as heritage, culture or history and locally produced traditional foods are positively perceived by consumers especially in the last decades. Kefalotyri is a traditional hard Greek cheese. It is manufactured from sheep or goat milk or mixtures of them. Cow milk may also be used. Regarding the origin of its name, "Kefalotyri" owes it either from the Greek word "Kefali" which means head and the word "tyri" which means cheese or from the word "Kefalo" that means big and the word "tyri" that means cheese. A third possible origin of its name is that it may come from the cheeses' that resemble a Greek hat called "Kefalo" (Zerfiridis, 2001). Kefalotyri has a flat cylindrical shape, firm texture, salty taste, strong flavour and is usually consumed graded with spaghetti. It is considered to be the ancestor of many Greek hard cheeses. Before the twentieth century, all hard cheeses produced in Greece from sheep's and/ or goat's milk were called Kefalotyri. In the literature, few research papers have been published about Kefalotyri cheese (Anifantakis and Kaminarides, 1987; Litopoulou-Tzanetaki and Vafopoulou-Mastojiannaki, 1988; Litopoulou-Tzanetaki 1990; Govari *et al.*, 2020).

Kefalotyri cheese is manufactured in various parts of Greece with technologies that differ from area to area and it is named according to the district of its manufacture. A very popular, artisanal Kefalotyri cheese is still manufactured traditionally in the mountains of Pindos without the addition of starter cultures, during summer (Anifantakis, 1991; Caric, 1993; Zerfiridis, 2001; Litopoulou-Tzanetaki and Tzanetaki, 2007). Its manufacturing process has been recorded from the beginning of 1900 (Dimitriadis, 1900).

Due to the growing interest in the characterization of traditional products and because its production and demand have increased greatly over recent years, the purpose of the present work was to study the biochemical and microbiological characteristics of this artisanal Kefalotyri cheese manufactured in the mountains of Pindos. Moreover, according to our knowledge, the volatile compounds of Kefalotyri cheese are reported in this study for the first time.

2. Materials and methods

2.1 Cheese making technology

According to the traditional method, three batches of cheese were manufactured by the same producer in the mountains of Pindos, at the village Samarina (1450 m height) during summer. Raw sheep milk (90 kg) from a local native sheep population was used to make the

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cheese, without using starter cultures. At 34°C, rennet (1:10.000 strength, NATUREN Extra, Hansen, Hoersholm, Denmark) was added according to the supplier's instructions (3.30g rennet/90 kg milk) and the coagulation took place within 1 hour. Then the coagulum was cut into small particles; the temperature was raised at 45-50°C under continuous stirring for 10 mins. The curd particles were left to settle for 5 mins, and then they were gathered together and pressed by hand to remove part of the whey. The pieces were put into the moulds, lined with a cheesecloth and pressed by hand to assist draining. After 10 mins the curd was turned over. This was repeated three times and then the cheese was transferred into the ripening room (14-16°C, and 75-85% Relative Humidity) for 24 hrs. Cheeses were dry salted with 6 g salt/kg cheese which was spread in the whole cheese surface. This was done daily and for seven days' time. Then the cheeses were left to ripen at 10-15°C and 60-70% Relative Humidity in natural conditions until they were 90 days old. During ripening, the cheeses were turned over daily or every second day; when necessary, their surface was brushed with brine, to inhibit the growth of moulds. Afterwards, the cheeses were transferred to the cold room (2-3°C) and left for storage for up to 180 days. The experiment was repeated three times.

2.2 Biochemical analyses

pH, water activity (aw) and moisture, fat, salt, protein and ash contents were determined as described by Bontinis *et al.* (2008). 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) as described by Mallatou *et al.* (2004). The volatile compounds were determined as described by Kondyli *et al.* (2016).

2.3 Microbiological analyses

Total viable counts (TVC); mesophilic and thermophilic lactic acid bacteria (LAB); mesophilic cocci and thermophilic cocci; enterococci; total staphylococci; enterobacteria and yeasts were enumerated as described by Bontinis *et al.* (2008).

2.4 Statistical analysis

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

3. Results and discussion

3.1 Biochemical analyses

The gross composition of artisanal Kefalotyri cheese is presented in Table 1. It can be seen that the pH of the cheese ranged from 5.0 to 5.7 during ripening and storage. The pH values remained stable ($p >0.05$) from the 5th to the 90th day of ripening but increased ($p < 0.05$) from 90 to 180 days. This increase in pH values could be due to the buffer effects of milk salts transferred into the cheese and it has been also observed by others too (Fresno *et al.,* 1996). In this study, the greatest decrease in the water activity (aw) values were observed from day 5 to day 30. A high correlation between salt and aw was found (correlation coefficient -0.94; $p<0.05$) in this study. This was in accordance with the results of others (Cuesta *et al.,* 1996). The moisture content decreased ($p <0.05$) from day 5 to day 30 possibly due to the syneresis of cheeses. Thereafter it did not differ significantly ($p >0.05$), ranging from 39.0% to 40.4%. Table 1 shows that the fat content of artisanal Kefalotyri cheese varied during ripening and ranged from 27.5% to 29.3%. However, its protein content remained constant at all sampling dates (Table 1). Ash content increased during the first days of cheese ripening, while no statistical differences were observed from the 30th day until the 180 days. The salt content generally increased ($p<0.05$) during ripening and storage, reaching the value of 4.4%, at the end of storage (180th day).

Although in general similar composition of Kefalotyri cheese was observed by others (Anifantakis

Table 1. Physicochemical parameters of traditional Kefalotyri cheese during ripening and storage

Time days	pH	Moisture %	Fat %	Salt %	Proteins %	Ash %	aw
5	5.0±0.0^{a}	45.5±0.5^{b}	27.5±0.2^{a}	0.5±0.0^{a}	23.2±0.3^{a}	2.9±0.0^{a}	0.97±0.35^{c}
30	5.3±0.2^{a}	39.3±0.8^{a}	28.8±0.4^{ab}	3.1±0.1^{b}	24.2±0.7^{a}	3.8±0.2^{b}	0.92±0.50^{b}
60	5.2±0.0^{a}	39.0±0.5^{a}	29.3±0.5^{b}	3.3±0.0^{b}	23.9±0.6^{a}	3.6±0.2^{b}	0.92±0.79^{ab}
90$^{\#}$	5.2±0.0^{a}	40.4±0.6^{a}	28.8±0.4^{ab}	4.1±0.1^{c}	23.3±0.4^{a}	3.9±0.1^{b}	0.91±0.47^{ab}
180	5.7±0.0^{b}	40.4±0.7^{a}	28.5±0.4^{ab}	4.4±0.0^{d}	22.5±0.4^{a}	3.7±0.1^{b}	0.91±0.59^{a}

Values are presented as mean± Standard Error, n = 3. Values with different superscripts within the same column are significantly different ($p <0.05$).

$^{\#}$The day the cheeses entered the cold room, aw: water activity.

and Kaminarides, 1987; Caric, 1993) some differences, such as the higher moisture and salt values, could be attributed to differences in milk composition and different manufacturing conditions used (i.e., pressure).

The changes in the nitrogenous fractions during ripening and storage of traditional Kefalotyri cheese are shown in Table 2. The values of total nitrogen (TN%) did not change during ripening and storage and were between 3.5-3.8%. The values of WSN %TN increased ($p<0.05$) from 8.5% to 20.6%. The TCA content was found to be between 5.5 to 14.2% while the PTA content ranged from 1.8 to 4.5, the 5th day and the 180th day, respectively (Table 2). An increase in the soluble proteins content was also reported by Anifantakis and Kaminarides (1987) for Kefalotyri cheese.

Table 2. Proteolysis of traditional Kefalotyri cheese during ripening and storage

Time days	TN %	WSN %TN	TCA %TN	PTA %TN
5	3.6±0.0^{a}	8.5±2.3^{a}	5.5±0.1^{a}	1.8±0.6^{a}
30	3.8±0.1^{a}	10.2±0.4^{a}	5.1±1.8^{a}	2.3±0.7^{ab}
60	3.7±0.1^{a}	10.2±0.4^{a}	11.5±1.4^{b}	3.4±0.2^{bc}
90$^{\#}$	3.6±0.0^{a}	18.7±0.0^{b}	11.5±1.4^{b}	3.7±0.3^{cd}
180	3.5±0.0^{a}	20.6±1.3^{b}	14.2±1.6^{b}	4.5±0.3^{d}

Values are presented as mean± Standard Error, n = 3. Values with different superscripts within the same column are significantly different ($p<0.05$).
$^{\#}$The day the cheeses entered the cold room.

The volatiles in the headspace of mature (90-180 days) traditional Kefalotyri cheese were grouped by chemical class to simplify comparison and are reported in Table 3. Sixteen compounds, i.e., two ketones, four alcohols, five esters and five free fatty acids were identified. No significant ($p>0.05$) differences in the total volatile fraction content were observed in the 90th and 180th age. The most abundant group of volatiles was free fatty acids (FFA), while the most abundant compounds were butyric acid, ethanol, 3methyl butanoic acid and 3 methyl butanol (Table 3). Free fatty acids come mainly from lipolysis, i.e., hydrolysis of triglycerides into free fatty acids, during the ripening of the cheese; although their source is not limited to fat hydrolysis. The metabolic products of lactose metabolism, deamination of amino acids and possibly lipid oxidation may also contribute to the overall fatty acid pool (Collins *et al.,* 2003). In the literature butyric acid was also found to be an important component of Kefalotyri cheeses (Georgala *et al.,* 2006) and in abundance in other cheeses (Castillo *et al.,* 2007).

3.2 Microbiological analyses

The changes in the population of the main microbial types of Kefalotyri cheeses are shown in Table 4. In the first 5 days of ripening, Kefalotyri cheese contained high counts of mesophilic lactic acid bacteria (LAB) and cocci, but lower of thermophilic LAB and cocci. This reflects the absence of starter cultures during its manufacture. As the ripening and storage process was going on, there were significant decreases in the microbial groups perhaps due to the specific composition of this cheese i.e., its high salt content. These results are in accordance with the findings of Bontinis *et al.* (2008) for Xinotyri cheese made from raw milk.

In this study, enterococci remained stable at all sampling dates ranging from 7.2-7.6 log CFU/g. This was due to their high resistance to salt content (Sharpe, 1979). Similarly, Litopoulou-Tzanetaki (1990) found high counts of enterococci throughout the ripening and storage of Kefalotyri cheese that was made from pasteurized milk. According to Aquilanti *et al.* (2006) enterococci represent a typical microflora of ewe milk and play an important role in the late-ripening of several cheese varieties. They constitute part of the normal food microbiota and play an important role in the manufacture of typical cheeses from Mediterranean countries.

Yeasts also remained stable at all sampling dates ranging from 3.6-2.6 log CFU/g. The population of total staphylococci were present at high levels (6.8 log CFU/ g) at the beginning of ripening and although they exceeded the 5-log threshold level specified in Commission Regulation No 1441/2007 (European Commission, 2005) at the 5th day, they did not show lecithinase activity on Baird-Parker agar. However, they decreased ($p<0.05$) at 30 days and remained <5log until the end of ripening and storage. This means that the cheese meets the requirements specified in Commission Regulation No 1441/2007 (European Commission, 2005).

Total enterobacteria were high (7.4 log CFU/g) at day-5, fell to 1 log CFU/g when cheeses entered the cold room and remained to that level until the 180th day of ripening and storage. The fact that Enterobacteriaceae did not completely disappear could be associated with the pH values which were 5.7 at the end of the storage period. It is known that pH values lower than 5.0-5.2 are necessary to achieve the inhibition of the Enterobacteriaceae (Nunez *et al.,* 1985).

The Greek Codex Alimentarius (2009) permits the manufacture of hard cheeses from raw milk provided that the cheeses are ripened for 90 days or longer at a temperature of not less than 10^{o}C. The cheeses of this study were in accordance with the Greek legislation. The microbiological data revealed a rather satisfactory hygienic sanitary condition of the evaluated mountainous Kefalotyri cheese samples.

Table 3. Relative abundance (peak area × 10^3) of volatile compounds of mature traditional Kefalotyri cheese during storage

Volatiles compound	Day-90#	Day-180
Ketones		
2Butanone	259.2±123.7[a]	270.9±99.5[a]
Acetoin	219.4±44.6[a]	103.9±32.1[a]
Total ketones	478.7±111.4[a]	374.9±68.5[a]
Alcohols		
Ethanol	295.2±81.2[a]	216.1±42.5[a]
1 Butanol 3methyl	399.4±81.3[a]	337.7±69.1[a]
2Butanol	70.5±43.5[a]	183.7±131.9[a]
1 Butanol	76.9±59.4[a]	35.6±22.6[a]
Total alcohols	842.0±97.2[a]	773.1±53.4[a]
Esters		
Butanoic acid ethyl ester	284.1±171.7[a]	273.8±141.3[a]
Propanoic acid 2methyl propyl ester	25.8±11.1[a]	35.6±9.4[a]
Butanoic acid butyl ester	48.7±0.0[a]	30.9±0.0[a]
Hexanoic acid ethyl ester	75.5±17.1[a]	97.4±10.0[a]
Propanoic acid 2methyl butyl ester	47.7±0.4[a]	66.7±2.5[b]
Total esters	482.0±177.0[a]	504.5±145.3[a]
Free Fatty Acids		
Acetic acid	225.5±28.3[a]	270.2±45.3[a]
Butyric acid	2238.3±826.2[a]	1629.6±518.8[a]
Butanoic acid 2 methyl	251.3±75.8[a]	201.3±286.6[a]
Butanoic acid 3 methyl	595.5±187.4[a]	744.8±139.3[a]
Hexanoic acid	262.9±40.1[a]	247.5±3.0[a]
Total free fatty acids	3573.7±787.6[a]	3093.5±394.6[a]
Total volatile compounds		
Total volatile compounds	5376.5±1155.1[a]	4746.1±456.5[a]

Values are presented as mean± Standard Error, n = 3. Values with different superscripts within the same row are significantly different ($p < 0.05$).

#The day the cheeses entered the cold room.

Table 4. Microorganisms (log CFU/g) of traditional Kefalotyri cheese during ripening and storage

Time (days)	Total Viable Counts	Mesophilic lactic acid bacteria	Thermophilic lactic acid bacteria	Mesophilic cocci	Thermophilic cocci	Enterococci	Total Staphylococci	Yeasts	Total Enterobacteriaceae
5	8.8±0.3[c]	9.1±0.1[d]	7.9±0.0[c]	9.0±0.1[d]	8.7±0.1[b]	7.2±0.1[a]	6.8±0.3[b]	3.6±0.2[a]	7.4±0.3[c]
30	8.4±0.0[bc]	8.4±0.0[c]	7.8±0.1[bc]	8.9±0.1[cd]	7.9±0.1[a]	7.6±0.1[a]	5.0±0.4[a]	3.5±0.1[a]	5.6±0.7[b]
60	8.0±0.0[ab]	8.0±0.0[b]	7.7±0.1[b]	8.7±0.1[bc]	7.9±0.0[a]	7.4±0.1[a]	4.3±0.3[a]	3.4±0.5[a]	4.6±0.8[b]
90#	8.0±0.0[a]	7.7±0.0[ab]	7.4±0.1[a]	8.5±0.0[b]	7.8±0.1[a]	7.6±0.0[a]	4.7±0.3[a]	2.6±0.5[a]	1.0±0.0[a]
180	7.8±0.0[a]	7.6±0.1[a]	7.6±0.0[ab]	8.2±0.0[a]	7.8±0.1[a]	7.4±0.0[a]	4.3±0.5[a]	3.3±1.1[a]	1.0±0.0[a]

Values are presented as mean± Standard Error, n = 3. Values with different superscripts within the same column are significantly different ($p < 0.05$).

#The day the cheeses entered the cold room.

4. Conclusion

Traditional Kefalotyri cheese has good acceptance in the local market. As its production and demand have increased greatly over recent years, the data of this study would be useful in order to standardize its cheese-making procedure, establish and improve its quality. Knowledge of cheese changes during ripening is of great interest for standardization of this traditional product, in order to produce it with constant characteristics, securing a fair return for farmers and producers and sustaining the socio-economic vitality of local rural communities. Additional studies might be necessary and pasteurized milk could be used in order to produce Kefalotyri cheese with high hygienic quality and safety; however, more work must be done in order to specify the microbiological and biochemical differences between raw and pasteurized cheese.

Conflict of Interest

The authors declare no conflict of interest.

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