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Research Article

Proteolysis in kashkaval cheese stored at different temperatures

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Abstract

The aim of the present study was to evaluate the effect of refrigerated storage temperature on the proteolysis in cow's milk Kashkaval cheese. The Kashkaval samples were stored for 12 months at four different temperature regimes - cooled at $4.0 \pm 1.0^\circ\text{C}$, cooled at $1.0 \pm 1.0^\circ\text{C}$, superchilled at $-7.5 \pm 0.5^\circ\text{C}$ and frozen at $-18.0 \pm 1.0^\circ\text{C}$. The proteolysis in cheese samples was evaluated by determining the non-casein nitrogen (NCN/TN), non-protein nitrogen (NPN/TN) as a percentage of total nitrogen and content of free amino groups. It was found that the storage temperature had a significant impact on the hydrolysis of cheese paracasein. Complete inhibition of the proteolysis was established only in frozen stored Kashkaval. Slight, but statistically significant ($P < 0.05$) increase in the NCN/TN values indicating for retarded proteolysis in samples stored at $-7.5 \pm 0.5^\circ\text{C}$ was established. The increasing of the storage temperatures results in increased NCN/TN, NPN/TN values and free amino groups content. The highest proteolysis rate was observed at the refrigeration temperatures of $4.0 \pm 1.0^\circ\text{C}$. The samples stored at this temperature regime had the highest values of both high molecular weight protein fractions (NCN/TN) and products of deep proteolysis (NPN/TN and free amino groups).

Keywords: kashkaval cheese, proteolysis, nitrogen fractions, cold storage, freezing, superchilling

Abbreviations: BSS - Bulgarian State Standard; NCN – non-casein; NPN – non-protein; MANOVA - multivariate analysis of variance; TN – total nitrogen

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Introduction

Proteolysis is one of the most important and complex biochemical processes that take place during the ageing and storage of the cheese. It greatly affects both texture formation - through the breakdown of casein and the taste of cheeses - through the formation of low molecular weight compounds (Fox et al., 1996). The active proteolytic enzymes involved in this process are proteolytic enzymes from milk (plasmin, cathepsin D, acid phosphatase), rennet enzymes (pepsin and chymosin) and enzymes released from the starter, secondary and non-starter microflora (Hayalog et al., 2004).

The process of proteolysis is influenced by a number of factors, such as the content of proteolytic enzymes, the growth of the starter and non-starter microflora, pH, water content, salt content, temperature and storage time, etc.

Park et al. (1995) stored 5 types of goat and 2 types of cow cheese at different temperature regimes (4°C, 13°C and 22°C) for 6 months. In all samples, the process of proteolysis was found to accelerate with increasing temperature and storage time. Low storage temperatures and in particular freezing significantly retarded the proteolysis of the cheese casein. A number of other data support the claim that low temperatures inhibited the proteolytic processes and do not significantly alter the texture and organoleptic characteristics of most types of cheeses (Bertola et al., 1996; Park, 2001; Van Hekken et al., 2005; Osman et al., 2009; Chen et al., 2010; Hamad et al., 2012).

The present study aims to investigate the effect of storage temperature on the proteolysis in cow's milk Kashkaval cheese.

Materials and Methods

Cheese making. Kashkaval samples were produced at a local dairy plant (Bor-Chvor -RK Ltd, Plovdiv) from a single vat of milk according to the following procedure. Cow's milk of 3.9% fat was heat-treated at $65 \pm 1^\circ\text{C}$ for 15 s and cooled to $33 \pm 1^\circ\text{C}$, pumped into a cheese vat, and inoculated with a thermophilic culture which consisted of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. Subsequently, calcium chloride and

commercial animal rennet were also added. Following a 30 min set, the curd was cut and allowed to heal for 5 min. Next, the curd was stirred gently without heat for another 20 min, followed by heating to $39 \pm 1^\circ\text{C}$ for 40 min with continuous agitation. After that, the whey was drained from the curd, which was partially molded. The curd was kept at cooking temperature for cheddaring until the pH reached 5.3 (about 2 h). Then the curd was milled and stretched on a blade mixer under a concentrated salt solution (13%) at 72°C . The Kashkaval loaves were molded into 1 kg parallelepiped forms. After 15 h the Kashkaval loaves were vacuum-packaged in polyethylene foil under 90 - 99.8 Pa and left to ripen at three different regimes - $9 \pm 1^\circ\text{C}$, $11 \pm 1^\circ\text{C}$ and $13 \pm 1^\circ\text{C}$ and relative humidity of 75 - 80% for 60 d. The gaseous permeability of the polyethylene foil was 175 (pO₂) cm³/d.m².Pa and 1000 (pCO₂) cm³/d.m².Pa, and water vapor permeability was 4 g/cm².d at 23°C .

The samples were analyzed at 0, 3, 6, 9 and 12 month of refrigerated storage. The measurements of every one examined indicator were repeated three times. The mean values of those three independent determinations were indicated in the tables and figures.

Physicochemical analyses.

Water content was determined by weight, by drying the samples at $t = 102 - 105^\circ\text{C}$ for 7 h.

Milk fat was estimated by Gerber - Van Gulik method (BSS 1671-89).

Sodium chloride was determined by the method of Moor (BSS 8274-82).

Active acidity was assessed potentiometrically.

Titrate acidity was estimated by the Turner method, according to BSS 1111-80.

Total nitrogen (TN), non-casein (NCN) (soluble at pH = 4.6) and non-protein (NPN) (soluble in trichloroacetic acid) were determined by Vakaleris and Price (1959) method modified according to the specific conditions of the assay.

Free amino groups - the samples were extracted with distilled water. An aliquot of the water-soluble extract was diluted with 1 mL of distilled water and 2 mL of the cadmium ninhydrin reagent was added.

The mixture was heated to 84°C for 5 min, then cooled. Spectrophotometrically measure the absorbance of the sample at 507 nm against a sample containing no water-soluble extract. The results are presented as absorbance units at 507 nm (A507) or as mg Leu/g cheese, standard line.

Statistical analysis. Statistical analyses were carried out on the averages of the triplicate results. Two-way multivariate analysis of variance (MANOVA) and multiple comparison tests were carried out to study the effect of both ripening time and temperature on the physicochemical characteristics and texture parameters of Kashkaval samples (Box et al., 1978). Differences in the averages and F tests were considered significant when the computed probabilities were less than 0.05. All statistical procedures were computed using the Microsoft Excel and Sigma Plot 2001 software.

Results and Discussion

The Kashkaval cheese samples were ripened at $9.0 \pm 1.0^\circ\text{C}$ for 60 d and after that were stored for twelve months at four different temperature regimes - cooled at $4.0 \pm 1.0^\circ\text{C}$, cooled at $1.0 \pm 1.0^\circ\text{C}$, superchilled at $-7.5 \pm 0.5^\circ\text{C}$ and frozen at $-18.0 \pm 1.0^\circ\text{C}$. The data for the main physico-chemical parameters of Kashkaval cheese samples at the beginning and end of storage are presented in Table 1. In the present study, no significant ($p < 0.05$) changes in the values of water content, dry matter content, total protein, total fat content and salt content during the storage period of Kashkaval cheese (for 12 months) were found. Controversially, the pH and titratable acidity of Kashkaval samples stored at $4.0 \pm 1.0^\circ\text{C}$ and $1.0 \pm 1.0^\circ\text{C}$ changed significantly ($p < 0.05$). Most significant ($p < 0.05$) were those changes in the cheese test samples stored at higher temperatures ($4.0 \pm 1.0^\circ\text{C}$). Similar results have been reported by other authors who stored cheeses in chilled and frozen conditions (Sendra et al., 1999; Tejada et al., 2002; Elsamani et al., 2014; Diezhandino et al., 2015).

For evaluation of the proteolysis in the Kashkaval samples in the present study, the ratios of the different nitrogen fractions, i.e. non-casein (soluble at pH = 4.6) to total nitrogen (NCN/TN), non-protein (soluble in trichloroacetic acid) to total nitrogen (NPN/TN), as well as the changes in the free amino groups were established. The NCN/TN

provided information on the share of the milk proteins that had undergone hydrolytic changes in the process of cheese ageing. They are commonly used for evaluation of the cheese ripening degree since they provide a general characteristic of the proteolysis. The results obtained for the change of NCN/TN ratio during the storage of Kashkaval cheese are presented in Fig. 1.

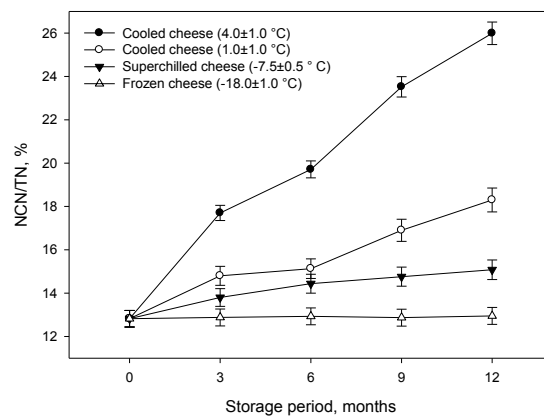


Figure 1. Changes in NCN/TN ratio of Kashkaval cheese during refrigerated storage at $1.0 \pm 1.0^\circ\text{C}$, $4.0 \pm 1.0^\circ\text{C}$, $-7.5 \pm 0.5^\circ\text{C}$ and $-18.0 \pm 1.0^\circ\text{C}$.

From the data presented, it can be seen that the NCN/TN values of the frozen-stored samples did not undergo significant ($p < 0.05$) changes during the 12 months of the storage. At the end of storage, the NCN/TN values of these samples were maintained at $12.65 \pm 0.52\%$. With the increase of the storage temperature an increase in NCN/TN values of Kashkaval cheese was observed indicating for more pronounced proteolysis. A minimal, but significant ($p < 0.05$) increase in the NCN/TN values of Kashkaval samples stored at $-7.5 \pm 0.5^\circ\text{C}$ was found. The NCN/TN of these samples reached $15.08 \pm 0.42\%$ at the end of storage. A more substantial increase in NCN/TN, indicating for more intense proteolytic processes, was observed in the cold stored Kashkaval.

During the 12-month storage period of the samples at $1.0 \pm 1.0^\circ\text{C}$, the NCN/TN values increased from $12.82 \pm 0.51\%$ to $18.31 \pm 0.44\%$. For Kashkaval stored at $4.0 \pm 1.0^\circ\text{C}$, the increase in NCN/TN was from $12.82 \pm 0.51\%$ to $25.99 \pm 0.35\%$. The results obtained (Fig. 1) indicate for the strong

Table 1. Mean values for the main physicochemical parameters of Kashkaval samples

Parameter	Temperature and period of storage, °C							
	4.0 ± 1.0°C		1.0 ± 1.0°C		-7.5 ± 0.5°C		-18.0 ± 1.0°C	
	1 st month	12 th month	1 st month	12 th month	1 st month	12 th month	1 st month	12 th month
Moisture content, %	41.9±0.5 ^a	41.4±0.7 ^a	41.8±0.4 ^a	41.3±0.6 ^a	41.7±0.4 ^a	41.4±0.6 ^a	41.9±0.5 ^a	41.2±0.6 ^a
Protein content, %	21.8±0.7 ^c	21.9±0.6 ^c	22.1±0.7 ^c	21.9±0.6 ^c	22.2±0.7 ^c	22.0±0.6 ^c	22.3±0.7 ^c	22.2±0.6 ^c
Fat in dry matter, %	34.5±0.7 ^d	35.0±0.6 ^d	34.7±0.6 ^d	34.9±0.8 ^d	35.0±0.7 ^d	35.1±0.6 ^d	34.9±0.7 ^d	34.8±0.8 ^d
NaCl, %	2.10±0.04 ^c	2.20±0.05 ^c	1.90±0.03 ^c	2.10±0.05 ^c	2.00±0.04 ^c	2.20±0.03 ^c	2.10±0.03 ^c	2.20±0.05 ^c
pH	5.55±0.02 ^a	5.40±0.03 ^b	5.55±0.01 ^a	5.50±0.01 ^b	5.55±0.02 ^a	5.59±0.03 ^a	5.55±0.02 ^a	5.55±0.02 ^a
Titrateable acidity, °T	175.0±2.0 ^a	226.0±2.0 ^b	175.0±2.0 ^a	184.0±3.0 ^b	175.0±2.0 ^a	178.8±2.0 ^a	175.0±2.0 ^a	176.0±3.0 ^a

a, b, c - Means within the same row bearing a common superscript did not differ significantly ($p < 0.05$). $n = 3$

interdependence of proteolytic activity in cheese and storage temperature. Similar tendencies were reported by other authors (Kasprzak et al., 1994; Bertola et al., 1996; Tejada et al., 2002; Sousa et al., 2001).

It is noteworthy that complete inhibition of proteolysis is achieved only by freezing and storage the Kashkaval cheese at temperatures below $-18.0 \pm 1.0^\circ\text{C}$. The slight increase in NCN/TN values found in the Kashkaval stored at $-7.5 \pm 0.5^\circ\text{C}$ indicated for residual proteolytic activity in the samples stored at this temperatures. The intensity of proteolysis in chilled Kashkaval cheese is largely influenced by temperature. It can be seen that even a small temperature difference of 3°C by the two experimental regimes ($1.0 \pm 1.0^\circ\text{C}$ and $4.0 \pm 1.0^\circ\text{C}$) leads to significant differences in the NCN/TN values. This indicates that for improving the Kashkaval cheese quality, a strict control and monitoring of the temperature in the storage facilities should be performed. The results of the present study indicate that even minimal temperature variations could have a significant effect on the biochemical processes occurring in Kashkaval cheese during its refrigerated storage. The results obtained for the change of NPN/TN and the content of free amino groups during the storage of Kashkaval cheese are presented in Fig. 2 and Fig. 3. The changes in the values of these two indicators

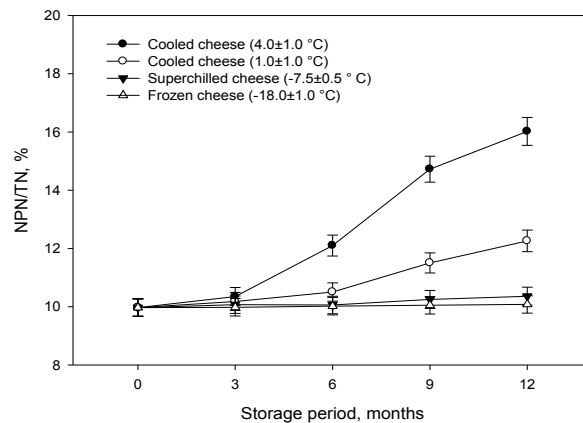


Figure 2. Changes in NPN/TN ratio of Kashkaval cheese during refrigerated storage at $1.0 \pm 1.0^\circ\text{C}$, $4.0 \pm 1.0^\circ\text{C}$, $-7.5 \pm 0.5^\circ\text{C}$ and $-18.0 \pm 1.0^\circ\text{C}$.

reflected the deep hydrolysis of the proteins in the cheese matrix to low molecular weight compounds such as dipeptides, free amino acids, biogenic amines, ammonia, etc. (the depth of cheese ripening).

From the data presented, it can be seen that the values of NPN/TN and the content of free amino groups did not change significantly ($p < 0.05$) during the storage of Kashkaval samples at $-18.0 \pm 1.0^\circ\text{C}$ and $-7.5 \pm 0.5^\circ\text{C}$. At the end of storage, the

values of NPN/TN and the content of free amino groups in these cheese samples were maintained at levels of $10.23 \pm 0.35\%$ and 22.86 ± 0.62 mgLeu/100g, respectively. Controversy, a significant increase ($p < 0.05$) of NPN/TN values and the content of free amino groups of cold stored Kashkaval was observed, indicating for the deep hydrolysis of proteins in the cheese matrix.

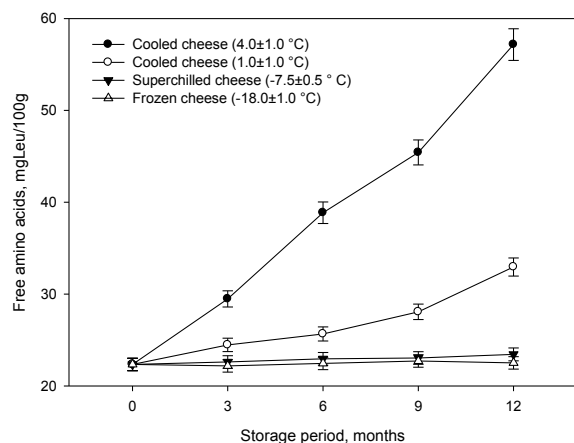


Fig. 3. Changes in free amino groups content of Kashkaval cheese during refrigerated storage at $1.0 \pm 1.0^\circ\text{C}$, $4.0 \pm 1.0^\circ\text{C}$, $-7.5 \pm 0.5^\circ\text{C}$ and $-18.0 \pm 1.0^\circ\text{C}$.

During the 12-month storage period of the samples at $1.0 \pm 1.0^\circ\text{C}$, the NPN/TN values and the content of free amino groups increased from $9.97 \pm 0.37\%$ to $12.26 \pm 0.41\%$ and from 22.34 ± 0.52 mgLeu/100g to 32.95 ± 0.48 mgLeu/100g, respectively. Kashkaval samples stored at $4.0 \pm 1.0^\circ\text{C}$ had the highest increase in NPN/TN values and the content of free amino groups - from $9.97 \pm 0.37\%$ to $16.02 \pm 0.38\%$ and from 22.34 ± 0.52 mgLeu/100g to 57.17 ± 0.38 mgLeu/100g, respectively. The pronounced proteolysis found in the present study in the Kashkaval cheese stored at $4.0 \pm 1.0^\circ\text{C}$ is a prerequisite for the appearance of some taste defects. The results obtained in the present study were in agreement with the findings of other authors investigating the effect of storage temperature on the proteolysis in different types of cheeses (Tavaria et al., 2003; Park et al., 2013; Setyawardani et al., 2019). Simov and Ivanov (2005) investigate the proteolytic activity of *Lactobacillus delbrueckii ssp. bulgaricus* and *Streptococcus thermophilus* in frozen-stored

Kashkaval cheese. The authors found that the content of non-casein and non-protein nitrogen in the cheese samples stored in frozen state for 12 months was low. According to the authors the retarded proteolysis is probably due to the inhibition of the growth of lactic acid bacteria and the activity of proteolytic enzymes in the frozen cheese.

Conclusion

During the 12 months of storage at four temperature regimes ($4.0 \pm 1.0^\circ\text{C}$, $1.0 \pm 1.0^\circ\text{C}$, $-7.5 \pm 0.5^\circ\text{C}$ and $-18.0 \pm 1.0^\circ\text{C}$) the main physicochemical parameters of Kashkaval cheese as water content, dry matter content, total protein, total fat content and salt content did not change significantly ($p < 0.05$). Some other cheese parameters as pH and titratable acidity were significantly affected by the storage temperature. With the increase of the storage temperature, the process of glycolysis in the cheese matrix proceeds at a higher rate. The proteolysis in Kashkaval cheese was highly influenced by the storage temperature. In cheese samples stored at temperatures higher than 1.0°C , the hydrolysis of casein proceeds at a much higher rate than in samples stored at lower temperatures. The results obtained prove that temperature is a major factor that must be controlled in order to maintain a high quality of cheeses throughout their storage period.

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