



Comparison of some functionalities of water soluble peptides derived from Turkish cow and goat milk Tulum cheeses during ripening

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Abstract

In this study, profiles and functional properties such as antioxidant, mineral binding, and antimicrobial activities of water-soluble peptides from Turkish goat milk Tulum cheese and cow milk Tulum cheese were examined during the ripening period. According to the results of RP-HPLC, the number of peptides increased as the ripening days progressed due to proteolysis. DPPH results indicated that the antioxidant activity of peptides increased as the ripening days progressed for these cheeses. However, the highest antioxidant activity of peptide extracts was found in goat milk Tulum cheese according to the DPPH assay. The highest Iron (II) binding activity of peptide extracts was determined in goat milk Tulum cheese on the 60th day. Peptide extracts obtained from goat milk Tulum cheese on the 90th day demonstrated an inhibitory effect against *Salmonella typhimurium* ATCC 14028.

Keywords: antimicrobial activity; antioxidant activity; bioactive peptide; mineral binding activity; Tulum cheese.

Practical Application: Determination of the effect of ripening time on the functional properties of water soluble peptides in Tulum cheese.

1 Introduction

A great number of cheese types are manufactured in certain geographic areas in the world and are consumed regionally in vast amounts. It was identified that more than 150 types of cheeses were found in Turkey (Akın, 2010). The most consumed and produced cheeses in Turkey are White pickled, Kaşar, and Tulum cheeses (Akın, 2010). According to the data of the Turkish Statistical Institute (2014), the annual production amount of Turkish Tulum cheese was 14,179 tonnes. Its production has increased considerably, and this growth in production has been kept up by a large number of cheese plants and mostly individual farmers in recent years.

Tulum cheese is one of the most famous Turkish cheeses. It has a granular or open texture; is semi-hard and white or cream in color; and has a buttery taste and tangy flavor. All these properties can change with the processing and ripening conditions. Tulum, which means “goat’s skin bag” in Turkish, is made from goat’s skin. The skin bag is utilized as a packaging material that has a small pore to control air and moisture transfer, thus improving microbial activity. Wild flora of raw milk is used in Tulum cheese production in order to obtain a traditional flavor, and therefore, starter cultures are not used. Fermentation starts from the drainage of whey and continues until the end of the pre-ripening period for about 15 days. In this fermentation process, salt is also added to about 2.5%, and lactic acid is produced from lactose to control microbial growth. Thus, microorganisms that are selected under these conditions become dominant in the cheese media, and they start to produce some flavor components through glycolysis,

proteolysis, and lipolysis. After pre-ripening, the Tulum cheese is transferred into a cooled room at about 2 °C - 4 °C to control fermentation and enzymatic reaction.

Cheese ripening occurs as a result of various microbiological, biochemical, and metabolic processes (Farkye, 2004). Proteolysis, which occurs for as long as the cheese ripening, is the most complicated and significant biochemical process (Sousa et al., 2001). Large, medium-size, and small-size peptides, as well as free amino acids are formed in consequence of all proteolytic reactions that are brought about during ripening. Small-size peptides and amino acids caused by further proteolytic reaction are effective for the development of cheese flavor (McSweeney, 2004).

Bioactive peptides are special protein components consisting of 2 - 20 amino acids in length, and most of them demonstrate multifunctional features (Rutherford-Markwick & Moughan, 2005). Most investigations (Korhonen & Pihlanto, 2006; Pritchard et al., 2010) reported that bioactive peptides had a variety of physiological functions, such as immunomodulator, antithrombotic, antioxidative, mineral binding, antihypertensive, and antimicrobial effects.

Bioactive peptides are obtained from various dairy products, especially fermented foods such as cheese, kefir, and yoghurt. Owing to their high protein content, cheeses can be a natural source of peptides derived from milk proteins. A variety of proteolytic regulations and the effectiveness of proteolytic reactions during cheese ripening give rise to the formation of milk-derived peptides (Park et al., 2007). Secondary proteolysis that takes place in course of the ripening period may cause the occurrence of

Received 25 Apr., 2017

Accepted 06 Aug., 2017

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different peptides, and bio-functional activities occur depending on the ripening stage of the cheese (Haque et al., 2008).

Various enzymes, such as milk enzymes, rennet enzyme (chymosin), and microorganisms' or starter cultures' enzymes, found in cheese flora lead to the hydrolysis of casein (α_{s1} -, α_{s2} -, β - and κ -caseins). Cheese may have rich bioactive peptides depending on the variety of the enzymes (Pihlanto, 2006). However, these enzymes and microflora are affected by heat treatment (Raynal-Ljutovac et al., 2007). The numbers, varieties, and functions of bioactive peptides that take place during the ripening period can be affected by this condition. Pisanu et al. (2015) determined that specific sequences, functions, and relative numbers of peptides differed significantly in raw and pasteurized sheep milk cheeses. The peptides occur through proteolysis during cheese ripening. Proteolysis in cheese is influenced by various parameters, including milk varieties used in the cheese production, enzymes such as plasmin from milk, chymosin from rennet, proteases from microbial flora or starter culture, cheese production process, acidity of the curd, salt to moisture proportion, and ripening time and temperature (Park & Jin, 1998).

There are no investigations on the bioactive properties of peptides isolated from Turkish Tulum cheeses. Turkish Tulum cheese produced with raw milk was considered to be likely to present peptides with different amounts of bioactivities. In this study, Turkish Tulum cheeses produced with raw cow and goat milk were investigated to determine whether the peptides had antimicrobial, antioxidative, and mineral binding or chelating properties. The main objective of this research was to evaluate the effects of milk type and ripening periods on profiles and functional properties of water-soluble peptides isolated from Turkish Tulum cheeses. Cow and goat milk Tulum cheeses were compared in terms of potential bioactivity and abundance of peptides during the ripening period. Novel bio-functional dairy peptides, which are more active and functional than formerly studied bioactive peptides, were explored to determine which one provides better health advantages to consumers.

2 Materials and methods

2.1 Experimental design and Turkish Tulum cheese making

Goat and cow milk were fermented by its wild microflora. The calf rennet (Peyma-Chr. Hansen's Inc., Istanbul, Turkey) whose coagulation strength had 1:16000 MCU mL⁻¹ was added at a level of 0.1% (v/v), and coagulation took place at approximately 32 ± 2 °C, for about 60 min. The coagulum was cut into pieces (about 3 cm³) and moved into cotton bags for whey drainage. Drainage was performed at room temperature for 12 h, and the curd was put under pressure. After this stage, the curd was broken into pea-sized pieces, salted (3.5%, w/w), and kept for 2 - 3 days at room temperature. Afterward, goat skin bags (the skin bags prepared and cleaned specially for this process) were tightly filled with salted and pea-sized curd at 2 different dairies (as cow milk cheese and goat milk cheese samples), and the samples packaged in goat skin were ripened for 120 days. Tulum cheeses were manufactured in duplicate, and the Tulum

cheese samples were analyzed once in every 0, 15, 30, 60, 90, and 120 days.

The Tulum cheeses were stored and ripened at 4 °C and were sampled from the different skin bags (4 kg) at each sampling time (0, 15, 30, 60, 90, and 120 d) after the surface area (around 0.5 to 1.0 cm) was thrown out.

2.2 Evaluation of physicochemical properties of cheese milk

In determination of the compositional characteristics of goat milk and cow milk samples used in cheese production, pH, titratable acidity, dry matter, protein, fat and ash analyses were performed by using Kirk & Sawyer (1991) method.

2.3 Determination of physicochemical changes of Tulum cheeses during ripening period

Dry matter content was examined by the gravimetric method, drying the sample to a constant weight at 103 ± 2 °C, following the International Dairy Federation (1982) standard. The Gerber-Van Gulik method using the cheese butyrometer (Funke Gerber, Germany) was utilized for examining fat content (International Organisation for Standardization, 2008). Ash was determined using the AOAC International method (Association of Official Analytical Chemists, 2002), whereas the Fox (1963) method was employed for determining Sodium chloride content. The pH meter WTW-315i (Germany) was used for pH determination. Titratable acidity was measured according to Richardson (1985). Total nitrogen (TN) in the Tulum cheeses was measured by the Kjeldahl method (Association of Official Analytical Chemists, 2000). The CR400 color measurement equipment (Minolta, China) was used for performing color analysis. According to CIELAB color scale, L*, a*, and b* values were measured (Pinho et al., 2004). Water activity was measured using the AquaLab 3 TE (America) equipment (Lang & Steinberg, 1980). All determinations were performed in duplicate.

2.4 Extraction of Tulum cheese peptides

Water-soluble extracts (WSEs) were obtained pursuant to the modified version of the method identified by Kuchroo & Fox (1982). Tulum cheese samples (10 g) were homogenized in 20 mL of deionized water for 10 min using a Stomacher (Mayo, homogeneous, Italy). The resulting homogenates were held at 4 °C for 1 h in a water bath (Memmert WB22, Germany). The insoluble materials were then separated by centrifugation (Nuve, NF800R, Germany) at 10,000 × g for 30 min at 4 °C. Finally, the acquired supernatants were filtered by a membrane filter with 0.45 µm diameter to remove residual suspended fat.

2.5 Profiling of Tulum cheese peptides by RP-HPLC

WSEs prepared from cheese samples during the ripening period were mixed (1:1, v/v) with deionized water containing 0.2% trifluoroacetic acid, and filtered through a 0.45 µm cellulose acetate filter. After filtration, samples were made ready for analysis, after being put into HPLC vials. To this end, the ThermoFinnigan Spectrasystem HPLC system was used. Chromatographic separation was done using a Phenomenex

Jupiter C18 (250 mm × 4.6 mm, 5 µm, 300 Å) (Phenomenex, Torrance, CA, USA) RP-HPLC column. Mobile phase A was deionized water containing 0.1% trifluoroacetic acid (v/v), and mobile phase B was acetonitrile containing 0.1 trifluoroacetic acid (v/v). The measurements were made at 214 nm. Finally, the gradient elution was performed (Topçu & Saldamli, 2006).

2.6 DPPH radical scavenging method for WSE

The 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) was performed by modifying the method of Bhandari et al. (2010) to examine antioxidant activity of water-soluble peptides. 0.1 mL of a sample, 0.9 mL tris hydrochloride (HCl) buffer, and 2 mL of 0.1 mM DPPH were added in spectrophotometer cuvettes. The cuvettes were then kept at room temperature for 30 min without light. Control value was determined by using distilled water (free extract). After 30 min, the scavenging activity was monitored spectrophotometrically by the reduction in absorbance at 517 nm. All experiments were carried out in triplicate. The results were calculated according to Equation 1.

$$\text{Scavenging rate(\%)} = [1 - (\text{Absorbans}_{\text{sample}} / \text{Absorbans}_{\text{control}})] \times 100 \quad (1)$$

2.7 Iron (II) chelating/mineral binding activity

In order to determine mineral binding activity, ferrous ion chelating ability of water-soluble extracts was examined. Ferrozine method with some modifications was used for the ferrous ion chelating ability (Tang et al., 2002). A volume of 2 mL of WSE was mixed with 0.1 mL of 2 mM iron / ferrous (II) chloride and 0.2 mL of 5mM ferrozine (3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4- triazine) in spectrophotometer cuvettes. The cuvettes were then incubated in the dark at room temperature for 10 min. After 10 min, the absorbance was read at 562 nm. All experiments were carried out in triplicate. Distilled water was used as a control. Chelating activity of sample mixture was calculated as Equation 2:

$$\text{Chelating activity(\%)} = [1 - (\text{Absorbans}_{\text{sample}} / \text{Absorbans}_{\text{control}})] \times 100 \quad (2)$$

2.8 Antimicrobial activity

The pH values of WSEs were adjusted to 4.6 by using 1 N HCl before antimicrobial activity analysis. A disk-diffusion assay was used with modifications to observe the antimicrobial activity of the WSEs (Bauer et al., 1966). *Escherichia coli* ATCC 25922, *Listeria monocytogenes* ATCC 7644, *Staphylococcus aureus* ATCC 25923, *Clostridium perfringens* ATCC 13124, *Bacillus cereus* ATCC 11778, and *Salmonella typhimurium* ATCC 14028 were used to determine antimicrobial activity. All cultures belonged to Provincial Control Laboratory in Konya, Turkey. Water-soluble extracts (5, 10 and 20 µL) were moved into paper disks with 6 mm diameter (Whatman No. 1), and allowed to dry at room temperature in the laminar flow cabin. Deionized water was used as a negative control. Cultures of the different strains were activated using Brain-Heart broth. Two hundred microliters of cultures were added into 10 mL of Brain-Heart broths and the cultures were incubated overnight

at 37 °C. Thereafter, they were implemented at the surface of a petri dish containing the Brain-Heart agar medium, and dishes were allowed to dry in the laminar flow cabin. Whatman disks including water-soluble extracts were placed at the surface of inoculated petri dishes. Eventually, petri dishes were incubated at 37 °C for 24 h - 48 h to identify possible inhibition zones. In order to examine the antimicrobial activity, diameters of inhibition zones were measured.

2.9 Statistical design

The means of results were analyzed with repeated measures design and the means were compared through the Duncan's multiple range test with a confidence interval set at 95% (Minitab Inc., 1991).

3 Result and discussion

3.1 Compositional characteristics of cheese milk

The compositional characteristics of milk used in the cheese production are shown in Table 1. According to the results, titratable acidity and pH values of milk samples were between 0.14 - 0.16 and 6.65 - 6.71, respectively. Cow and goat milk were determined to be within the limits of literature in terms of dry matter, fat, protein and ash contents (Haenlein & Caccese, 1984; Jenness, 1980).

3.2 Physicochemical profile of Tulum cheeses during ripening period

Physicochemical parameters of the Tulum cheeses at determined ripening period has been demonstrated in Table 2. It was examined that cow milk Tulum cheese had a higher fat content ($P < 0.01$) due to the milk composition. The fat ratio did not differ in both cheeses during the ripening period. Changes in the salt content during the ripening period demonstrated varied statistical significants ($P < 0.01$) and the salt content was higher in goat milk Tulum cheese ($P < 0.05$) as was the Ash content ($P < 0.01$). This could have been due to higher mineral matter and salt content in goat milk. Furthermore, different ash values in the ash analysis, as an indicator of mineral and salt content, could have resulted from the different titratable acidity and pH values, which affected the absorption of salt. After the 15th ripening day, water losses were insignificant for each cheese, and dry matter content was higher in cow milk Tulum cheese ($P < 0.01$), especially due to the high fat content. Goat milk Tulum cheese had a higher total nitrogen content ($P < 0.05$). Generally, total nitrogen content remained stable during the ripening period. The pH values of both cheeses were not the

Table 1. Compositional characteristics of cheese milk (n=2).

Characteristics	Cow milk	Goat milk
Dry matter (g 100 g ⁻¹)	11.80 ± 0.17	12.05 ± 0.37
Fat (g 100 g ⁻¹)	3.50 ± 0.10	3.24 ± 0.06
Protein (g 100 g ⁻¹)	3.12 ± 0.09	3.37 ± 0.18
Ash (g 100 g ⁻¹)	0.80 ± 0.03	0.75 ± 0.04
pH	6.65 ± 0.05	6.71 ± 0.03
Titratable acidity (% lactic acid)	0.16 ± 0.02	0.14 ± 0.01

Table 2. Physicochemical changes in cow and goat milk Tulum cheeses during ripening period (n=2).

Physicochemical properties	Samples ¹	Ripening period (days)						Overall mean
		0	15	30	60	90	120	
Dry matter (g 100 g ⁻¹)	CMTC	63.50 ± 1.12 ^{abc}	65.43 ± 0.00 ^{***}	66.01 ± 0.00 ^{bc***}	67.31 ± 0.16 ^{ab***}	68.21 ± 0.00 ^{***}	67.12 ± 0.00 ^{ab***}	66.26 ± 0.08 ^{***}
	GMTC	47.90 ± 0.07 ^{***}	51.41 ± 0.30 ^{***}	53.90 ± 0.69 ^{***}	53.01 ± 0.00 ^{ef***}	52.05 ± 0.00 ^{***}	54.19 ± 0.81 ^{c**}	52.08 ± 0.08 ^{b***}
Fat (g 100 g ⁻¹)	CMTC	33.50 ± 0.35 ^{ns}	34.17 ± 1.18 ^{ns}	32.88 ± 0.18 ^{ns}	33.00 ± 0.00 ^{ns}	33.00 ± 0.00 ^{ns}	34.88 ± 0.18 ^{ns}	33.57 ± 0.13 ^{**}
	GMTC	13.38 ± 0.18 ^{ns}	13.42 ± 0.12 ^{ns}	13.13 ± 0.18 ^{ns}	13.20 ± 0.00 ^{ns}	13.25 ± 0.07 ^{ns}	13.50 ± 0.71 ^{ns}	13.31 ± 0.13 ^{**}
Ash (g 100 g ⁻¹)	CMTC	4.62 ± 0.55 ^{ab}	5.25 ± 0.11 ^{ghb}	6.16 ± 0.00 ^{ef*}	5.55 ± 0.13 ^{fg*}	6.80 ± 0.00 ^{**}	5.01 ± 0.08 ^{gh*}	5.57 ± 0.09 ^{***}
	GMTC	7.64 ± 0.47 ^{d*}	8.05 ± 0.17 ^{cd*}	8.79 ± 0.19 ^{bc*}	8.99 ± 0.79 ^{b*}	11.27 ± 0.00 ^{**}	8.34 ± 0.05 ^{bcd*}	8.85 ± 0.09 ^{***}
Salt (g 100 g ⁻¹)	CMTC	3.18 ± 0.35 ^{cd***}	3.33 ± 0.52 ^{cd***}	3.16 ± 0.00 ^{cd***}	2.81 ± 0.16 ^{d**}	3.94 ± 0.00 ^{bc***}	3.83 ± 0.04 ^{cd**}	3.37 ± 0.10 ^{**}
	GMTC	4.95 ± 0.78 ^{ab**}	4.88 ± 0.16 ^{ab**}	3.42 ± 0.13 ^{cd**}	5.30 ± 0.17 ^{a**}	4.84 ± 0.00 ^{ab***}	4.06 ± 0.06 ^{bc**}	4.57 ± 0.10 ^{**}
Titratable acidity (% lactic acid)	CMTC	1.17 ± 0.05 ^{c**}	1.29 ± 0.13 ^{bc**}	1.26 ± 0.00 ^{bc**}	1.46 ± 0.04 ^{b**}	1.41 ± 0.00 ^{b**}	1.87 ± 0.10 ^{a**}	1.41 ± 0.01 ^{a**}
	GMTC	0.63 ± 0.00 ^{d**}	0.68 ± 0.00 ^{d**}	0.58 ± 0.01 ^{d**}	0.74 ± 0.00 ^{d**}	1.11 ± 0.00 ^{c**}	1.14 ± 0.11 ^{c**}	0.81 ± 0.01 ^{b**}
pH	CMTC	4.99 ± 0.01 ^{d**}	5.00 ± 0.01 ^{d**}	5.01 ± 0.00 ^{d**}	5.00 ± 0.02 ^{d**}	5.01 ± 0.00 ^{d**}	5.10 ± 0.13 ^{d**}	5.02 ± 0.01 ^{b**}
	GMTC	5.68 ± 0.00 ^{c**}	5.70 ± 0.03 ^{c**}	6.03 ± 0.01 ^{a**}	5.99 ± 0.00 ^{ab**}	5.97 ± 0.00 ^{ab**}	5.85 ± 0.06 ^{b**}	5.87 ± 0.01 ^{a**}
L* value	CMTC	83.41 ± 0.58 ^{abc**}	85.32 ± 0.04 ^{ab**}	80.56 ± 0.00 ^{de**}	77.75 ± 1.48 ^{g*}	83.42 ± 0.00 ^{abc**}	80.41 ± 0.06 ^{**}	81.81 ± 0.19 ^{**}
	GMTC	85.99 ± 0.93 ^{***}	83.08 ± 1.05 ^{bcd**}	84.21 ± 0.07 ^{abc**}	82.59 ± 1.35 ^{cde**}	83.90 ± 0.35 ^{abc**}	85.41 ± 0.59 ^{ab**}	84.19 ± 0.19 ^{**}
a* value	CMTC	-2.16 ± 0.09 ^{c**}	-2.67 ± 0.11 ^{b**}	-3.35 ± 0.00 ^{***}	-3.50 ± 0.08 ^{a**}	-3.73 ± 0.00 ^{a**}	-3.44 ± 0.01 ^{a**}	-3.14 ± 0.03 ^{b**}
	GMTC	-2.45 ± 0.13 ^{bc**}	-2.63 ± 0.91 ^{bc**}	-2.46 ± 1.20 ^{bc**}	-2.69 ± 0.08 ^{b**}	-3.44 ± 0.08 ^{a**}	-2.59 ± 0.33 ^{bc**}	-2.71 ± 0.03 ^{b**}
b* value	CMTC	16.38 ± 0.13 ^{cd**}	16.02 ± 0.24 ^{cd**}	19.03 ± 0.00 ^{***}	17.84 ± 0.43 ^{ab**}	18.06 ± 0.00 ^{ab**}	16.93 ± 0.11 ^{bc**}	17.38 ± 0.04 ^{b**}
	GMTC	11.83 ± 0.08 ^{***}	13.77 ± 0.83 ^{f**}	12.91 ± 0.87 ^{fg**}	15.30 ± 0.02 ^{de**}	13.99 ± 0.26 ^{ef**}	13.26 ± 0.01 ^{f**}	13.51 ± 0.04 ^{b**}
Water activity (a _w)	CMTC	0.897 ± 0.00	0.889 ± 0.00	0.864 ± 0.00	0.881 ± 0.00	0.860 ± 0.00	0.873 ± 0.00	0.877 ± 0.00
	GMTC	0.908 ± 0.00	0.896 ± 0.02	0.882 ± 0.00	0.864 ± 0.04	0.889 ± 0.00	0.892 ± 0.00	0.889 ± 0.00
Total nitrogen (g 100 g ⁻¹)	CMTC	4.03 ± 0.01 ^{cd**}	4.31 ± 0.15 ^{abc**}	4.03 ± 0.25 ^{cd**}	3.78 ± 0.00 ^{d**}	3.92 ± 0.00 ^{cd**}	3.92 ± 0.19 ^{cd**}	3.40 ± 0.07 ^{b**}
	GMTC	4.15 ± 0.22 ^{bcd**}	4.53 ± 0.04 ^{ab**}	4.27 ± 0.10 ^{abc**}	4.64 ± 0.26 ^{g**}	4.51 ± 0.00 ^{ab**}	4.68 ± 0.12 ^{**}	4.46 ± 0.07 ^{**}

^{a-g}Values marked with different letters are statistically different from each other; ^{ns}No significant difference; ¹CMTC: cow milk Tulum cheese; GMTC: goat milk Tulum cheese. *P < 0.05; **P < 0.01.

same ($P < 0.01$), with the goat milk Tulum cheese having a higher pH value around 5.87. While the pH value of cow milk Tulum cheese was constant during the ripening period, in goat milk Tulum cheese it increased on the 30th ripening day. Alonso et al. (1987) reported that acid degradation performed by yeasts and molds, and amphoteric peptides and amino acids, which occurred by proteolysis at the progressive ripening days, brought about the increase of pH value. The titratable acidity was higher in cow milk Tulum cheese ($P < 0.01$), and the highest titratable acidity in both cheeses was observed at the end of the ripening period ($P < 0.01$).

No differences were found in the water activity of Tulum cheeses, but a reduction was determined in accordance with the initial water activity value during the ripening period. McSweeney (2004) reported that the water activity value fell as a result of the hydrolysis of the casein matrix by proteolysis, which took place during the cheese ripening. Goat milk Tulum cheese had a higher L^* value ($P < 0.01$) that may have resulted due to the low content of β -carotene in goat milk, making it appear whiter in color. Values of a^* were negative for the Tulum cheeses. Cow milk Tulum cheese had a higher a^* value ($P < 0.01$), which may be in consequence of high fat content and color pigments such as β -carotene in cow milk. Cow milk Tulum cheese was yellower ($P < 0.01$) because of high fat content.

3.3 Chromatographic profiles of Tulum cheese peptides by RP-HPLC

The peptide profiles of the WSEs obtained from cow and goat milk Tulum cheeses changed with the progression of ripening days. Chromatographic peaks of the WSEs increased with the progression of days as shown in Figure 1 and 2. It was reported that hydrophilic peptides were generally determined between 20 - 40 retention times (Yoshida, 2004). Thus, as shown in Figure 1 and 2, the hydrophilic peptide content of goat milk Tulum cheeses was higher than that in cow milk Tulum cheeses. The amounts of all water-soluble peptides at the beginning of

the ripening period were considerably poor. However, they increased with the advancement of ripening days ($P < 0.01$). Sforza et al. (2012) reported that the formation of oligopeptides in Parmigiano-Reggiano cheeses showed parallelism with ripening days. Diezhandino et al. (2015) determined an increase in the peptide amount through the ripening days in Spanish blue cheese. Increment of the peak number during the ripening period was resulted from hydrolysis of peptides and proteins. Depending on the number of ripening days, a series of large and intermediate sized peptides derived from caseins began to occur due to plasmin, other proteolytic enzymes, microbial enzymes, and residual coagulant activity in the cheese. Afterward, these constituents converted to shorter peptides and amino acids by secondary microflora (Gupta et al., 2009).

The highest peak numbers were observed in WSEs from cow and goat milk Tulum cheeses on the 120th ripening day. The number of peaks were found to be significantly different upon comparing the cow and goat milk Tulum cheeses. WSEs acquired from goat milk Tulum cheese had higher number of peptide peaks in the ripening period, except on the 120th day. Raw cows' and goats' milk have the potential to a diverse bacterial population as reported previously (Alonso-Calleja et al., 2002; Quigley et al., 2011). Hence, these results can be indicative of the diversity of wild flora in the composition of raw milk. Pisanu et al. (2015) reported that the peptide profile differed in ripened sheep milk cheese, which was produced using raw and pasteurized milk. This was because raw milk had a higher content of bio-functional peptides.

3.4 Antioxidative activity

Inhibition results of DPPH radical demonstrated that the antioxidant activity increased with the progression of ripening days ($P < 0.01$) for both the Tulum cheese samples (Table 3). Gupta et al. (2009) examined that DPPH inhibition (%) of WSEs isolated from Cheddar cheese increased until 4 months as well. Abadía-García et al. (2013) determined an increase of

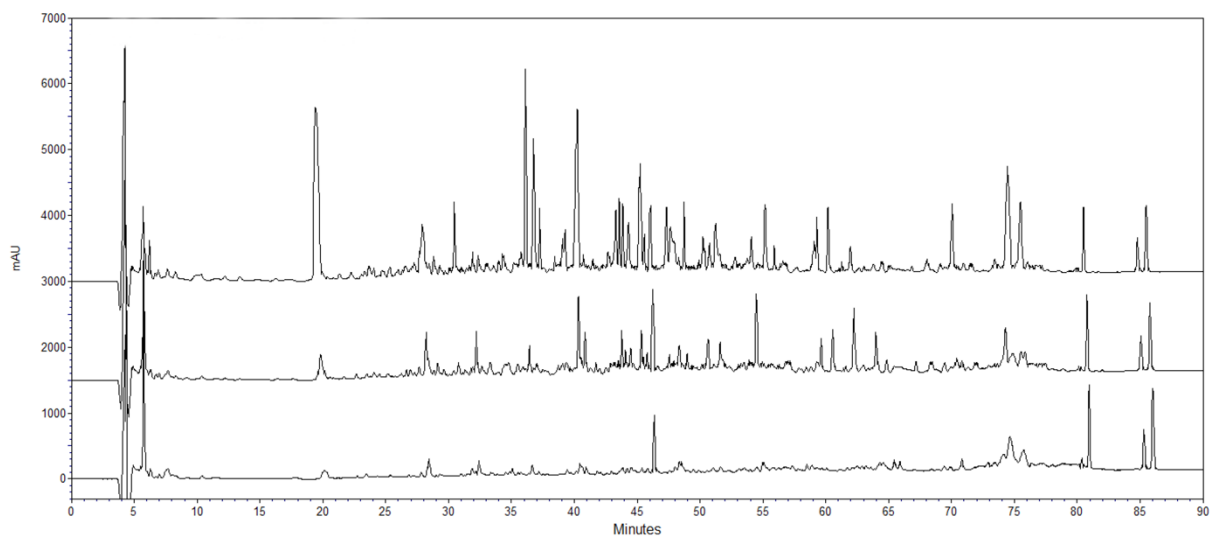


Figure 1. Peptide profiles of the WSEs of cow milk Tulum cheeses. Detection was at 214 nm. Profiles were determined by reversed-phase chromatography using a Phenomenex Jupiter C18 column. Chromatogram results are given from the 0th, 60th, and 120th days from bottom to top.

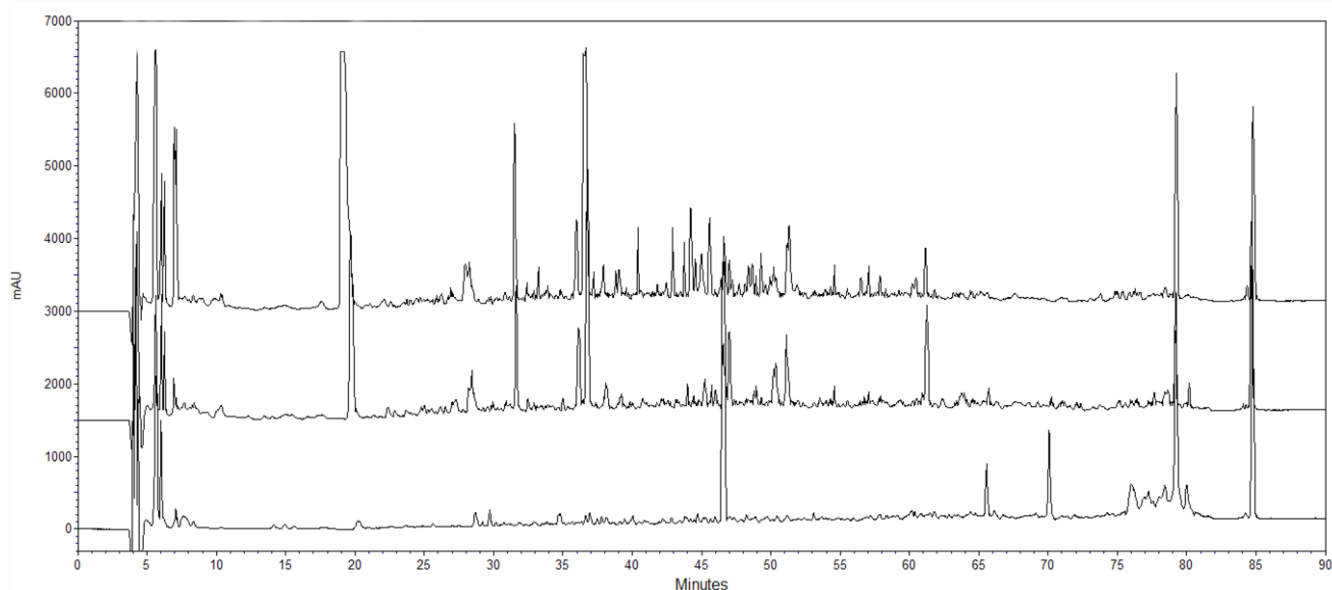


Figure 2. Peptide profiles of WSEs of goat milk Tulum cheeses. Detection was at 214 nm. Profiles were determined by reversed-phase chromatography using a Phenomenex Jupiter C18 column. Chromatogram results are given from the 0th, 60th, and 120th days from bottom to top.

Table 3. Antioxidant and mineral binding properties of WSE obtained from cow and goat milk Tulum cheeses during ripening period (n=2).

Ripening period (days)	DPPH		Chelating activity (%)	
	% Inhibition			
	CMTC	GMTC	CMTC	GMTC
0	13.25 ± 1.56 ^{c**}	21.58 ± 2.04 ^{d**}	4.68 ± 0.04 ^{**}	22.20 ± 0.04 ^{h**}
15	17.03 ± 2.16 ^{bc**}	23.47 ± 0.64 ^{cd**}	13.61 ± 0.05 ^{**}	56.58 ± 0.03 ^{d**}
30	18.41 ± 1.37 ^{bc**}	25.04 ± 0.09 ^{bcd**}	36.94 ± 0.02 ^{**}	64.51 ± 0.02 ^{b**}
60	20.94 ± 3.37 ^{b**}	27.50 ± 0.71 ^{abc**}	30.19 ± 0.02 ^{**}	65.99 ± 0.02 ^{a**}
90	21.46 ± 2.96 ^{b**}	28.40 ± 0.56 ^{ab**}	35.31 ± 0.03 ^{**}	56.24 ± 0.02 ^{d**}
120	30.96 ± 3.35 ^{a**}	29.87 ± 1.14 ^{a**}	63.30 ± 0.42 ^{**}	62.99 ± 0.02 ^{c**}
Overall mean	20.34 ± 0.70 ^{b*}	25.97 ± 0.70 ^{a*}	30.67 ± 0.04 ^{b**}	54.75 ± 0.04 ^{a**}

^{a-d}Values marked with different letters are statistically different from each other; CMTC: cow milk Tulum cheese; GMTC: goat milk Tulum cheese. *P < 0.05; **P < 0.01.

inhibition (%) of DPPH radical with the increase in storage days in WSEs isolated from Cottage cheese. These results were caused by further proteolysis occurred in the ripening period. Timón et al. (2014) reported that the DPPH inhibition capacity of the WSEs isolated from Burgos-type cheese became higher as the peptide concentration increased.

Goat milk Tulum cheese had a higher antioxidant activity with regards to the DPPH method (P < 0.05). This case could have been caused due to higher hydrophilic peptides content in goat milk Tulum cheese. According to the DPPH inhibition (%), antioxidant activities of WSEs in cow and goat milk Tulum cheeses were higher than those observed in Himalayan cheese produced using raw buffalo milk (Mushtaq et al., 2015), and commercial Australian Cheddar cheeses (Pritchard et al., 2010).

3.5 Mineral binding activity

Table 3 presents values (%) for the chelating activities of WSEs from cow and goat milk Tulum cheeses. Iron (II) chelating ability took place at distinct values for WSE samples during ripening period. The chelating activities of WSEs isolated from

cow and goat milk Tulum cheeses were higher than Roquefort, Cerrillano, and Pecorine-type cheeses produced using sheep milk (Meira et al., 2012). Goat milk Tulum cheese had significantly higher chelating activity than cow milk Tulum cheese (P < 0.01). The highest iron chelating activity of WSEs was observed in goat milk Tulum cheese on the 60th ripening day (P < 0.01). After the 60th day, iron chelating activity of WSEs decreased in goat milk Tulum cheese (P < 0.01). The changes observed in the chelating activity results of WSEs could be due to the fact that novel peptides with different functional properties were created by proteolysis, and the peptides having the chelating ability were hydrolyzed during the ripening period. Corrêa et al. (2011) asserted that the hydrolysis time had an inverse impact on Fe²⁺ binding ability of the WSEs, and longer hydrolysis time decreased metal chelation activity.

3.6 Antimicrobial activity

The ability of WSEs to inhibit the growth of *Escherichia coli* ATCC 25922, *Listeria monocytogenes* ATCC 7644, *Staphylococcus aureus* ATCC 25923, *Clostridium perfringens* ATCC 13124,

Bacillus cereus ATCC 11778, and *Salmonella typhimurium* ATCC 14028 was investigated. An antimicrobial activity against *Salmonella typhimurium* ATCC 14028 was only observed on the 90th ripening day in goat milk Tulum cheese. The inhibition zone was measured as 44 mm and the zone was observed in a disk using 10 µL of WSE.

Corrêa et al. (2011) determined antimicrobial activity against *Bacillus cereus* and *Corynebacterium fimi* in sheep caseinate hydrolysates for 3h and the diameters of the inhibitory zones were measured as 9.3 and 11.5 mm respectively. In another study on Italian cheese varieties, Rizzello et al. (2005) detected antimicrobial activities against *Lactobacillus sakei* A15, *Listeria innocua* DSM 20649, *Bacillus megaterium* F6, and *Escherichia coli* K12 in WSEs obtained from Caprino del Piemonte (goat milk and no starter) and Crescenza cheeses (cow milk and freeze dried thermophilic starter), and the diameters of the inhibitory zones were determined between 3 mm - 6 mm for indicator *Lactobacillus sakei* A15. Pritchard et al. (2010) identified antimicrobial activities against *Escherichia coli* ATCC 8739 and *Bacillus cereus* ATCC 11778 in WSEs isolated from Australian Cheddar cheese. Meira et al. (2012) examined no antimicrobial effect in WSEs obtained from ripened sheep cheeses. Rizzello et al. (2005) determined no antimicrobial activity in WSEs isolated from Fossa cheese produced with sheep milk as well. Generally, while antimicrobial activity against *Bacillus* strains (Gram-positive) was determined in many studies carried out using peptides isolated from cheese, antimicrobial activity against *Salmonella typhimurium* (Gram-negative) was identified in this study. In addition to this, the diameter of the inhibitory zone identified in this study was higher than the diameters of the inhibitory zones determined in the previous studies.

None of the WSEs obtained from cow milk Tulum cheese during the ripening period demonstrated antimicrobial activity against indicator microorganisms. Furthermore, antimicrobial activity of WSEs isolated from the goat milk Tulum cheese could not be observed during the ripening period, with the exception of the 90th day. It could have resulted from the changes in the peptidomics structure of the cheeses during the ripening period. When chromatographic results were compared, the most extensive hydrophobic peptide content was observed in the goat Tulum cheese on the 90th ripening day. Rizzello et al. (2005) identified antimicrobial activity in Caprino del Piemonte and Crescenza cheeses, which contained the majority of peptides in the hydrophobic site. However, they determined that none of the Parmigiano-Reggiano, Pecorino Romano, Fossa, and Caciocavallo cheeses, which contained abundant peptides in the hydrophilic site, showed antimicrobial activity.

Antimicrobial peptides derived from milk proteins have an inhibitory impact on a wide range of microorganisms, including pathogens and spoilage microorganisms (Corrêa et al., 2011). However, in this study, only Gram-negative bacteria was inhibited. Differences in the bacterial membrane compositions between Gram-positive and Gram-negative bacteria might have influenced the inhibition action and the bacterial specificity of the antimicrobial extract. This study has shown that goat milk Tulum cheese peptides displayed antimicrobial activity.

4 Conclusion

Consequently, the peptide profile of ripened cow and goat milk Tulum cheeses produced using raw milk demonstrated statistically significant differences ($P < 0.01$). It was determined that the quantity of peptides in WSEs was higher in goat milk Tulum cheese. These different results were most likely appertaining to the alteration of raw milk constituents, such as microbial or proteolytic enzymes, and varieties of appropriate proteins that underwent enzymatic breakdown by proteolysis for the formation of the peptides during the cheese ripening. WSEs obtained from cow and goat milk Tulum cheeses presented antioxidant and mineral binding activities. In addition to this, WSEs from goat milk Tulum cheese showed antibacterial activity against *Salmonella typhimurium* ATCC 14028. Changes in the functional properties of cheese peptides were examined during the ripening period, and generally, the defined functional activities increased with the progression of ripening days. Goat milk Tulum cheese has been found to have higher bioactivity of functional peptides during the ripening period.

Several cheeses are still produced using raw milk; therefore, the use of goat milk in the cheese production may be more beneficial for health, in terms of diversity and functional activity of the bioactive components. It was determined that Tulum cheeses ripened for a long time was more healthful for consumers.

Acknowledgements

This research was taken from my master's thesis titled "Determination of some quality characteristics, contents and functional properties of bioactive peptides of Tulum cheeses produced by traditional method" and supported by Selcuk University Scientific Research Projects Coordinatorship (Project number: 14201033).

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