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Characterization and comparative profiling of milk protein from dairy animals.

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Abstract:
Milk proteins are chief constituents that give numerous nutritional and functional properties to milk and other dairy products. Having complexed nature, their analysis requires several methods to determine the protein content. In this study, comparative and quantitative study of milk proteins of four species (buffalo, camel, cow and goat) was executed. Milk samples were randomly collected from different regions of Balochistan mainly from Quetta, Zhob and Lasbela. SDS-PAGE gel electrophoresis and Bradford method were applied to enable the accurate determination of the total protein content in all milk samples. Highest concentration of total proteins was observed in buffalo milk 65 mg/ml while 62 mg/ml, 52 mg/ml, 32 mg/ml protein content was found in goat, cow and camel milk respectively. Electrophoretic results of four species were almost same but camel milk protein bands were somewhat different from other three species. It is concluded that highest concentration of total proteins was found in buffalo milk followed by goat and cow milk respectively however the least protein content was observed in camel milk. αs1-casein that is considered as an allergy causing protein was absent in camel milk making it suitable for the development of non-allergic dairy products and this αs1-casein deficient property is making it an appropriate substitute for individuals with αs1-casein sensitivity and can be consumed with proper consultation of professional healthcare.

Keywords; Milk proteins, αs1-casein, Electrophoresis, SDS-PAGE

Introduction:
The dietary benefit of proteins is increasing and valued over time. Milk is a significant source of protein secreted through female mammary glands, which provides a vital supply of proteins to their infants (Yang *et al.*, 2013). Milk proteins are not only responsible for nutritional benefits but possess metabolic, anti-carcinogenic antihypertensive, immune modulatory, and other properties as well (Warakaulle *et al.*, 2023). Human milk is the primary source of protein for their infants that provides all the essential amino acids in perfect proportion. It generally displays some convergent and divergent properties compared to the milk of other species in composition and concentration. To find a substitute of human milk, a comprehensive analysis is needed to study the milk composition of other species such as buffalo, cow, camel, donkey and mare etc. Cow is the largest milk producing source in the world followed by buffalo and goat (Gantner *et al.*, 2015). According to FAO's 2010 report buffalo is the largest milk producing source in the world and produces 63% of milk nationally in Pakistan (Ahmad *et al.*, 2013). The most dominant source of milk in Pakistan is buffalo, which provides roughly 67.04%, 31.56% from cows, 1.81%, 1.65%, 1.65% from camels, sheep, and goats, respectively.

Total milk production of country is about 47.951 million tons. Therefore, complete protein profile of milk from cows, buffalo, sheep, goats and camels must be examined (Huma *et al.*, 2018).

Milk proteins are majorly composed of two types of proteins; one is casein, an insoluble protein which makes up about 80% of total milk proteins, and the other is whey protein which is a soluble protein and contains 20% of total protein. Casein possesses sub classes αs1, αs2, β and ϰ –caseins. The supernatant or soluble proteins known as whey proteins have sub types β-lactoglobulin, α-lactoglobulin and minor proteins such as lactoferrin, immunoglobulin and bovine serum albumin (BSA) (Pereira, 2014). The composition and concentration of all of these proteins varies from species to species depending on the type of milk taking. Recent studies have revealed that cow milk has certain proteins that cause allergic reactions in children and these proteins are not found in human and camel milk (El-Agamy, 2007). Our country mostly consumes buffalo milk followed by cow, goat and camel. The purpose of current study is to quantify and compare the milk proteins of four species (buffalo, cow, goat, camel) to help out the selection of good protein source that can be used as a substitute of human milk. which enables to select the good milk source that contains the proteins in appropriate amount required not only for the growth of children but for adults too.

Materials and Method:
In the current study SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis) has been used to characterize the milk proteins due to its high resolution capacity. And for quantification of proteins Bradford method was used. That is based on Coomassie blue G-250 which is brown in color, but when it binds with protein its color changes to blue and it depends on the protein concentration. It is very sensitive and rapid method that can quantify the concentration even in micrograms (Bradford, 1976).

Sample collection:
60 milk samples of four species (Buffalo, Camel, Goat and Cow) were collected randomly from different regions of Balochistan particularly Quetta, Zhob and Lasbela. All samples were collected in sterilized bottles, carefully labeled, stored in an ice box and then immediately transferred to the laboratory for further analysis.

Sample preparation:
Initially, degreasing of milk was executed. For degreasing of milk, 10 ml of milk samples were taken in centrifuge test tubes and centrifuged it at 10,000 rpm for 10 min at 4 ºC through *BCKMAN* COULTER Allegra 64R centrifuge. After centrifugation the upper fat or creamy layer was removed with the help of spatula and the remaining aqueous part or skimmed milk was put into other test tubes and then this extract was used for protein characterization (Sharma *et al.*, 2017).

Quantification:
The quantity of total proteins was determined according to the Bradford method (1976). The initial step was to prepare the Bradford reagent, and to do this, in a measuring umber color glass bottle, 100 mg of Coomassie blue G-250 were added and 100 ml phosphoric acid and 50 ml ethanol was dissolved in it and the volume was brought up to 200 ml with distilled water and filtered through what-man filter paper.

Preparation of stock solution:
Bradford assay is based on a standard where the accurate concentration of protein is known. In the current study Bovine serum albumin (BSA) was used as standard protein, and a 0.1 mg/ mL stock solution of BSA was prepared.

Solution preparation for calibration curve:
10 to 100 µl of stock solution was taken in 10 test tubes and volume was brought up to 800 µl by adding distilled water and 200 µl Bradford reagent was added and solution was mixed properly. In separate 8 test tubes 2 µl in four test tubes and 4 µl of unknown samples in the remaining test tubes were added and then volume was brought up to 800 µl by adding distilled water and finally, 200 µl of Bradford reagent was added and left for few minutes at room temperature.

Within 60 min the absorbance was measured at SHIMADZU spectrophotometer at 595 nm wavelength (λ max). A standard curve was generated by plotting a graph between absorbance on Y-axis and concentration (mg/ml) on x-axis. And the absorbance of unknown samples was compared with standard absorbance to determine the concentration of proteins in unknown samples.

Characterization of protein on SDS-PAGE:
For comparative study of proteins SDS-PAGE was used by using the skimmed milk samples. Briefly, 15 µl of milk samples along with 15 µl marker DIAMOND™ protein marker (Page Ruler # 26616) was loaded in 10% gel and the electrophoresis was performed at 170 Volts, 15A for 3 hours. When the running was accomplished, the gel was stained with acetic acid and methanol containing Coomassie Brilliant Blue solution (0.0625g/250ml) and then destained with the same mixture until the bands were clear. Following that, photographs of gel were taken through epoxy gel scanner and protein bands were determined by using gel analyzer software 2010.

Heterogeneity of protein:
By cluster analysis using MVSP software, the protein pattern heterogeneity among four samples was compared (Khoshroo *et al.*, 2013).

Results:
The results of this comparative and quantitative research provide valuable understanding into the nutritional composition of four different species, enlightening the difference in protein content and their potential health benefits. Results obtained from the analysis of four different (cow, buffalo, goat, camel) milk samples were presented in figure 1 which displays the graphical appearance of the values achieved from analysis.

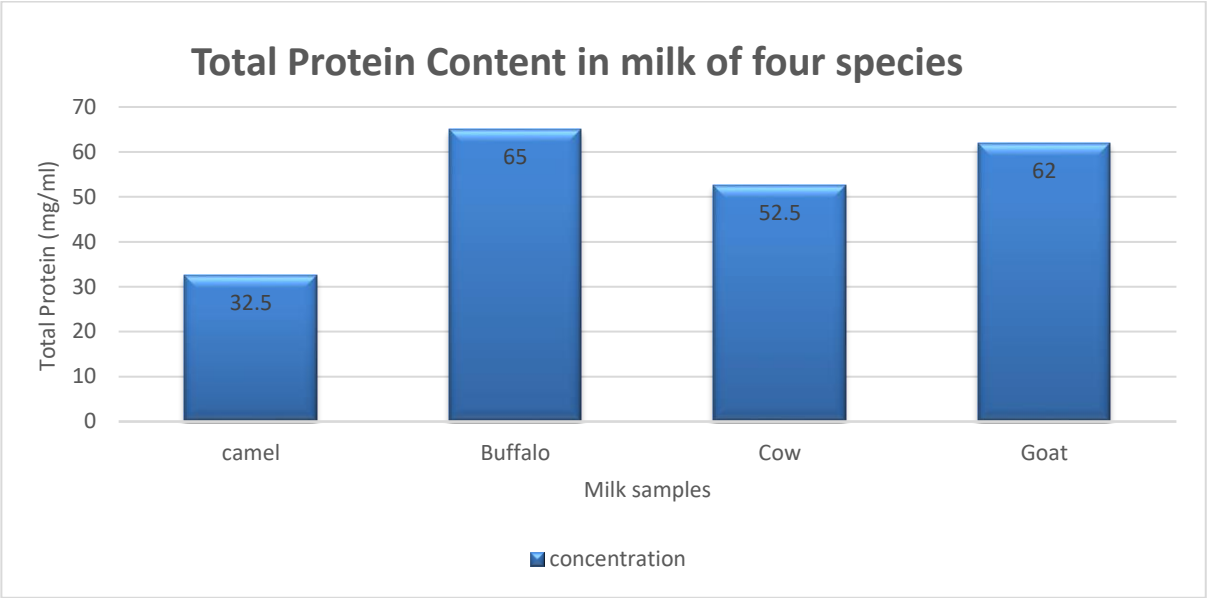

Figure 1 Total protein content (mg/ml) in all milk samples

The comparative protein profile of four species buffalo, camel, cow and goat milk showed the highest concentration of protein in buffalo milk with 65 mg/ml followed by goat milk 62 g/ml, cow milk 52.5 mg/ml and less concentration was obtained in camel milk 32 mg/ml. The current results are similar to the findings of a previous study in which determined concentration of protein by Bradford method in Nili Ravi buffalos ranges from 58.267-84.667 mg/ml (R Kausar *et al.*, 2015). Followed by goat milk (62 mg/ml) and cow milk (52.5 mg/ml), these obtained results are similar to the previous study that reported total milk protein concentration in the range of 63.87 mg/ml for goat milk and in cow milk about 54.71 mg/ml (Cozma *et al.*, 2011). The current investigation observed least protein content in camel milk and the obtained concertation of protein was 32 mg/ml which is in agreement with a previous study with a concentration of protein ranges from 31-33 g/l (Ryskaliyeva *et al.*, 2018).

Characterization of protein on SDS-PAGE:

Characterization was performed using (Laemmli, 1970) method and clear bands appeared on 10% acrylamide gel. By passing the gel through gel analyzer software 2010© usually helps in determining the molecular weight of proteins of unknown samples. The unknown molecular weight of four samples was determined by comparing it with the known molecular weight ranging from 15-180 KDa proteins marker (Page Ruler # 26616) showing in Figure 2 to7.

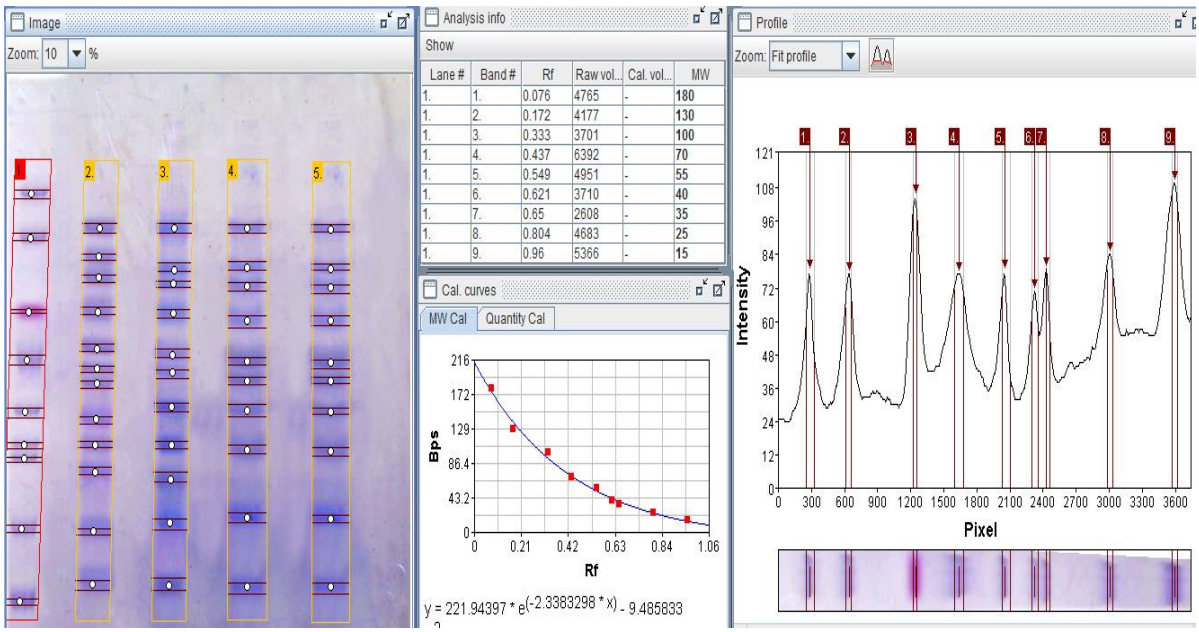

Figure 2 Molecular weight and Rf analysis of protein marker in lane 1 by Gel analyzer 2010a©

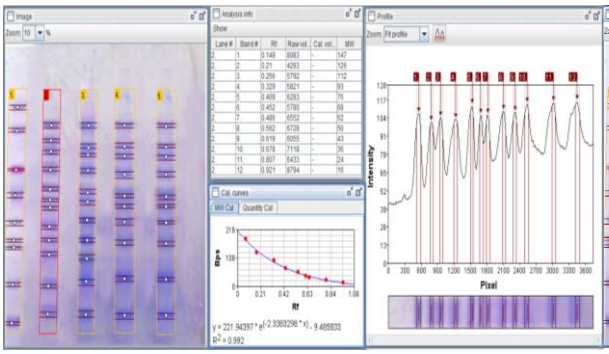

Figure 3 Molecular Weight and Rf analysis of protein bands in Camel milk

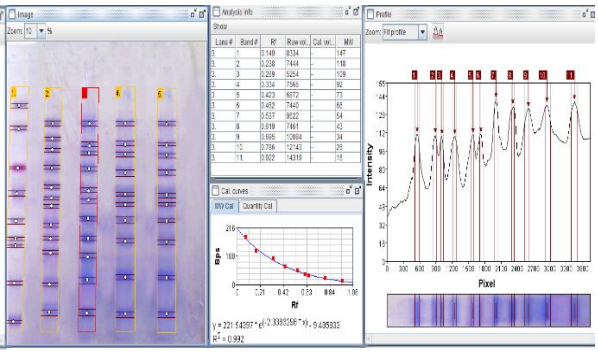

Figure 4 Molecular weight and Rf analysis of protein bands in Buffalo milk

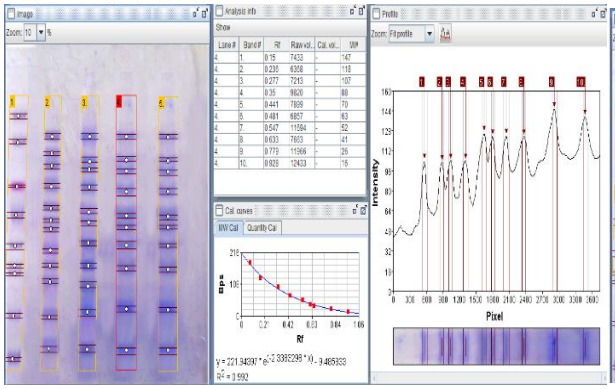

Figure 5 Molecular weight and Rf analysis of protein bands of goat milk in lane 4

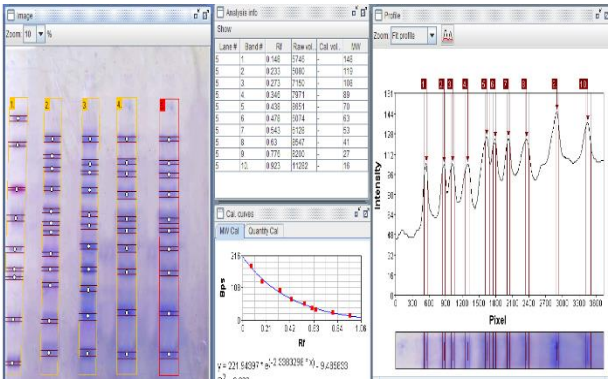

Figure 6 Molecular weight and Rf analysis of protein bands of cow milk in lane 5

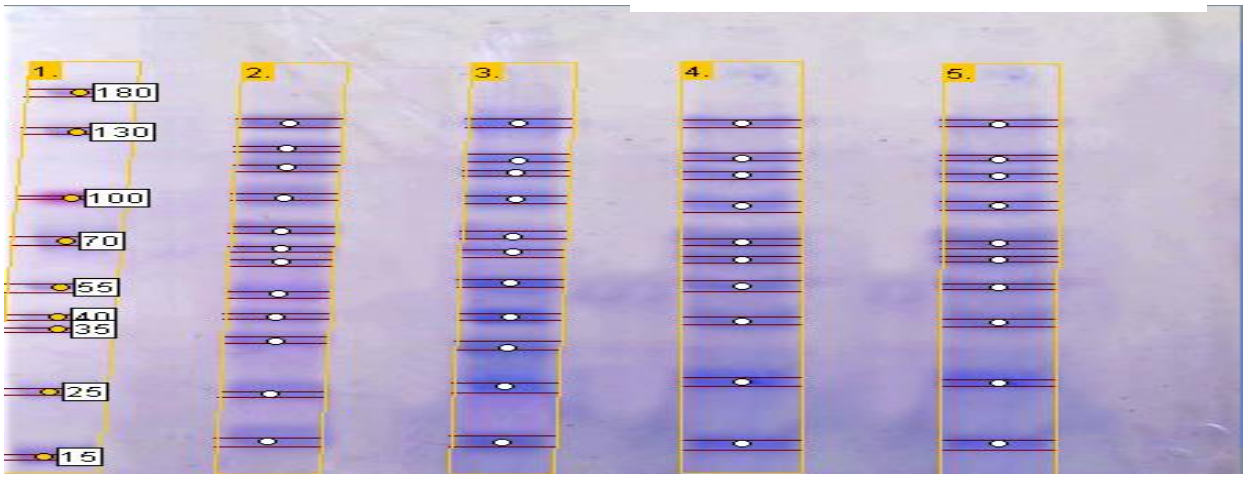

Figure 7 SDS-PAGE of protein extract of milk of four species. Lane 1 represent the DIAMOND™ protein marker ranging from 15-180 KDa, Lane 2 Camel, Lane 3 Buffalo, Lane 4 Goat and Lane 5 Cow milk proteins.

The bands having molecular weight of 16 KDa present in all samples is may be β-lactoglobulin and these findings are in accordance with a previous research where the similar bands were detected in cow and goat milk samples (Dos Santos *et al.*, 2018). Bands having molecular weight of 24- 27 KDa present in lower region in all milk samples is casein. In camel milk 24 kDa and 26 kDa in goat and buffalo milk are may be β-casein because in previously described studies, 24 KDa and 26 KDa bands were observed as β-casein (Razia Kausar *et al.*, 2017) (Salmen *et al.*, 2012). 27KDa in cow milk is may be αs1-casein since in a previuos study same resluts were observerd and considered as major allargy causing protein (Dos Santos *et al.*, 2018). Current study revealed that 27KDa band that is considered as αs1-casein and a major allergen was absent in camel milk sample so it might be regarded as a great option for making different protein-based items free from allergies. Previous work observed same results and detected camel milk as an αs1-casein deficient milk and also considered it as a good choice for the manufacturing of allergy free dairy products (Yasmin *et al.*, 2020). 50-54 KDa bands found in all milk samples are immunoglobulins and these bands are in accordance with a previuos work (Madureira *et al.*, 2007). Bands 66-68 KDa were detected and considered as serum albumin in camel and buffalo milk, and these bands were in agreement with previuos investigations where same bands were identified in buffalo milk using chromatographic techniques (Buffoni *et al.*, 2011). Bands ranging from 70-76 KDa are may be lactoferrin in cow and buffalo milk that were similar to the findings of a previuos observed

study (Razia Kausar *et al.*, 2017). Bands 88-89 KDa, 118-119 KDa and 147-148 KDa were unidetified proetins bands and appeared at different zones. Unidentified bands at 112 KDa and 126 KDa were only present in camel milk making it different from other milk samples.

Table 1 Calculated molecular weight of four different milk samples

Marker (M.W)	Camel	Buffalo	Goat	Cow
M1	147	147	147	148
M2	126	-	-	-
M3	112	-	-	-
M4	0	118	118	119
M5	93	92	-	-
M6	0	0	88	89
M7	76	73	70	70
M8	68	66	-	-
M9	62	63	63	-
M10	50	54	52	53
M11	43	43	-	-
M12	-	-	41	41
M13	36	34	-	-
M14	24	26	26	27
M15	16	16	16	16

Milk proteins hetrogenity amoung four species:
The current study used SDS-PAGE and cluster analysis to relate protein heterogeneity among four milk samples (buffalo, camel, cow, and goat). The electrophoretic profiles showed two clusters: Cluster 1 (cow and goat) and Cluster 2 (buffalo, camel, and goat). Particularly, camel milk exhibited a different composition due to the presence of 112 KDa and 126 KDa protein bands. Cow and goat milk were almost identical and showed resemblances. This study helps in determining protein heterogeneity based on band number and intensity revealing the utility of SDS-PAGE.

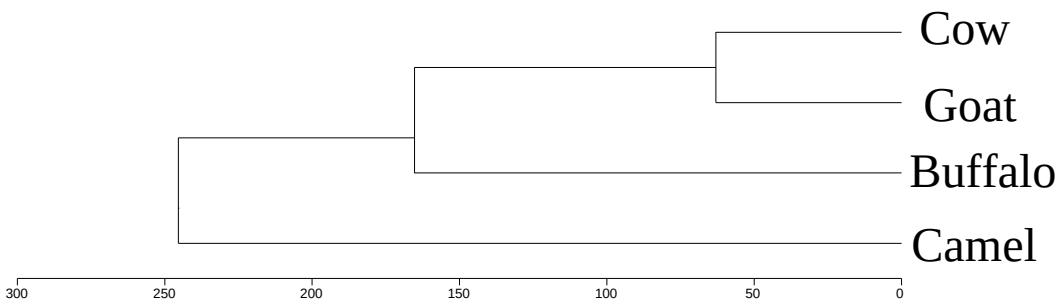

Figure 8 Dendogram showing the relationship among four species

Conclusion:
Milk is considered as a complete food. It not only serves as a source of essential proteins but also confers nutraceutical properties. Milk and other dairy products are an essential part of human diet as they support in the growth and development. The current study revealed valuable insights into determining the protein profile in different species. Obtained results concluded that highest protein content (65 mg/ml) was found in buffalo milk, 62 mg/ml in goat milk, 52.5 mg/ml in cow milk and 32 mg/ml in camel milk. The electrophoretic study of four different milk samples displayed almost the same results. αs1-casein band only appeared in cow milk that is considered as an allergy causing protein. However, camel milk showed some peculiar results. Major allergens were not found in camel milk samples making it a good choice for individuals with αs1-casein sensitivity or intolerance. The individuals may consume this milk with the recommendation of a healthcare professional with proper precautions. Furthermore, it can be used to make allergy free dairy products.

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Conflict of Interest: The authors have no conflict of interest.

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