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The effect of in vitro digestion on antioxidant properties of water-soluble and insoluble protein fractions of traditional Serbian white- brined cheeses

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Abstract

The influence of simulated in vitro digestion on antioxidant potential of protein fractions of traditional Serbian white-brined cheeses was investigated. Water-soluble (WSF) and water- insoluble fractions (WINF) of three cow's and three ovine white-brined cheeses were fractionated. Total antioxidant capacity, reducing power and iron (II) chelating properties of these fractions before and after in vitro digestion were assayed. The investigated protein fractions had different antioxidant properties. WSFs had a better total antioxidant capacity and reducing power and less pronounced iron (II) chelating properties than WINFs. A strong negative correlation (-0.818, $P<0.05$) between the total antioxidant capacities of undigested WSF and WINF of traditional cheeses were observed. *In vitro* digestion greatly improved the total antioxidant capacities of WINFs (by 16.61-34.18 times), their reducing power (up to 95.77 %) and except in the case of Svrljig ovine cheese, the iron (II) chelating ability as well. A less pronounced increase (up to 71.29 %) of the total antioxidant capacity of WSFs was induced by in vitro digestion. In vitro digestion reduced reducing power of WSF of investigated ovine cheeses as well as reducing power of WSF of Homolje cow's cheese. Since there was no significant correlation between the investigated antioxidant properties of digested WSFs and their free amino acids and mineral contents the observed differences should be attributed to different composition and properties of low molecular weight peptides. Thus, further investigations related to their isolation and characterization needs to be conducted. However, these results indicate that Serbian white-brined cheeses have great potential as source of antioxidant peptides.

Key words: **cheese, protein fractions, antioxidant properties, in vitro digestion**

Introduction

Free radicals and reactive oxygen species produced in cells during oxidative metabolism are involved in initiation or progress of several degenerative diseases including cancer, atherosclerosis, Alzheimer's, Parkinson's and diabetes (Aluko, 2012). Control of oxidative stress seems to be one of the crucial steps in slowing down the progress of these diseases or preventing their complications. Besides other well-known natural food compounds (such as vitamin C, polyphenols, flavonoids and carotenoids), proteins and peptides from different sources of animal and plant origin have been recognized as antioxidants.

Research conducted over the past twenty years have shown that cheeses are potentially good sources of proteins and peptides with antioxidant activities (Gomez-Ruiz et al., 2002; Corrêa et al., 2011; Meira et al., 2012; Pattom and Hongsprabhas, 2013; Timón et al., 2014, 2018; Pisanu et al., 2015; Erkaja and Sahingil, 2015). A part of these proteins and peptides originates from milk itself, but most of them are released during cheese production, especially during ripening (Gupta et al., 2009; Mushtaq et al., 2015, Meira et al., 2012; Kumar et al., 2013; Pisanu et al., 2015). It is also known that the level of their formation is determined by numerous factors including conditions of milk heat treatment, cheese ripening conditions (type and activity of proteolytic agents, conditions and duration of ripening process) (Hernández-Galán et al., 2017; Barać et al, 2017; Basilicata et al., 2018). Cheese peptides with antioxidant properties are usually low molecular weight peptides (up to 20 amino acids) and commonly belong to water-soluble protein fraction. Therefore, there is a large number of studies related to antioxidant properties of this fraction. However, it is known that pure caseins and serum proteins exert antioxidant properties (Hernandez-Lendesma et al,, 2011; Power et al., 2013; Çekiç et al., 2015) which were explained by their ability to free radical scavenging. Barac et al (2016, 2019a) showed that water-insoluble protein fractions of cow`s and goat white cheeses prepared from high heat-treated milk expressed antioxidant properties. In the work of Barac et al. (2019b) it was shown that traditional Serbian white-brined cheeses had significant antioxidant potential. However, cheese is a complex food system composed of protein matrix in which different components including fat, fatty acids, vitamins, minerals and polyphenolic compounds are chemically, non-covalently or physically incorporated. In such complex systems antioxidant activity cannot be considered as simple summation of the activity of their constituents (Apak et al., 2016). Thus, it can be assumed that antioxidant properties of whole cheese are somewhat different than those of isolated protein fractions. The aim of this investigation was to characterize antioxidant properties of purified protein fractions of previously investigated traditional Serbian white-brined cheeses and the effect of in vitro digestion on these properties. These findings could be helpful for better understanding their role in antioxidant potential of white-brined cheeses.

Material and methods

This study covers water-soluble (WSF) and water-insoluble nitrogen fractions (WINF) of four two-month old Serbian traditional white-brined cheeses prepared from ovine and cow`s milk. Cheeses are produced in households and named according to the region of production (Sjenica, Homolje, Zlatar and Svrljig cheese). Whole raw milk was used for production without the application of heat treatment or the addition of starter culture. Traditionally liquid rennet is used for milk coagulation. Further processing of curd includes self-pressing, pressing, salting and ripening in brine. All of these operations differ among individual cheese makers regarding used pressure and the amount of salt. Thus, variations in chemical composition of cheeses can be observed. Nevertheless, all types of Serbian white-brined cheeses are characterized by high acidity and sharp and salty flavour. Gross composition and other characteristics of these cheeses were reported previously by Barac et al. (2019b). The protein fractions of investigated cheeses were isolated according to the following procedure: a grounded cheese (15 g) was extracted in 45 mL of Ultrapure water (Ultrapure water system, SG ver.1.11, Waters, Milford, MA, USA) tempered at 40 °C. To preserve the extract, a drop of formaldehyde was added. The extraction was

carried out in an ultrasound bath (Clifton, UK) for 90 min. After that period, the extract was cooled in the freezer for 1 h and centrifuged for 15 min at 4000 g (Janetzki, Prague, Czech Republic). Then, the upper layer was carefully removed, and the supernatant was filtered through Whatman No 1. To further remove any impurities, the obtained filtrate (WSF) was filtered through a 0.45-µm-pore-size filter (Millipore, Billerica, MA, USA) and lyophilized. The precipitate (WINF) was rinsed out with three portions of 5 mL of Ultrapure water. To remove any residual lipids, the WINF was treated with n-hexane for one hour, filtered through Whatman No.1, dried at room temperature overnight and lyophilized.

In vitro simulated digestion

Isolated cheese protein fractions were subjected to in vitro gastrointestinal digestion as described by Petrat-Melin et al. (2015). Briefly, the digestion consisted of a two-step static system with a simulated gastric phase using porcine pepsin (Sigma Aldrich, St. Louis, MO, USA) at pH 2.0 for 60 min, followed by a simulated duodenal phase. In the duodenal phase, the pH was increased to 6.5 by the addition of 55 mM of $NaHCO_3$, and digestion was carried out for 120 min with porcine pancreatin (Sigma Aldrich). Equal enzyme activities were used for both steps, corresponding to a w/w ratio of enzyme to protein of approximately 1 to 200. After digestion, the enzymes were inactivated by a heat treatment at 90 °C for 5 min and immediately cooled in an ice bath. Before digestion and after each step of digestion, 50 µL of the reaction mixture was sampled and diluted with a sample buffer (Tris-HCl pH 6.6) for SDS-PAGE electrophoresis and then frozen at -20 °C.

The digestion process was monitored by an SDS-electrophoresis according to the method of Fling and Gregerson (1986) on 12.5 % resolving gels and 5 % stacking gels as previously reported by Barac et al. (Barac et al., 2013; 2019a). The total free amino acid levels of protein fractions before and after *in vitro* digestion were determined as suggested by Hayaloglu (2008) and reported by Barac et al. (2019b). The results were expressed as mg Leu per gram of lyophilized sample by reference to a standard curve which was first prepared using Leu (Sigma Chemical Co., St Louis, MO, USA) at various concentrations (0.050-0.500 mg Leu mL^{-1} water).

Mineral profiles of digested fractions

The mineral content in protein digested fractions was determined by atomic absorption spectroscopy (AAS). Contents of Fe, Cu and Zn in lyophilized digested fractions were detected after the destruction in concentrated HNO_3 and $HClO_4$ with the addition of 33 % H_2O_2 by wet ashing (Jones, 2001). Contents of Ca and Mg were detected after the destruction with concentrated acids HNO_3 and HCl by dry ashing (Jones and Case, 1990). Contents of P and K in lyophilized samples were determined after the destruction with concentrated acids H_2SO_4 and $HClO_4$ by dry ashing. Phosphorus was determined by the colorimetric method, whereas K was determined by the flame-photometry method (Jones, 2001).

Antioxidant properties of digested and undigested cheese protein fractions

The total antioxidant capacity (TEAC) of WSF and WINF of the investigated cheeses was measured according to the QUENCHER method by Serpen et al. (2008), as previously described in detail (Barac et al., 2019a, b). The Trolox equivalent antioxidant capacity (TEAC) was expressed in mmol of Trolox per kg of DM. The reducing power of undigested and digested fractions was assessed as suggested by Meira et al. (2012) whereas their ferrous ion chelating ability was determined using ferrozine method (Lee et al., 2007) and expressed as the concentration (mg/mL) of proteins that is required for 50 % of ferrous ions chelating (IC_{50} value).

Statistical analysis

All measurements were performed at least in triplicate. Data were subjected to one-way analysis of variance (ANOVA) with IBM-SPSS v20 software (IBM Corp., Armonk, NY, USA) and the comparison of means was done by Tuckey's test at $P<0.05$. Pearson's correlation was performed to appreciate and interpret interactions between variables through the linear correlation coefficient.

Results and discussion

Antioxidant properties of WSF and WINF of traditional cheeses before and after *in vitro* digestion were evaluated by three different methods: radical scavenging activity, reducing power and iron (II) chelating ability. The obtained results are presented in Table 1.

Total antioxidant capacity of cheese protein fractions

Protein fractions of traditional Serbian white-brined cheeses had significantly (P<0.05) different radical scavenging capacity. Also, significant differences (P<0.05) between TEAC values of investigated protein fractions before and after in vitro

TABLE 1. The radical scavenging capacity, reducing power and iron (II) chelating ability of undigested and in vitro digested water-soluble and water-insoluble protein fractions of Traditional Serbian white-brined cheeses.

Cheese	Total antioxidant capacity (TEAC)*				
	mmol Trolox Eq/kg				
	WSF		WINF		
	B.D.	A.D.	B.D.	A.D.	
Cow					
	Sjenica	44.98±1.63^{a,C}	47.62±0.32^{d,B}	16.95±0.61^{c,D}	425.90±9.44^{a,b,A}
	Homolje	46.20±0.35^{a,C}	51.98±0.91^{b,B}	3.06±0.11^{e,D}	323.02±1.86^{d,A}
	Zlatar	40.46±2.08^{b,C}	48.32±0.38^{c,B}	9.76±0.29^{d,D}	333.64±8.79^{d,A}
Ovine					
	Sjenica	32.48±0.96^{c,C}	54.59±1.39^{a,B}	19.7±0.65^{b,D}	447.44±10.12^{a,A}
	Homolje	30.88±1.13^{c,C}	49.22±0.96^{c,B}	21.71±0.52^{a,b,D}	360.65±7.24^{c,A}
	Svrljig	31.63±0.38^{c,D}	54.18±1.05^{a,C}	23.43±1.45^{a,B}	409.81±13.45^{b,A}
	Reducing power (A_{700})				
Cow					
	Sjenica	0.263±0.002^{a,C}	0.298±0.001^{a,B}	0.331±0.01^{a,B}	0.648±0.02^{a,A}
	Homolje	0.215±0.004^{d,B}	0.191±0.007^{c,C}	0.157±0.003^{d,D}	0.282±0.07^{c,A}
	Zlatar	0.226±0.001^{c,C}	0.282±0.007^{b,A}	0.176±0.002^{b,D}	0.265±0.001^{d,B}
Ovine					
	Sjenica	0.234±0.008^{c,A}	0.206±0.015^{b,B}	0.163±0.006^{c,C}	0.230±0.01^{e,A}
	Homolje	0.254±0.003^{b,B}	0.207±0.012^{b,C}	0.167±0.003^{c,D}	0.274±0.002^{c,A}
	Svrljig	0.235±0.005^{c,B}	0.189±0.003^{c,C}	0.168±0.009^{c,D}	0.314±0.003^{b,A}
	Fe-chelating properties IC_{50} (mg/mL)				
Cow					
	Sjenica	41.76±0.11^{c,A}	39.73±0.21^{b,B}	21.85±0.25^{e,C}	18.95±0.19^{d,D}
	Homolje	45.75±0.1^{b,B}	40.09±0.3^{b,C}	63.56±0.4^{a,A}	37.50±0.32^{a}
	Zlatar	37.20±0.36^{d,B}	33,6±0.22^{c,C}	47.29±0.39^{b,A}	21.97±0.17^{c,D}
Ovine					
	Sjenica	85.85±0.45^{a,A}	45.96±0.64^{a,B}	37,68±0.10^{c,C}	25.44±0.62^{b,D}
	Homolje	13.09±0.14^{f,D}	17.72±0.42^{d,C}	27.34±0.13^{d,B}	25.00±0.26^{b,A}
	Svrljig	17.46±0.11^{e,B}	10.07±0.24^{e,C}	10.98±0.44^{f,C}	24.94±0.58^{b,A}

*TEAC, Trolox equivalent antioxidant capacity; IC50, protein concentration needed to chelate half of the added Fe(II) ions; B.D., before digestion; A.D., after digestion; data within the same column marked with different lowercase letters are significantly different at P<0.05; data within the same row and the same parameter marked with different uppercase letters are significantly different at P<0.05

digestion were detected. Generally, undigested WSF of investigated cheeses had higher ability to scavenging free radicals than undigested WINF; TEACs of WSF were ranged from 30.88 to 46.20 mmol Trolox/kg (Table 1) whereas TEACs of undigested WINF were 3.06 to 23.43 mmolTrolox/kg (Table 1). However, insoluble fraction represents approximately about 80 % of ripened cheese proteins and according to the obtained results the antioxidant capacity of this fraction could not be ignored. Furthermore, it is interesting to note that the cheeses characterized by higher TEAC values of the WSF fraction had a lower antioxidant capacity of the water-insoluble fraction. Thus, correlation analysis showed strong negative correlation (-0.818, P<0.05; Table 2) between TEAC values of undigested WSF and WINF of traditional cheeses.

According to data presented in Table 1 undigested WSFs of cow cheeses had higher TEAC potential (40.46 mmolTrolox/kg - 46.20 mmolTrolox/kg) than WSFs of traditional ovine cheeses (30.88 mmolTrolox/kg - 32.48 mmolTrolox/kg) which is in a agreement with the results of Öztürk and Akin (2018) who reported the influence of milk type on radical scavenging capacity of WSF of traditional Tulum cheeses. But, bearing in mind that: (a) processing history of investigated autochthonous cheeses differs (Dozet et al., 2006) and (b) the autochthonous non-starter bacteria play an essential role in the ripening of traditional Serbian white-brined cheeses (Barać et al., 2013, Smiljanić, 2014), the observed differences should be at least in part attributed to these factors. This is consistent with the observations of Revilla et al. (2016) who showed that total antioxidant capacity was significantly correlated with season of manufacturing and time of ripening but not with animal species providing the milk and Sieber et al (2010) who pointed to non-starter lactic acid bacteria as the main cause of the different functional effects of traditional cheeses.

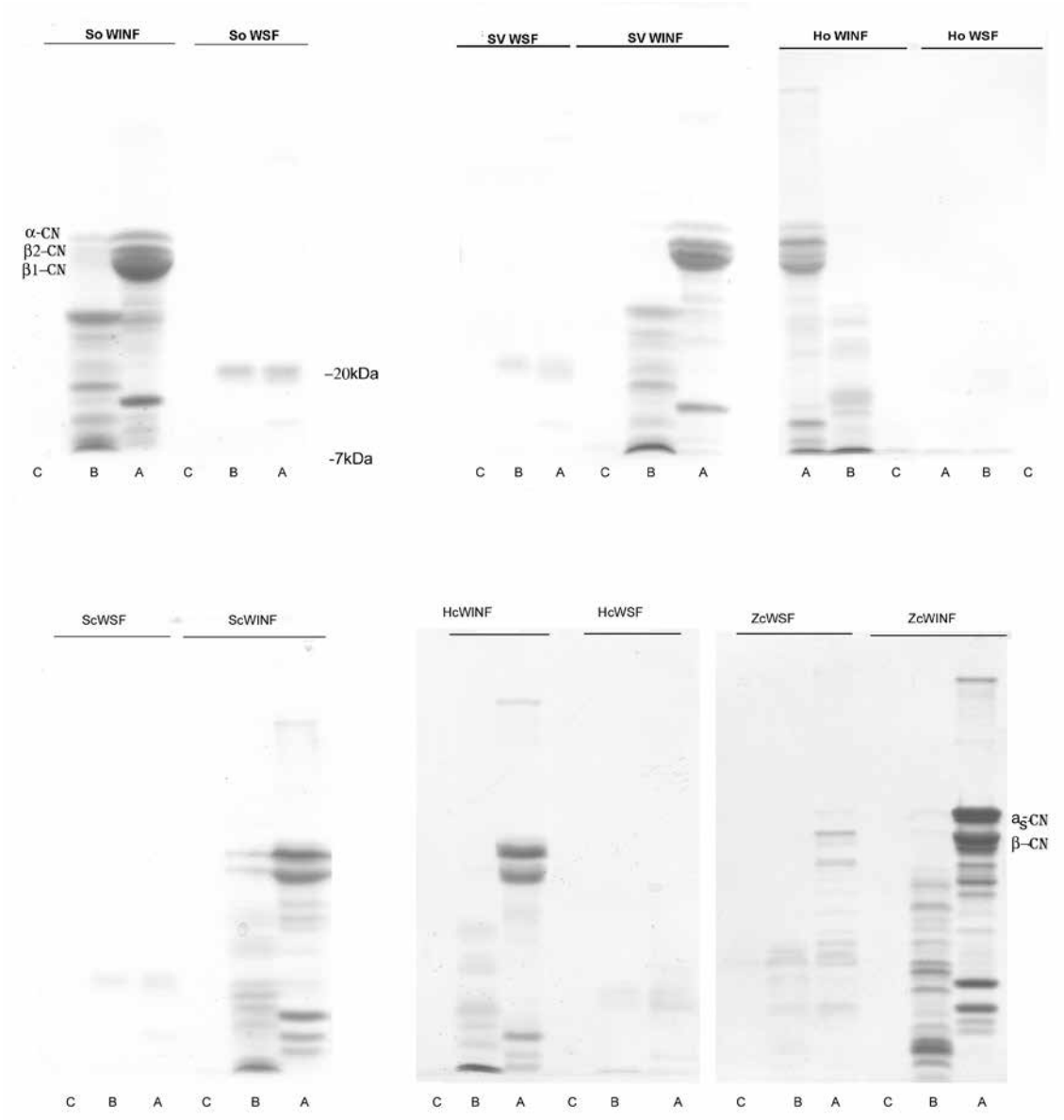
The observed difference between total antioxidant capacities of undigested WSF and WINF fractions as well as between samples within the same fraction can be attributed to their compositions. Namely, both investigated fractions are complex systems. WSF fractions are composed of residual whey proteins, low molecular weight peptides derived from casein hydrolysis, free amino acids, weakly bounded caseins and minerals which can also act as antioxidants (Pavia et al., 2000; Malatou et al., 2004; Barac et al., 2016; Revilla et al., 2016). According to current knowledge the major contributors to radical scavenging of WSF are low molecular weight peptides and free amino acids released during ripening (Power et al., 2013; Santiago-López et al., 2018). Fig. 1 shows that the SDS–PAGE profiles of WSF peptides before digestion, except WSF of Zlatar cow`s cheese, are quite similar and consist of two diffused low molecular weight peptides (7 kDa, 20 kDa). Considering that, different antioxidant capacity of WSFs should be attributed to different properties and composition of small peptides which cannot be detected using

TABLE 2. Statistically significant (P<0.05) correlations between investigated parameters

Parameters		Coefficient of correlation (r)
Content of Ca in WSF a.d.	Content of P in WSF a.d.	0.951
TEAC of WSF b.d.	TEAC of WINF b.d.	-0.818
TEAC of WINF b. d.	Chelating ability of WINF b.d.	-0.890
RP of WINF b.d.	RP of WINF a.d.	0.981
Chelating ability of WINF bd.	Chelating ability of WINF a.d.	0.855
Content of P WINF a.d.	Content of Zn in digested WINF	0.959
Content of K in WINF a.d.	RP of WINF a.d.	0.984
Content of Cu WINF a.d.	Chelating ability of WINF a.d.	0.896

TEAC - total antioxidant capacity; RP - reducing power; WINF - water-insoluble fraction; WSF - water-soluble fraction; b.d. - before digestion; a.d. - after digestion

SDS-PAGE and free amino acids. These compounds are formed during secondary proteolysis due to activity of non-starter lactic bacteria (Abd El Salam et al., 1993). Figure 3 shows that free amino acid contents of undigested WSF of cheeses are significantly ($P<0.05$) different. WSF of ovine cheeses had higher free amino acid contents (17.10-22.30 mg/g) than WSF of cow cheeses (7.10-10.20 mg/g; Figure 2). But, no significant correlation between free amino acid content and total antioxidant capacity was observed. Moreover, from Table 1 and Figure 2 it can be seen that WSF of ovine cheeses with lower TEAC than WSF of cow cheeses had higher content of free amino acids. This indicates that free amino acids are not as effective free radical scavenging agents as low molecular weight peptides. This is consistent with the observation of Elias et al (2008). The effectiveness of these peptides compared with free amino acids, Samaranayaka and Li-Chan (2011) attributed to the unique chemical



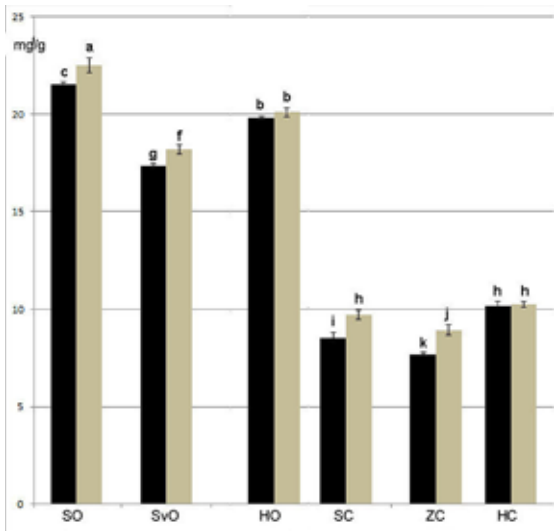
*So - Sjenica ovine cheese, Sv - Svrljig ovine cheeses, Ho - Homolje ovine cheese, Sc - Sjenica cow`s cheese, Hc - Homolje cow`s cheese, Zlatar cow`s cheese

FIGURE 1. SDS-PAGE of water-soluble (WSF) and water-insoluble fractions of Traditional cheeses before and after in vitro digestion

and physical properties conferred by their sequences, especially the stability of resultant peptide radicals that do not initiate or propagate oxidative reactions. The SDS-profile of undigested WSF of Zlatar cow cheese (Figure 1) contained several high molecular weight peptides and β-CN which can also contribute to antioxidant capacity but to the lesser extent than small peptides (Pritchard et al., 2010). The presence of these compounds in WSF of Zlatar cheese can partly explain its lower TEAC value (40.46 mmolTrolox/kg) comparing to the other investigated cow cheeses (44.98 and 46.20 mmolTrolox/kg).

Opposite to WSF, antioxidant capacity of WINF encourages from proteins and peptides tightly incorporated to complex gel matrix. Table 1 shows that undigested WINF of ovine cheeses had significantly higher (P<0.05) TEAC values (19.70- 23.43 mmolTrolox/kg) than their cow`s cheese counterparts (13.06-16.05 mmolTrolox/kg). However, the more pronounced differences were observed within WINFs of cow`s then within WINFs of ovine cheeses. This is in consistence with the SDS-profiles of these fractions. As evident from Figure 1 protein profiles of undigested WINF of ovine cheeses are quite similar whereas qualitative difference between profiles of cow`s WINFs can be observed. Although undigested WINF of Zlatar cheese had more polypeptides than the WINF fractions of other cow cheeses (Figure 1) the ability of this sample to scavenge free radicals was the lowest. This can be attributed to different influence of several factors including amino acid composition, amino acid position in protein sequence, three-dimensional structure and molecular weight of protein and their hydrophobicity (Aluko, 2012; Zilic et al., 2012; Liu et al., 2018).

In vitro simulated gastrointestinal digestion improved TEAC of both fractions. The average TEAC values of digested WSF and WINF fractions were in the range of 47.62-54.59 mmol Trolox Eq/kg and 323.02-447.40 mmol Trolox Eq/kg, respectively (Table 1). In other words, in vitro digestion improved the initial TEACs of WSF by 5.87 %-71.29 % and those of WINF by 16.61-34.18 times. In vitro digestion had particularly favorable effect on insoluble fractions whose antioxidant capacity became more than six times higher than the corresponding digested WSFs. Consequently, it seems that insoluble cheese fractions have a key antioxidant role during white cheese consumption. Similar effect of proteolysis and digestion on antioxidant capacity of different type of cheeses and their WSN fractions are documented (Liu et al., 2018). However, the improved radical scavenging capacity observed after simulated digestion of WSF of traditional white cheeses was in disagreement with the results reported by Bottesini et al. (2013). These authors reported that antioxidant capacity of WSF of ripened Parmigiano-Reggiano cheese was unaffected by simulated gastrointestinal digestion. This inconsistency could be attributed to different composition of WSF extracts which is in turn result of several factors including processing history of cheese and ripening conditions. Undigested WSF of Parmigiano-Reggiano cheese was mainly composed of free amino acids whereas WSF of investigated cheeses contained peptides within the range of 7-20 kDa. These peptides completely disappeared after digestion with pancreatic enzymes (Figure 1). This induced small increase of FAA content in most of digested WSF extracts (Figure 2). Thus, higher values of TEAC of digested WSFs could not be attributed only to the increase of FAA content



*data marked with different lowercase letters are significantly different at p<0.05; So - Sjenica ovine cheese, Sv - Svrljig ovine cheeses, Ho - Homolje ovine cheese, Sc - Sjenica cow`s cheese, Hc - Homolje cow`s cheese, Zlatar cow`s cheese.

FIGURE 2. The change of free amino acid content of water-soluble fractions of Traditional cheeses induced by in vitro digestion

but also to small peptides released during digestion and those resistant to further degradation. This is supported by the absence of significant correlation ($P<0.05$) between FAA content and TACs of digested WSFs.

Table 1 shows that despite the lower initial TEAC values, both digested fractions of ovine cheeses generally had higher total antioxidant capacity than corresponding digested fractions of cow cheeses. TEAC value of digested WSN fractions of cow and ovine cheeses was in the range of 47.62-51.98 mmol Trolox Eq/kg and 49.22-54.59 mmol Trolox Eq/kg, respectively. The average TEAC of WINF of ovine cheeses was 360.65-447.44 mmol Trolox Eq/kg whereas TEAC of digested WINFs of cow cheeses was 333.64-425.90 mmol Trolox Eq/kg. More intensive increase of WSF of sheep cheeses (59.39 %-60.07 %) than cow cheeses (5.87 %-12.51 %) suggests different resistance of low molecular peptides responsible for free radicals scavenging. It seems that a part of initial and/or newly released antioxidant peptides of WSF of cow cheeses are degraded further which causes lower TEAC values than might be expected. In addition, Figure 1 shows that SDS-profiles of undigested WINF of 60-days ripened cow and ovine cheeses differ. The SDS-profiles of WINF of cow cheeses are composed of almost equal ratio of α_s-CN and β-CN whereas insoluble fractions of ovine cheeses are mainly composed of β-CN and to a lesser extent of α_s-CN. In electrophoretic system used in this study residual β-CN of cow`s cheeses is registered as a single band whereas ovine β-CN is detected by two bands which correspond to multi-phosphorylated forms: dominant β1 and less intensive β2-casein. β1-casein has greater electrophoretic mobility at alkaline pH and higher phosphorus content than β2-casein (Selvaggi et al., 2014). However, after pepsin digestion both residual caseins almost completely disappeared (except in the case of Sjenica cow and ovine cheeses) and profiles are mainly composed of high- and medium-size peptides which were completely hydrolysed due to pancreatin activity. The results of SDS-PAGE suggest that the major contributors to free radical scavenge capacity of digested WINFs are low molecular weight peptides and free amino acids. The SDS-PAGE also suggests that the most of these peptides and free amino acids in digested WINFs of ovine cheeses arise from β-CN, especially β1-CN. Since free amino acids had low ability to scavenge free radicals (Khan et al., 2019) these peptides are the main cause of the higher TEAC values of ovine digested WINFs in comparison to WINFs of cow cheeses.

Reducing power and chelating ability of undigested and digested protein fractions

The investigated protein fractions of traditional cheeses had significantly different ($P<0.05$) reducing power and Fe (II) chelating ability. In general, WSFs had higher reducing power than WINFs (Table 1). The only exception was WIN fraction of Sjenica cow cheese which had higher reducing power (0.331) than WSF of all cheeses. Moreover, the less pronounced differences between reducing power of WSFs (0.215-0.263) than between WINF values (0.157-0.331) were observed. Reducing power of WSF of investigated cheeses was in the range or better than those reported for WSF of several sheep cheeses from Brazil and Uruguay (0.131-0.306; Meira et al., 2012). In current literature there are no reports related to reducing power of WINF of cheeses. In opposite to reducing power, WINF of almost all cheeses were more capable to chelate Fe^{2+}-ions than WSF. IC_{50} of WINF ranged from 10.98-63.56 mg/mL whereas IC_{50} of WSF was in the range of 13.09 to 85.85 mg/mL. Furthermore, WINF of ovine cheeses had more favourable chelating ability than WINF of cow`s cheeses; the IC_{50} of WINF of ovine cheeses was 10.98-37.68 whereas IC_{50} of WINF of cow's was 21.85-63.56 mg/mL. Better chelating ability of ovine WINFs can be attributed to the high content of β1-CN form, which is highly phosphorylated form of caseins. Studies have shown that phosphoserine residues and inorganic phosphate present in casein expressed high capability to bind iron and other divalent metal ions (Kitts, 2005; Rival, 2001). The best ability to chelate iron had WINF of Svrljig ovine cheese and WSF of Homolje cow cheese.

In vitro digestion differently influenced reducing power of cheese protein fractions. Digestion slightly (by 13.31-23.83 %) improved reducing power of WSF of Zlatar and Sjenica cow cheeses whereas values of other digested WSFs were approximately 10-20% lower than undigested counterparts. On the contrary, digestion improved reducing power of

all WINFs by 9.59 %-95.77 %, indicating that digestion of WINF with pepsin and pancreatin released peptides that were more capable to act as electron donors (Sarmadi and Ismail, 2010; Correa et al., 2014). Thus, correlation analysis showed very strong positive correlation (0.981; P<0.05, Table 2) between reducing power of undigested and digested WINFs. The highest reducing power had digested WINF of cow cheese from Sjenica (0.648, Table 1).

After digestion, Fe(II) chelating ability of both fractions of the most of traditional cheeses increased. However, decreased Fe(II) chelating ability of digested WSF of Homolje and WINF of Svrljig ovine cheese were registered. Regarding Fe(II) chelating ability, literature reports for digested protein of cheese are scarce. Furthermore, current literature reports different data about effect of digestion on Fe(II) chelating ability of milk products. The improved Fe(II) chelating ability was observed for digested whey concentrate, heat-denatured whey concentrate, skim milk protein hydrolysates, casein hydrolysates, whey protein isolate (Peng et al., 2010; Wang et al., 2011) and cheese (Liu et al, 2018). This was attributed to increased concentration of carboxylic acid groups and increased exposure of certain amino acids, such as histidine, which is a well-known metal-chelation amino acid (Ramirez et al., 2005). On the other hand, Correa et al. (2011) and Meira et al. (2012) showed that short peptides induced by extensive proteolysis might lose their ability to complex with Fe^{2+}-iron. Since the extensive hydrolysis induces lower total antioxidant capacity (Botesini et al., 2016), a strong negative (-0.890; P<0.05) correlation between total antioxidant capacities and the IC_{50} values obtained for undigested WINF fractions is consistent with the observation of these authors. Namely, it can be assumed that WINF with the lower TEAC value will have also the lower chelating ability. Regardless of the observed differences, both fractions showed an effective capacity for Fe^{2+} chelating, suggesting that it may have potential as an effective antioxidant.

Mineral content of digested fractions

The studies of Lucas et al. (2008) and Revilla et al. (2016) showed impact of some of minerals on TEAC of water extracts of cheese. Thus, we investigated mineral distribution but into digested protein fractions and their possible influence on antioxidant properties of water-soluble and water-insoluble protein fractions. The obtained results are shown in Table 3.

As evident, during separation of fractions most of investigated minerals were distributed mainly into water-soluble fractions. The only exception was Fe in the case of Homolje and Svrljig ovine cheeses; the content of Fe in digested WINFs of these ovine cheeses was 52.32 mg/kg and 51.60 mg/kg whereas in their WSFs the content of Fe was 18.41 mg/kg and 49.06 mg/kg, respectively (Table 2). It seems that in these cheeses Fe was more tightly incorporated into casein matrix than in other cheeses and the most of this mineral retained in water-insoluble fraction. Also, this could be attributed partly to the higher pH observed in these cheeses (4.88-5.18; Barac et al., 2019b) which can contribute to lower solubility of iron.

According to Table 2 digested WSFs of cow cheeses had higher contents of Ca, P and K than digested WSFs of sheep cheeses. In contrast, the contents of trace elements in digested WSFs were quite uniform. Also, relatively similar content of both macro and microelements in digested WINFs can be observed. The only exceptions were the slightly higher content of Fe detected in digested WINF of sheep cheeses (16.49-52.32 mg/kg) than in digested WINFs of cow cheeses (10.52-11.96 mg/kg) and the much higher content of K detected in WINF of Sjenica cow cheese (0.91 %) than in the other digested WINFs.

A correlation analysis showed only a few significant correlations between mineral contents and investigated antioxidant properties (Table 3). Within digested WSFs only very strong correlation (0.951, P<0.05) between contents of Ca and P was observed whereas no particular significant influence of any investigated minerals can be noticed. In opposite, significant positive correlation (0.959, P<0.05) between the contents of P and Zn of digested WINFs was observed. Also, the content of K was significantly (0.984; P<0.05) correlated with reducing power of digested WINFs as well as the content of Cu and Fe (II) chelating ability (0.896, P<0.05).

TABLE 3. Mineral content of digested water-soluble and insoluble protein fractions

Cheese		WSF						WINF					
	P	K	Ca	Fe	Cu	Zn	P	K	Ca	Fe	Cu	Zn	
		%		mg/kg			%			mg/kg			
Ovine cheese	Sjenica	1.58^{b}	0.78^{b}	2.49^{d}	27.60^{d}	3.87^{c}	85.47^{a}	0.59^{b}	0.04^{b}	0.16^{b}	16.49^{b}	2.08^{b}	11.29^{e}
	Homolje	1.2^{c}	0.78^{b}	1.61^{e}	18.41^{e}	2.51^{d}	75.92^{b}	0.60^{b}	0.08^{b}	0.12^{b}	52.32^{a}	1.96^{c}	14.13^{d}
	Svrljig	1.42^{b}	0.45^{c}	2.57^{d}	49.06^{b}	4.08^{b}	43.99^{f}	0.73^{a}	0.04^{b}	0.34^{a}	51.60^{a}	2.02^{b}	33.41^{a}
Cow cheese	Sjenica	2.03^{a}	1.03^{a}	4.77^{a}	62.48^{a}	4.47^{a}	68.38^{c}	0.70^{a}	0.91^{a}	0.22^{b}	11.96^{c}	1.09^{e}	23.48^{c}
	Homolje	2.23^{a}	0.95^{a}	4.30^{b}	21.82^{e}	3.89^{c}	55.94^{e}	0.71^{a}	0.04^{b}	0.31^{a}	10.52^{c}	2.86^{a}	31.34^{b}
	Zlatar	2.01^{a}	0.96^{a}	4.10^{c}	32.10^{c}	3.70	60.20^{d}	0.70^{a}	0.04^{b}	0.35^{a}	11.40	1.67^{d}	31.40^{b}

*data marked with different lowercase letters are significantly different at P<0.05; WSF- water-soluble fraction, WINF-water-insoluble fraction.

Conclusions

Results of this study showed different antioxidant properties of protein fractions of Serbian traditional white-brined cheeses. Major source of antioxidant peptides before digestion was WSF. Undigested WSFs had superior total radical scavenging capacity and reducing power than WINFs whereas undigested WINFs of most of investigated cheeses had better chelating properties. In vitro simulated gastrointestinal digestion improved TEAC values of both fractions but to the different extent. As result of digestion, TEAC of WSFs increased by 5.87-71.29 % whereas the digested WINs had 16.61-34.18 times higher radical scavenging capacity than undigested counterparts. Despite the lower initial values, both digested fractions of ovine cheeses had higher total antioxidant capacities than cow`s counterparts. Digestion improved reducing power of all WINFs (by 9.59 %-95.77%) as well as reducing power of WSF of Zlatar and Sjenica cow cheeses. Digestates of almost all fractions, except WSF of Homolje and WINF of Svrljig ovine cheese had an improved Fe(II) chelating ability. These results clearly indicate that besides nutritive value traditional white cheeses may have a significant role in the maintenance of human antioxidant defense systems. However, further investigation related to characterization of peptides responsible for antioxidant activities should to be conducted.

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Utjecaj in vitro digestije na antioksidativna svojstva u vodi topljivih i netopljivih proteinskih frakcija tradicionalnih srpskih bijelih sireva u salamuri

Sažetak

Ispitan je utjecaj simulirane *in vitro* digestije na antioksidativni potencijal proteinske frakcije tradicionalnih srpskih bijelih sireva u salamuri. Iz bijelih sireva u salamuri (tri kravlja i tri ovčja) frakcionirane su u vodi topljive (VTF) i u vodi netopljive proteinske frakcije (VNTF). Analiziran je ukupni antioksidativni kapacitet, sposobnost redukcije i svojstva keliranja željeza (II) ovih frakcija prije te nakon *in vitro*

digestije. Ispitane proteinske frakcije imale su različita antioksidativna svojstva. VTF su imale bolji ukupni antioksidativni kapacitet, sposobnost redukcije i manje izražena kelirajuća svojstva željeza (II) u odnosu na VNTF. Uočena je snažna negativna korelacija (-0,818, P<0,05) između ukupnih antioksidacijskih kapaciteta neprobavljenih VTF i VNTF tradicionalnih sireva. *In vitro* digestija uveliko je poboljšala ukupan antioksidativni kapacitet VNTF (16,61-34,18 puta), njihovu sposobnost redukcije (do 95,77 %), i osim u slučaju svrljiškog ovčjeg sira, sposobnost keliranja željeza (II). Manje izražen porast (do 71,29 %) ukupnog antioksidativnog kapaciteta VTF izazvan je *in vitro* digestijom. Smanjenje sposobnosti redukcije nakon *in vitro* digestije uočeno je kod svih VTF ispitivanih ovčjih sireva, kao i kod homoljskog sira od kravljeg mlijeka. Kako nije bilo značajne korelacije između ispitivanih antioksidativnih svojstava digestivnih VTF i sadržaja slobodnih aminokiselina i minerala, uočene razlike treba pripisati različitom sastavu i svojstvima peptida niske molekulske mase. Stoga je potrebno provesti daljnja ispitivanja vezana za njihovu izolaciju i karakterizaciju. Međutim, ovi rezultati ukazuju da srpski bijeli sirevi u salamuri imaju veliki potencijal kao izvor antioksidativnih peptida.

Ključne riječi: **sir, proteinske frakcije, antioksidativna svojstva, *in vitro* digestija**

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