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Assesment of antimicrobial, antioxidant and ACE-inhibitory activities of reduced sodium chloride Kačkavalj cheese

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Abstract

Excessive consumption of sodium chloride has negative effects on the consumers' health. Considering the World Health Organisation's recommendation to reduce salt intake in nutrition, the microbiota, proteolytic, antimicrobial, antioxidant and angiotensin converting enzyme (ACE) inhibitory activity of following Kačkavalj cheeses were analysed during six months: control cheese produced according to the usual technological production process; cheese with 30 % reduced sodium chloride content; cheese with 30 % sodium chloride replaced by potassium chloride. The lactic acid bacteria number increased in all cheeses on the 30th day of ripening and until the 90th day remained at a level of 7-8 log units. On the 180th day of ripening significant differences were found in the number of thermophilic species. The reduction and substitution of sodium chloride in Kačkavalj cheese showed no significant effect on the tested parameters of proteolysis. The best antimicrobial activity was observed in the sodium chloride reduced cheese on day 30 and increased until day 180. The reduction of sodium chloride achieved significantly better antioxidant activity in the sixth month ripened Kačkavalj cheese by methods for determination of antioxidant capacity by inhibiting 2,2-azino-bis/3-ethyl-benothiazoline-6-sulfonic acid (ABTS) radicals and the determination of free-radical capacity using 2,2-diphenyl-picrylhydrazyl (DPPH) radicals. ACE inhibitory activity increased during ripening, but there were no significant differences between produced cheese variants. The present study highlights the great potential for the development of a product that contributes to consumer health and corresponds to modern trends in human nutrition.

Keywords: cheese; sodium chloride reduction; antimicrobial activity; antioxidant activity; ACE inhibition

Introduction

The World Health Organization (WHO, 2016) has recognized the reduction of sodium chloride (NaCl) content in food as one of the five priority actions for the prevention and control of non-communicable diseases. Therefore, in recent years, the possibility of reducing the NaCl content in food has been researched, either by reducing the added NaCl or by partially or completely replacing NaCl with other suitable salts, such as potassium chloride (KCl) or magnesium chloride (Miocinovic et al., 2022). The WHO (2023) recommended consuming less than 5 g of salt per day and choosing foods with lower-sodium content. The WHO also suggested replacing regular table salt with lower-sodium salt substitutes that contain potassium and reformulating food products to contain less sodium. This was the reason for reducing the NaCl content in this study by 30 %, as well as replacing NaCl with KCl and investigating the characteristics of such Kačkavalj cheese.

Considering the fact that the quality of cheese is mostly formed during ripening, proteolysis is one of the most important biochemical processes and has a great influence on the final product. Proteolysis is influenced by many factors: the activity of enzymes such as plasmin, peptidases and proteinases originating from lactic acid bacteria (LAB), time, humidity and temperature during ripening, pH and cheese composition, especially salt content (Miocinovic et al., 2014). During cheese ripening and proteolytic changes, bioactive peptides or compounds are formed. The specific sequences of the released peptides can potentially exhibit biological activity, including antioxidant and antimicrobial, as well as ACE inhibition. Moreover, the enzymatic hydrolysis of casein can create antioxidant compounds, and therefore the antioxidant properties of cheese are largely related to the degree of casein degradation (Chen et al., 2019). The antimicrobial role is reflected in the stimulation of the body's natural defense against pathogenic microorganisms. Looking at the bioactivity of dairy products, it can be assumed that the overall antibacterial effect against a broad spectrum of pathogenic bacteria is significant.

Kačkavalj cheese is characterized by a unique technology, in particular the processing of the curd, which is acidified with lactic acid, produced by LAB and then kneaded in hot water, contributing to the plastic consistency characteristic of this type of cheese (Niro et al., 2014). The most popular Kačkavalj cheese is produced in the municipality of Pirot in south-eastern part of Serbia. This cheese is produced without the addition of starter culture. Therefore, the influence of the native microbiota on the processes of proteolysis and the release of bioactive peptides during ripening is of great importance, with the emphasis on LAB that survive high temperatures and tolerate higher salt concentrations. Additionally, in the production of traditional Kačkavalj cheese, the hot curd is kneaded by hand, which strongly affects the texture of the cheese. According to Pappa et al. (2020), supporting the maintenance of the traditional cheese production is of great importance, as the traditional products are the result of several factors, such as the raw material, processing and sensory characteristics.

Kačkavalj cheese was chosen as the subject of the study for several reasons. First, it is one of the most well-known and consumed *pasta filata* cheeses in the Balkan and Mediterranean regions. Moreover, it has different names depending on the country of production such as Kačkavalj, Caciocavallo, Kasar, Kasseri, Kashkaval and Cascaval (Satric et al., 2024). The second reason is that the production stages and characteristics of Kačkavalj are defined by the Institute for Standardization of Serbia through the National Standard for Quality Requirements (Institute for Standardization of Serbia, 1997) which provides Kačkavalj cheese great significance. The third reason is that Kačkavalj cheese is a traditional cheese which is produced without the addition of the starter culture. Furthermore, Kačkavalj cheese is traditionally characterised by a high salt content (Satrić et al., 2023). Therefore, it is important to analyse the effects of salt reduction on microbiota and bioactivity in order to protect the health of consumers. The research of the low sodium chloride Kačkavalj cheese in this study focused mainly on the determination of the microbiota and the antimicrobial and antioxidant activity as well as the ACE-inhibitory activity of this traditional cheese. To our knowledge, this type of research had not been conducted.

Material and method

Production of Kačkavalj cheese

The production of Kačkavalj cheese to investigate the reduction of sodium chloride content and the partial replacement of sodium chloride with potassium chloride was carried out in three repetitions. The Kačkavalj cheese was traditionally made from raw cow's milk which originates from the municipality of Pirot without the addition of a starter culture. The milk was coagulated by adding rennet at a temperature of 32 °C for 40-45 minutes, followed by cutting and stirring, and final scalding of the mixture at 38-42 °C for approximately 40 minutes. The whole mixture was transferred to the pressing machine, where the curd grains were separated from whey and the curd was pressed at a pressure of 5–10 kg of cheese per kg of weight. After pressing, the curd was formed into pieces called "baskija", which had to reach a pH value of 5.2. After, the curd was cut into small pieces and placed in a wooden basket where the thermal treatment took place. The basket with the curd pieces was then placed in a vat of hot water at 75 °C and manually stirred with a wooden stick until the curd was streached and the desired homogeneous cheese mass was achieved. Furhtermore, the cheese mass was transferred to the table to be streached and manually salted. The salt content and

type of salt were added according to the cheese variant. The cheese mass was then shaped and placed in molds. The ripening conditions for Kačkavalj cheese were at a temperature of 15-18 °C and a relative humidity of 75-85 %. The cheese ripening was carried out for 6 months. The following cheeses were produced: control cheese (C) produced according to the usual technological production process with the addition of sodium chloride (2 %); cheese with 30 % reduced sodium chloride content (R); cheese with 30 % sodium chloride replaced by potassium chloride (M).

Microbiological and bioactivity (ABTS, DPPH and ACE inhibition) cheese analysis

Microbiological analysis was conducted according to Mirkovic et al. (2021). To investigate the antioxidant activity of the obtained lyophilized cheese protein extracts, methods for determination of antioxidant capacity by inhibiting ABTS radicals according to Re et al. (1999) and the determination of free-radical capacity using DPPH radicals with a modified method according to Xiao et al. (2020) was used. Antimicrobial testing of lyophilized cheese protein extracts was performed using a modified method according to Campos et al. (2022). The angiotensin converting enzyme inhibition assay was performed according to the method of Sahingil et al. (2014), which is based on the potential of the protein extract to inhibit ACE activity. Cheese analyzes were performed in triplicate during six months of cheese ripening.

Analysis of total nitrogen, water-soluble nitrogen, phosphotungstic acid soluble nitrogen and pH value

The analysis of the content of water-soluble nitrogen (WSN) was carried out according to the method of Kuchroo and Fox (1982). The content of nitrogen soluble in 5 % (v/v) phosphotungstic acid (PTA-SN) was analysed according to the method of Stadhousers (1960). Total nitrogen (TN) content was determined according to the Kjeldahl method (FIL-IDF, 2014). Digital pH meter Consort C 931 (Consort, Turnhout, Belgium) was used to determine the pH value of cheese samples. All analyses were performed in triplicate.

Statistical analysis

One-way and two-way ANOVA was performed to analyze data using the SPSS program (IBM SPSS Statistics 21). The salting conditions (reduction of NaCl and replacement of NaCl by KCl) and the ripening time were considered as fixed factors. A two-way ANOVA was performed to determine the effect of these fixed factors. A post-hoc Tukey test was performed with a significance level of 0.05.

Results and discussion

Microbiological analysis of cheese

The changes in LAB number showed an increase in the number of bacteria in all cheeses from day 1 to day 30 of ripening (Table 1). The number remained at a high level, from 7.26 to 7.86 log on 30th day and from 7.30 to 7.98 log on 90th day of ripening. Only on the 180th day, a significant decrease in the number of LAB was observed in all cheeses, around 1-2 log units, where significant differences were found in the number of thermophilic LAB species. On day 30, the number of thermophilic and mesophilic LAB species equalizes and remains the same until the 90th day. On the 180th day, the number of LAB decreased to 6 log and the ratio between thermophilic and mesophilic bacteria changes and mesophilic bacteria become dominant.

This change/shift of thermophilic and mesophilic bacteria is expected in view of the technological process. Considering the fact that the cheeses were produced without the addition of starter cultures, thermally treated with 72 to 75 °C, and stretched, the dominance of thermophilic LAB species was expected. But at ripening stages, which took place at lower temperatures of 15 to 18 °C, it is clear that these conditions are not favourable for thermophilic LAB, so mesophilic LAB dominate. It was also found that there were no significant differences in the number of LAB between cheeses, with the exception of thermophilic lactobacilli, where significant differences were observed in cheeses M (cheese with NaCl partially replaced by KCl) and R (cheese with reduced salt content) compared to C (control cheese) at day 180. Changes in the number of mesophilic and thermophilic LAB are understandable taking into consideration that the autochthonous microbiota consists mainly of LAB that are more robust and can withstand much more extreme growth conditions such as the ability to grow at 4 % and even 6.5 % salt (Radulović, 2010). Milosavljevic (2015) showed an increase of thermophilic and mesophilic cocci in cheese until the 30th day and the level of 7.2 to 7.3 log was maintained until the end of ripening of 60 days. Further, regarding lactobacilli, author noted a slight decrease at the beginning, but they also reached their maximum on day 30 and a high level of 7.0 to 7.4 log was also maintained until the end of ripening. Similar to our results, the author also observed stagnation of the LAB count until the end of the ripening process of up to 60 days, while in our results it was found that this stagnation can last even 90 days.

Danilović et al. (2020) examined the microbiota of Kačkavalj cheese from Pirot and found a decrease in the LAB number during first 5 days, thereafter the number increased and remained at a level of about 7 log units. Additionally, the number of thermophilic LAB was lower compared to mesophilic LAB, especially in the later stage of ripening, which is in accordance with our results. Niro et al. (2014) determined an increase in the number of

Table 1. Changes in the number of mesophilic and thermophilic LAB during cheese ripening

Days of ripening	Significance*	C	M	R
Mesophilic LAB (lactobacilli)				
Significance*		a	a	a
1	a	6.67±0.25	6.72±0.24	6.81±0.12
30	b	7.69±0.31	7.79±0.55	7.64±0.19
90	b	7.52±0.26	7.55±0.22	7.49±0.22
180	a	6.44±0.31	6.51±0.29	6.56±0.28
Thermophilic LAB (lactobacilli)				
Significance*		a	b	ab
1	a	7.22±0.10	7.30±0.35	7.37±0.23
30	a	7.26±0.29	7.68±0.35	7.44±0.17
90	a	7.30±0.20	7.51±0.11	7.43±0.31
180	b	5.57±0.43	6.29±0.59	5.90±0.39
Mesophilic LAB (cocci)				
Significance*		a	a	a
1	a	6.85±0.12	7.07±0.19	7.22±0.23
30	b	7.84±0.80	7.85±0.26	7.86±0.14
90	b	7.73±0.27	7.57±0.34	7.55±0.51
180	c	6.54±0.23	6.62±0.26	6.71±0.24
Thermophilic LAB (cocci)				
Significance*		a	a	a
1	a	7.28±0.17	7.41±0.14	7.66±0.22
30	a	7.86±0.30	7.47±0.58	7.81±0.31
90	a	7.98±0.40	7.53±0.48	7.57±0.48
180	b	5.99±0.48	6.01±0.31	6.21±0.42

C - control cheese; M - cheese with 30 % NaCl replaced by KCl; R - cheese with 30 % reduced NaCl.
Values shown are arithmetic mean ± standard deviation.
Different letters (a-c) in the same column or row indicate a statistically significant difference ($p<0.05$).

mesophilic LAB in Caciocavallo cheese, reaching a level of 7 log cfu/g, while thermophilic LAB showed a decreasing trend. Similar to our results, the authors explained this phenomenon with lower ripening temperatures, which are more favorable for the growth of mesophilic LAB (Niro et al. 2014). Samelis et al. (2009) showed that even lower thermal treatments of milk at 60-67 °C for 30 seconds caused a significant reduction in the number of native microbiota LAB in traditional Greek hard cheese. Simov et al. (2006) determined that in the production of Kashakaval, thermal treatment at 72 °C for 2 minutes prior stretching can destroy 84.5 % of the starter culture. The authors showed that despite the high temperatures, the ripening process started with a high number of starter bacteria above 8 cfu/g (Simov et al., 2006). Pappa et al. (2019) investigated the microbiota of cheeses produced from raw and pasteurized milk and showed that bacterial growth is much more pronounced in cheeses made from raw milk. Similar to our results, these authors observed that the number of both thermophilic and mesophilic LAB exceeded the level of 8 logs on the 30th day of ripening and continued to grow until the 180th day when the number decreases significantly. Simov et al. (2006) showed that when *Lb. casei* is used as a starter for cheese production, this species remains dominant up to 95.2 % on the day 90. In contrast, Balabanov et al. (2023) showed that the initial number of LAB in the production of cheese with different NaCl content (0.7 %, 1.5 % and 3.1 %) was about 4 log cfu/g and that the number of LAB increased sharply until day 30 of ripening and decreased until the end (day 45). Furthermore, the authors showed that the increase of LAB was partially inhibited in cheese samples with a higher NaCl content. Ivanova et al. (2021) showed that the growth of autochthonous bacteria in cheese also depends on different ripening temperatures, which is explained by gradual fermentation and slightly higher pH values.

Proteolytic and pH changes during cheese ripening

During proteolysis, many chemical changes occur, primarily the breakdown of proteins, which are first broken down into primary and then secondary products. Degradation primarily produces polypeptides, then low molecular weight peptides and finally amino acids. The amount of WSN increases during ripening in accordance with protein degradation. In order to observe the extent of proteolysis, it is necessary to monitor the nitrogen fractions formed during ripening. The determination of WSN and the determination of nitrogen soluble in 5 % PTA are methods suitable for the extraction of peptides

Table 2. Proteolytic parameters and pH value during cheese ripening

Days of ripening	Significance*	C	M	R
WSN (%)				
Significance*		a	a	a
1	a	0.46±0.03	0.41±0.09	0.44±0.01
30	b	0.76±0.10	0.86±0.03	0.85±0.10
90	c	0.93±0.15	1.06±0.07	1.05±0.17
180	d	1.33±0.10	1.42±0.25	1.44±0.13
PTA-SN (%)				
Significance*		a	a	a
1	a	0.02±0.00	0.02±0.01	0.03±0.01
30	ab	0.05±0.01	0.06±0.00	0.06±0.02
90	b	0.07±0.01	0.09±0.02	0.09±0.03
180	c	0.17±0.05	0.23±0.11	0.21±0.07
WSN/TN (%)				
Significance*		a	a	a
1	a	12.88±0.83	10.82±2.56	12.36±0.61
30	b	20.08±2.23	22.01±1.32	22.03±2.55
90	c	23.72±4.38	26.88±3.34	26.99±4.70
180	d	31.79±2.16	33.42±6.20	33.31±3.28
PTA-SN/WSN (%)				
Significance*		a	a	a
1	a	5.31±0.14	5.69±2.30	6.08±1.84
30	a	6.49±0.26	6.90±0.49	7.25±2.00
90	a	7.97±0.14	8.47±2.24	8.23±2.19
180	b	12.77±3.28	15.95±5.90	14.07±3.60
PTA-SN/TN (%)				
Significance*		a	a	a
1	a	0.68±0.06	0.64±0.35	0.75±0.23
30	ab	1.30±0.10	1.51±0.07	1.59±0.47
90	b	1.89±0.38	2.23±0.32	2.23±0.78
180	c	4.05±1.23	5.53±2.72	4.76±1.68
pH value				
Significance*		a	b	ab
1	a	5.28±0.04	5.31±0.05	5.30±0.02
30	ab	5.33±0.06	5.36±0.04	5.37±0.03
90	b	5.32±0.09	5.44±0.04	5.40±0.02
180	ab	5.31±0.05	5.35±0.05	5.32±0.01

Values shown are arithmetic mean ± standard deviation.
Different letters (a-b) in the same column or row indicate a statistically significant difference (p<0.05).
WSN – water-soluble nitrogen content; PTA-SN - 5% (v/v) phosphotungstic acid-soluble nitrogen; TN – total nitrogen.

produced during proteolytic changes. The later was used for the extraction of peptides with a low molecular weight of less than 600 Dalton. The content of WSN and nitrogen soluble in 5 % PTA of cheeses with reduced NaCl content and replaced by KCl during ripening are shown in Table 2.

In general, the reduction and substitution of sodium chloride content in Kačkavalj cheese had no significant effect on the analysed parameters of proteolysis. The results for all investigated parameters of proteolysis: WSN (%), PTA-SN (%), WSN/TN (%), PTA-SN/WSN (%), PTA-SN/TN (%) showed that there are no significant differences between variants C, M and R ($p>0.05$). These results indicate that proteolysis proceeded uniformly in the control cheese and the cheese variants with reduced NaCl content and NaCl replaced by KCl. In the study by Yalcin et al. (2021), the PTA-SN (%) of the investigated Kashar cheese was 0.05 % on day 30, which is in accordance with the results in our study, which ranged from 0.05 to 0.06 % for all analysed varieties of cheese. The parameter WSN/TN (%) is also known as the ripening index and represents the content of WSN expressed as a percent of TN. This parameter increases as the ripening of the cheese progresses: from 10.82 to 12.88 % on day 1 to 31.79 to 33.42 % on day 180 for all varieties of cheese. Moreover, the pH value of the cheese also has an effect on the soluble nitrogen content. Therefore, the determination of WSN is suitable for cheeses with small changes in pH during ripening. The cheese ripening index (WSN/TN (%))

on day 30 when cheese reached its maturity was 20.08 %, 22.01 % and 22.03 % for varieties C, M and R, respectively. Pappa et al. (2019) determined that the ripening index of mature cheese ranged from 16.6 to 22.9 %, which is in accordance with our results. Similar results were also observed in the gradual increase of WSN (%) which was around 0.99 % for Kashar cheese on the 60th day of ripening (Yalcin et al., 2021). In the study of Talevski et al. (2017), the pH of Kačkavalj cheese on the 30th day of ripening ranged between 5.32 and 5.40, which is in accordance with our results, as the pH ranged from 5.33 to 5.37 on the 30th day of ripening. Furthermore, in accordance with the results in our study, according to Santa and Srbinovska (2014), the pH of the cheese was in the range of 5.28 to 5.47 during 6 months of ripening. Similar results were shown by Ruzic-Muslic et al. (2011), where the pH of cheese ranged from 5.30 to 5.47, and in the study of Sahan et al. (2008), where the pH of cheese was 5.42 on the 90th day of ripening. A slight increase in pH from day 1 to day 30 during cheese ripening is associated with the activity of LAB that survived after cheese processing. Additionally, the pH increases during this initial ripening period due to protein degradation, resulting in a higher buffer capacity (Talevski et al., 2017).

Antimicrobial activity

On the 30th day of ripening, antimicrobial activity was detected in the most of the R cheeses, followed by the M cheeses, while the lowest antimicrobial activity was observed in C cheeses (Table 3). The best antimicrobial activity (+++) was obtained with R cheeses towards 5 bacterial species: *Pseudomonas aeruginosa, Listeria monocytogenes, Candida albicans, Bacillus spizizenii* and *Staphylococcus aureus*, which was achieved with the lowest concentrations of 25 mg/mL protein extract. In M cheeses, the lowest concentration was sufficient to inhibit only *Bacillus spizizenii.* Regarding the C cheese, it was found that a concentration of 25 mg/mL was insufficient to inhibit any bacteria, and only a concentration of 50 mg/mL inhibited four strains of bacterial species: *Pseudomonas aeruginosa, Yersinia enterocolitica, Candida albicans* and *Staphylococcus aureus*. On day 180, the antimicrobial activity increased for all cheeses, again with the best results obtained for the R cheese, and then the M cheese, while the antimicrobial activity was the least observed in the C cheese (Table 3).

Finally, it was shown that the MIC of 25 mg/mL of R cheese after 180 days was sufficient to inhibit all bacteria

Table 3. *The antimicrobial activity of the investigated Kačkavalj cheese variants expressed as MIC (mg/mL) on 30th day and 180th day of ripening*

Bacterial strain	MIC mg/mL	C 30	M 30	R 30	C 180	M 180	R 180
Escherichia coli	100	+	+	+	+	+	+
	50	-	-	+	-	+	+
	25	-	-	-	-	-	+
Shigella sonneri	100	+	+	+	+	+	+
	50	-	+	+	+	+	+
	25	-	-	-	-	-	+
Pseudomonas aeruginosa	100	+	+	+	+	+	+
	50	+	+	+	+	+	+
	25	-	-	+	-	+	+
Yersinia enterocolitica	100	+	+	+	+	+	+
	50	+	+	+	+	+	+
	25	-	-	-	-	-	+
Listeria monocytogenes	100	+	+	+	+	+	+
	50	-	+	+	+	+	+
	25	-	-	+	-	+	+
Candida albicans	100	+	+	+	+	+	+
	50	+	+	+	+	+	+
	25	-	-	+	-	+	+
Bacillus spizizenii	100	+	+	+	+	+	+
	50	-	+	+	+	+	+
	25	-	+	+	-	+	+
Staphylococcus aureus	100	+	+	+	+	+	+
	50	+	+	+	+	+	+
	25	-	-	+	-	+	+
Enterococcus faecalis	100	+	+	+	+	+	+
	50	-	+	+	-	+	+
	25	-	-	-	-	-	+
Salmonella enteritidis	100	+	+	+	+	+	+
	50	-	-	-	-	+	+
	25	-	-	-	-	-	-

C - control cheese; M - cheese with 30 % NaCl replaced by KCl; R - cheese with 30 % reduced NaCl. MIC - minimum inhibitory concentration.

except *Salmonella enteritidis*, for which the MIC was 50 mg/mL. The MIC values of cheese M on day 180 were almost identical to those of cheese R on day 30, where MIC of 25 mg/mL was inhibitory for five bacterial species, and the MIC was 50 mg/mL inhibitory for the other five. The antimicrobial activity of C cheese was still the weakest observed, although it improved compared to day 30. However, the lowest inhibitory concentration of 25 mg/mL was not present in the C cheese. Such results were to be expected, considering that the number of bacteria was already very high on day 30. The microbiota contributed greatly to the formation of bioactive peptides that showed an antimicrobial effect on day 30 of ripening. Although the number of LAB decreased by day 180, their number was still as high as 5-7 log cfu/g, resulting in even better antimicrobial activity at day 180. Furthermore, the decrease in the number of present bacteria from day 30 to day 180 is explained by their death, releasing intracellular enzymes from the lysed cells that contributed to the further breakdown of larger peptides into smaller ones, resulting in formation of bioactive peptides with antimicrobial activity. It can be concluded that in the later stages of ripening, there is no direct dependence between the number of cultivable LAB cells and antimicrobial activity, as the present enzymes play a key role. The accumulation of bioactive peptides takes time (in our case 30 to 180 days) to increase antimicrobial activity, and the decrease in the number of living cells is a logical consequence. Furthermore, antimicrobial activity in C cheese could be possibly due to the normal salt content that might be also slightly antimicrobial towards autochthonous bacteria.

Several studies have found that the type of milk used for cheese production influences the formation of antimicrobial peptides. For example, Silva et al. (2019) examined the antimicrobial activity of protein extracts from fresh cheese made from buffalo milk, determing the inhibitory activity against *Enterococcus faecalis* (12.5 mg/mL) and *Bacillus subtilis* (25 mg/mL). In contrast, Arruda et al. (2012) found out that casein peptides from cow's milk showed antimicrobial activity against previously mentioned bacteria, but at concentrations 5 and 2.5 times higher, respectively. Silva et al. (2012) also investigated the antimicrobial activity of protein extracts obtained from traditional Brazilian hard cheeses and found that a 4- and 2-times higher concentrations, respectively, of the protein extracts were required to inhibit the mentioned bacteria than for those obtained from buffalo milk. Rizzello et al. (2005) examined the antimicrobial activity of different types of Italian cheese and determined the presence of 32 water-soluble peptides that exhibited antimicrobial activity against the pathogenic bacterial strains *Escherichia coli, Yersinia enterocolitica, Bacillus megaterium, Listeria innocua, Staphylococcus* and *Salmonella* spp. The authors showed that water-soluble extracts from Parmigiano Reggiano, Fossa and Gorgonzola cheeses had no antibacterial effect, while fractions from Pecorino Romano, Canestrato Pugliese, Crescenza and Caprino del Piemonte cheeses contained a mixture of peptides with a high degree of homology and the presence of antibacterial peptides was demonstrated in pasta filata cheeses such as Caciocavallo and Mozzarella. Fialho et al. (2018) extracted and identified antimicrobial peptides from traditional Canastra cheese. 30 identified soluble peptides showed pronounced antimicrobial activity against various pathogenic bacteria, including *Escherichia coli*.

Antioxidant activity

The results of the antioxidant activity showed that the antioxidant capacity of protein extracts from cheese

Table 4. *Antioxidant activity of Kačkavalj cheese using the ABTS and DPPH methods on 30 day and 180 days of ripening*

ABTS			
30 days	**100 mg/mL**	**50 mg/mL**	**25 mg/mL**
C	65.76±9.58^{a}	62.21±0.68^{a}	43.57±2.83^{a}
M	74.95±3.79^{a}	66.58±3.53^{a}	49.60±1.39^{a}
R	76.29±7.35^{a}	66.33±3.92^{a}	49.65±3.55^{a}
180 days			
C	85.27±3.55^{a}	72.66±3.38^{a}	51.88±0.29^{a}
M	87.13±2.76^{a}	77.07±3.32^{a,b}	54.92±7.25^{a,b}
R	90.39±0.23^{a}	81.73±1.80^{b}	64.64±1.99^{b}
DPPH			
30 days	**100 mg/mL**	**50 mg/mL**	**25 mg/mL**
C	53.35±2.13^{a}	51.12±1.52^{a}	29.41±2.56^{a}
M	55.61±4.66^{a,b}	52.48±0.77^{a}	30.11±1.15^{a,b}
R	61.48±1.09^{b}	57.54±2.77^{b}	35.72±3.17^{b}
180 days			
C	68.03±0.99^{a}	60.50±2.59^{a}	39.98±2.50^{a}
M	70.26±2.05^{a}	63.82±2.27^{a,b}	42.10±3.23^{a,b}
R	74.78±1.77^{b}	67.13±2.21^{b}	48.33±1.99^{b}

Values shown are arithmetic mean ± standard deviation.
Different letters ($^{a-b}$) in the same column indicate a statistically significant difference ($p<0.05$).

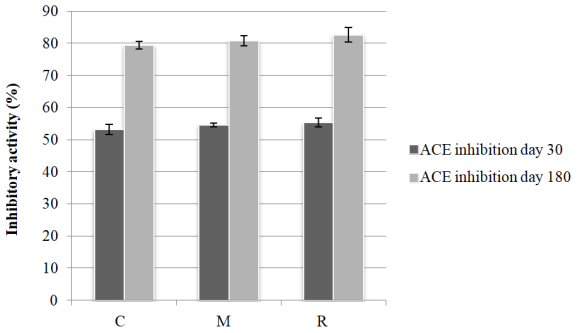
samples was directly proportional to the peptide concentration, reaching values of 43.57 to 49.65 % at a concentration of 25 mg/mL; 62.21 to 66.58 % at a concentration of 50 mg/mL and 65.76 to 76.29 % at a concentration of 100 mg/mL on the 30th day of ripening (Table 4). During ripening, a significant increase in antioxidant activity was observed in all cheeses. Namely, values of 51.88 to 64.64 % at a concentration of 25 mg/mL; 72.66 to 81.73% at a concentration of 50 mg/mL and 85.27 to 90.39 % at a concentration of 100 mg/mL on the 180th day of ripening were detected. The results showed that although the antioxidant values for the R cheeses were the highest, followed by M cheeses, and the lowest for the C cheeses, these differences were not significant at the 30th day of ripening when cheeses reached commercial maturity. However, after 180 days of ripening, significant differences were found at concentrations of 50 and 25 mg/mL, as the R cheeses were significantly different from the C cheeses. These results suggest that the reduction of NaCl content in the six months ripened Kačkavalj cheese leads to significantly better antioxidant activity than in the C cheese.

Perna et al. (2015) obtained similar results by investigating the antioxidant capacity of cheese over 150 days, with the highest values being reached on the last day. Gupta et al. (2009) also found an increase in antioxidant capacity in Cheddar cheese, reaching a maximum in the fourth month of ripening. Yang et al. (2021) showed that the antioxidant capacity of Cheddar cheese increased during ripening and reached the maximum of 84.92 % in the fifth month of ripening. It is known that a greater number of antioxidant peptides was produced during the ripening of cheese, mainly due to proteolytic changes in casein in the first five months of ripening, which contributes to better antioxidant properties. However, proteolytic changes continue during longer ripening processes, allowing active peptides to be degraded to amino acids, which brings hydrolysis and peptide formation to a stable state and thus stabilises the antioxidant properties of protein extracts (Rafiq et al., 2016). The results of antioxidant activity using the DPPH method showed the same trend as the ABTS method, where an increase in antioxidant activity was observed during ripening (Table 4). On day 30, the antioxidant activity ranged from 29.41 to 35.72 % at a concentration of 25 mg/mL; 51.12 to 57.54 % at a concentration of 50 mg/mL and 53.35 to 61.48 % at a concentration of 100 mg/mL. During ripening, the antioxidant capacity increased so that on day 180 the values ranged from 39.98 to 48.33 % at a concentration of 25 mg/mL; 60.50 to 67.13 % at a concentration of 50 mg/mL and 68.03 to 74.78 % at a concentration of 100 mg/mL. Similar results were obtained by Yang et al. (2021) using the DPPH method as the authors determined the maximum values in the fifth month of ripening of Cheddar cheese in the range of 55.90 % and 60.32 %. The analysis of the Kačkavalj cheeses determined that the R cheeses showed the highest values at all concentrations on the 30th and 180th day of ripening. The values of the investigated M cheeses were in between C and R cheeses, while the C cheeses showed the lowest antioxidant properties. Statistical analysis revealed that variant R of cheese differed significantly from the C.

Although similar results were obtained with either ABTS or DPPH methods, it was noted that the values obtained with the DPPH method were slightly lower, which is not unusual. Other authors also reported that the values obtained with the ABTS method were higher than those obtained with the DPPH method (Floegel et al., 2011). The differences between the two methods may be due to the inequality between the structures of the radicals that may react differently depending on the peptides present in the water-soluble extract (Ramirez-Rivas et al., 2022). The application of both methods to test antioxidant activity is justified by the fact that bioactive peptides behave differently towards different radicals depending on the solubility medium. The ABTS radical is known to be water-soluble and easily removed by hydrogen-donating peptides, while DPPH accepts hydrogen in hydrophobic (lipophilic) systems (Phanturat et al., 2010). In our study, it was found that both methods for determining antioxidant activity, ABTS and DPPH, showed results with a similar trend of changes, revealing that the R variant of cheese with reduced NaCl content had the best antioxidant activity during cheese ripening.

ACE-inhibitory activity

The results showed that all three cheese samples exhibited ACE-inhibitory activity on day 30 of ripening, ranging from 53.15 to 55.28 % (Figure 1), with no significant differences. During ripening, ACE-inhibitory activity increased and on day 180 was 79.38 % for the C cheeses, 80.74 % for the M cheeses and 82.57 % for the R cheeses. There were no significant differences between the investigated cheese variants during ripening. The increase in ACE inhibition during ripening was to be expected, considering that proteolysis increases during ripening.



C - control cheese; M - cheese with 30 % NaCl replaced by KCl; R - cheese with 30 % reduced NaCl.
Values shown are arithmetic mean ± standard deviation.

***Figure 1**. ACE inhibitory activity of Kačkavalj cheese on 30 day and 180 days of ripening*

ACE inhibition in the first stages of ripening occurs as a result of the formation of bioactive peptides that remain resistant during further peptide degradation in the later stages of ripening and new ones are additionally formed, leading to an overall higher bioactivity in the later stages of ripening (Meyer et al., 2009). Meisel et al. (2005) showed that ACE-inhibitory activity in medium-aged Gouda cheese is higher compared to young and mature Gouda cheese. In contrast, Saito et al. (2000) showed a slightly higher ACE inhibition in two-year-old Gouda cheese (78.2 %) compared to eight months-old cheese (75.5 %). The great diversity in ACE-inhibitory activity was also showed by Meyer et al. (2009), who compared ACE inhibition in different Swiss cheeses and found that Emmentaler showed a continuous increase in ACE inhibition during 400 day ripening period, while Gruyere showed the highest ACE inhibition in the first stage of commercial maturity and a further decrease in this activity after 9 months of ripening. There is not much data on the influence of NaCl content on the ACE-inhibitory activity of cheeses. Gandhi and Shah (2016) investigated the effect of salt reduction in Akawi cheese and found that the release of the essential amino acids phenylalanine, tryptophan, valine and leucine improved in NaCl reduced samples. However, they did not find the significant differences in the ACE-inhibitory activity of bioactive peptides when the NaCl content was reduced. Similarly, Ayyash et al. (2012) investigated the effect of partial replacement of NaCl with KCl in Akawi cheese and also found no significant differences in ACE-inhibitory activity during ripening. However, Ayyash and Shah (2011) showed significant differences in Mozzarella cheese when part of NaCl was replaced by KCl.

Conclusion

The minimum inhibitory concentration of 25 mg/mL antimicrobial activity was the best for cheeses with reduced sodium chloride in relation to five pathogenic bacteria on day 30 and nine on day 180 of ripening. Reduction of sodium chloride and substitution with potassium chloride in Kačkavalj cheese showed no significant effects on the parameters of proteolysis. The results of the ABTS and DPPH methods showed that the reduction of sodium chloride leads to significantly better antioxidant activity in the sixth month of ripening of Kačkavalj cheese. All cheeses showed no significant differences in ACE-inhibitory activity, which ranged from 53.15 to 55.28 % on day 30 and from 79.38 to 82.57 % on day 180 of ripening, respectively. In conclusion, the cheese variant with 30 % reduced sodium chloride content can be successfully used for the production of Kačkavalj cheese, as it does not deviate in terms of proteolytic changes and shows the best antimicrobial and antioxidant activity. The reduction of sodium chloride in Kačkavalj cheese resulted in a product with health benefits that is even recommended for consumption by consumers, even those from the category of sensitive groups of people. These findings are particularly important for achieving health goals related to salt intake reduction.

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Procjena antimikrobne, antioksidativne i ACE-inhibitorne aktivnosti kačkavalj sira reduciranog natrij klorida

Sažetak

Prekomjerna konzumacija natrij klorida negativno utječe na zdravlje potrošača. Uzimajući u obzir preporuku Svjetske zdravstvene organizacije da se smanji unos soli u prehrani, mikrobiota, proteolitička, antimikrobna, antioksidativna i angiotenzin konvertujući enzim (ACE) inhibitorna aktivnost sljedećih kačkavalj sireva analizirane su tijekom šest mjeseci: kontrolni sir proizveden prema uobičajenom tehnološkom procesu proizvodnje; sir sa 30 % smanjenim sadržajem natrij klorida; sir sa 30 % natrij klorida zamijenjenim kalij kloridom. Broj bakterija mliječne kiseline kod svih se sireva povećao 30. dana zrenja i do 90. dana ostao je na razini od 7-8 log jedinica. Tijekom 180. dana zrenja utvrđene su značajne razlike u broju termofilnih vrsta. Redukcija i supstitucija natrij klorida u kačkavalj siru nije pokazala značajan utjecaj na ispitivane parametre proteolize. Najbolja antimikrobna aktivnost uočena je kod sira reduciranog natrij klorida 30. dana i povećavala se do 180. dana. Redukcija natrij klorida postigla je značajno bolju antioksidativnu aktivnost u šestom mjesecu zrenja kačkavalj sira metodama za određivanje antioksidativnog kapaciteta pomoću inhibicije 2,2'-azinobis/3-etilbenzotiazolin-6-sulfonska kiselina (ABTS) radikala i određivanje slobodno-radikalskog kapaciteta pomoću 2,2-difenil-1-pikrilhidrazil (DPPH) radikala. ACE inhibitorna aktivnost povećavala se tijekom zrenja, ali nije bilo značajnih razlika između proizvedenih varijanti sira. Ova studija ističe veliki potencijal za razvoj proizvoda koji doprinosi zdravlju potrošača i koji odgovara suvremenim trendovima u prehrani ljudi.

Ključne riječi: sir; redukcija natrij klorida; antimikrobna aktivnost; antioksidativna aktivnost; ACE inhibicija

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