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PROTEOLYSIS DURING RIPENING OF TRADITIONAL MONTENEGRIN WHITE BRINED PLJEVLJA CHEESES

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Abstract

Pljevlja cheese is known traditional Montenegrin product that belongs to brined cheeses group. Due to its high nutritional value and long tradition of production and consumption, Pljevlja cheese is a candidate for receiving the sign of protected origin (PDO).

The objective of this study was to analyze composition and proteolysis of Pljevlja cheeses, supplied from 10 producers that produce cheese by traditional technology. Collected cheese samples were analyzed in duplicate for dry matter by the oven drying method, fat by Van Gulik, total protein by Kjeldahl and sodium chloride by Volhard method. The level of proteolysis was monitored by urea polyacrylamide gel electrophoresis (Urea PAGE) and water-soluble nitrogen content (WSN) according to the method of Kuchroo and Fox. All analysis were done at first, 20th, 40th and 60th day of cheese ripening. The differences among cheeses parameters during ripening were determined by LSD test at a 0.05 statistical level using Statistica software (Stat Soft. Inc., Tulsa, USA).

Parameters of proteolysis increased during 60 days of ripening and ranged from 7.45 to 14.23% for water-soluble nitrogen (WSN) as a percentage of total nitrogen (WSN/TN) and from 8.38 to 11.13% for nitrogen soluble in 5% phosphotungstic acid as a percentage of WSN (PTAN/WSN). Degradation of αs casein was more pronounced than of β casein but still not very extensive during all ripening period. Preliminary results of this study indicate that high salt content of cheeses was the main cause of limited proteolysis, observed in all Pljevlja cheese samples. Also, the high variability of the gross composition and proteolysis parameters of Pljevlja cheeses are due to the lack of standardized cheese-making technologies.

It is necessary to continue with additional research on chemical, microbiological and sensory analysis, together with proteolysis, in order to obtain enough relevant data that could be useful for achieving the constant quality of Pljevlja cheese, i.e. to create the necessary preconditions for the development of its standard, as a final aim.

***Key words**: Pljevlja cheese, Proteolysis, Electrophoresis, Soluble nitrogen matter.*

1. Introduction

Traditional food, including cheeses, is a result of accumulated empirical knowledge transfered from generation to generation and represents a cultural heritage of each country. Montenegro is well known by a wide range of traditional dairy products (cheeses, Skorup, and fermented milk). Unfortunately, there isn't any traditional Montenegrin dairy product with standardised technology, which often varies from manufacturer to manufacturer.

White brined cheeses (WBCs) are the most popular cheeses consumed in East Mediterranean region [1]. In Montenegro, WBCs are named according to geographical origin e.g., town or village where they are produced such as: Polimsko-Vasojevički, Ulcinjski, Sozinski, Kučki etc. [2]. However, the most famous WBC in Montenegro is Pljevlja cheese produced mainly in households and small craft dairy plants in Pljevlja municipality, north part of Montenegro. The cheeses are made from whole sheep's, cow's or mixed (sheep's and cows) milk. Negative trends in sheep breeding have caused that the majority of Pljevlja cheese is produced from cow's

milk nowadays [3]. A few studies have been published showing a considerable variation in technology and chemical composition of this type of cheese [3, 4, and 5]. In recent study [3], the certain parameters of raw milk and Pljevlja cheese quality were analysed and traditional technology in cheese making households were, also, recorded. Authors mentioned that despite considerable variations, obtained data are very useful for the development of Pljevlja cheese standard that could serve as a base for Pljevlja cheese to be proposed for Protected Designation of Origin.

In last 20 years, special attention was given to the protection of origin of different traditional food, including cheeses which are connected with some specific regions. As mentioned above, traditional cheeses are characterised with unique composition and sensory properties that are associated to the territory of their origin including the: production methods, climatic conditions, habits, environmental conditions, etc. EU Regulation [6] defines all terms and different levels of food origin protection such as: Protected Designation of Origin (PDO), Protected Geographical Indication (PGI) and Traditional Specialty Guaranteed (TSG). These terms identify the value of food in the specific region and clearly define quality and authenticity of such products. Use of corresponding symbols on the labels of specific food products encourage the diversification of agricultural production, protect the product names from misuse and imitation, and provide consumers with clear and concise information on their origin. The introduction of these terms benefits the rural economy since it boosts farmers' income and maintains the population in less favoured or remote areas [7].

In Montenegro, National regulation about geographical signs of origin was introduced in the Law on protected designation of origin, protected geographical indication and traditional speciality guaranteed [8]. Within all Montenegrin dairy products, Pljevlja cheese is the first in line to be characterised with the sign of PDO or PGI. However, in order to get the protection of origin sign, as well as high price on the market, this product must have standardized technological procedure, as the primary requirement for constant quality and safety of products.

Proteolysis is the most complex and, in most varieties, the most important of the three primary processes that occur during cheese ripening. Ripening agents that contribute to these changes include: rennet, natural raw milk enzyme such as plasmin, and, in the case of traditional products, the most important are enzymes of natural microbiota that originate from raw milk, environment, equipment etc. Rennet and/or plasmin usually participate in primary proteolysis (hydrolysis of caseins to large and medium peptides), while secondary proteolysis is done by enzymes of autochthonous microbiota, which usually consist lactic acid bacteria [9]. Hence, in order to get better knowledge about traditional cheeses, it is important to investigate their ripening process that is responsible for the formation of specific sensory properties.

The useful tools for determination of proteolysis in cheese are urea-polyacrylamide gel electrophoresis (urea-PAGE) of the casein fractions, as well as extraction of the nitrogen fractions soluble in different solutions that released during cheese ripening and their quantification [10]. Also, several examples from the recent literature are given to demonstrate the applicability of electrophoretic techniques as valuable tools for authentication of animal foods with protected geographical status [11]. Studies of proteolysis were conducted for several brined cheeses such as: Feta [12], Turkish Beyaz Penir [13, 14], Iranian WBC [15], Teleme [16], as well as for many other cheese types as: Tounj cheese [17], Beaten cheese [18], etc.

The study represents preliminary investigations of proteolysis parameters during 60 days of Pljevlja cheese ripening. Until now, only studies of the basic chemical composition of cheese milk and Pljevlja cheeses have been carried out, and there has been no research dealing with biochemical changes such as proteolysis during ripening of this cheese. It is considered that the information that this work provide could be the useful tool for determination of cheese ripening stages. Also, it could serve for preventing the insufficiently ripened cheeses from appearing on the market.

2. Materials and Methods

2.1 Materials

Cheeses were collected from 10 households from the area of Pljevlja municipality that are engaged in the production of Pljevlja cheese during several generations. The technology of Pljevlja cheese was previously recorded in details [3]. Briefly, raw milk is used immediately after milking, no starters were added and coagulation lasted 45 - 60 minutes. After slow draining and 6 hours of pressing, the curd is dry salted and put in brine (15 - 18% NaCl) where ripened 2 - 4 weeks at max. 18 ^{0}C. All the analysis were done during 60 days of ripening (after 1, 20, 40 and 60 days).

2.2 Methods

2.2.1 Cheese composition analysis

Grated cheese samples were analysed in duplicate for dry matter (DM) by the oven drying method at 102 ± 2 ^{0}C [19], fat (MF) by Van Gulik method [20], total protein (TP) by Kjeldahl method [21] on a Kjeltec System S3 (Behr, Germany), and sodium chloride (NaCl) by Volhard method [22].

2.2.2 Assessment of proteolysis

Urea polyacrylamide gel electrophoresis (Urea PAGE) was performed according to Andrews's method [23]. Electrophoresis was performed using a vertical slab unit TV200YK (Consort, Belgium) with 100 x 200 x 1 mm slabs, Tris-glycine electrode buffer, a constant current of 60 mA, a maximum voltage of 300V for 3 h, with 4% stacking gel (pH 7.6), and 12 % separating gel (pH 8.9).

Detected polypeptides were identified using the standards of α- and β-casein (Sigma, USA). The gels were stained with a staining solution (0.23% Coomassie Brilliant blue R-250, 3.9% TCA, 17% methanol, 6% acetic acid) for 1.5 h and destained in a de-staining solution (8% acetic acid, 18% ethanol). The gel images were recorded using a scanner HP ScanJer 300 (HP Development Company, L.P. USA).

Scans of the urea electrophoretograms were used to quantify bands using densitometric software ImageJ (National Institute of Health, Bethesda MD, USA). Quantification was based on the measurement of the areas of each peak of β-casein (β-CN), α_s - casein (α_s-CN) and their degradation products as relative percentages. Two electrophoretic ripening indexes were calculated: beta index (sum γ-CN/ β-CN) (Mayer *et al.*, [27]) and, as a modified version of the one proposed by Kalit *et al.*, [17] - alpha index (αs-CN degradation products/αs-CN).

Cheeses were also analysed for water-soluble nitrogen content (WSN) according to the method of Kuchroo and Fox [24], 5% phosphotungstic acid soluble nitrogen (PTAN) according to Stadhousers method [25], and expressed as a percentage of WSN and PTAN of the total nitrogen matter (WSN/TN and PTAN/TN), and also as a percentage of PTAN of the WSN (PTAN/WSN).

2.2.3 Statistical methods

Analysis of variance (one way ANOVA) was done using Statistica 10.0 software (Stat Soft. Inc., Tulsa, USA) and the mean comparisons of parameters during cheese ripening were performed by LSD test, with the level of significance at 0.05.

3. Results and Discussion

3.1 The chemical composition of Pljevlja cheese

The chemical composition of Pljevlja cheese during 60 days of ripening is shown in Table 1.

According to fat in dry matter and moisture in non-fat basis (MNFB), Plejvlja cheeses analysed in this study belongs to full fat and soft cheeses. Changes of compositional parameters of Pljevlja cheeses were not statistically significant except for MNFB that decreased during first 20 days of ripening. The results are in agreement with literature data [3, 4, and 5]. However, as in previous studies, our findings also showed wide variations in the chemical composition of the Pljevlja cheeses produced in different households which were most likely related to the lack of standardised cheese making technology and variation in the milk quality [2, 3].

3.2 Proteolysis

The proteolysis of WBC is affected by the: type of the milk and coagulant used, salt concentration, storage temperature, ripening period and other conditions. High moisture in the curd and a low degree of heat treatment during production of white brined cheese, caused the high level of retained coagulant, compared with other cheese types [26].

Table 1. Chemical composition of Pljevlja cheese during 60 days of ripening

Ripening time (days)	Parameters	n	Proteins (%)	NaCl (%)	Fat in dry matter (%)	Moisture in non-fat basis (%)
1	± Sd	10	14.78 ± 2.11^{a}	2.94 ± 0.63^{a}	49.91 ± 4.91^{a}	70.93 ± 2.60^{a}
	Xmin.		12.53	2.06	42.42	66.66
	Xmax.		19.02	4.28	55.75	74.31
20	± Sd	10	18.00 ± 2.30^{b}	2.79 ± 0.97^{a}	52.51 ± 5.00^{a}	67.28 ± 2.85^{b}
	Xmin.		14.64	1.42	46.42	63.62
	Xmax.		20.83	4.69	60.50	71.43
40	± Sd	10	17.50 ± 2.25^{b}	2.64 ± 1.27^{a}	52.21 ± 4.71^{a}	68.71 ± 4.17^{b}
	Xmin.		13.06	0.58	47.08	62.62
	Xmax.		20.22	4.38	62.07	77.79
60	± Sd	10	17.95 ± 2.50^{b}	2.30 ± 1.78^{a}	54.70 ± 6.37^{a}	66.94 ± 4.03^{ab}
	Xmin.		14.37	0.19	46.97	62.30
	Xmax.		23.19	5.30	66.83	72.66

*Values are expressed as mean ± standard error of means; Means in each column with the same letter did not differ significantly (P > 0.05)

Urea-PAGE electrophoretograms of the protein fractions of the Pljevlja cheese samples after 1, 20, 40 and 60 days of ripening are shown in Figure 1. Changes of β-CN and α_s-CN indexes during ripening are shown in Figure 2.

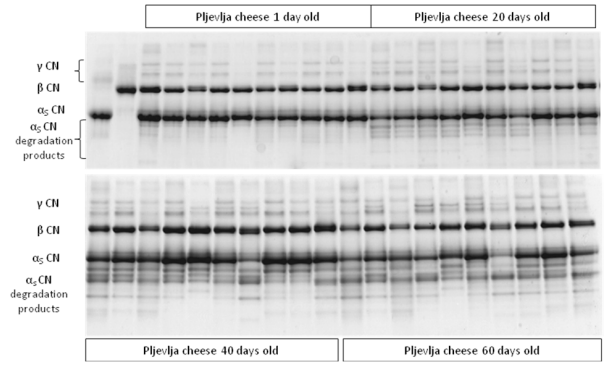

Figure 1. UREA PAG electrophoretogram of Pljevlja cheeses after 1, 20, 40 and 60 days of ripening

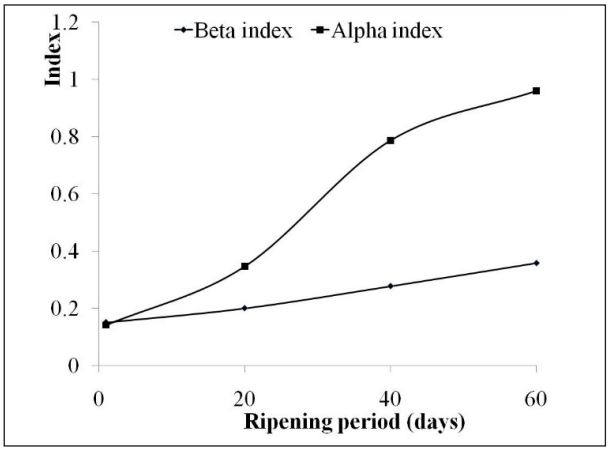

Figure 2. Changes of beta and alpha indexes during 60 days of ripening of Pljevlja cheese. Beta index = γ/β casein; alpha index = α_s degradation products/α_s casein

Protein profiles of Pljevlja cheeses showed two major bands, representing α_s-CN and β-CN. It could be seen from the Figure 2 that both α_s-CN and β-CN contents were reduced during ripening but to a different extent. However, degradation of neither β-CN nor α_s-CN was extensive compared to the other WBC [12, 13, and 16].

In all cheeses, β-CN degradation progressed more slowly and beta index varied from 0.15 at the beginning of ripening to 0.36 at the end of ripening (Figure 3). The average level of residual β-casein after 60 days of ripening (as a % of that of the 1st day) was 71.98%. In all analyzed Parmesan type cheeses [27], where the extensive hydrolysis of β-CN and concomitant accumulation of γ-caseins is typical due to high plasmin activity, beta index is at least 1.3 for 12 months old cheeses which is more than 3 times higher than in 60 days old Pljevlja cheese (0.36). However, the resistance of β-CN to hydrolysis during ripening was found for the many other cheese varieties, especially WBC [13, 18, and 26]. The assumption is that the high salt content and the low pH (both typical for WBC), as well as ripening conditions (temperature, short ripening period etc.), are the reasons for the inhibited plasmin activity, and therefore limited proteolysis of β-CN during ripening. However, for other cheeses made without a starter, slightly higher concentrations of γ-caseins caused by higher plasmin activity were reported in the literature [14].

Degradation of α_s-CN is more pronounced but still not very extensive compared with some other brined cheeses such as Feta and Turkish WBC [12, 13].

The level of residual α_s-CN at the end of ripening was 63.6% while alpha index varied from 0.14 to 0.96. During 60 days of ripening of WBC made from raw cow milk, the content of α_s-CN continually reduced to 63.40% of the initial value of fresh cheese [28]. The degradation of α_s-casein is carried out mainly by residual coagulant and enzymes of non-starter microorganisms after a certain period of ripening, taking into consideration that the traditional cheeses were produced without starter culture. The rate of αs-CN proteolysis may be different because of the usage of different clotting enzymes which could vary in their proteolytic activities [29], as well as different cheese making technologies [30]. The high percentage of salt in the cheese, which inhibits microorganisms and their enzymes, slows down the proteolysis and it is considered to be the biggest obstacle in the ripening of WBC [18]. Parameters analysed in this study also showed that proteolysis differs among the Pljevlja cheeses from producer to producer.

Proteolysis parameters such as: WSN/TN, PTAN/WSN and PTAN/TN are shown in Figure 3. As it was expected, the level of WSN/TN increased in cheeses throughout ripening. The percentage of the WSN/TN fraction increased 1.9 times within 60 days of ripening of Pljevlja cheeses and varied from 7.45 to 14.23% at the end of ripening. The cheeses contained a small quantity of WSN compounds at the beginning of ripening which indicates that proteolytical changes started during curd processing [28]. Data about proteolysis parameters of traditional WBC in West Balkan region are still rare and often show great variability [31, 32]. Miocinovic *et al.* [32], showed that authentic Zlatar white brined cheese has the highest ripening index (37.49%) compared to the other WBC cheeses purchased on market (from 7.85 to 24.57%). This indicates that Zlatar cheese is being placed on the market before the end of its ripening time, most likely due to the economic reason. WSN/TN of Feta cheeses after 60 days of ripening (minimum ripening period according to Greek Codex Alimentarius) varied from 15.88% to 19.58% [12, 26]. Abd El Salam *et al.*, [26], reported that the ripening index (WSN/TN) of numerous WBC usually ranges from 12 to 20% (max. value is 25%) and our data are within this range.

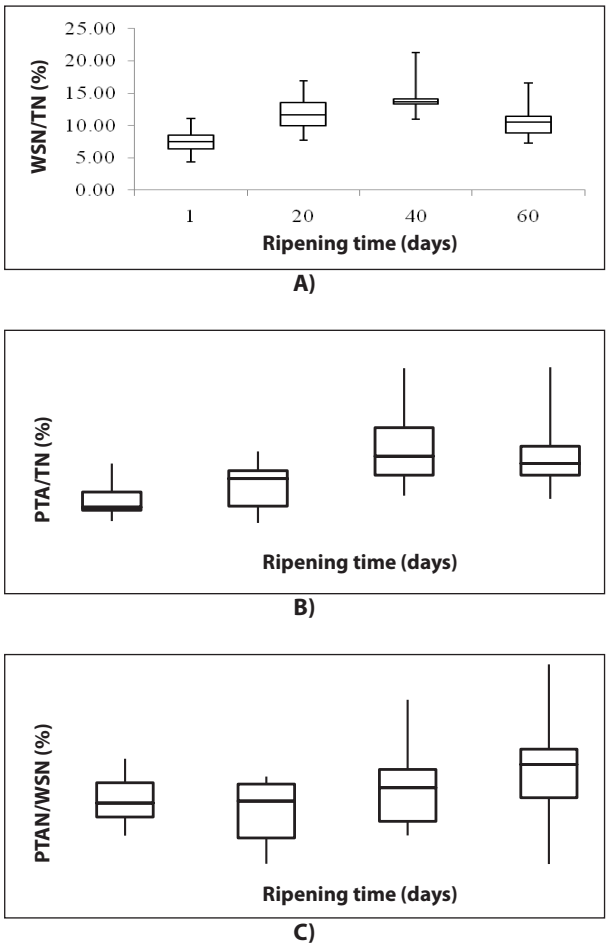

Figure 3. Boxplots. Ripening index (WSN/TN) (A), PTA nitrogen fraction as a percentage of water soluble nitrogen (B), and total nitrogen (C) of Pljevlja cheeses, determined after 1, 20, 40 and 60 days of ripening.

Data in the boxplots in Figure 3. represent: minimum, 75 percentile, median, 25 percentile and maximum. The PTAN fraction in cheese contains small peptides and amino acids mainly from the proteolytic activity of starter (if it is used), and non-starter bacteria and to a lesser extent rennet. PTAN/WSN and PTAN/TN of Pljevlja cheeses were within 8.38 - 11.13% and 0.61 - 1.27%, respectively. The PTAN/TN content in white brined cheeses is usually within the range of 3 - 5% and presented results correspond to this interval [26]. Moatsou *et al*., [12] found that after 60 days of Feta cheese ripening 13.3 - 23.6% of PTAN/WSN. The results of the present study indicate that primary and secondary proteolysis parameters (WSN/TN, PTAN/WSN and PTAN/TN) were lower then found in literature for the other WBCs [12, 13, and 26]. Secondary proteolysis rate depends on proteolytic agents that originate from cheese microbiota, salt concentration as well as ripening conditions such as temperature. Cold ripening may also contribute to the low level of PTAN as a parameter of the accumulation of amino acids and low molecular mass nitrogenous compounds [12].

4. Conclusions

- The study provides information on composition and proteolysis parameters during 60 days of ripening of Pljevlja cheeses produced in 10 different households. Pljevlja cheeses belong to full fat and soft cheeses, and its composition did not change significantly during ripening.

- The level of proteolysis parameters such as WSN/TN PTAN/TN and PTAN/WSN determined in this study are in agreement with literature data for brined cheeses. Preliminary results of this study indicate that relatively low level of both primary and secondary proteolysis was mostly due to high salt content. The high variability of the gross composition and proteolysis parameters of Pljevlja cheeses are due to the lack of standardized cheese-making technologies.

- It is necessary to continue with additional research on chemical, microbiological and sensory analysis, together with proteolysis, in order to obtain enough relevant data that could be useful for achieving the constant quality of Pljevlja cheese, i.e. to create the necessary preconditions for the development of its standard, as a final aim.

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