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Comparative Analysis of Cow and Water Buffalo Milk Casein Fractions by Western Blotting *

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This study was planned to investigate the electrophoretic profiles of casein fractions in cow and water buffalo milk. Water buffalo and cow milk samples were collected during November 2018 from the milk processing plants in Kayseri. After being tested for each of their components, the milk samples were analysed for the molecular weight of their casein fractions by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western blot test. According to the SDS-PAGE and Western blot test findings of the α_s, β, κ- casein fractions, the average molecular weight were 24.39 daltons (Da), 25.13 Da, 28.66 Da in cow milk and 26.12 Da, 27.85 Da, 26.88 Da in water buffalo milk, respectively. Statistically a significant difference was found between the molecular weights of cow and water buffalo milk's α_s, β, κ- casein fractions. Due to the higher average molecular weights of buffalo milk's casein fractions than cow's milk, it has been concluded that the water buffalo milk could positively affect the rheological properties and the yield of the milk product.

Key Words: *Water Buffalo milk, molecular weight, SDS PAGE, western blot*

Sığır ve Manda Sütü Kazein Fraksiyonlarının Western Blot ile Karşılaştırılmalı Analizi

Bu çalışma, inek ve manda sütlerinde bulunan kazein fraksiyonlarının elektroforetik profillerinin incelenmesi amacı ile planlanmıştır. İnek ve manda sütü örnekleri 2018 Kasım ayı boyunca Kayseri'de bulunan süt işletmelerinden temin edilmiştir. Genel bileşimleri belirlenen süt örneklerine ait kazein fraksiyonlarının moleküler ağırlıklarının değerlendirilmesi için Sodyum Dodesil Sülfat Poliakrilamid Jel Elektroforezi (SDS-PAGE) ve Western blot testleri kullanılmıştır. α_s, β, κ kazein fraksiyonlarına ait SDS-PAGE ve western blot testi bulgularına göre ortalama moleküler ağırlık değerleri inek ve manda sütlerinde sırasıyla 24.39 dalton (Da), 25.13 Da, 28.66 Da ile 26.12 Da, 27.85 Da, 26.88 Da olarak bulunmuştur. İnek ve manda sütü α_s, β, κ kazein fraksiyonlarının moleküler ağırlıkları arasında istatistiksel bakımdan anlamlı bir farklılık olduğu saptanmıştır. Manda sütü kazein fraksiyonlarına ait ortalama moleküler ağırlıkların inek sütünden yüksek olması sebebiyle, son üründeki reolojik özellikleri olumlu yönde etkileyeceği ve süt ürünleri imalatında verim artışına olanak sağlayacağı sonucuna varılmıştır.

Anahtar Kelimeler: *Manda sütü, moleküler ağırlık, SDS PAGE, western blot*

Introduction

Milk and milk-derived products are considered to be an important component of a balanced diet. Milk is capable of fractionation of the components for industrial applications due to its liquid nature. By adjusting the pH of the milk to 4.6, milk proteins are divided into two groups as 'casein' and 'serum proteins' (1). Casein micelles directly affect the physicochemical properties and functionality of milk (2). Because of the wide use in industrial areas, the interest in casein micelles is increasing. New information about the protein profile associated with casein micelles and the functionality of the casein micelles helps to understand the physiological importance of the proteins that make up the micelles and the effect on the rheological properties of the product (3). Even though various models on casein micelles have been proposed, their detailed structure is still not fully understood (4). The yield and quality of a product depends on both composition and technological properties of the milk. In particular, the quality of the cheese coagulum is closely related to the physical and chemical properties of the casein micelles and their molecular weight. For the processing and production of innovative dairy products, information on the general composition of buffalo milk and the characteristics of its structural elements needs to be critically analyzed and updated (5). Cow's milk is the most widely used and most universal raw material in the milk industry, while buffalo milk is suitable for technological use due to its high protein content. Over the years monitoring of changes in buffalo milk composition is important as a general index for the combined effects of environmental and genetic factors (3, 5).

In this study, it is aimed to reveal electrophoretic profiles of fractions by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and compare the molecular weight of casein micelles in cow and water buffalo milk by Western blot method.

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Materials and Methods

A total of 6 samples of 500 mL (3 cows and 3 buffalo milk) were tested. Materials were used for the determination of general components of milk, chemical isolation of casein fractions, preparation of samples and SDS-PAGE, blotting and imaging. Protein, fat, non-fat dry matter, density values of cow and water buffalo milk taken three times in November with 10 day intervals in Kayseri province were determined by milk analyzer (Mayasan, Turkey). After being tested for each of their components, the milk samples were analysed for the molecular weight of their casein fractions by SDS-PAGE and Western blot tests. The process was carried out in three replicates and two in parallel.

Obtaining Casein Fractions: Milk fat was removed by centrifugation at 4°C, 5000 g x 10 minutes. Skimmilks portions were collected in separate beakers and subjected to precipitation. The precipitation procedure was performed according to the method proposed by Behera et al. (6).

Isolation of α-Casein Fractions: The coagulum was homogenized in the mixer with 6.6 M urea solution. The solution molarity was adjusted to 4.63 M by adding distilled water. As the precipitate formed mainly contains α-casein, it was ensured to separate the supernatant and pellet by centrifugation. The pellet was used to purify α-casein. Urea was added to the pellet by adjusting to 6.6 M urea solution with distilled water and NaCl. The precipitate was separated by centrifugation and purified by washing in 4.7 M urea solution. The glue-like precipitate formed was removed to eppendorf tubes as α-casein. This method has been modified by the method of Hipp et al. (7).

Isolation of β-casein fractions: The supernatant obtained from 4.63 M urea solution used for the isolation of α-casein was used to isolate β-casein by adding distilled water to 3.3 M. The insoluble and not fully fractionated precipitates were removed by centrifugation. The supernatant molarity was reduced to 1.7 M and the pH was adjusted to 4.7 with the addition of 0.1 M HCl. The precipitate was obtained as β-casein, purified in 4.6 M urea solution. The solution molarity was adjust to 3.3 M. The β-casein casein was adjusted to a concentration of 1.7 M in order to make it insoluble. The precipitate formed was the purest β-casein. This method has been modified by the method of Hipp et al. (7).

Isolation of κ-casein fractions: Isoelectric casein (15 g) was dissolved in 6.6 M urea solution, dispersed in 400 mL of distilled water. The solution is diluted to 4.6 M. To obtain precipitate, it was centrifuged by holding approximately 8 hours under ambient conditions. The α and κ-casein complexes were washed with distilled water and dissolved in 6.6M urea solution. The pH was adjusted to 8.5 by addition of 0.1 N NaOH. The solution was adjusted to pH 7 with the addition of 0.1 M HCl. The final solution was adjusted to 0.25 M by adding 4 M $CaCl_2$. The pH was maintained at 7 with the addition of 0.1 N NaOH. The resulting solution was centrifuged for 5 minutes at 5000 g. The supernatant containing the κ-casein was placed in a separate tube for dialysis against water. The κ-casein fraction was used from the dialysed solution for SDS-PAGE analysis. This method was modified by the method of Tripathi and Gerhrke (8).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis: The amount of protein was determined by using qubit fluorometer and qubit analysis kits (Thermo Fisher Scientific, ABD (9). The amount of protein in the liquid solution obtained was taken 50 µg for each protein.

The fractionated casein proteins in SDS-PAGE Bio-Rad Mini Protein II electrophoresis equipment (Bio-Rad, USA) was applied according to Laemmli (10) method. Each protein was homogenized in 1:1 ratio by adding 2x Laemmli sample buffer (Bio-Rad, USA). The proteins were heat-treated at 95 °C for 5 minutes.

Preparation of the Gel: The gel was prepared according to the Bio-rad stain free gel preparation procedure (11). A total of six proteins for each of the three fractions, respectively, were loaded with 10 µL in each well. 5 µL protein standard was applied to the beginning and the end. Proteins were run about 1 hour at 150 V. The applied gel was carefully removed from the system and transferred to the imaging device.

Western Blotting: The Polyvinylidene difluoride (PVDF) membrane was kept in methanol until it became transparent. Blot papers were kept in 1x transfer buffer for 3 minutes. The membrane was incubated in 1x transfer buffer solution (transfer buffer) for 3 minutes after methanol. In the trans-blot turbo transfer system, blot paper, membrane, gel, blot paper were settled respectively, blotting process was performed in 2.5 amperes, for 3 minutes. The membrane was taken to the Tris-buffered saline with Tween 20 (TBST) and placed on a shaker for 5 minutes and treated.

The membrane was taken to the blocking buffer (blocking buffer). It was treated in a shaker for 1 hour and then kept in shaker 3 times for 5 minutes in TBST. When the time was up, 10 mL blocking buffer was added to the primary antibody. 10 µL pimary antibody was added to the on the blocking buffer. The mixture was shaken overnight at 4 °C. It was then washed for 5 minutes, 3 times with TBST.

For imaging, 5 mL of chemiluminescent substrates were added to a container and the membrane was shaken for 5 minutes. Fusion Fx In the Western blot and chemiluminescent imaging system, the image of the membrane was examined (12).

Statistical Analysis: Statistical analyses were performed using IBM SPSS software ver. 22.0. The molecular weights of the fractionated samples and the value of milk components were subjected to statistical analysis using the package program. The difference in the comparison of the molecular weight of the general composition of cow and buffalo milk with casein fractions were checked by Independent Sample t-test (13).

Results

Cow and Buffalo Milk Component Quantities: The average amount of cow and buffalo milk components detected in Milk Analyzer device and statistical analysis results according to Independent Sample t-test are given in Table 1.

Gel Image by SDS-PAGE: The gel images transferred to the imaging system are shown in the Figure 1, Figure 2 and Figure 3**,** respectively.

Membrane Image by Western Blotting: According to the protein standard, protein molecular weights of fractions were determined in Western blot and Fusion Fx chemiluminescent imaging system. The membrane of the first Western blot application is shown in Figure 4, Figure 5 and Figure 6 as chemiluminescent, coomassie blue and 3D image, respectively. The membranes belong to the second and third Western blot application is shown in Figure 7 and Figure 8 as chemiluminescent, coomassie blue stained image. The results of statistical analysis of molecular weights of cow and buffalo milk casein fractions are given in Table 2.

Table 1. Statistical analysis findings of components of cow and buffalo milk according to independent sample t-test

	n	x̄ ± SD (Mean ± Standart Deviation)			
		Protein (%)	Fat (%)	Non-fat dry matter (%)	Density (g/mL)
Cow milk	6	3.31±0.77[b]	3.63±0.04[b]	8.77±0.20[b]	1.029±0.00
Water Buffalo Milk	6	3.75±0.08[a]	8.60±0.04[a]	9.82±0.02[a]	1.029±0.00
P value		0.000	0.000	0.000	1.000

[a,b] Different upper case superscript letters within a column indicates the differance of means are statistically significant (P<0.05).

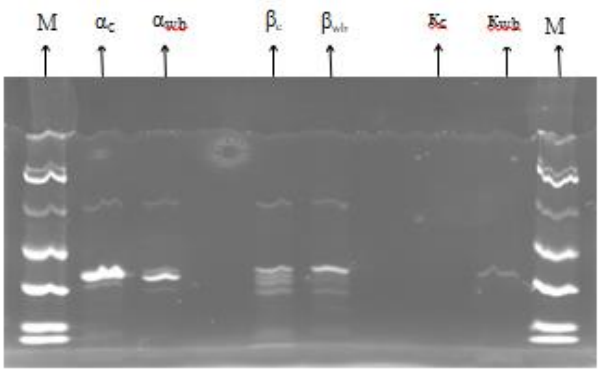

Figure 1. Image of Fractions in first applied SDS-PAGE (M: marker, α_c: cow milk α, α_{wb}: water buffalo milk α, β_c: cow milk β, β_{wb}: water buffalo milk β, κ_c: cow milk κ, κ_{wb}: water buffalo milk κ- casein fractions).

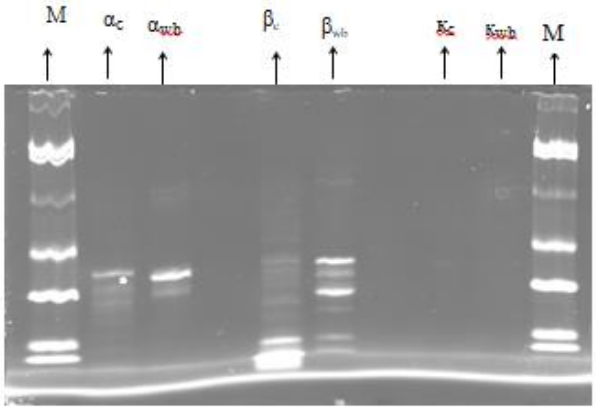

Figure 2. Image of Fractions in second applied SDS-PAGE (M: marker, α_c: cow milk α, α_{wb}: water buffalo milk α, β_c: cow milk β, β_{wb}: water buffalo milk β, κ_c: cow milk κ, κ_{wb}: water buffalo milk κ- casein fractions).

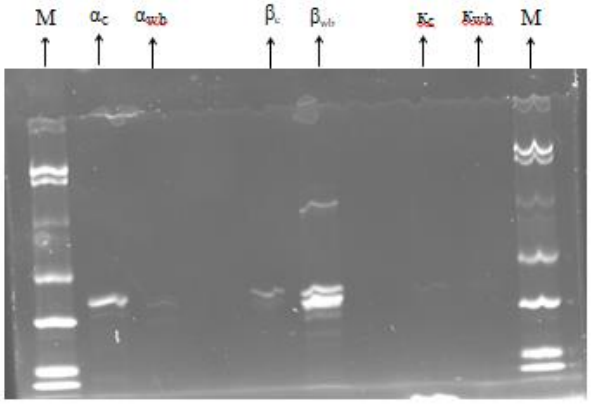

Figure 3. Image of Fractions in third applied SDS-PAGE (M: marker, α_c: cow milk α, α_{wb}: water buffalo milk α, β_c: cow milk β, β_{wb}: water buffalo milk β, κ_c: cow milk κ, κ_{wb}: water buffalo milk κ- casein fractions).

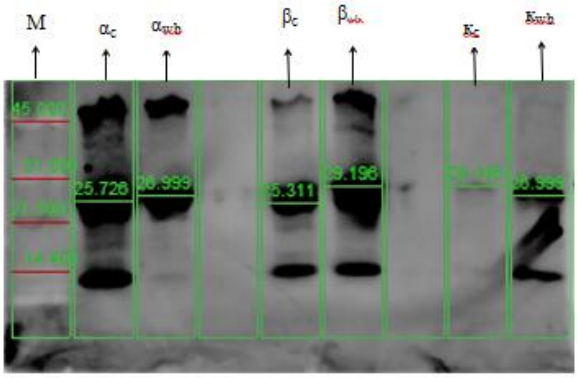

Figure 4. Chemiluminescent image of casein fractions with molecular weights in the membrane of the first Western blot application (M: marker, α_c: cow milk α, α_{wb}: water buffalo milk α, β_c: cow milk β, β_{wb}: water buffalo milk β, κ_c: cow milk κ, κ_{wb}: water buffalo milk κ- casein fractions) P<0.05.

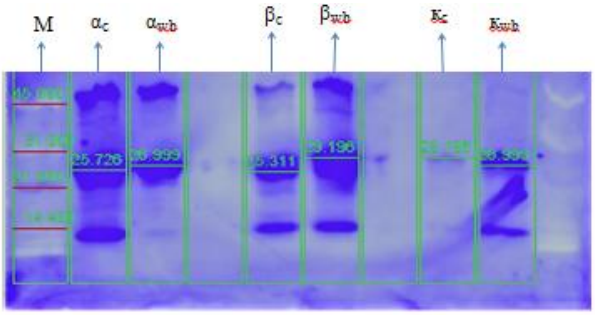

Figure 5. Coomassie blue image of casein fractions with molecular weights in the membrane of the first Western blot application (M: marker, α_c: cow milk α, α_{wb}: water buffalo milk α, β_c: cow milk β, β_{wb}: water buffalo milk β, κ_c: cow milk κ, κ_{wb}: water buffalo milk κ- casein fractions) P<0.05.

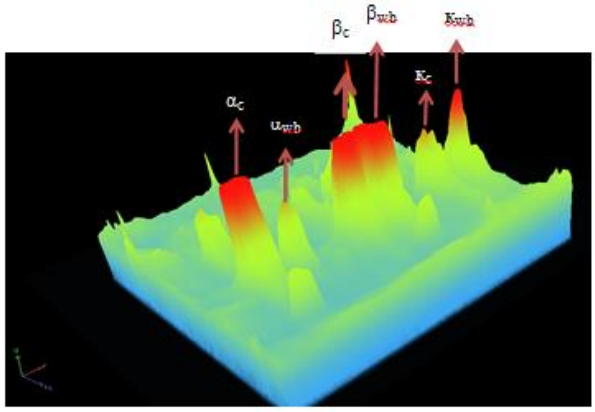

Figure 6. 3D image of proteins transferred to the membrane of the first Western blot application (M: marker, α_c: cow milk α, α_{wb}: water buffalo milk α, β_c: cow milk β, β_{wb}: water buffalo milk β, κ_c: cow milk κ, κ_{wb}: water buffalo milk κ- casein fractions) P<0.05.

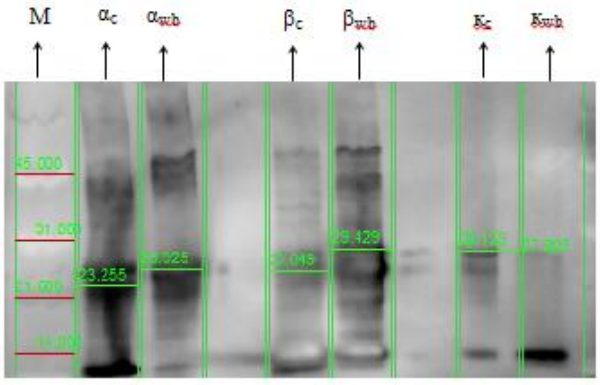

Figure 7. Image of the membrane in the second applied Western Blot method (M: marker, α_c: cow milk α, α_{wb}: water buffalo milk α, β_c: cow milk β, β_{wb}: water buffalo milk β, κ_c: cow milk κ, κ_{wb}: water buffalo milk κ- casein fraction) P<0.05.

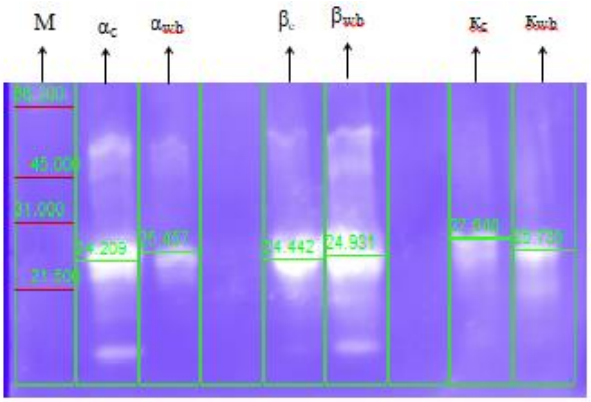

Figure 8. Coomassie blue stained image of the membrane in the third Western blot method (M: marker, α_c: cow milk α, α_{wb}: water buffalo milk α, β_c: cow milk β, β_{wb}: water buffalo milk β, κ_c: cow milk κ, κ_{wb}: water buffalo milk κ- casein fraction) P<0.05.

Table 2. Statistical analysis results of molecular weights by cow and buffalo milk according to ındependent sample t-test

		x̄ ± SD (Mean ± Standard Deviation)		
	n	α- casein (Da)	β- casein (Da)	κ- casein (Da)
Cow milk	6	24.39±1.11[b]	25.13±0.55[b]	28.66±0.78[a]
Water buffalo milk	6	26.12±0,70[a]	27.85±2.26[a]	26.88±0.98[b]
P value		0.009	0.017	0.006

[a,b] Different upper case superscript letters within a column indicates the differance of means are statistically significant (P<0.05).

Discussion

In this study, the general composition of cow milk and buffalo milk was determined and molecular weights of casein fractions was exhibited by SDS-PAGE and Western Blotting techniques.

The average fat values of cow and buffalo milk were found to be 3.63% and 8.60%, and the non fat dry matter values were 8.77% and 9.82%, respectively. These values were higher in buffalo milk. According to the results of the independent sample t-test statistical analysis; a significant difference was found between fat and non fat dry matter contents of cow and buffalo milk (P<0.05).

Salman et al. (14) showed that buffalo milk had a considerable amount of dry matter, fat, protein, lactose and ash content compared to cow milk, and that the calorific value in buffalo milk was significantly higher than that of cow's milk. Enb et al. (15) and Mahmood and Sumaira(16) stated that the dry matter content of buffalo milk was more than that of cow's milk.

When the average protein values of cow and buffalo milk were compared, these values were 3.31% and 3.75%. According to the results of the independent sample t-test statistical analysis; a significant difference

was found between the amount of protein of cow and buffalo milk ($P<0.05$). This result was similar reported by Salman et al. (14) and Mahmood and Sumaira (16).

In addition to calorific values, buffalo milk is a rich source of macro nutrients and has an important place in dairy products. Salman et al. (14) suggested that cow's milk with low fat compared to buffalo milk could be used as low-fat milk drinks or low-fat dairy products.

In general, milk has a complex structure with many chemical and physical components. Although milk of different animal species contains the same type of components, the amounts of these components are different. Within a given species, environmental factors such as genetic factors, climate and lactation period affect the composition (17).

In this study; according to SDS-PAGE and Western Blotting results of α-casein fraction, mean molecular weight values were found as 24.397 Da for cow's milk and 26.127 Da for buffalo milk. According to the statistical analysis findings, it was observed that there was a significant difference between the molecular weights of cows and buffalo milk α-casein fraction ($P<0.05$). Kuasar et al. (18) found that cow's milk α_{s1}-casein and a_{s2}-casein molecular weights were 27 and 29 kDa, buffalo milk a_{s2}-casein molecular weight was 29 kDa.

In the study, mean values of β-casein fraction were 25.134 and 27.852 Da in cow and buffalo milk respectively. According to Independent Sample T-Test; it was determined that the difference between β-casein fraction molecular weights of cow and buffalo milk was significant ($P<0.05$). Kuasar et al. (18) found the molecular weights of β-casein fraction in cows and buffalo milk to be 24 and 25 kDa, respectively. The results obtained from the study were found to be relatively higher than the results of Kuasar et al. (18).

The average molecular weight of the κ -casein fraction was found to be 28.665 and 26.886 Da in cow and buffalo milk. The difference was statistically significant ($P<0.05$). In the study of Kuasar et al. (18), the molecular weight of the cow and buffalo milk κ-casein fraction was determined to be 22 kDa. It can be said that this result is higher compared to the study of Kuasar et al. (18) and this finding may be due to the difference in fractionation methods.

The molecular weight of buffalo milk casein fractions is higher than that of cow's milk and it affects the physicochemical and rheological properties of the milk products. Especially for the production of mozarella type cheese, cow and buffalo milk can be used as long as this information is shown on the label.

In the comperative study of Hussain et al. (5) about mozzarella cheese rheology made from cow and buffalo milk, when cow's milk was used (pH 6.7-6.5), the coagulation with rennet was slower than that of buffalo milk. It has been also concluded that the coagulum takes longer to form the maximum firmness and that the hydrolysis of the κ-casein in cow's milk takes longer. Therefore the stability of the casein micelles is prolonged and the coagulum development is delayed. The casein micelles in buffalo milk rapidly increase the coagulum structure due to the fact that the fraction volume is higher than cow's milk. Therefore, a smoother structure is formed within the coagulum made from buffalo milk and it is stated that the coagulum obtained from buffalo milk is generally stronger and more frequent (5).

Analysis of milk protein expression, identification, characterization and quantification of milk proteins, determination of structure and modifications containing genetic variations, changes in phosphorylation levels (naturally occurring during milk processing and storage), glycosylation and other post-translational modifications (PTMs) will contribute to the development of proteomics in the determination of PTM domains on milk proteins, the acquisition of many valuable information for the dairy industry, and a better understanding of the structure and properties of buffaloes and other species' milk. In this respect, proteomic studies will guide the standardization and development of product quality (19).

In conclusion, the molecular weight of casein fractions in cow and water buffalo milk were significantly different. Casein micelles contribute to the physicochemical properties of milk and affect its functionality. It was concluded that the molecular weight of buffalo milk casein and its subunits is larger than that of cow's milk, it will improve the physicochemical properties of dairy products in a positive way and it will increase the yield. It was emphasised that the spread of buffalo milk will increase the quality and product diversity.

In addition, the importance of buffalo milk was emphasized with the study of buffalo milk casein and casein fraction molecular weight was found higher than cow's milk. The electrophoretic profiles of casein fractions in both types were revealed.

References

1. du Puis EM, Lang JJ. Nature's perfect food: How milk became America's drink. Contemp Sociol 2003; 32: 341.
2. Fox PF, Brodkorb A. The casein micelle: Historical aspects, current concepts and significance. Int Dairy J 2008; 18: 677-684.
3. Wang X, Zhao X, Huang D, et al. Proteomic analysis and cross species comparison of casein fractions from the milk of dairy animals. Sci Rep 2017; 7: 1-16.
4. Dalgleish DG. On the structural models of bovine casein micelles- review and possible improvements. Soft Matter 2011; 7: 2265-2272.
5. Hussain I, Bell AE, Grandison AS. Comparison of the rheology of mozzarella-type curd made from buffalo and cows' milk. Food Chem 2017; 128: 500-504.
6. Behera P, Kumar R, Kapila S, et al. Fractionation and characterisation of α, β and κ-casein trypsin hydrolysates

from Buffalo (Bubalus bubalis) milk. Milchwissenschaft 2012; 67: 2-8.

7. Hipp NJ, Groves ML, Custer JH, et al. Separation of α-, β- and γ-casein. J Dairy Sci 1952; 35: 272-281.
8. Tripathi KK, Gehrke CW. Chemical and chromatographic isolation of κ-casein J Chromatogr 1970; 46: 280-285.
9. Anonymous. "Thermofisher user guide". https://www.thermofisher.com/order/catalog/product/Q332/ 29.12.18.
10. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227: 680-685.
11. Anonymous. "Bio-rad protein blotting guide". http://www.biorad.com/webroot/web/pdf/lsr/literature/Bulle Bul_2895.pdf?source_wt=s180516000180/ 29.12.2018.
12. Mahmood T, Yang PC. Western blot: Technique, theory, and trouble shooting. N Am J Med Sci 2012; 4: 1-12.
13. Düzgüneş O. İstatistik Metodları. Ankara: İstatistik Genel Müdürlüğü Yayınları, 1952.
14. Salman M, Khaskheli M, Ul-Haq I, et al. Comparative studies on nutritive quality of buffalo and cow milk. IJRANS 2014; 2: 69-78.
15. Enb A, Abou Donia MA, Abd Rabou NS, et al. Chemical composition of raw milk and heavy metals behavior during processing of milk products. Glob Vet 2009; 3: 268-275.
16. Mahmood A, Sumaira U. A comparative study on the physico-chemical parameters of milk samples collected from buffalo, cow, goat and sheep of Gujrat. Pakistan. Pak J Nut 2010; 9: 1192-1197.
17. Walstra P, Wouters JTM, Geurts TJ. Dairy science and technology. 2nd Edition, Florida: CRC Press, 2006.
18. Kausar R, Muhammad G, Qureshi ZI. Comparative protein profiling of milk of nili-ravi buffaloes, sahiwal and cross bred cows by SDS-PAGE. J Anim Plant Sci 2015; 25: 645-649.
19. Manso MA, Léonil J, Jan G, et al. Application of proteomics to the characterisation of milk and dairy products. Int Dairy J 2005; 15: 845-855.