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Compositional Profiling and Proteolytic Activities in Cow and Buffalo Milk Cheddar Cheese

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ABSTRACT

Milk composition is an imperative aspect influencing the quality of dairy products. The study was aimed to compare the cheddar cheese made from buffalo and cow milk with respect to composition and proteolysis (water-soluble nitrogen, non-protein-nitrogen) during ripening. The assessment of proteolysis in cheese through RP-HPLC was also core part of present exploration. Considerable differences were found in compositional profile of both cheeses. Buffalo milk cheese was relatively higher in fat (30.32%) and protein (31.91%) contents throughout storage period. The rate of proteolysis and peptide's production was also comparatively higher in buffalo milk cheese as evident from water-soluble nitrogen (16.86%) and non-protein-nitrogen (8.44%) contents. Significant (p<0.01) increase in proteolysis index was observed over the ripening period of 180 days. RP-HPLC studies demonstrated that cheeses expressed peptide peak developments from the very first day till the end of ripening. However, slight differences were observed concerning the number, area and height of peaks in cheese extracts during ripening. Hence, it was concluded that buffalo milk is superior with regard to compositional profile and proteolytic activities.

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Authors' Contribution
SR conceived the project, executed the experimental work and wrote the article. Others helped in designing the experiments and analysis of data.

Key words
Cheddar cheese, buffalo milk, cow milk, proteolysis.

INTRODUCTION

Globally, consumers pay great attention towards the food and its composition due to pivotal relationship between diet and human health (Granato *et al.*, 2010). Milk is extensively used for development of value added products. Composition of milk is an imperative aspect which influences the quality of end product. However, quantity and relative proportion of milk constituents fluctuate markedly among different dairy animals depending on species, breeds, stage of lactation, milking methods, environment, season, diet and feeding system (Kittivachra *et al.*, 2007).

Pakistan is the 2nd largest producer of buffalo milk contributing 67.04%, while the share of cow milk is 31.56% of total production (GOP, 2013-14; Tahira *et al.*, 2014). The buffalo milk receives an increasing research attention and investment in many countries owing to its unique nutritional profile (Murtaza *et al.*, 2014). It is richer in fat, lactose, protein, total solids, vitamins and minerals (Ahmed *et al.*, 2008). The dairy products especially cheeses prepared from buffalo milk are becoming increasingly popular throughout the world (Hofi, 2013).

Cheddar cheese is mixture of moisture, fat, salt, protein, peptides, amino acids, lactose, minerals and other minor constituents, occluded within a casein matrix (Murtaza *et al.*, 2008). It is a biochemically dynamic product which undergoes significant changes during ripening. The cheese ripening is a very complex microbiological and biochemical process which involves the enzymatic digestion of curd components. It mainly includes the fermentation of lactose proteolysis and the degradation of fats. The resultant products and active molecules like peptides, free fatty acids and others from ripened cheeses may perform several physiological roles in the body (Yasuda *et al.*, 2012). The quality of cheddar cheese depends on manufacturing technology, starter cultures and composition of milk.

Special nutritional characteristics have been claimed for various types of non-bovine milk and milk products (Al Haj and Al Kanhal, 2010). These underutilized resources are of great significance to milk producers, processors, and consumers for designing the innovative products with versatility, taste and functionality. Therefore, valorization of non-bovine milk and milk products requires intensive research, particularly in the area of proteins and peptides. Keeping in view the production of buffalo milk in Pakistan and its composition, present research work was carried out to manufacture cheddar cheese. Moreover, cheeses were subjected to compositional profiling and proteolysis during different stages of ripening.

MATERIALS AND METHODS

Procurement of raw material

Buffalo and cow milk samples were collected from Dairy Farm, University of Agriculture, Faisalabad-

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Pakistan. The starter culture *Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *cremoris* (Rohan Industrial Estate, Co. Cork, Ireland) and rennet (Chr. Hansen, Denmark) were used for cheese production. The acetonitrile and trifluoroacetic acid (Sigma-Aldrich, MO, USA) were used for RP-HPLC studies.

Physico-chemical analysis of milk

The buffalo and cow milk samples were analysed for pH using pH meter (Hanna, HI-99161), acidity (AOAC, 2000), protein (Kjeldahl's method; AOAC, 2000), fat (Marshall, 1993), solid-not-fat (SNF) contents (David, 1977), total solids (AOAC, 2000) and moisture (AOAC, 2000).

Manufacturing of cheddar cheese

The cheddar cheese was manufactured (triplicate batches) in cheese vat (FT20-MkII, Armfield) after standardization of buffalo and cow milk at 4% fat level, according to the method described by Ong *et al.* (2006). The prepared cheese was stored for ripening at 8°C for 6 months.

Compositional profiling of cheddar cheese

The buffalo and cow milk cheddar cheeses were subjected to compositional assays at different stages of ripening. The pH of cheese slurry was measured using pH meter (Hanna, HI-99161) after calibrating with fresh pH 4.0 and 7.0 standard buffers (Ong *et al*., 2007). Acidity percentage was calculated by titration (Method No. 920.124; AOAC, 2000). The moisture content in cheddar cheese was determined by drying samples in oven at 103 ± 5°C till the constant weight is obtained (Method No. 926.08; AOAC, 2000). Fat content in cheese was measured according to method No. 933.05 (AOAC, 2000). The protein (nitrogen) contents were determined by Kjeldahl method using kjeltec system (D-40599, Behr Labor Technik, Germany) as given in FIL-IDF (1993).

Determination of proteolysis

The degree of proteolysis in the cheddar cheeses was evaluated by water-soluble nitrogen (Kuchroo and Fox, 1982) and non-protein-nitrogen (NPN) according to FIL-IDF (1993) Kjeldahl method using Kjeltec system (D-40599, Behr Labor Technik, Germany).

RP-HPLC profiling of cheddar cheese

The cheeses were profiled on an RP-HPLC system (Agilent, Series 1100, USA) consisted of C_{18} column (Ascentis™, 25cm × 4.6 mm, 5.0µm) equipped with a pump, an auto-sampler (Agilent, Model G1313) and UV-visible detector (Hewlett Packard HP 1050). Purposely, the cheese extracts (40 mg) were dissolved in solvent A (1 mL) containing 0.1% TFA, centrifuged (Eppendorf AG 22331 Hamburg, Germany) at 14000 × *g* for 10 min and filtered. The gradient elution was used with solvent A (0.1% TFA in water) and solvent B (0.1% TFA in acetonitrile). The sample (50 µL) was injected into column and chromatographic separation was conducted at room temperature, 1.0 mL/min flow rate and 215 nm wavelength.

Statistical analysis

The analyses were performed in triplicate to investigate the impact of milk source (buffalo, cow) and ripening time on biochemical changes and proteolysis in cheddar cheese. The resultant data analyzed statistically by ANOVA using Minitab statistical package and Tukey's test was used for multiple comparisons (α= 0.05) between means.

RESULTS AND DISCUSSION

Physico-chemical analysis of milk

Cheddar cheese manufacturing commences with the selection of milk of high microbiological and chemical quality. Purposely, the milk samples were subjected to different compositional assays to ascertain the suitability of milk for cheese production. Table I shows that proteins (4.25%), fat (6.58%), solid-not-fat (10.09%) and total solid (16.67%) contents were relatively higher in buffalo milk. Higher moisture (87.96%) percentage was observed in cow milk whilst, buffalo milk showed comparatively lower (85.34%) moisture. Slight differences were observed for % acidity and pH values of milk samples of both species.

The buffalo milk has relatively higher content of fat, protein, lactose and total solids than cow which makes it a highly suitable ingredient for manufacturing of various milk products, especially cheese (Murtaza *et al.,* 2008). The milk's pH is most critical factor for manufacturing of various dairy products. Many previous findings regarding pH, acidity, fat and protein contents of buffalo and cow milk (Imran *et al.,* 2008; Ozrenk and Inci, 2008; Ahmad *et al.* (2013) are in agreement with the current investigation. The lower pH of fresh milk may be due to bacterial action and higher one indicates the udder infection or mastitis (Uallah *et al.,* 2005).

Compositional profiling of cheddar cheese

The pH values of buffalo milk cheddar cheese (BCC) and cow milk cheddar cheese (CCC) were increased significantly (p<0.01) over the ripening period of 180 days. The slight drop in pH was noticed in both cheeses at early stages of ripening. This may be attributed to the conversion of residual lactose into lactic acid. The

Table I.- Physico-chemical composition of buffalo and cow milk.

Species	pH	Acidity (%)	Protein (%)	Fat (%)	SNF (%)	TS (%)	Moisture (%)
Buffalo	6.65±0.01^{a}	0.12±0.01^{a}	4.25±0.07^{a}	6.58±0.02^{a}	10.09±0.03^{a}	16.67±0.05^{a}	85.34±1.02^{b}
Cow	6.63±0.02^{a}	0.13±0.01^{ab}	3.32±0.05^{b}	4.17±0.03^{b}	9.13±0.02^{ab}	13.32±0.06^{b}	87.96±0.91^{a}

Values are expressed as means ± standard deviation; Means sharing similar letter (superscript) in a row or in a column are statistically non-significant (P> 0.05); SNF= Solid- not- fat; TS= Total solids

Table II.- Compositional profiling of buffalo and cow milk cheddar cheese during ripening.

	Buffalo milk cheese				Cow milk cheese			
	1day	60 day	120 day	180 day	1day	60 day	120day	180 day
pH	5.25±0.01^{e}	5.27±0.01^{d}	5.32±0.02^{c}	5.36±0.02^{ab}	5.23±0.02^{e}	5.27±0.02^{d}	5.31±0.01^{c}	5.38±0.01^{a}
Acidity	0.88±0.01^{de}	0.89±0.02^{cd}	0.91±0.02^{abc}	0.92±0.01^{ab}	0.89±0.01^{cd}	0.90±0.02^{bc}	0.91±0.02^{abc}	0.93±0.01^{a}
Moisture	37.50±0.5^{abc}	36.68±0.54^{bc}	36.59±0.55^{bc}	36.53±0.58^{c}	38.50±0.4^{a}	38.01±0.2^{ab}	37.45±0.5^{abc}	37.17±0.3^{abc}
Fat	30.29±0.7^{a}	30.31±0.62^{a}	30.33±0.49^{a}	30.34±0.50^{a}	29.79±0.51^{ab}	29.82±0.6^{ab}	29.90±0.4^{a}	29.90±0.5^{a}
Protein	32.00±0.39^{a}	31.99±0.36^{a}	31.86±0.32^{a}	31.78±0.29^{a}	25.82±0.33^{b}	25.57±0.29^{b}	25.53±0.3^{b}	25.50±0.2^{b}

Analysis were performed in triplicate and results are expressed as means ± standard deviation. Means sharing similar letter (superscript) in a row or in a column are statistically non-significant (P> 0.05)

pH continued to increase during the rest of the ripening phases for both cheddar cheeses. Further increase in pH was credited to the destruction of lactic acid, limitation of lactose and proteolysis resulted in the liberation of numerous degradation products which were basic in nature (Gupta *et al.,* 2009). Likewise, Fenelon and Guinee (2000) reported the increase in pH of cheddar cheese due to production of ammonia via deamination of free amino acids. Similar increase have been investigated in ripened hard cheeses, semi hard cheeses (Guinee *et al.,* 2000) and cheddar cheeses (Gupta *et al.,* 2009).

The increase in acidity (%) of cheddar cheese was also observed during ripening; however, this increase was not significant (p>0.05) in both the BCC and CCC as shown in Table II. Increasing trend in titratable acidity during cheese ripening was also expounded by many previous findings (Marth and Steele, 2001; Aly and Galal, 2002). The higher acidity in cheeses may be attributed to the activity of starter culture because the acidification of cheese milk to the desired pH during manufacture is basic function of starters (Amarita *et al*., 2006).

The decrease in moisture content during ripening was recorded as 37.5%, 36.68%, 36.59% and 36.53% at 1, 60, 120 and 180 days in case of BCC, respectively (Table II). The present study is related with the findings of Srivastava (2002) and O'Connor and O'Brien (2000) demonstrating 34-38% and 36% moisture content in normal cheddar cheese respectively, while Kucukoner and Haque (2006) found 40% moisture in full fat cheddar cheese.

The cheese prepared from buffalo milk contained significantly higher (30.32%) fat content as compared to cow (29.75%). It may be due to the fact that the loss of buffalo milk fat with whey is relatively less than that in case of cow milk. However, the slight increase in fat level during storage period may also be attributed to the decrease in moisture content (Aly and Galal, 2002).

The protein content of buffalo milk Cheddar cheese was found to be significantly higher (31.91%) as compared to cow (25.61%) milk. However, the non-significant decrease in protein content was recorded as 32.00%, 31.99%, 31.86% and 31.78% at different stages of BCC ripening, respectively (Table II). The present study is justified by the findings of Fenelon and Guinee (2000), Ong *et al.* (2007) and Kucukoner and Haque (2006). The considerable difference between protein content in cheeses is because buffalo milk is richer in protein, especially caseins than cow (Murtaza *et al.,* 2008) milk. Significant inter-species differences in the concentration and types of caseins contents of milk are reflected in the characteristics of the cheeses produced from them (Tahira *et al.,* 2014). Non-significant decrease in protein content during ripening is generally accompanied with increase in fat and reduced moisture contents (Rehman *et al.,* 2004).

Proteolysis in cheese

Proteolysis is the most important chemical and biochemical change occur during cheese ripening. The degree of primary proteolysis is measured by monitoring the concentration of water-soluble nitrogen (WSN) which includes mainly peptides with molecular mass ≤ 10kDA (Fenelon and Guinee, 2000). In the present study, ripening

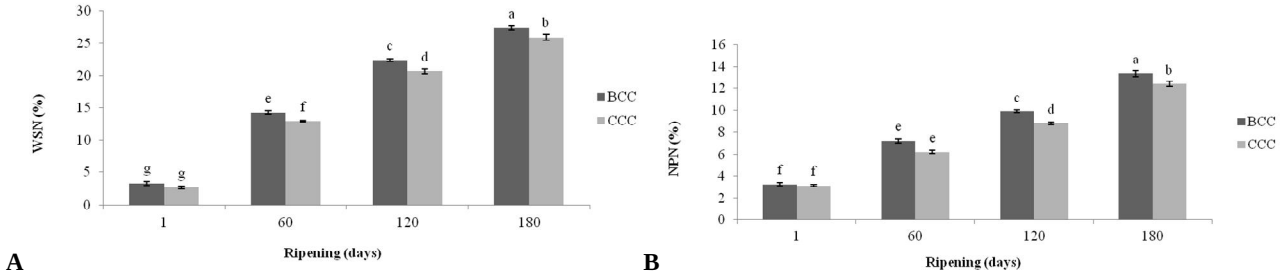

Fig. 1. Effect of milk source and ripening days on WSN and NPN contents (%) of cheddar cheeses. Results are expressed as means ± standard error. NPN, non-protein-nitrogen (expressed as percentage of total nitrogen); WSN, water-soluble nitrogen (expressed as percentage of total nitrogen); BCC, buffalo cheddar cheese; CCC, cow cheddar cheese.

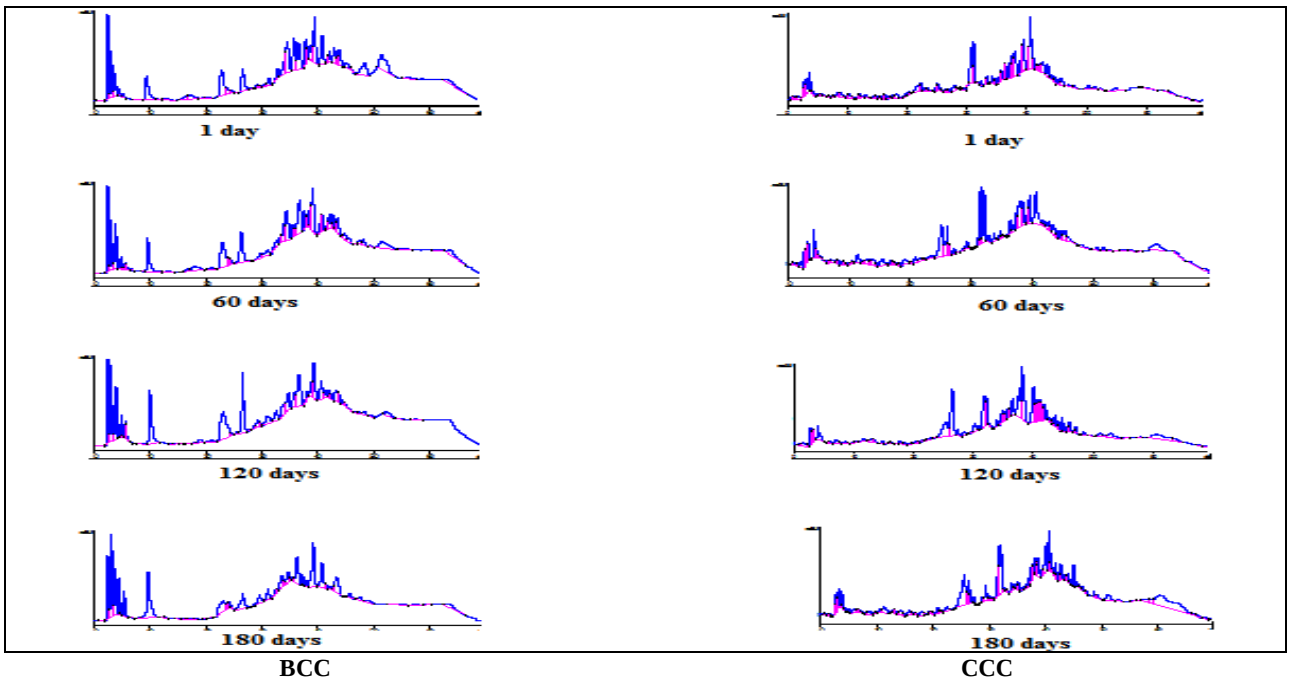

Fig. 2. RP-HPLC chromatograms of buffalo cheddar cheese (BCC) and cow cheddar cheese (CCC) at different stages of ripening.

was accompanied by a significant (p<0.01) increase in WSN contents. The increase in mean value of which ranged from 2.62±0.35% at 1 day to 27.44±0.08% at 180 day of cheese maturation. The significant difference was also observed for the milk source (p<0.05) indicating means values 16.86% for BCC and 15.55% for CCC (Fig. 1A). The amount of NPN contents was also increased progressively throughout the 180 days of investigation period. However, the BCC demonstrated relatively higher (8.44%) levels of NPN than CCC (7.63%) during maturation (Fig. 1B).

Many previous studies (Kiernan *et al.*, 2000); Guinee *et al.*, 2000; Fenelon *et al.*, 2002; Ong *et al.*, 2007) are in accordance with the present work regarding proteolysis. Starter and non-starter bacterial proteinases are principally responsible for the formation of NPN contents. More products of the primary proteolysis became available as substrates for the subsequent proteolysis by peptidases thus resulted in the increased levels of NPN of cheeses (Rynne *et al.*, 2004; Kenny *et al.*, 2005; Hannon *et al.*, 2005).

RP-HPLC profiling of cheddar cheese extracts

The RP-HPLC chromatograms of BCC and CCC extracts obtained at day 1, 60, 120 and 180 are shown in Figure 2. Both cheeses expressed peak developments

during whole ripening period. Slight differences were observed concerning the number of peaks, area and peak height throughout the ripening. The chromatograms changed and became more complex as the cheeses matured due to increase in number of peaks and area. Overall, the increase in peak height was noticed throughout ripening time of BCC but the peaks eluted at 9, 23, 26 retention time developed maximum at 150 days and decreased at 180 days.

The slight deviation in RP-HPLC profiling of cheese extracts could be attributed to variability related to treatment or cheese sample obtained in replicate cheese making trials. However, variation due to the analytical technique including sampling, extraction and storage of the extracts, and chromatographic separation may also significantly affect the HPLC profiles obtained (Coker *et al.*, 2005). Previous studies (Ong and Shah, 2008; Ong *et al.*, 2007) revealed that more peptides liberated into the cheeses as the ripening period proceeded. They also observed increase in number of peaks and total area during the first 12 weeks which continued to increase slowly until 24 weeks and then remained constant in all Cheddar cheeses. The RP-HPLC profiles are comparable to those found in the Cheddar cheese investigations by many researchers (Law *et al.*, 1993; De Wit *et al.*, 2005).

Hence, it was found that buffalo milk cheese has greater rate of proteolysis owing to its compositional profile and buffering capacity when compared with cow.

Conflict of interest statement

The authors have no conflict of interest to declare.

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