J Food Sci Technol (August 2017) 54(9):2737–2745
DOI 10.1007/s13197-017-2710-9

ORIGINAL ARTICLE

Effect of quality of milk on maillard reaction and protein oxidation during preparation of cow and buffalo milk *khoa*

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Revised: 16 May 2017 / Accepted: 16 May 2017 / Published online: 23 May 2017

Abstract The browning indicators (Lactulose, HMF and Furosine) and protein bound carbonyls were used to assess the effect of developed acidity and subsequent neutralization of milk at various stages of *khoa* (heat desiccated milk product) preparation. Available lysine was also analyzed in raw milk and final product i.e. *khoa*. Available lysine decreased as milk progressed to *khoa* preparation. Present study indicated that increase in heating intensity resulted in increased concentration of browning indicators and protein bound carbonyls (PC) in boiled milk and *khoa*. Concentration of browning indicators was found to be significantly higher in buffalo milk and *khoa* samples whereas, PCconc. was higher in cow milk and *khoa* samples. Neutralization of milk significantly affected Maillard reaction by elevating concentration of browning indicators and PC in both milk and *khoa*.

Keywords *Khoa* · HMF · Furosine · Lactulose · Protein bound carbonyls

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Introduction

Heating of milk resulted in many chemical changes which significantly affects the nutritional quality of milk and milk products. *Khoa* is a heat desiccated traditional Indian milk product with semi solid consistency, prepared by heat concentration of milk in an open pan with continuous stirring and scrapping. Depending upon the end use, *khoa* can be further classified as Pindi, Dhap, and Danedar which differs in composition, texture and quality. All of these varieties are in demand and required for making value added *khoa* based products e.g. *burfi, peda, kalakand, gulabjamun* etc. (De 2004; Londhe et al. 2012; Choudhary et al. 2015). These products may be potential source for export. *Khoa* is also used for stuffing vegetables in many food items. It is a major intermediate base for a variety of sweets e.g. *burfi, peda, kalakand, gulabjamun* etc. (De 2004; Londhe et al. 2012; Choudhary et al. 2015). The application of heat during *khoa* manufacture inevitably gives rise to reactions between the milk constituents. Maillard reaction is considered among one of most important heat induced modifications. It involves reaction between amino acids and reducing carbohydrates which can result in a loss in nutritive value of foods. Hydroxyl methyl furfural (HMF) is potentially a polymer building block and is a well-known decomposition product of 3-deoxyglucosones and has often served as an indicator of severity of heat intensity applied to milk. Evaluation of early stages of Maillard reaction can be achieved by determination of Furosine (ε-N-2-furoylmethyl-L-lysine). Furosine is an artificial amino acid formed during acid hydrolysis of the Amadori compounds, originated in the interaction of the ε-amino groups of lysine with glucose, lactose, and maltose. It is a useful indicator of damage in milk, prolonged heating or inadequate storage results in

increased level of Furosine (Guerra-Hernández et al. 2002). Other reactions that can take place include isomerization and the crystallization of lactose. Lactulose is a disaccharide produced from lactose by isomerization in alkaline solutions and during heat treatment of milk. Lactulose itself is absent in raw milk but heating of milk results into conversion of lactose to Lactulose by Lobry de Bruyn–Alberda van Ekenstein (LA) transformation (*Beach and Menzies* 1983; Hashemi and Ashtiani 2010). The amount of Lactulose formed depends on extent of heating and may be used as an index of heating. Lactulose may be formed as indirect consequences of Maillard reaction or by isomerization of lactose catalyzed by amino group of protein (Amine et al. 2000; Guerra-Hernández et al. 2002). HMF, Furosine and Lactulose are known as browning indicators and also the indicator of intensity of heat treatment in milk. Prolonged storage results in increased concentration of these browning indicators (Guerra-Hernández et al. 2002). Protein oxidation occurs as a result of either direct attack by reactive oxygen species or photo oxidation or indirectly through peroxidation of lipid that further degrades and attack protein. When secondary lipid oxidation products reacts with amino acids it forms protein bound carbonyl groups in the proteins. Protein-bound carbonyls (PC) are routinely used as biomarker of protein oxidation (Fedele and Bergamo 2001; Balestrieri et al. 2002). Protein oxidation in terms of protein carbonyl has been reported in milk and dairy products such as cheese and milk powder (Fedele and Bergamo 2001; Balestrieri et al. 2002; Scheidegger et al. 2012, Fernandez et al. 2014).

In recent years, the milk adulteration has become a significant problem in India. Due to comparatively higher ambient temperature and tropical climate conditions of India, acidity is rapidly developed in milk at ambient temperature which results in unsuitability of such milk for processing (Choudhary et al. 2016). As neutralization is one of common practices of adulteration in India therefore, neutralization of such milk makes it acceptable/suitable for heating during sweets preparation. The consumption of sweets is an integral part of Indian dietary system. Historically, surplus milk in the rural areas is converted into a variety of traditional dairy products (Londhe et al. 2012) and a considerable share of milk production is converted to *khoa* preparation. As *khoa* is a heat desiccated milk product, interactional changes occur during heating at various stages. These are also affected by change in consistency of *khoa* starting from liquid to solid. Most of the work on *khoa* in past was focused on standardization of manufacturing methods, chemical composition, sensory evaluation, changes in microstructure, preservation and improvement in keeping quality (Rajorhia et al. 1990; De 2004; Choudhary et al. 2017). Limited information is available regarding the effect of developed acidity and subsequent neutralization of milk on heat induced changes as a result of interaction between protein-lactose (Maillard reaction) and protein-lipid (protein bound carbonyls) at various stages during preparation of *khoa*. Therefore, this study was designed to evaluate the effect of developed acidity and subsequent neutralization of milk on heat induced Maillard reaction and protein oxidation in *khoa* at various stages of preparation i.e. raw, boiled milk and *khoa* stage during preparation of *Khoa*.

Materials and methods

Materials

Sodium bi carbonate, 5-hydroxy methyl furfural, Lactulose, L-lysine monohydrochloride and dichloromethane were procured from Sigma Aldrich, St. Louis, Missouri, USA. Dinitrophenyle hydrazine and resorcinol were procured from Rankem, RFCL Ltd., New Delhi, India, Furosine was procured from polypeptide laboratory, Strasbourg, France. Water, methanol, O-phosphoric acid of HPLC grade were procured from Thermo Fisher Scientific India Pvt. Ltd., Delhi, India.

Methods

Good quality pooled cow and buffalo milk was obtained from the experimental dairy of the ICAR-National Dairy Research Institute, Karnal. The gross composition of fresh cow and buffalo milk is given in Table 1. Fresh milk samples were incubated at 30 °C in an incubator (Narang Scientific Works Pvt. Limited, Delhi, India) for rapid development of acidity up to 0.18% lactic acid (LA). Acidity of milk was evaluated hourly until it reaches up to 0.18% LA. Developed acidity was neutralized with the addition of calculated amount of neutralizer (sodium bicarbonate) at required rate to adjust the acidity to 0.14% LA (~acidity of fresh milk). The fresh, acidic and neutralized cow and buffalo milk samples were used to prepare *khoa*. *Khoa* was prepared according to the method of De (2004). The concentration of milk was carried out in batches of 4–5 L in an open jacketed stainless steel kettle at a steam pressure of 2 kg/cm^2. Stirring and scraping with the help of a flat iron ladle was continued to avoid burning of milk solids during boiling. This process was continued until the entire mass reached a pasty consistency, and the steam pressure was reduced to 1 kg/cm^2. Vigorous stirring was pursued with the help of an iron ladle with simultaneous scraping of the pan bottom sides, more so at the last stage. The steam was closed when the milk solids started leaving the sides of the kettle and all the condensates were removed. The pasty mass was then worked up and down to

Table 1 Chemical characteristics of cow and buffalo milk

Samples	Parameters (%)					
	Fat	Protein	Lactose	Ash	SNF	pH
Cow milk	3.93 ± 0.15	3.63 ± 0.05	4.79 ± 0.10	0.74 ± 0.02	8.67 ± 0.21	6.46 ± 0.11
Buffalo milk	7.56 ± 0.12	4.34 ± 0.05	5.10 ± 0.11	0.82 ± 0.02	9.79 ± 0.21	6.62 ± 0.14

Data represented as Mean ± SEM, n = 3

form a pat. During preparation of *khoa,* samples were analyzed at three different stages i.e. raw milk stage, boiled milk stage and final stage which is finished product i.e. *khoa*. The proximate composition of *khoa* in terms of fat, lactose, ash, total solids pH and acidity of *khoa* was determined by the method described in Indian Standard (IS: SP (Part XI) 1981). Moisture and protein content was determined by Indian Standard (IS 16072 2012 and AOAC 1970), respectively. The gross composition of three types of *khoa* prepared from buffalo and cow milk is presented in Table 2a, b.

Lysine estimation

Available lysine in raw milk and *khoa* casein samples was measured according to the method of Friedman et al. (1984). Casein samples were prepared from fresh, acidic and neutralized raw milk and *khoa*samples by following the protocol of Gupta and Ganguli (1965). Standard curve of lysine concentration was drawn in a range of 50–500 µ mole/l. Absorbance of the reaction mixture was measured at 570 nm using spectrophotometer. Final lysine content was expressed as lysine in g/100 g of protein.

Lactulose

Lactulose in milk and *khoa* was estimated by the method of Amine et al. (2000). 10 g of *khoa* was reconstituted in 60 ml of warm distilled water and volume was made up to 100 ml. 4 ml of this reconstituted *khoa* sample was transferred to a 10 ml volumetric flask and 2 ml of zinc sulphate and 2 ml of potassium ferrocynate was added, volume was finally made up to 10 ml with distilled water. Sample was shaken vigorously and filtered through whatman no 42 filter paper (Whatman International Ltd., Kent, England) to obtain a clear filtrate. 0.5 ml of *khoa* filtrate or 0.5 ml of milk sample was transferred to glass-capped test tubes to which 2 ml of seliwanoff's reagent (Aqueous solution of 4 M HCl/L containing resorcinol 0.1%) was added. These test tubes were placed in a water bath at 90 ± 0.1 °C for 10 min and cooled under running tap water. The resulting solution was filtered using a disposable filter (0.22 µm porosity syringe filters, Millipore India Pvt. Ltd., Bangalore, India) and the absorbance was measured at 482 nm against distilled water. A blank run was also conducted simultaneously substituting water for milk/*khoa*. Standard curve of Lactulose concentration was drawn by substituting distilled water with raw cow and buffalo milk using a range of 0.5–5 mg/L and results are expressed mg/100 ml of milk or 100 g of *khoa*.

Hydroxy methyl fufural (HMF) Potential

PotentialHMF content in *khoa* was determined according to the spectrophotometric method of Keeney and Bassette (1958). 2.0 g of *khoa* was made in slurry using warm distilled water and volume was made up to 10 ml in volumetric flask. This sample was transferred to a glass stoppered test tube, 5 ml of oxalic acid (0.3 N) was added to it and the tube was placed in boiling water bath for 1 h, after which it was cooled under running tap water. 5 ml of 40% trichloroacetic acid was added and then mixture was filtered through What Man no. 42 filter paper. An aliquot of filtrate (4 ml) was transferred to a test tube and 1 ml of 0.05 M 2-thiobarbituric acid (TBA) was added to it. The contents were heated at 40°C for 30–40 min in water bath and then cooled to room temperature (30 °C). A blank run was also conducted simultaneously substituting water for *khoa*. Same protocol was followed for the standard solutions as in case of *khoa* samples. Optical density was measured at 443 nm and a standard curve was prepared. Standard curve of HMF concentration was drawn for a range 0.5–50 µM/l and results were expressed as micromoles HMF.

Furosine

Furosine in *khoa* was determined using the method of Birlouez-Aragon et al. (1997) for milk with slight modifications. 1.0 g of *khoa* was defatted using ethanol (20 ml), diethyl ether (25 ml) and petroleum ether (25 ml) following the Mojonnier method as described in IS: SP:18 part XI (1981). The process was repeated thrice and defatted *khoa* was finally lyophilized. 10 mg of lyophilized *khoa* sample was hydrolysed by adding 8 ml of 7.8 N HCl followed by flushing with nitrogen and finally placing it in muffle furnace at 100 °C for 18 h. Dried residue was then reconstituted with 0.6 ml of HPLC grade

Table 2 Gross composition and physico chemical characteristics of fresh, acidic and neutralized (a) Buffalo milk *khoa*, (b) Cow milk *khoa* (Choudhary et al. 2016)

Samples	Parameters						
	Fat	Protein	Lactose	Moisture	Ash	Total solids	PH	Acidity (% LA)
(a) Buffalo milk khoa								
FBMK	35.13 ± 1.06A	17.56 ± 0.17A	20.59 ± 0.78B	23.94 ± 0.41B	2.74 ± 0.025B	76.06 ± 0.41B	6.48 ± 0.003B	0.57 ± 0.003B
ABMK	35.50 ± 1.53A	17.70 ± 0.14A	17.66 ± 1.02A	22.43 ± 0.55A	2.64 ± 0.034A	77.57 ± 0.55C	6.38 ± 0.005A	0.62 ± 0.002C
NBMK	35.41 ± 1.35A	17.76 ± 0.29A	17.03 ± 1.32A	25.44 ± 0.70C	2.85 ± 0.040C	74.56 ± 0.70A	6.68 ± 0.005C	0.55 ± 0.005A
(b) Cow milk khoa								
FCMK	26.46 ± 0.50A	18.30 ± 0.12A	24.87 ± 0.18B	29.65 ± 0.62B	3.24 ± 0.017B	70.35 ± 0.62B	6.40 ± 0.005B	0.63 ± 0.002B
ACMK	26.13 ± 1.08A	18.43 ± 0.29A	21.53 ± 0.44A	27.07 ± 0.37A	3.17 ± 0.012A	72.92 ± 0.37C	6.22 ± 0.005A	0.68 ± 0.006C
NCMK	25.19 ± 0.80A	19.03 ± 0.04A	20.80 ± 0.50A	30.75 ± 0.30C	3.46 ± 0.019C	69.25 ± 0.30A	6.51 ± 0.003C	0.58 ± 0.006A

Data are presented as mean ± SEM (n = 3)

ABMK Acidic Buffalo Milk *Khoa*, *FBMK* Fresh Buffalo Milk *Khoa*, *NBMK* Neutralized Buffalo Milk *Khoa*, *ACMK* Acidic Cow Milk *Khoa*, *FCMK* Fresh Cow Milk *Khoa*, *NCMK* Neutralized Cow Milk *Khoa*

$^{A–C}$ Means within column with different upper case superscript are significantly different ($p < 0.05$) from each other

water and filtered through 0.45 µm membrane. 20 µl of this sample was injected into HPLC (Waters High Pressure Liquid Chromatography 515 Milford, Massachusetts, USA). Standard curve of Furosine was drawn for a range 0.5–200 mg Furosine/l and the final results were expressed as mg Furosine/100 g of protein.

HPLC conditions includes single column HPLC–UV system: C18 (5 µm, 100Å, 250 × 4.6 mm, Phenomenex, Torrance, CA, USA) with guard column C18 (5 µm, 100Å, Waters, Massachusetts, USA). The mobile phase consisted of 5.6 mM*0*-phosphoric acid, pumped at flow rate of 1 ml/min. Column temperature was maintained at 40 °C using a column heater chamber. The sample volume injected was 20 µl and a PDA detector at 280 nm was used to monitor the eluate.

Protein bound carbonyl

Protein bound carbonyls (PC) in milk and *khoa* were evaluated by method prescribed by Fedele and Bergamo (2001) for cheese (*khoa* has protein and fat within range of cheese). 2 g of *khoa* was carefully weighed and transferred into glass centrifuge tubes containing 10 ml of 0.2 M sodium citrate-NaOH (pH 8). The tubes containing samples were homogenized at 4 °C, just before analysis to ensure proper mixing. Particulate material was removed by centrifugation (10,000*g*, 10 min, 4 °C) and supernatants were used for the PC assay. *Khoa* homogenates or milk aliquots (0.1 ml) were incubated for 15 min at room temperature in the presence of 0.3 ml of 10 mM di-nitro phenyl hydrazine in 2.5 M HCl. Samples treated with 2.5 M HCl were prepared and used as blanks. At the end of the incubation, 1 ml of ice-cold 20% trichloroacetic acid (TCA) was added and the mixture was vigorously shaken. Proteins were precipitated by centrifugation (2 min, 10,000*g* at 30 °C) and the pellets were washed once with 400 ml of ice cold 10% TCA and 3 more times with 1 ml of ethanol/ethyl acetate mixture (1:1). Protein precipitates were finally dissolved in 1 ml of 6 M guanidine HCl in 10 mM phosphate buffer (pH 2.3). The amount of hydrazone formed was determined by measuring sample absorbance at 370 nm. The titer (concentration) of protein-bound carbonyls was calculated using the molar extinction coefficient (22,000 M^{-1} cm^{-1}) on blank subtracted data. PC concentration was finally expressed as nmoles/mg of proteins.

Statistical analysis

Data reported were expressed as mean values with standard errors. Means and standard error mean (SEM) were calculated using Microsoft excel (2007) (Microsoft Corp., Redmond, WA). Significant difference between values was verified by one way or two way analysis of variance and

comparison between means was made by critical difference value (Snedecor and Cochran 1994).

Results and discussions

Effect of developed acidity and subsequent neutralization of milk on Maillard reaction and protein oxidation was evaluated in terms of Available lysine, HMF, Furosine, Lactulose and protein bound carbonyls during preparation of *khoa* at various stages. The nutritive value of milk proteins is altered by heating (Fedele and Bergamo 2001). Under mild conditions Maillard products can be formed by reaction of epsilon-amino groups of lysine with the reducing group to form biologically unavailable lysine-sugar complex (Van Teeffelen et al. 2005). Virtually, Liquid milk appears to be unaffected by the industrial pasteurization and only slightly affected by autoclaving and canning but *Khoa* is a product manufactured under rigorous heating conditions. Therefore, the effect of acidity and neutralization was analyzed on available lysine content in raw milk and *khoa* samples prepared from buffalo and cow milk are given in Table 3a, b. Lysine content of buffalo milk was higher than cow milk which might be due to protein content of respective milk. Significant ($p < 0.05$) difference was observed in lysine content of fresh buffalo and cow milk as compared to their respective, acidic and neutralized milk samples, however non-significant ($p > 0.05$) difference was reported within in acidic and neutralized milk samples. Available lysine content was highest in fresh milk *khoa* samples followed by acidic and neutralized milk *khoa* samples owing to the fact of developed acidity and addition of neutralizers to milk in respective sample. Lower values were also observed in acidic *khoa* samples as acidic milk was used for *khoa* preparation, which contained both glucose and galactose (formed during souring of milk) which are more prone to Maillard reaction. Prakash and Sharma (1984) reported that lysine content was higher in *khoa* prepared from lactose unhydrolysed milk than *khoa* prepared from hydrolysed milk. Greater loss of available lysine in *khoa* samples made from lactose hydrolysed milk is due to extensive reaction of lysine with both glucose and galactose which are formed on hydrolysis (Reynolds 1965). Similarly, Janssons et al. (2014) also reported that more favorable conditions for Maillard reactions were observed in lactose-hydrolyzed milk compared to conventional UHT milk.

In case of Lactulose (Fig. 1a, b) significant difference ($p < 0.05$) was observed in fresh, acidic and neutralized buffalo and cow milk during preparation of *khoa* at various stages i.e. boiled milk and *khoa* stage as Lactulose itself is absent in raw milk. Similarly, significant difference ($p < 0.05$) was observed in HMF (Fig. 1c, d) and Furosine content (Fig. 1e, f) of fresh, acidic and neutralized buffalo and cow milk during preparation of *khoa* at various stages i.e. raw milk, boiled milk and *khoa* stage. On heating Lactulose, HMF and Furosine concentration increased gradually in both the cow and buffalo milk and *khoa* samples. Lactulose, HMF and Furosine content of buffalo milk was higher than cow milk and their respective *khoa* samples; this can be attributed to higher initial lactose and protein content in buffalo milk. Neutralized samples contained significantly higher ($p < 0.05$) Lactulose content as compared to fresh and acidic samples due to the presence of neutralizers which shifted the pH of milk towards alkaline values leading to the formation of higher

Table 3 Lysine content in raw milk casein and TCA precipitated *khoa* protein of fresh, acidic and neutralized (a) Buffalo milk, (b) Cow milk

Samples	Lysine (g/100 g of protein)	
	Raw milk casein	TCA precipitated *khoa* protein (pH 4.6)
(a) Buffalo milk		
FBM	12.73 ± 0.76^{bB}	11.08 ± 0.54^{aC}
ABM	10.76 ± 0.45^{bA}	9.36 ± 0.29^{aB}
NBM	10.48 ± 0.24^{bA}	8.15 ± 0.27^{aA}
(b) Cow milk		
FCM	11.83 ± 0.24^{Bb}	9.59 ± 0.28^{Ca}
ACM	9.96 ± 0.32^{Ab}	8.89 ± 0.06^{Ba}
NCM	9.55 ± 0.61^{Ab}	7.76 ± 0.28^{Aa}

Data are presented as mean ± SEM (n = 3)

FBMK Fresh buffalo milk *khoa*, *ABMK* Acidic buffalo milk *khoa*, *NBMK* Neutralized buffalo milk *khoa* and *FCMK* Fresh cow milk *khoa*, *ACMK* Acidic cow milk *khoa*, *NCMK* Neutralized cow milk *khoa*

[A–C] Means within column with different upper case superscript are significantly different ($p < 0.05$) from each other

[a, b] Means within row with different lower case superscript are significantly different ($p < 0.05$) from each other

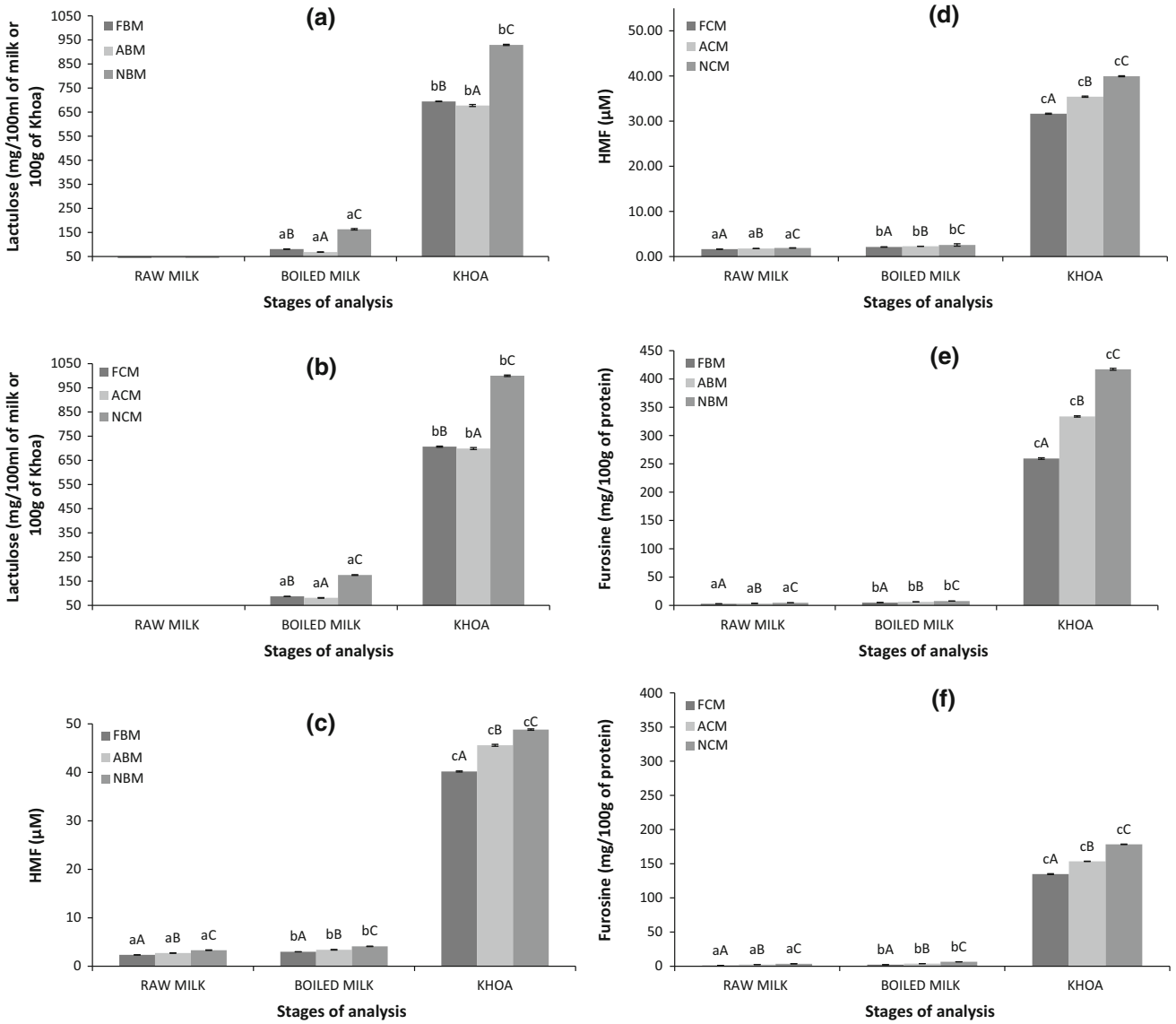

Fig. 1 Lactulose, HMF and Furosine concentration of milk during preparation of *khoa* at raw milk, boiled milk and *khoa* stage. **a** Lactulose concentration in Buffalo milk. **b** Lactulose concentration in Cow milk. **c** HMF concentration in Buffalo milk. **d** HMF concentration in Cow milk. **e** Furosine concentration in Buffalo milk. **f** Furosine concentration in Cow milk. $^{a–c}$ Different lowercase letters denote significant difference ($p < 0.05$) between groups (raw milk, boiled milk and khoa). *Error bars* show the variations of three determinations in terms of standard error of mean. $^{A–C}$ Different uppercase letters denote significant difference ($p < 0.05$) across subgroups [(FBM, ABM and NBM) and (FCM, ACM and NCM)]. *FBM* Fresh buffalo milk, *ABM* Acidic buffalo milk, *NBM* Neutralized buffalo milk and *FCM* Fresh cow milk, *ACM* Acidic cow milk, *NCM* Neutralized cow milk)

concentration of Lactulose (Fig. 1a, b). Lower Lactulose content in acidic samples can be explained by the fact that Lactulose was obtained by alkaline isomerization of lactose hence lower values for Lactulose was observed under acidic conditions. Our results were well correlated with Hashemi and Ashtiani (2010) who reported significant increase in Lactulose concentration with increase in pH values. Beach and Menzies (1983) observed that in-container sterilized milk and liquid infant feed had Lactulose in range of 55–469 mg/100 ml and explained that the higher values of Lactulose was attributed to increase in concentration of lactose and protein, and prolonged heating during sterilization process. Guerra-Hernandez et al. (2002) reported the Lactulose concentration was observed up to 83.16 mg/100 ml in infant milk formula. As these milk formulas contain milk, the milk lactose isomerizes to Lactulose during heat treatment.

HMF and Furosine content of neutralized buffalo milk and *khoa* samples was the highest among the acidic and fresh samples (Fig. 1c, e). Similar trend was observed for the fresh, acidic and neutralized cow milk and *khoa* samples (Fig. 1d, f). The higher rate of formation of HMF and

Furosine in buffalo milk can be explained due to higher concentration of milk protein and lactose in buffalo milk than in cow milk. This induces the condensation reaction between epsilon group of lysine and the aldehyde group of lactose during Maillard reaction, leading to a faster rate of HMF and Furosine formation after the milk coagulates and during the pat stage of *khoa*. Maillard reaction is generally favoured under alkaline conditions (Owusu-Apenten 2004) which may have resulted in higher HMF and Furosine content in neutralized milk and *khoa* samples. During the souring of milk, lactose is broken down to glucose and galactose by lactic acid bacteria, the glucose and galactose can be fermented to lactic acid (O'Connor 1995). These monosaccharides are also more prone to Maillard browning, contributing towards higher HMF and Furosine in acidic milk as compared to fresh milk. Our results were in accordance with Rajorhia et al. (1990) who reported that HMF values for neutralized buffalo milk *khoa* samples were higher as compared to fresh and acidic buffalo milk *khoa* samples. Nagendera et al. (1991) reported that HMF content increased significantly during preparation of *khoa*. However, HMF content was higher in *khoa* prepared in a shallow iron pan than *khoa* prepared in steam pan. Masotti and De Noni (2005) reported Furosine content of 4.4 mg/100 g of protein in raw milk utilized for the preparation of Taleggio cheese. Van Renterghem and De Block (1996) reported Furosineup to 372 mg/100 g of protein in sterilized milk and up to 8.3 mg/100 g of protein in UHT milk. Guerra-Hernandez et al. (2002) also reported Furosine concentration up to 701 mg/100 g of protein in powdered infant formula. However, the Furosine content in infant formula was comparatively higher than *khoa* samples due to the intense Maillard reaction occurring in these low moisture foods.

Protein bound carbonyls determines the extent of protein oxidation in milk and milk products. Protein bound carbonyl differed significantly ($p < 0.05$) in all three samples i.e. fresh, acidic and neutralized buffalo and cow milk at various stages during *khoa* preparation (Table 4a, b). Present study revealed a positive connection between intensity of heating, Maillard reaction and protein bound carbonyls. As milk progressed to boiling during heating an increase was observed in the concentration of protein bound carbonyls. However, the extent of increase of protein bound carbonyls was non-significant ($p > 0.05$) up to the boiling stage during *khoa* preparation. In final stage of *khoa* preparation significant increase ($p < 0.05$) was observed in concentration of protein bound carbonyls as compared to raw and boiled milk stages. Results indicated that highest values for PC were contributed by neutralized milk and *khoa* samples followed by acidic and fresh samples. Maillard reaction was initiated by the presence of neutralizers which might be the reason for higher PC content in neutralized samples. Fedele and Bergamo (2001) also confirmed the Maillard reaction remarkably influenced the formation of protein bound carbonyls during cheese manufacturing. It was also concluded from results that the PC content of cow milk *khoa* was higher than buffalo milk *khoa* which might be due to higher poly unsaturated fatty acids (PUFA) content, arginine (presence of free amino, amide and hydroxyl group susceptible to protein oxidation) and low fat content (Kolakowska and Bartosz 2013). Liang (1999) reported that free amino group content of protein decreased due to the interaction with lipids.

Lal and Narayanan (1984) reported that cow milk has higher PUFA content as compared to buffalo milk, which strengthens the fact of higher protein bound carbonyl concentration in cow milk as compare to buffalo milk and *khoa* samples. It has also been reported that reduced fat dairy products often are more sensitive to protein oxidation compared to dairy products with higher lipid content (Mestdagh et al. 2011). Protein bound carbonyl was found to be significantly higher in bovine mozzarella cheese (1.71–15.00 nmol/mg protein) than in buffalo mozzarella cheese (Balestrieri et al. 2002). The reported protein bound carbonyl concentration in the literature ranges from 1.5 to 4.5 nmol/mg protein for different cheese varieties (Fedele and Bergamo 2001) and 3.8 to 5.5 nmol/mg protein in pulsed light treated processed cheese (Fernandez et al. 2014). However, the protein bound carbonyl concentration in *khoa* was higher than reported values for cheese as intense heating of milk was involved during preparation of *khoa*.

Balestrieri et al. (2002) also reported that the Maillard reaction occurring during processing of food and storage is also involved in mechanisms of protein oxidation. Since protein glycosylation mainly takes place in the presence of free sugar thus, the further carbonyl production might depend on the enhanced production rate of amadori derived deoxysones. It was also observed that temperature, metal ions and hydroxyl radicals synergistically contribute to the amadori group conversion rate, which resulted in higher yield of protein bound carbonyls (Kawakishi et al. 1990a, b).

Conclusion

It is evident that neutralization of milk adversely affected available lysine, heat induced protein-lactose (HMF and Furosine) and protein-lipid interactions (protein bound carbonyl). Heating of milk during *khoa* preparation resulted in increase in Lactulose, Maillard indicators (HMF and Furosine) and protein bound carbonyls indicating highest values in neutralized sample as alkaline pH favours Maillard reaction. Lysine content was found highest in fresh

Table 4 Protein bound carbonyl concentration of milk at various stages during preparation of *khoa* (a) Buffalo milk, (b) Cow milk

Samples	Stages of analysis	Protein bound carbonyls (nmoles/mg of protein)		
		Raw milk	Boiled milk	*Khoa*
(a) Buffalo milk				
	FBM	$0.105 \pm 0.01^{a,A}$	$0.590 \pm 0.02^{a,A}$	$27.284 \pm 0.73^{b,A}$
	ABM	$0.285 \pm 0.00^{a,B}$	$0.635 \pm 0.01^{a,B}$	$41.074 \pm 0.16^{b,B}$
	NBM	$0543 \pm 0.01^{a,C}$	$1.046 \pm 0.09^{a,C}$	$91.838 \pm 4.81^{b,C}$
(b) Cow milk				
	FCM	$0.690 \pm 0.01^{a,A}$	$1.114 \pm 0.03^{a,A}$	$56.96 \pm 2.77^{b,A}$
	ACM	$1.127 \pm 0.08^{a,B}$	$1.186 \pm 0.09^{a,B}$	$84.26 \pm 3.20^{b,B}$
	NCM	$1.238 \pm 0.03^{a,C}$	$2.149 \pm 0.06^{a,C}$	$126.83 \pm 1.30^{b,C}$

Data are presented as mean ± SEM (n = 3)

FBM Fresh buffalo milk, *ABM* Acidic buffalo milk, *NBM* Neutralized buffalo milk and *FCM* Fresh cow milk, *ACM* Acidic cow milk, *NCM* Neutralized cow milk)

[A–C] Means within column with different upper case superscript are significantly different ($p < 0.05$) from each other

[a–c] Means within row with different lower case superscript are significantly different ($p < 0.05$) from each other

milk *khoa* samples and lowest in neutralized milk *khoa* samples. The remarkable influence of neutralization on Maillard reaction and protein oxidation during *khoa* manufacture was evidenced by elevated concentration of HMF, Furosine, Lactulose and protein bound carbonyls in neutralized samples.

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