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The Influence of Heat Treatment in Liquid Whey at Various pH on Immunoglobulin G and Lactoferrin from Yak and Cows' Colostrum/Milk

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Abstract

Yak milk is gaining popularity yearly, as a source of nutritious, immune, less likely to cause allergies than cows' milk and a means of generating income, though ranked behind bovine milk in China. The main objective of the research was to compare the Immunoglobulin G (IgG) and Lactoferrin (LF) concentrations of liquid whey from Yak and dairy Cows' colostrum or milk. Thereafter, the thermal stability of liquid whey was evaluated by measuring the concentration change in IgG and LF using an ELISA technique, as influenced by temperature and pH after centrifugation.

IgG and LF concentrations in liquid whey from colostrums both in yak and cow were significantly higher ($p<0.05$) compared to milk. IgG did not differ significantly ($p>0.05$) between yak and cow's milk, as well as between yak and cow's colostrum. Also, LF content was not significant different between yak and cow milk. However, LF concentration was significantly higher in cows' colostrum than in yak. With reference to IgG content, yak breed observed to produce high quality colostrum for all samples tested compared to cows.

The extent of IgG and LF denaturation confirmed to increase with increased protein concentration, temperature and pH change near to pI. Colostral IgG and LF were more denatured compared to milk in both yak and dairy cows. There was significant ($p<0.05$) influence of pH changes resulted in either partial or complete denaturation or increased tendency to aggregate, which was removed during centrifugation. Liquid whey less affected at lower pH and at mild heat temperatures.

Keywords: ELISA; Yak colostrum/milk; Heat stability; Liquid whey; Immunoglobulin G; Lactoferrin

Introduction

Whey, a soluble fraction of milk and by-product liquid from cheese or casein manufacturers is widely recognized to contain many valuable constituents such Lactoglobulin (β-Lg), Lactalbumin (α-Lac) and BSA. IgG and LF are natural occurring antimicrobial proteins available in colostrum and milk as the major antimicrobial agent for a wide range of pathogens or spoilage micro-organisms [1-6]. Colostrums contain natural defensive antibodies from mother to neonate. It is highly concentrated with immune, growth and tissue repair factors, with a distinct level of protein concentration particularly in Immunoglobulins (IgG, IgA and IgM) and nutritive components. IgG being the major Immunoglobulin present in ruminant milk, and IgA being the major Immunoglobulin present in human milk [7,8].

LF, a glycoprotein with molecular weight of about 80 kDa (~700 amino acids) belongs to the transferrin family, commonly known as lactotransferrin [9]. It is an iron (Fe^{+3} ions) binding glycoprotein which is considered as an essential element of innate immunity with antigen-nonspecific defense mechanism after exposure to an antigen [10,11]. Antibacterial activities of LF is effective against gram positive and gram negative bacteria, viruses (non-capsular and capsular) and several parasites and fungi [12].

Processing conditions applied during production involving heating, adjustment of pH, minerals, sugars, protein composition and processing time determine retentions of nutritional value and physiological importance of whey proteins [6,13,14]. Whey proteins are comparatively thermal labile, therefore; heat treatment of milk contribute to denaturation and aggregation of the denatured proteins with caseins [15].

The high level of intramolecular disulphide bridges plus other whey components including fats, lactose, carbohydrates, salts, and other proteins assist to stabilize antibodies during heat treatment [16]. Immunoglobulins thermal resistance varies between classes, i.e. high stability in a decreasing order from IgG, IgA and IgM as well as varies among species [17-19]. The conformation changes in the IgG molecule which occurs during heat exposure reduces antigen-binding activity of this protein Calmettes et al. [20] and antigen-binding site of IgG is more sensitive to heat compared to other part of the molecule [21,22]. On the other hand, LF are denatured by heat treatment too, therefore pasteurizing milk might affect LF unless mild heat treatment is applied [23,24].

Yaks (*Bos grunniens*) are distributed at high altitudes ranging from 2000 m to 4500 m. Though the milk yield is low but with good quality due to high level of fat, proteins, sugar content as well as carotene and vitamin E compared to cows' milk. Yak milk are easily digested and absorbed by infants due to low α-caseins (about 40%) and higher β-casein (more than 45%) with small increase in κ-casein (15%) compared to cows' milk which contains higher α-caseins (50%) and lower β-casein (35%) [25-27].

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In China, Yaks and their products are mostly dominated in northwestern China [13,14,28]. Due to the increase demand in new functional food development and therapeutic value of LF and IgG (such as infant formulas fortification from different milk sources), more studies are needed from different animal species. Therefore, we aimed to compare the IgG and LF concentrations from Yak and dairy Cows' milk or colostrum. Furthermore, we evaluated the effect of heat at various pH on antibacterial proteins (IgG and LF) in liquid whey from yak colostrum and milk in comparison to cows' as applied during dairy processing temperatures.

Apart from IgG and LF in liquid whey, other major whey proteins namely β-Lg, α-Lac, BSA and total whey proteins (TWP) also were analyzed as might contribute to denaturation of IgG and LF. But, our discussion focused only on antimicrobial proteins (IgG and LF). Turbidity and free sulfhydril (-SH) on liquid whey supernatants were also evaluated to monitor the effect of heat, pH. Therefore, optimization of environmental processing conditions during extraction of liquid whey expected to retain reasonable amount of antimicrobial proteins which is beneficial in new functional food development.

Materials and Methods

Materials

Collection of samples: Random sampling of colostrum and milk samples from individual yak were collected from Gannan and Qinghai Plateau (Gansu and Qinghai Provinces, China); while Holstein Friesian cow milk and colostrum were collected from Lanzhou dairy Farmer. A total of 80 samples (20 samples each group) were collected for this study. Approximately 80 mL sample for each animal was collected immediately after milking into sterile plastic test tubes and kept on ice before sent to the laboratory. All samples were stored at -80°C (Ultra Low Temperature Freezer, Haier-Biomedical, China) before extraction and analysis.

Standard ELISA kits for IgG and LF were purchased from Biotechnology Co. Ltd, Shanghai-China. A Coomassie (Bradford) Protein Assay Kit for total protein analysis and all other analytical grade chemicals were purchased from Sigma Aldrich (St Louis, MO, China).

Methods

Liquid whey preparation: Liquid whey samples were prepared according to Chen et al. [29] and Cozma et al. [30] with some modifications. Frozen colostrum/milk samples were thawed in running tap water; then, 40 mL of colostrum/milk samples were skimmed by centrifuge (Biofuge stratus centrifuge, Thermo, Germany) at 5000 rpm for 15 min at 4°C. Skimmed samples were adjusted to pH 4.6 using 1 M HCL or 1 M NaOH, warmed at 40°C in water bath (Julaba F12, Germany) for 30 min. and centrifuged at 5000 rpm for 15 min at 4°C. Furthermore, liquid whey supernatants obtained (1.5 mL) were centrifuged at 17,000 rpm for 30 min at 4°C to obtain the clear supernatants which were stored in fridge at 4°C (Ronshen, BCD-209YMB, China) for short-term or frozen at -18°C (Sanyo Medical Freezer, Japan) for long term analysis, respectively.

Total whey proteins assay: The Total Whey Protein (TWP) concentration was assayed by using the Modified Bradford Assay protocol as applied by Cozma et al. [30] using Coomassie (Bradford) Protein Assay Kit Manufacturer's instructions. The liquid whey samples extracted from milk and colostrum were diluted 50 times and 1000 times with PBS, respectively. For each sample, 20 µL aliquot was transferred into microplate wells followed by addition of 200 µL Bradford reactive and incubated for 10 min at room temperature.

Thereafter, with 15 minutes, absorbance was measured at 595 nm (λ max) using microplate reader (Multiskan FC, version 1.00.96, Finland). The BSA standard was serially diluted with PBS according to Manufacturers' instructions at the range of 10-150 μgmL^{-1} to prepare a standard curve for quantifying TWP for each sample.

ELISA assays: IgG and LF concentrations in liquid whey samples were quantified by Enzyme-Linked Immunosorbent Assay (ELISA) technique using Bovine ELISA quantification kits according to the Manufacturer's protocols with minor modifications pertaining sample dilutions. 50 µL of each diluted specific Bovine standard and liquid whey sample were transferred into a 48-microplate wells coated with primary antigen, incubated in oven (THZ-C-1, China) at 37°C for 30 min., followed by washing five times using 20-fold washing buffer solution. Then, 50 µL HRP conjugate reagent was added to each microplate well except blank and incubated at 37°C for 30 min. After 30 min of incubation, microplate wells were washed five times using 20-fold washing buffer solution followed by addition of 50 µL Chromogenic agent A and B, respectively, to each well and incubated at 37°C for 10 min. in dark place. The reaction was stopped by addition of 50 µL stop solution reagent after incubation, whereby colour changed from blue to yellow. Within 15 min. samples were assayed at 450 nm using microplate reader (Multiskan FC, version 1.00.96, Finland). Using specific Bovine standards, the same technique was applied to quantify β-Lg, α-Lac and BSA in liquid whey samples from milk and colostrum.

Bovine standards for IgG (range; 5-80 μgmL^{-1}) and LF (range; 12.5-800 μgL^{-1}) were diluted as per Manufacturer's instructions to prepared calibration curves for quantification in each sample and all samples were analyzed in duplicate.

Heat treatment of liquid whey: From each five liquid whey samples either colostrum or milk, 2 mL were selected randomly and mixed together to obtain one homogenous sample (approximate 10 mL). Heat stability of liquid whey from yak and cow's milk/colostrum were evaluated according to Chevalier et al. [31] and Laleye et al. [32] with some modifications. Liquid whey samples were adjusted to pH 3.5, 4.6, and 5.5 (pH meter 55, Martini instruments, Mauritius) by 1 M HCL or 1 M NaOH. After pH adjustment, 5 mL samples in 15 mL capacity closed plastic test tube was heated in digital temperature-controlled water bath (Julaba F12, Germany) at 60°C/30 min, 63°C/30 min, 72°C/15 sec and 90°C/5 min. Different heating up times were allowed to equilibrate to reach the water bath temperature before measuring experimental time. One extra sample was used to monitor the temperature by inserting a thermometer. Immediately, heat treated samples were cooled in an ice water and at each pH studied; a control sample was maintained at room temperature (22°C). An aliquot of heat treated sample (1.5 mL) was centrifuged at 10,000 rpm for 15 min at 4°C and each thermal treatment was done in duplicate.

Turbidity: Optical density (absorbance at 20°C) of the supernatant was measured the 280 nm wavelength by UV/VIS 2550 Spectrophotometer (Shimadzu, Japan) to evaluate the denatured proteins with comparison to untreated sample as applied by Laleye et al. [32].

Free sulfhydryl content: Free sulfhydryl (-SH) content was determined according to Monahan, et al. [33] and Hoffmann and Mil [34]. 0.02 mL of 10 mM DTNB was added to 2 mL of the liquid whey supernatant and the absorbance (at 20°C) measured by UV/VIS 2550

Spectrophotometer (Shimadzu, Japan) at 412 nm. Free sulfhydryl content was calculated according to Manufacturer's protocol using Molar absorptivity (E=14,150 $M^{-1}cm^{-1}$).

Statistical analysis

All samples were analyzed in duplicate, and the results were reported as the means ± SD. Results were evaluated statistically using the Statistical Package for Social Sciences, v16.0 (SPSS, Chicago, IL, USA). The data for IgG and LF concentration from different sources of liquid whey were analyzed statistically by one-way analysis of variance (ANOVA). A three factor analysis of variance with interaction was used to evaluate the different sources of liquid whey, effect of heat treatment and various pH on denaturation of proteins in liquid whey. Thereafter, multiple comparisons of means were analyzed using Least Significance Difference (LSD) and Duncan comparison at a α-level of 5%.

Results and Discussion

IgG concentration in liquid whey

Results for liquid whey from yak and cows'colostrum as well as milk respectively are presented in Figure 1. Colostral IgG concentration were 59.40 ± 4.44 $mgmL^{-1}$ and 65.99 ± 25.90 $mgmL^{-1}$ from yak and cows' liquid whey samples, respectively. IgG did not differ significantly ($p>0.05$) between yak and cows' colostrum. Yak and cows' milk IgG concentration were 0.58 ± 0.16 $mgmL^{-1}$ and 0.49 ± 0.08 $mgmL^{-1}$, respectively. IgG content both yak and cows' milk were not significantly differently ($p>0.05$). However, results indicated that colostral IgG concentration were significantly higher ($p<0.05$) compared to milk both in yak and cow.

Analyses of colostrum and milk from individual animal species (yak and cow) indicated considerable variations in their IgG content. The colostral IgG concentration ranged from 54.44-72.72 $mgmL^{-1}$ (70.98% of TWP) and 18.95-102.21 $mgmL^{-1}$ (83.88% of TWP) for yak and cows' colostrum, respectively. The milk IgG concentration of liquid whey ranged from 0.35-0.87 $mgmL^{-1}$ (8.72% of TWP) and 0.39-0.65 $mgmL^{-1}$ (9.50% of TWP) for yak and cow, respectively (Figure 1).

Our results are in agreement with other studies, bovine colostrum contain high IgG content compared to normal milk which has been reported to vary from 40-200 $mgmL^{-1}$ IgG and 0.7-1.0 $mgmL^{-1}$ in colostrum and normal milk, respectively [3,8,35-37].

Several factors may be contributed to concentration variations of IgG in colostrum and milk, such as species, breed, health status, pathogen exposure, feeding practices and environmental conditions (season), prepartum diet/nutritional management and the stage of lactation/parity [38-43].

Other factors which might be attributed to variation of IgG content within yak breed or between yak and cows includes number of lactation and length of the non-lactating [42,44,45]. Heat stress also reported to affect the animal that attribute to reduction of colostral levels of IgG and IgA, fat, lactose, protein, and energy [39].

According to Hansen [46] and Godden [47], high quality colostrum should contain more than 50 gL^{-1} of IgG. From our results showed that 100% of yak produced high quality colostrum compared to cow Holstein Friesian cow which was 73.33%.

LF concentration in liquid whey

Experimental results are presented in Figure 1, LF content measured by ELISA technique were 0.58 ± 0.16 $mgmL^{-1}$ and 0.49 ± 0.08 $mgmL^{-1}$ from yak and cows' milk, respectively. Both yak and cows' milk were not significant different ($p>0.05$). Colostral LF concentration were 1.52 ± 0.56 $mgmL^{-1}$ and 2.48 ± 0.63 $mgmL^{-1}$ from yak and cows' liquid whey supernatants, respectively. Cows' colostrum contained significantly higher LF content ($p<0.05$) compared to yak colostrum. In both yak and cow, colostrum contained significant higher ($p<0.05$) LF concentration compared to milk.

The mean LF concentration from yak colostrum ranged 0.94-2.47 $mgmL^{-1}$ (1.84% of TWP) and 1.04-3.11 $mgmL^{-1}$ (3.15% of TWP) for cows. While, mean LF concentration ranged from 0.24-0.43 $mgmL^{-1}$ (4.69% of TWP) and 0.15-0.35 $mgmL^{-1}$ (4.32% of TWP) for yak and cows' milk respectively.

Our results obtained were in agreement with other studies reported from different bovine, whereby LF concentration is significantly higher in colostrum than in milk. According to Haenlein, [48] the LF concentration was 1.5 $mgmL^{-1}$ and 0.02-0.5 $mgmL^{-1}$ in cow colostrum and milk respectively. The same results of LF content in cows' milk were reported by Zimecki and Kruzel [36]. Different factors may contribute to variation of LF concentration such as different animal health status, level of BSA, lactation stage as well as daily milk production, some studies reported a big variation of LF content in milk among individual cows, for example from 0.06-1.0 $mgmL^{-1}$ LF [28,49].

TWP composition

The TWP were 6.70 $mgmL^{-1}$ and 5.20 $mgmL^{-1}$ for yak and cows'

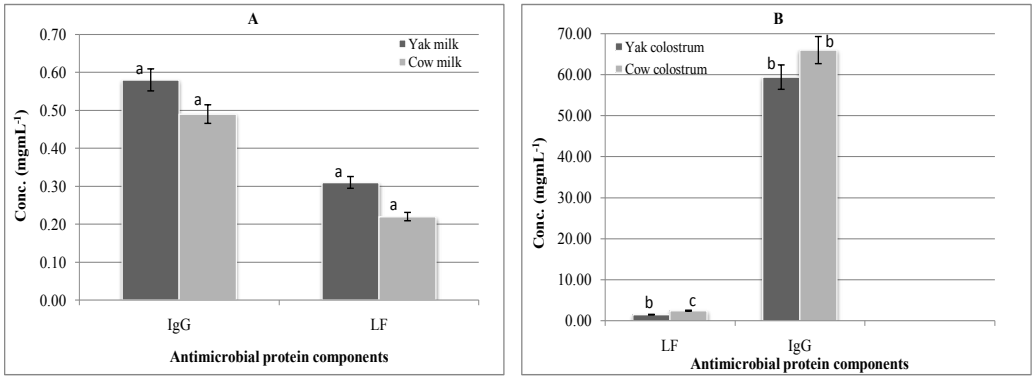

Figure 1: (a) Yak and cows' milk (b) Yak and cows' colostrum: Concentration of IgG and LF in liquid whey supernatant from yak and cows' milk as well as colostrum respectively after centrifugation. [a,b,c]Different superscripts within one protein type in both graphs indicate significant difference between different source ($P < 0.05$). Each value is the average of 20 samples determinations, error bars indicates standard errors.

milk, respectively, whereas 83.68 mgmL^{-1} and 78.67 mgmL^{-1} for yak and cows' colostrum respectively. High content of TWP observed may be attributed to high content of IgG in colostrum than in milk. The study revealed that, the TWP was significantly higher ($p<0.05$) in colostrum compared to milk in both yak and cows. By comparison of means, TWP between yak and cows' colostrum did not differ significantly ($p>0.05$), the same applies to yak and cows' milk. These results obtained from cows' milk are in agreement with literatures [50].

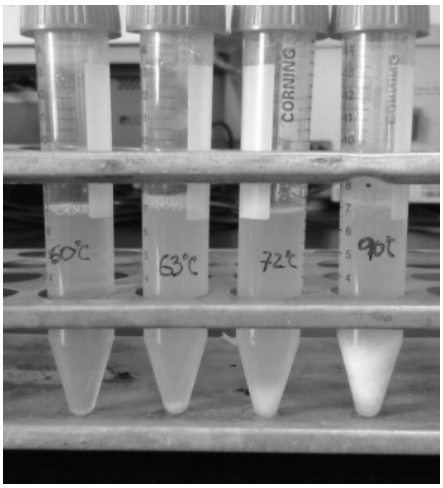

Figure 2: Appearance of liquid whey from yak colostrum after heat treatment at 60°C/30 minutes, 63°C/30 minutes, 72°C/15 seconds and 90°C/5 minutes heat treatment at pH 5.5 before centrifugation.

Effect of heat on liquid whey turbidity at various pH

Appearance and results of turbidity as the apparent optical density at 280 nm of liquid whey are presented in Figures 2 and 3, respectively. Slightly turbid and transparent (whitish in colour-viscous) and some small soluble aggregates increased with temperature increase particularly at 72°C/15 sec and 90°C/5 min., indicating whey proteins were denatured with temperature increase. Statistics analysis on heating liquid whey at each pH treatment followed by centrifugation of the supernatants showed that 60°C/30 min had no significant effect on turbidity. Also, comparison between 63°C/30 min v/s 60°C/30 min treatment showed no significant different on turbidity ($p>0.05$) too. However, more effect noted in both samples at 72°C/15 sec and 90°C/5 min, though were not significantly different between the two treatments ($p>0.05$), suggesting that denaturation temperature for β-Lg probably was reached.

Except cows' colostrum was more affected, probably due to high content of IgG and β-Lg that was noted on raw samples, turbidity results showed that comparison between different sources of liquid whey processed did not differ significantly ($p>0.05$) among other sources.

The pH change on the liquid whey influenced turbidity decrease significantly ($p<0.05$) when supernatants centrifuged after heat treatment at various pH, as the sign of denaturation and aggregation [51]. Turbidity (%) decreased significantly from pH 3.5 to pH 5.5 in both milk and colostrum supernatants. The liquid whey supernatant from yak/cows' milk or colostrum treated at 60-63°C at pH 3.5-5.5 after centrifuge, the turbidity reduced by 2.89-26.51% but high effect was noted at pH 5.5. Serious effect was noted 72°C/30 min and 90°C/5 min reduced the turbidity from 20.74-67.40% when supernatants were

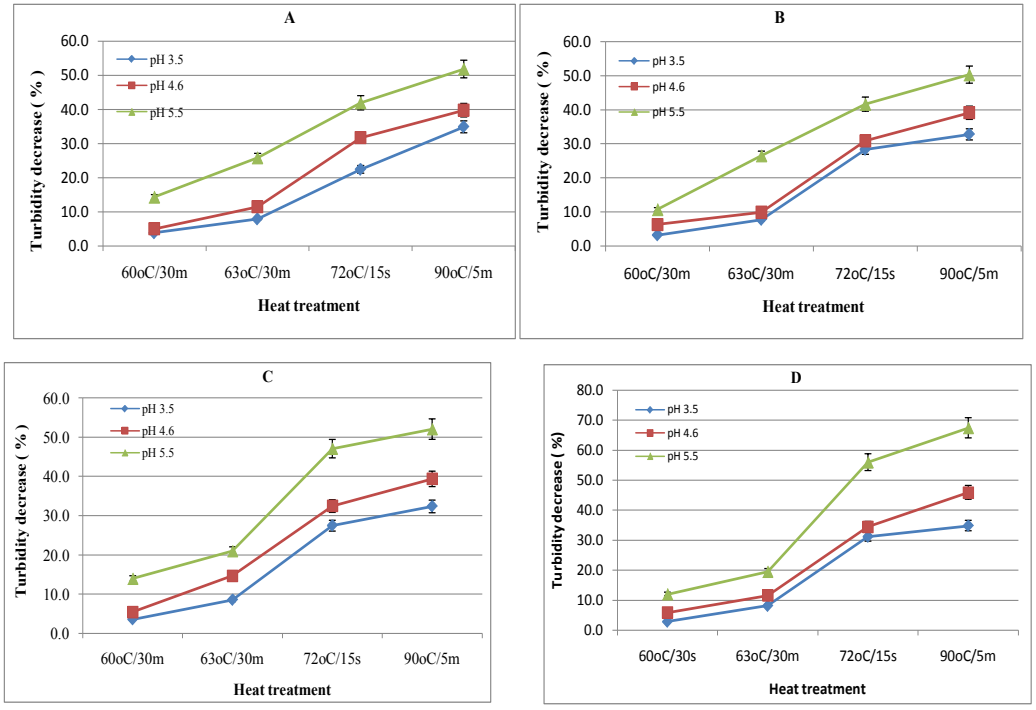

Figure 3: (a) Cows' milk (b) Yak milk (c) Yak colostrum (d) Cows' Colostrum: Plots indicating turbidity decrease of liquid whey supernatant from yak and cow after heat treatment (60°C/30 minutes, 63°C/30 minutes, 72°C/15 seconds and 90°C/5 minutes) at various pH (pH 3.5, 4.6 and 5.5) followed by centrifugation. Each value is the average of two determinations, error bars indicates standard errors.

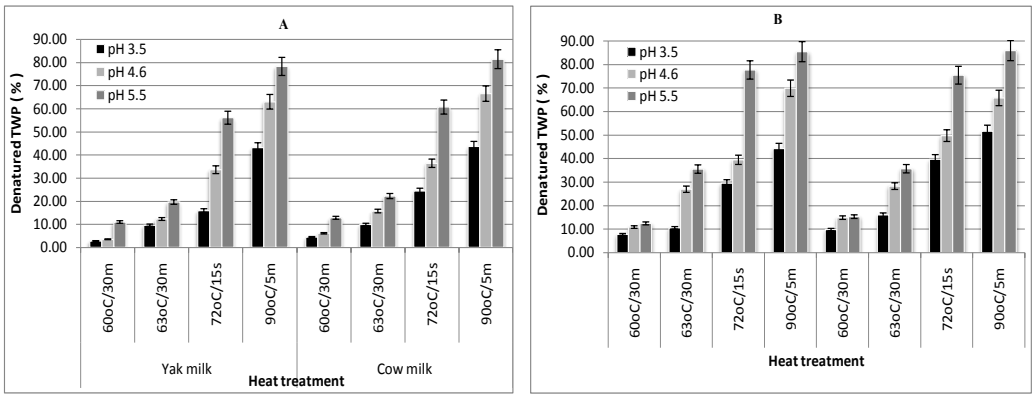

Figure 4: (A) Milk (B) Colostrum: Graphs indicating percentages reduction of Total Whey Proteins (TWP) in liquid whey supernatant from yak and cow after heat treatment (60°C/30 minutes, 63°C/30 minutes, 72°C/15 seconds and 90°C/5 minutes) at various pH (pH 3.5, 4.6 and 5.5) followed by centrifugation. Each value is the average of two determinations, error bars indicates standard errors.

centrifuged. Changes on liquid whey supernatant turbidity correlated with changes on TWP after heat treatment. TWP concentration in centrifuged supernatants was significantly reduced with increase pH and temperatures (Figure 4). The TWP concentration of Liquid whey from yak/cow milk or colostrum treated at 60-63°C/30 min at pH 3.5-5.5 reduced by 2.80-35.52%; while when the temperature raised to 72°C/15 sec and 90°C/5 min TWP reduced by 15.94-85.81%. High effect was noted at pH 5.5 and at high temperatures (72°C/15 sec and 90°C/5). An increase in pH influenced formation of soluble protein aggregates with large particle sizes which was removed out after centrifugation. The same explanation evidence reported recently by Chandrapala et al. [52].

The turbidity decrease of heated and centrifuged supernatants might be due to the processing condition near to pI of β-Lg. The temperature applied probably caused the native monomer β-Lg to reversibly interchange into a non-native monomer and non-native disulfide-bonded dimers of β-Lg and then interacted with other whey proteins such as α-LA, BSA [32,53-55] . Thereafter the denatured proteins were removed during centrifugation that attributed to reduce the liquid whey turbidity. The same phenomena was revealed when Bernal and Jelen [56] studied thermal stability of whey by a calorimetric at pH below pH 3.7, protein precipitation was prevented during heating at 95°C for 5 min; although protein denaturation occurred in various whey protein fractions. The protein concentration in the supernatant as a function of pH and temperature has been also reported by Chandrapala et al. [52], whereby soluble aggregates with large particles increased with pH and temperature increase as well as a decrease in proteins content by 7 to 20% noted from pH 3-10.5 at 15-90°C, respectively.

The effect of temperature and pH on free SH

It was observed that, denaturation and aggregation of liquid whey process as function of temperature and pH. Free -SH significantly decreased ($p<0.05$) with pH increase (pH 3.5 to 5.5) and temperature increase to indicate that more free -SH involved during denaturation process at pH 5.5 i.e. no more active sites after being denatured at pH 5.5 compared to low pH during heat treatment [57,58].

From our results it was observed that, liquid whey had low free -SH concentration at pH 5.5 compared to pH 3.5 and 4.6 (results not shown) indicating that more denaturation and aggregation occurred that involved SH and S-S bonds at pH 5.5.

Free -SH content decreased as proteins denaturation increased after heat treatment at various pH followed by centrifugation. Liquid whey studied comprised all major proteins including β-Lg, α-La, BSA, IgG and LF together, in that way heat treatment may easily induce interactions that result to sized aggregates by covalent (S-S) and non covalent bonds [59]. Presence of BSA on the liquid whey up on heating at pH 5.5 might be facilitated conformation of different monomers which expose -SH group to the outer structures with another reactive BSA to form a dimers and trimers through -SH/S-S interchange reactions [59].

Effect of heat on liquid whey IgG content at various pH

Results showed that IgG in milk was significantly ($p<0.05$) less denatured compared to colostrum in both yak and cows. Effect of heat treatment at various pH on liquid whey followed by centrifugation did not differ significantly ($p>0.05$) on heat stability between yak and cows' liquid whey (milk IgG concentration). However, the liquid whey from yak colostrum was less denatured significantly ($p<0.05$) compared to cow's colostrum. Probably, due to high IgG and β-Lg which were observed in cows colostrum though did not differ significantly. Generally, as reported previous, the extent of IgG denatured increased with increasing protein concentration, temperature and pH near to pI of IgG and β-Lg.

The pH sensitivity resulted in either partial or complete denaturation and increased the tendency to aggregate, which observed and were removed during centrifugation. The pH change in liquid whey significantly influenced denaturation ($p<0.05$) as approaching to pI. More serious aggregation observed and reflected with the calculated denatured IgG percent at 72°C/15 sec and 90°C/5 min (Figure 5). For mean IgG concentration in all liquid whey sources assayed, at 60°C/30 min treatment in all pH tested (pH 3.5-5.5) had no significant effect on IgG content reduction. Moreover, the effect of pH change from pH 3.5 to 5.5 during heat treatment of liquid whey reduced significantly IgG concentration as the temperature and pH increased. The mean reduction in IgG concentration as depicted in Figure 5 was less than 10% in milk for all pH tested at 60°C/30 min, except at pH 5.5 where reduction was higher in colostrum that rose to 12.95% and 13.48% in yak and cows' colostrum, respectively. When liquid whey from yak/cow milk or colostrum heated at 60-63°C/30 min. at pH 3.5-5.5, the IgG concentration reduced by 2.67-21.64% whereas at 72°C/15 sec and 90°C/5 min reduced by 14.45-55.67%.

High percent reduction in IgG concentration observed at pH 5.5

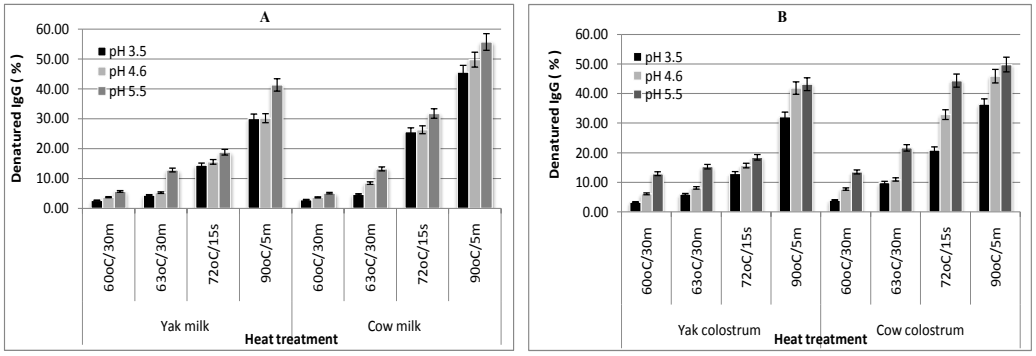

Figure 5: (A) Milk (B) Colostrum: Graphs indicating percentages of denatured IgG of liquid whey supernatant from yak and cow after heat treatment (60°C/30 minutes, 63°C/30 minutes, 72°C/15 seconds and 90°C/5 minutes) at various pH (pH 3.5, 4.6 and 5.5) followed by centrifugation. Each value is the average of two determinations, error bars indicates standard errors.

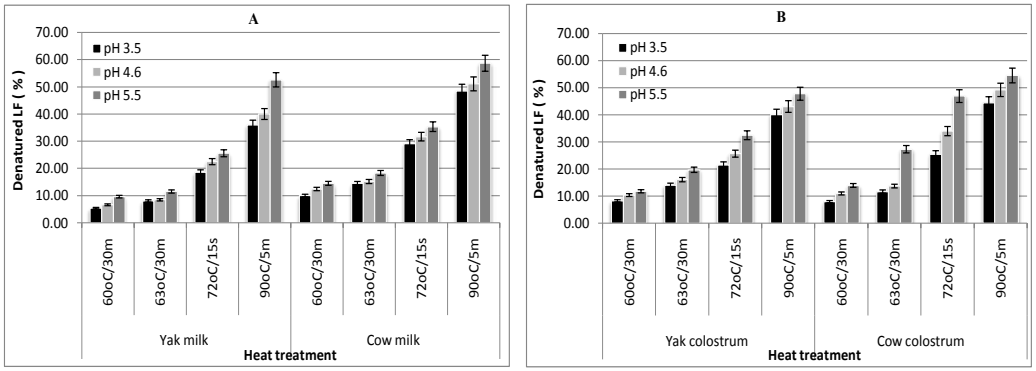

Figure 6: (A) Milk (B) Colostrum: Graphs indicating percentages of denatured LF of liquid whey supernatant from yak and cow after heat treatment (60°C/30 minutes, 63°C/30 minutes, 72°C/15 seconds and 90°C/5 minutes) at various pH (pH 3.5, 4.6 and 5.5) followed by centrifugation. Each value is the average of two determinations, error bars indicates standard errors.

at all temperatures used, suggesting that the effect was due to proteins concentration and processing condition near to pI of IgG and β-Lg which contributed to initiate coagulation and denaturation process [55,58].

High denaturation effect observed in colostrum liquid whey may be due to high IgG content and TWP compared to milk. Our results are in line with those reported by Levieux et al. [60], colostrum was more sensitive to heat treatment compared to milk due to high IgG content (12·6 $mgmL^{-1}$ v/s 0·5 $mgmL^{-1}$ for the mature milk) that influenced coagulation even at low temperature.

The decrease in IgG content, TWP assayed by ELISA technique and turbidity decrease (after centrifugation) may be due to aggregation and denaturation/unfolding of IgG molecules effected by heat treatment and pH increase (close to pI) that influences in loss of antigenicity [16,61]. The conformation change in the IgG molecule occurred during heat exposure reduces antigen-binding activity of this protein Calmettes et al. [62], whereas, antigen-binding site of IgG is more sensitive to heat compared to other part of the molecule [21,22]. Trujillo et al. [19] reported that Immunoglobulins, are species specific on heat sensitivity particularly IgG such as on bovine, caprine and ovine. It may be concluded that less effect IgG denaturation observed in acidic medium.

The presence/absent of casein or fat and different processing technique applied to prepare liquid whey might also influence the reduction of IgG and other whey proteins [62]. Walstra et al. [63] demonstrated the effect of removal of fat from colostrum. Heat treatment of colostrum at 60°C/30 min followed by centrifugation reduced 28% IgG due to flocculation of the fat globules by the immunoglobulins. According to Elfstrand et al. [17], reduction of IgG and other growth factors influenced by interaction of fat globules and high IgG concentration particularly in colostrum entrapped in the caseins matrix or cleaved by proteolytic enzymes.

On the other hand, Elfstrand et al. [17] showed that, heat treatment (60°C, 45 min) of liquid whey from Colostrum without the filtration process there was no change on concentration of IgG1 and TGF-b2. The same observation was noted even when whole colostrum was pasteurized (60°C/30 min), that no reduction on the concentration of the Immunoglobulins. Immunoglobulins in whole colostrum and colostrum whey were more resistant to heat processing temperatures compared to colostrum concentrate without fat, casein, salts and lactose, means that such components may protect the Immunoglobulins during heat processing treatment and filtration [16].

Effect of heat on liquid whey LF content at various pH

Heat treatment of liquid whey at various pH from different sources showed that LF in milk was less denatured ($p<0.05$) compared to liquid whey from colostrum both in yak and cows species. In comparison, the effect of heat treatment at various pH on liquid whey showed no significance difference ($p>0.05$) in LF denatured between yak and cows' milk liquid whey. However, LF in yak colostrum liquid whey was

less denatured compared to cow's colostrum liquid whey. The extent of LF denaturation increased with increased protein concentration, temperature and pH near to pI of LF and β- Lg; thus why colostrums were more denatured compared to milk in both yak and cow.

LF concentration decreased significantly as temperature increased ($p<0.05$), although there was no significant difference between 60°C/30 min and 63°C/30 min. The pH change of the liquid whey decreased significantly ($p<0.05$) from pH 3.5 to 5.5 or near to pI of LF and β-Lg, serious coagulation observed at such conditions which reflected with the calculated denatured LF percent (Figure 6). In general, the mean LF content showed significantly a gradual reduction of concentration from pH 3.5-5.5 for all liquid whey sources analyzed.

The mean reduction in LF concentration was about 10% in both milk and colostrum for all pH tested at 60°C/30 min, except at pH 5.5 where reduction was more noticed in colostrum that rose to 13.98% and 11.82% in cow and yaks' colostrum, respectively. When liquid whey from yak/cow milk or colostrum treated at temperature 60-63°C/30 min at pH 3.5-5.5, LF concentration reduced by 5.45-27.34%; Moreover, at 72°C/15 sec and 90°C/5 min reduced by 18.59-58.69%. High percent reduction in LF concentration noted at pH 5.5 at all temperatures used, suggesting that the effect was due to processing near to pI of LF and β-Lg which initiated coagulation and denaturation process.

Heat stability of LF is very crucial when it comes to bioactive components of foods. Several studies have been researched the influence of pH during pasteurization of LF in solutions. Saito et al. [64] studied the influence of pH (pH 2-11) observed that 5% bovine LF solution in distilled water at 80-120°C for 5 min gelled at neutral and alkaline pH while in acidic condition remained soluble and clear. Wakabayashi et al. [24] reviewed that bovine LF are comparatively stable to heating (90-100°C) at pH 4 pertaining iron binding capacity and antigenic activity.

Ueno et al. [65] studied the effects of temperatures (50-80°C for 10 min) on the iron-solubilizing capacity of LF in the presence of sodium bicarbonate observed that the temperatures above 70°C caused precipitation in the presence of Fe(III). The precipitation observed was related to the degree of thermal denaturation of LF, formed high-molecular-weight aggregates as disulphide bonds. Naturally, LF is a glycosylated protein, whereby the number and location glycosylation sites differ from one species to another. While the proportionality of glycan of oligomannosidic and of N-acetyllactosamine type also varies with lactation stage. Therefore, the primary structure of the specific glycans bound to LF determines the thermal stability [66].

However, discrepancies in our results obtained on effect of heat treatment at various pH of the liquid whey with other studies might be due to different methods to measure its denaturation and thermal treatment. Additionally, the effect of IgG and LF content in liquid whey may be influenced by sample volume and type of the container used to heat samples. Based on the processing condition, our study was conducted on unpurified proteins, whereas in the most mentioned and reported researches used pure, desalted proteins and in buffer solutions. Therefore, probably lactose, mineral, impurities and other proteins influenced our results.

To our knowledge this is the first time yak milk/colostrum liquid whey supernatants was heat processed at various pH to study the effect on antimicrobial proteins retained in comparison to cow.

Conclusion

According to the experimental results, liquid whey pH and thermal processing temperatures have significant effect on physical properties as well as antimicrobial proteins composition in manufacturing and formulation of functional foods. We confirmed that at low pH and mild heat treatment retained reasonable amount of IgG and LF. High level of aggregation and denaturation of the liquid whey proteins observed to be at pH 5.5 and at temperatures above 63°C. These results indicate that IgG and LF at pH 3.5 and 4.6 at mild heat temperatures condition is suitable as a practical method for pasteurization. Further studies are highly encouraged to verify the effects of heat treatment at various pH on purified IgG and LF from yak colostrum/milk liquid whey.

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