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Estimation of bio-active peptides content of casein hydrolysates of Cow, Buffalo and Goat milk

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Abstract
An investigation was undertaken for estimation of bio-active peptides content of casein hydrolystaes using neutrase enzyme, molecular weight markers for cow, buffalo and goat milk samples, The total ash and moisture content of BAPs obtained from hydrolyzed casein protein fractions were evaluated separately. The results revealed that, buffalo milk had higher protein (4.65%) fat (6.55%) ash (0.82%) and SNF (16.92%) content as compared to cow and goat milk. Goat milk had shown significantly higher protein content (3.90%) as compared to cow milk (3.38%). Higher yield of α -casein (54.31%) was observed in cow milk than buffalo milk (48.95%) and lower yield (20.36%) was found in goat milk. The enzymatic hydrolysis significantly improved functional properties besides increased level of bio- active peptides along with improved digestibility. Thus, physico-chemical attribute study of Buffalo milk and Goat milk along with fractionation of proteins were carried out to identify molecular weight, chemical and gross composition of buffalo cow and goat milk.

Keywords: Bioactive, casein, enzyme, milk, peptides

Introduction
India is the largest milk producer in the world contributing 24 Percent of global milk production in the year 2021- 22 and is the single largest agricultural commodity contributing 5 percent of the Indian national economy. India has been regarded as an extremely rich gold mine of Buffaloes as it harbours all the recognized, high producing breeds of indigenous / non-descriptive Buffaloes. The second highest share of contribution of milk in India is from Buffaloes with 44.84 percent of the total production (after Cows 51.85%), this feat places India as the milk capital of the world, out pacing its competitors (NDDB COOP 2021-22). Milk production in India increased to 221.0 million tonnes in the year 2021-22 with growth rate of 6.38 percent per annum. The share of milk contribution by Cow, Buffalo and Goat to India's milk production is 51.85 percent, 44.84 percent and 2.93 percent respectively. Among the species, indigenous Buffaloes have highest share of milk production in India with 32.13 percent in the fiscal year 2022, followed by cross breed cows accounting for over 29.31 percent of the total milk production in the country (FAOSTAT, 2022) [8]. Goat milk differs from cow or buffalo milk in having better digestibility, alkalinity, buffering capacity and certain therapeutic values in medicine and human nutrition. Goat milk is considered to be an ideal food for people suffering from cow milk allergies and other gastro-intestinal ailments. Feeding goat milk to infants provides significantly higher digestibility as compared to cow milk. The children fed on goat milk surpassed those on cow milk in weight gain, skeletal mineralization and blood serum content of vitamin A, calcium, thiamin, riboflavin, niacin and haemoglobin (Mahendra *et al.*, 2017) [18].

Mineral contents of goat milk are much higher than that of cow's milk. Goat milk has more calcium, phosphorous, magnesium, potassium and chlorides and less sodium and sulphur than cow milks. Among trace minerals, zinc is in greater amounts in goat than cow and human milk. Goat milk has higher amount of vitamin A, niacin, thiamin, riboflavin and panthothenate for human infants than cow milk. Hence, this investigation was taken up with the following objectives to suitably modify cow, buffalo and goat milk protein hydrolysates; to isolate, fractionate and characterize buffalo milk and goat milk proteins and to enzymatically modify and characterize buffalo milk and goat milk proteins.

Milk is biologically complex fluid, constituted mainly of water, proteins, lactose, fat and inorganic compounds. Caseins and whey proteins are the main proteins present in various milk of different species in different proportion. The ratio of casein and whey proteins are 40:60 in human milk, 50:50 in equine milk, while in milk of cow, sheep, goat and buffalo it is 80:20.

Proteins and peptides present in milk have important nutritional, functional, biological and technological properties (Hadohum *et al.*, 2017) [12]. Caseins are highly digestible than whey proteins and are important for growth and development of infants. (Holt *et al.*, 2016) [14]. Caseins in milk are present in the form of micelles which are composed of αSl- casein (αSl-CN), αS2-casein (αS2-CN), β-casein and *k*-casein.

Enzyme modified casein hydrolysates play crucial role in food industry. The extent of protein hydrolysis, which represents the extent of protein breakdown to peptides and amino acids, is expressed either as percent amino nitrogen or as degree of hydrolysis (DH). Degree of hydrolysis is the ratio of the number of peptide bonds cleaved and the total number of peptide bonds in the intact protein. DH is one of the important controlling factors, which reflects on the product quality. Proteolytic enzymes have the ability to hydrolyse proteins to peptides and amino acids. The chain length of peptides formed is dependent upon the extent of hydrolysis, condition of hydrolysis, type, concentration and activity of enzyme, and type of protein to be hydrolyzed.

Compositional difference of milk of different species

Acidity and pH were inversely proportional to each other with the production of acid pH decreases. Acidity can be determined as the amount of lactic acid produced in dairy products. Buffalo and cow milk had higher acidity values i.e., 0.16 percent followed by sheep, Goat and camel with values 0.12 percent, 0.11 percent, and 0.11 percent, respectively. Similar results of pH were observed previously for different species such as camel, buffalo and cow milk. (Asif *et al*., 2010) [4].

Goat milk contains a higher amount of Ca, Mg and P than cow and human milk but Vitamin D, Vitamin B12 and folate contents are less. Goat milk is recommended for infants, old and convalescent peoples. The comparison of cow and human milk with goat milk which contains higher amount of Ca, P, Mg and K than cow and human milk hence, goat milk is recommended for infants, old and convalescent people (Arora *et al.*, 2013) [3].

Physico-chemical properties of milk from different species: The physic-chemical properties studied for the various types of milk clearly indicate the bioavailability of some types of milk over the other. However, each type is valid for its own purposes and the requirements of feeding. A comparative study on complex combination of proteins, carbohydrates, fats, minerals, and elements can be conducted to show the immediate relationship between types of milk in order to enhance the formulation of milk being an essential food for newly born calves (Huma *et al*., 2018) [15].

Characterization of milk proteins from different species: Proteins and peptides present in milk have important nutritional, functional, biological and technological properties. The physicochemical characteristics of many dairy products depend on properties of milk proteins. Several techniques have been developed for the separation and characterization of milk proteins. These include mainly gel electrophoretic methods, such as polyacrylamide gel (PAGE) with urea (Urea- PAGE) or SDS-PAGE. The PAGE separates the proteins by molecular mass (Jovanovic *et al.*, 2007) [17].

Characterisation of casein fractions: Research findings of Bhatia *et al*., (2015) [9] revealed that the polypeptide chain of αs1 – casein consists of two predominantly hydrophobic regions (amino acid residues 1-44 and 90-199) and highly charged polar zone (45-89). According to them it binds about 8 moles of Ca^{2+}per mole to the ester phosphate groups. As2-CN has a remarkable dipolar structure and it binds Ca strongly. They were of the view that, β-CN has a strongly negatively charged N-terminal portion. It tightly binds about 5 Ca^{2+} per mole, consistent with its ester phosphate content. K-CN contains only one phosphate group (SerP-149) and binds about 2 moles of Ca^{2+} per mole of protein (Mohamad *et al.*,2017) [19].

Yield of Casein Fractions and Peptides: The whole casein obtained by precipitation of cow and buffalo milk with 10 percent Hcl was fractionated into αs, β and k-casein using urea. Among cow milk casein yield is more for cow αs (60%) compared to cow β casein (17.6%) and in case of buffalo milk yield is more for αs casein (54.50%) compared to β casein (26.30%) (Shanshan *et al*., 2016) [24] reported that the urea fractionation of acid precipitated whole casein of cow and buffalo milk yielded more amount of αs casein compared to β casein of about 58.66 percent and 59.7 percent whereas β caseins yields about 39.89 and 39.9 percent respectively.

Chemical Composition of casein fractions: Pticek *et al.*, (2016) [22] reported the chemical composition of casein fractions. According to them the nitrogen content of α and β casein was 15.58 and 15.53 percent. Enzymatic hydrolysis of buffalo milk and goat milk is carried out for modification of protein is referred to changes in conformational or structural features which subsequently alter the physico-chemical properties. Adil *et al.* (2015) [1] carried out the hydrolysis of whole casein using trypsin enzyme to obtain desired degree of hydrolysis at different enzyme substrate ratio 1:25, 1:50, 1:100 and concluded that enzyme substrate (E:S) ratio of 1:25 was found to be optimum and well suited to obtain 5 percent degree of hydrolysis within 3 hrs.

Goat milk proteins are similar to the cow milk proteins fractions such as α-casein, β-casein, k- casein, β-lactoglobulin, α-lactalbumin, but they differ widely in genetic polymorphisms and their frequencies in the goat population. Peptides formed from the enzymatic cleavage of caseins of goat milk have greater advantages than those from cow milk casein. Goat milk fat differs in contents of fatty acids profile significantly from average cow milk fat. Goat milk has higher content of monounsaturated fatty acids, poly unsaturated fatty acids, medium chain fatty acids, than cow milk which are proven to be beneficial for cardiovascular disorders. Goat milk is more digestible as compared to other bovine milk. Additionally, Goat milk protein exhibits antimicrobial activity that plays an active role in bio-preservation against pathogens. Mineral contents of goat milk are much higher than that of cow's milk. Goat milk has more calcium, phosphorous, magnesium, potassium and chlorides and less sodium and sulphur than cow milks. Among trace minerals, zinc is in greater amounts in goat than cow and human milk. Goat milk has higher amount of vitamin A, niacin, thiamin, riboflavin and panthothenate for human infants than cow milk.

Hence, this investigation was taken up with the following objectives to suitably modify buffalo and goat milk proteins to maximize the utilization share of buffalo milk and to characterize them enzymatically.

1. To isolate, fractionate and characterize buffalo milk and goat milk proteins.
2. To enzymatically modify and characterize buffalo milk and goat milk proteins.

Materials and Methods

The materials are used in this investigation were buffalo and goat milk samples, neutrase enzyme, molecular weight markers. Buffalo and goat whole milk samples were subjected to centrifugation in cooling centrifuge at 3000 rpm for 10 min at 10 °C. After centrifugation, skim milk and cream were separated. Acid whole casein (wet) was prepared by adjusting the pH of skim milk to 4.6 with 10 percent HCl at 20 °C, as per the method by Hipp *et al*., (1952) [13]. Whole casein and whey proteins were prepared by coagulation of buffalo and goat skim milk separately at pH 4.6 using 10 percent dilute hydrochloric acid. Cool the suspension to room temperature and leave it for 5 min. Filter through muslin cloth and casein precipitate was washed 2 to 3 times with cold distilled water to remove traces of acid. SDS-PAGE was carried out to assess the molecular weight ranges of casein fractions by following the method prescribed by Laemmli (1976). The following reagents were employed for analysis. The nitrogen content of casein fraction were estimated by micro kjeldhal method as per the procedure of AOAC (1980) [2]. Whole casein of Cow, Buffalo and Goat milk were dispersed separately in distilled water at 40 °C to give a 5 percent (w/v) protein concentration and the pH of the solutions was adjusted to optimum as that of the enzyme using 0.1N NaOH. The enzyme neutrase was added (1µl of enzyme/5g of protein) at pH 7.5 and temperature 45 °C was maintained. The total ash and moisture content of BAPs obtained from hydrolyzed casein protein fractions were evaluated separately as per the procedure of AOAC (1984) [2]. Whereas, nitrogen and protein content by micro-kjeldhal method as per the procedure of AOAC (1980) [2]. Bioactive peptides were analyzed for nitrogen and protein content by micro- kjeldhal method.

Results and Discussion

Cow, Buffalo and goat milk samples were subjected for analysis of gross composition. The results pertaining to this are presented in the Table 1.

Table 1: Gross composition of cow, buffalo and goat milk

Species	Moisture (%)	Fat (%)	Protein (%)	Lactose (%)	Ash (%)	SNF (%)	Total solids (%)
Cow	87.28^{a}	4.28^{a}	3.38^{a}	4.37^{a}	0.69^{a}	8.44^{a}	12.72^{a}
Buffalo	83.08^{b}	6.55^{b}	4.65^{b}	4.90^{b}	0.82^{a}	10.37^{b}	16.92^{b}
Goat	87.02^{a}	4.20^{a}	3.90^{c}	4.14^{a}	0.74^{a}	8.78^{a}	12.98^{a}
CD (p<0.05)	0.52	0.57	0.49	0.52	0.27	0.51	0.54

- All the values are average of three trails
- Similar superscripts indicate non-significant at corresponding critical difference (CD)

The results revealed that, buffalo milk had higher protein (4.65%) fat (6.55%) ash (0.82%) and SNF (16.92%) content as compared to cow and goat milk. Goat milk had shown significantly higher protein content (3.90%) as compared to cow milk (3.38%).

The physical characteristics of cow, buffalo and goat milk were studied, and the results are presented in Table 2 and Fig 1.

Table 2: Physical characteristics of cow, buffalo and goat milk

Species	Specific Gravity	pH	Acidity (%LA)	Surface Tension (dynes/cm)	Conductivity (S/m)	Viscosity (cP)
Cow	1.028^{a}	6.61^{a}	0.136^{a}	51.57^{a}	9.60^{a}	1.38^{a}
Buffalo	1.035^{b}	6.72^{a}	0.153^{a}	50.45^{b}	7.65^{b}	2.04^{b}
Goat	1.027^{a}	6.64^{a}	0.134^{a}	52.15^{a}	10.45^{c}	1.44^{ac}
CD (p<0.05)	0.03	0.19	0.29	0.50	0.49	0.48

- All the values are average of three trails
- Similar superscripts indicate non-significant at corresponding critical difference (CD)

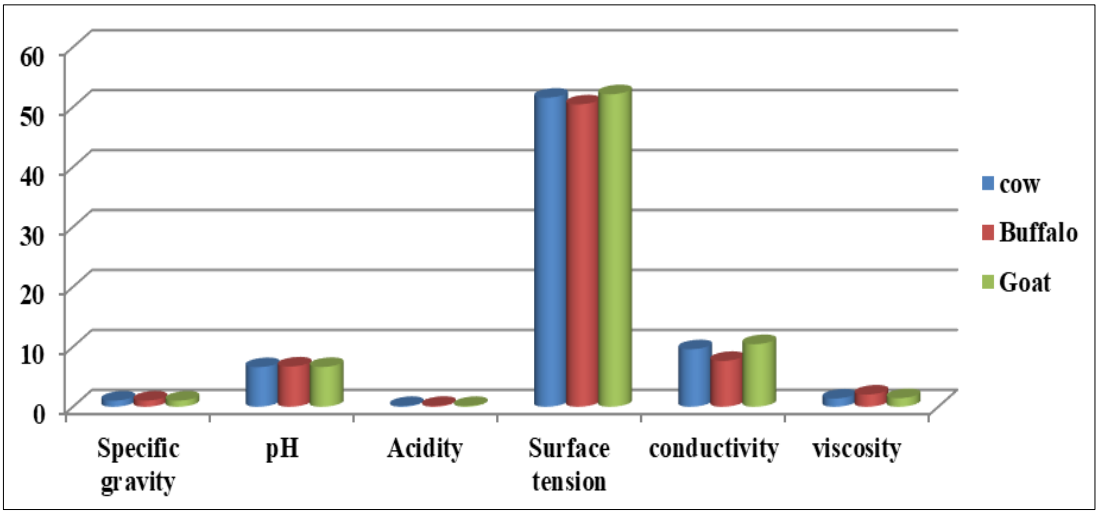

Fig 1: Physical characteristics of cow, buffalo and goat milk

Physico-chemical properties of cow, buffalo and goat milk used in this investigation revealed that, buf falo milk exhibited higher specific gravity (1.035), viscosity (2.04 cP) and acidity (0.153%) as compared to cow and goat milk. Higher surface tension (52.15 dynes/cm^2) and conductivity (10.45 S/m) in goat milk was observed as compared to cow and buffalo milk. Higher pH and lower acidity was noted in buffalo milk as compared to both cow milk and goat milk.

The effect of source of milk protein on yield of casein and whey protein fractions is presented in Table 3 and Fig 2. The effect of source of milk protein on yield of various casein fractions is presented in Table 4 and Fig 3.

Table 3: Effect of source of milk protein on yield of caseins and whey proteins

Source of Milk	Total Protein (g/l)	Protein (%)	Caseins (g/l)	Casein Yield (%)	Whey Proteins (g/l)	Whey Proteins Yield (%)
Cow	35.76^a	3.58^a	28.52^a	79.76^a	7.24^a	20.24^a
Buffalo	42.50^b	4.25^b	34.30^b	80.71^a	8.20^b	19.29^a
Goat	35.82^a	3.68^a	28.45^a	79.42^a	7.37^{ac}	20.58^a
CD (p<0.05)	0.57	0.51	0.55	0.53	0.53	0.58

- All the values are average of three trails
- Similar superscripts indicate non-significant at corresponding critical difference (CD)

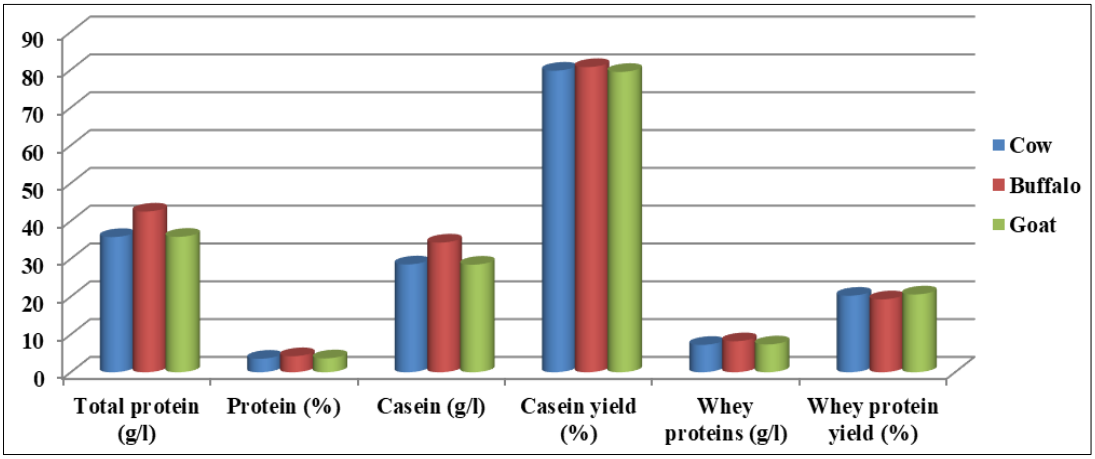

Fig 2: Effect of source of milk protein on yield of caseins and whey proteins

The sources of protein had significant influence on total casein and whey proteins content of different species. Highest quantity of total caseins (34.30g/l) and whey protein (8.87 g/l) were noted in buffalo milk than cow (28.52 g/l) and Goat milk (28.45 g/l). Total protein, casein and whey protein contents were greatly affected by the source of milk obtained.

Table 4: Effect of source of protein on yield of various fractions of casein

Source of Casein Fractions	Total Casein (g/l)	Yield (%)	Total α-Casein (g/l)	Yield (%)	Total β-Casein (g/l)	Yield (%)	Total k-Casein (g/l)	Yield (%)
Cow	27.47^a	76.83^a	14.92^a	54.31^a	9.38^a	34.14^a	3.17^a	11.53^a
Buffalo	34.13^b	76.70^a	16.64^b	48.75^b	12.30^b	36.03^b	5.19^b	15.20^b
Goat	26.62^c	72.33^b	5.42^c	20.36^c	17.85^c	54.05^c	3.35^{ac}	20.49^c
CD (p<0.05)	0.53	0.54	0.47	0.60	0.56	0.50	0.55	0.49

- All the values are average of three trails
- Similar superscripts indicate non-significant at corresponding critical difference (CD)

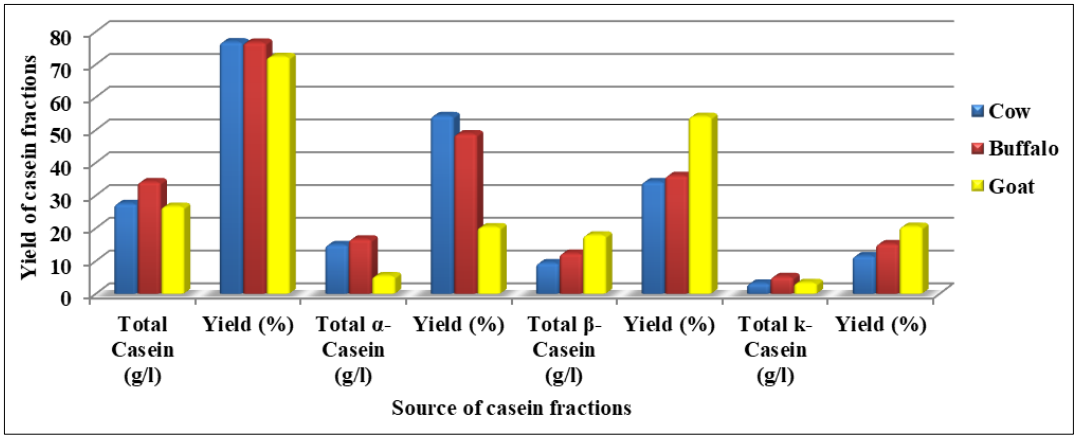

Fig 3: Effect of source of protein on yield of various fractions of casein

Results from the table revealed that, Casein obtained from cow, buffalo and goat milk were fractionated by urea solubility method and the fractions were quantified. The significant effect of source on protein yield and fractions of caseins was observed. Higher yield of α -casein (54.31%) was observed in cow milk than buffalo milk (48.95%) and lower yield (20.36%) was found in goat milk. Amongst the three species, highest percent of β casein was noted in goat milk (54.05%) followed by buffalo (36.03%) and cow milk (34.14%).

Buffalo and goat whole milk samples were subjected to centrifugation in cooling centrifuge at 3000 rpm for 10 min at 10 °C. After centrifugation, skim milk and cream were separated. Acid whole casein (wet) was prepared by adjusting the pH of skim milk to 4.6 with 10 percent HCl at 20 °C, as per the method by Hipp *et al.*, (1952) [13]. SDS-PAGE was carried out to assess the molecular weight ranges of casein fractions by following the method prescribed by Laemmli (1976). The results obtained are presented in Table 5 and 6, Fig 4.

Table 5: Physico-chemical characteristics of various fractions of cow milk casein

Type of casein fractions	Mol wt (kDa)	pI	pH	Phosphorous (%)	Nitrogen (%)
α-Casein	23.82[a]	4.55[a]	4.95[a]	0.69[a]	14.85[a]
β-Casein	24.31[a]	4.73[a]	5.44[b]	0.35[b]	12.55[b]
k-Casein	19.15[b]	5.50[b]	5.73[b]	0.10[c]	12.75[c]
CD (p<0.05)	0.63	0.50	0.45	0.22	0.55

- All the values are average of three trails
- Similar superscripts indicate non-significant at corresponding critical difference (CD)

Table 6: Molecular weight profile of casein fractions of cow, buffalo and goat milk

Molecular weight range Da	Cow milk			Buffalo milk			Goat milk		
	α Casein	β Casein	k- Casein	α Casein	β Casein	k- Casein	α Casein	β Casein	k- Casein
< 1800-19500	-	-	+	-	-	-	-	-	+
19500-21500	-	-	-	-	-	-	-	-	-
21500- 22500	-	-	-	-	-	+	-	-	-
22500-23500	-	-	-	+	-	-	-	-	-
23500-24500	+	-	-	+	+	-	+	+	-
24,500-25,000	-	+	-	+	-	-	-	-	-

+ Presence of bands
- Absence of bands

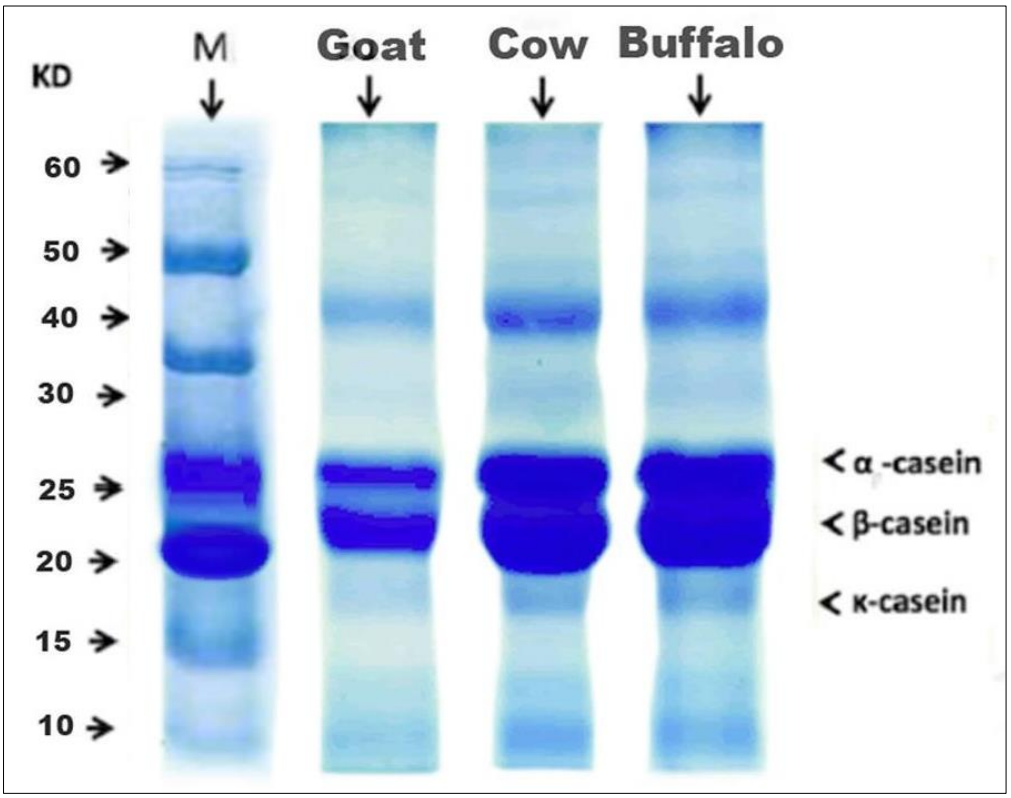

Fig 4: Characterization of casein fractions of cow, buffalo and goat milk proteins by SDS-PAGE

Cow, buffalo and goat milk were hydrolyzed by Neutrase enzyme by employing optimum enzyme: substrate (E: S) ratio of 1:25 at a pH of 7.5 and a temperature of 45 °C. The extent of hydrolysis was measured at an interval of every 5 minutes and the hydrolysis attained is expressed as degree of hydrolysis (DH). The results are presented in Table -7.

Table 7: Effect of duration of enzymatic hydrolysis on extent of hydrolysis (DH)

Hydrolysis Time (Min)	Cow milk casein	Buffalo milk casein	Goat milk Casein
	% Degree hydrolysis (DH) by Neutrase enzyme		
5	2.70	2.60	2.20
10	3.82	3.14	2.98
15	4.12	4.65	3.82
20	4.92	4.49	4.16
25	5.32	5.01	5.23
30	6.15	5.89	5.92
35	6.89	6.75	6.58
40	7.46	7.65	7.71
45	7.89	8.02	8.31
50	8.41	8.54	9.01
55	9.03	9.31	9.84
60	10.2	10.32	10.45
65	10.50	11.07	11.40
70	11.10	11.90	12.03
75	11.95	12.75	12.95
80	12.76	13.50	13.95
85	13.96	14.20	14.65
90	14.72	15.01	15.32

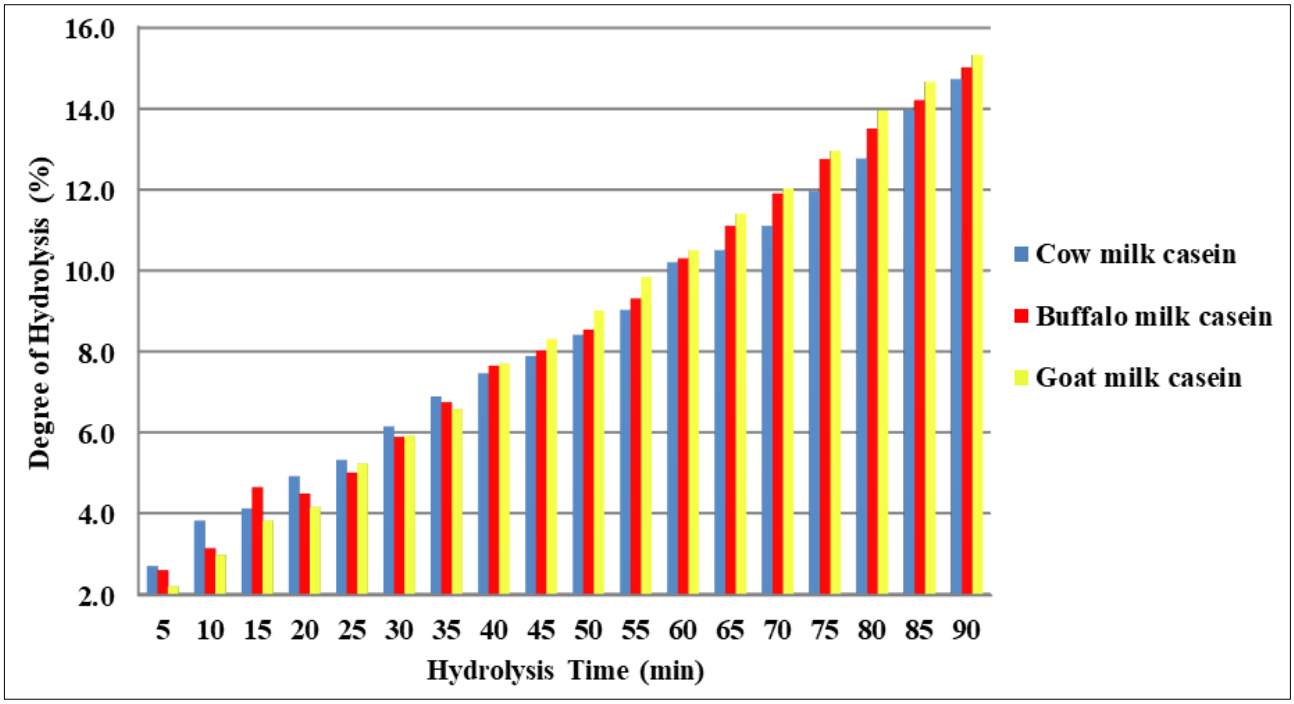

Fig 5: Effect of duration of enzymatic hydrolysis on extent of hydrolysis (DH)

Conclusion

Buffalo milk, Goat milk and Cow milk were subjected to enzymatic hydrolysis using Neutrase enzyme, to attain hydrolysis of 5, 10 and 15% Degree hydrolysis (DH). The enzymatically hydrolysed milk (5, 10 and 15% DH) was further subjected to physico-chemical and functional attribute studies. The enzymatic hydrolysis significantly improved functional properties besides increased level of bio- active peptides along with improved digestibility. Thus, physico-chemical attribute study of Buffalo milk and Goat milk along with fractionation of proteins were carried out to identify molecular weight, chemical and gross composition of buffalo cow and goat milk.

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