# Principal Components of Buffalo and Cow Milk from Bangladesh

**Proteolysis and Lipolysis of Buffalo, Cow and Omega-3 Enriched Milk by *Ex Vivo* Digestion**

## Karakterisering av hovedkomponentene i bøffel- og kumelk fra Bangladesh

**Proteolyse og lipolyse av bøffel-, ku- og omega-3 beriket melk ved *ex vivo* fordøyelse**

Philosophiae Doctor (PhD) Thesis

Mohammad Ashiqul Islam

Department of Chemistry, Biotechnology and Food Science
Norwegian University of Life Sciences

Ås 2014

Thesis number 2014:52
ISSN 1894-6402
ISBN 978-82-575-1213-2

**This thesis is dedicated in memory of my Father**

**Table of contents**

**Acknowledgements**

Thanks to the Almighty Allah for his unlimited kindness; unconditional and never ending love to me. I found Him always with me in every turn, ups and down all the time.

The work presented in this thesis was carried out at the Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Ås-1432, Norway (2009-2014), Bangladesh Livestock Research Institute (BLRI), Savar, Dhaka, Bangladesh (2012), and at the Tromsø University Proteomic Platform (TUPP) (2012-2013), Tromsø-9037, Norway. Milk samples were collected from BLRI and Central Cattle Breeding Station and Dairy Farm, Savar, Dhaka, Bangladesh. Financial support was provided by Norwegian state loan fund (Lånekassen) and Food and Health programme, IKBM, NMBU, Norway.

From the very bottom of my heart, many thanks to my main supervisor Professor Gerd Elisabeth Vegarud for all the support, no matter whether it is academic/family/social. Thanks Gerd for trusting in what I was doing and your nice enthusiastic guidance and constant encouragement all through this work. Whenever I remember you, I feel proud as your student with a pleasant mode. Thank you so much.

I would also like to thank my co-supervisor Associate Professor Dag Ekeberg for his help and support, especially regarding the lipid analysis and gas chromatography with mass spectrometry. You always ask about small things that are often neglected but important in making things strong.

I have to thank Professor Elling-Olav Rukke for sharing his expertise through all the work. I will never forget your lesson of “Red Thread”, though I often failed to construct that. Thank you so much for being with us in the project as my co-supervisor.

Thanks to Professor Einar Jensen to give a nice introduction to the world of “Proteomics”. Professor Ragnar Flengsrud, thank you very much to teach me the sequence alignment. I would also like to extend my thanks to all of my superb colleagues in the group GV-1921, “Food

Proteins; Structure and Biological Function" and in other collaborating groups. Thank you, Sara Arroyo Sanz, ERASMUS exchange student of Bachelor in Food Science, IKBM, NMBU for your contribution in the *ex vivo* digestion results presented in the appendices. Dr. Hanne, Ragnhild, Dr. Ashoka and Dr. Mestawet, thanks to you for making the lab more comfortable to me. Dr. Ellen, thanks for always being smiley to me with helping attitude. Irene, you always made lab essentials available in quick time and did the aspiration and its subsequent preparation, thank you very much. Thanks Tove for your quick informative reply whenever needed; sometimes I knocked you before using online scholar search! Kari and Maye you were excellent in lab, thanks for your support.

Thanks to my teachers and colleagues at the Department of Dairy Science, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh for their continuous inspiration and administrative support. My thanks are also to Mr. M. K. Alam of BLRI and M. M. Sarkar, PKSF, Dhaka for providing the animal's photograph.

I would also like to extend thanks to "The Bangladeshi Community" in Ås-1432, Norway, for providing my family such a wonderful social life. That helps to settle Norway herein and to focus on my study.

Finally, my family. My wife, such a wonderful lady I have as my better half who provides me all supports even making a maximum sacrifice in her personal life. I am proud of you my dear. My two little princes, they always provide a shower of their smile to me that wash out all of my frustrations and make me ready for the challenges on the coming day. I must mention my parents, brothers, sister and my uncle, Mr. Badiuzzaman who provides me all sorts of support from the beginning to see me in a place where I am today.

Ås, May 2014 Mohammad Ashiqul Islam

**Abbreviations**

| | |
|---|---|
| α | Alpha |
| β | Beta |
| $BF_3$ | Boron tri-fluoride |
| Ca | Calcium |
| $CH_3OH$ | Methanol |
| CLA | Conjugated linoleic acid |
| DHA | Docosahexaenoic acid |
| EPA | Eicosapentaenoic acid |
| f | Peptide fragment |
| FAMEs | Fatty acid methyl esters |
| FID | Flame Ionization Detector |
| GC | Gas chromatography |
| γ | Gamma |
| HCL | Hydrochloric acid |
| $H_2SO_4$ | Sulphuric acid |
| IgE | Immunoglobulin E |
| κ | Kappa |
| MFGM | Milk fat globule membrane |
| P | Phosphorus |
| PUFA | Polyunsaturated fatty acid |
| Q-TOF | Quadrupole-Time of Flight |
| SDS-PAGE | Sodium dodecyl sulfate- Polyacrylamide gel electrophoresis |
| UPLC-MS | Ultra Performance Liquid Chromatography-Mass Spectrometry |

**Summary**

The aim of the study was to characterize and compare the Bangladeshi buffalo, Holstein cross, Indigenous cattle and Red Chittagong Cattle milk based on milk's principal components, and further to investigate the proteolysis and lipolysis of the milk with a focus on the degradation of the allergenic milk proteins, $\alpha_{s1}$-casein and β-lactoglobulin. Proteolysis was monitored by protein degradation and peptide generation and lipolysis by the generation of free fatty acids. Most of the principal milk components showed considerable variation. Higher protein, casein, whey protein, β-casein, lactose, total mineral and P were detected in Red Chittagong Cattle and Indigenous cattle milk. Most of the other components were almost similar in these two types of milk. The casein number, content of $\alpha_{s2}$-, and κ-casein, α-lactalbumin, fat, unsaturated fatty acids, Ca and Ca:P were higher in buffalo milk than the cow's milk. Casein micelles and milk fat globules were also found larger in the buffalo milk. Lower casein to whey protein ratio, higher β-lactoglobulin content and richness in naturally occurring peptides were found in Holstein cross milk.

Buffalo milk showed rapid and complete digestion of caseins and β-lactoglobulin during the *ex vivo* digestion. Milk fat, or added cod liver oil, had no effect on the buffalo milk protein degradation. Whereas, full fat Red Chittagong Cattle milk still showed intact β-lactoglobulin after 120 min duodenal digestion, but it was not shown in the full fat milk of Holstein cross and Indigenous cattle. Probably the genotype and β-lactoglobulin concentration in milk contribute to this variation. Considering the number of identified peptides, the descending order of the protein was β-, $\alpha_{s1}$-, κ- and $\alpha_{s2}$-casein and β-lactoglobulin. The presence of proline and/or hydrophobic amino acids in the identified peptides was remarkable.

A sharp increase in the lipolysis (ca. 30%) was observed during the first 30 min duodenal digestion, thereafter, leveled off in buffalo milk fat and cod liver oil; while cow milk continued the lipolysis up to 60 min duodenal digestion. The lipolysis of total saturated fatty acids and unsaturated fatty acids were almost similar in cow milk and cod liver oil. However, 9% more lipolysis was observed in the saturated fatty acid than the unsaturated fatty acid of buffalo milk fat. More lipolysis was observed in short chain fatty acids followed by long chain and medium

chain fatty acids. In the cod liver oil, monounsaturated fatty acids hydrolyzed more (7%) than the polyunsaturated fatty acids. The eicosapentaenoic acid (C20:5n-3) and docosahexaenoic acid (C22:6n-3), both the omega-3 fatty acids showed 23% lipolysis.

The conclusion of the study is that the buffalo milk is preferable for individuals with cow milk allergy. Nevertheless, cow milk from/of certain genotype could also be useful in this regard. Enrichment of milk with cod liver oil could provide a good nutritional source of omega-3 fatty acids. Red Chittagong Cattle milk had a higher protein and casein content than buffalo and other cows' milk. So, it may be the best choice for dairy product development.

**Sammendrag**

Formålet med dette studiet var å karakterisere og sammenlikne melk og melke komponenter fra ulike ku- og bøffelraser i Bangladesh og undersøke proteolyse og lipolyse i denne type melk med fokus på nedbryting av de allergene melkeproteinene, $\alpha_{s1}$-kasein and β-Laktoglobulin. Rasene som ble benyttet var Bangladesh Bøffel, Holstein cross (krysning av Holstein og lokal rase), Indigenous (lokal rase) og Red Chittagong storfe. Proteolyse ble undersøkt ved protein degraderingsprofiler og peptidsekvenser, og lipolyse ved frigivelse av frie fettsyrer. Melkekomponentene i nevnte raser varierte betydelig. Høyere innhold av protein, kasein, myse protein, β-kasein, laktose, total mineral og fosfat ble observert i melk fra Red Chittagong og i den lokale rase, Indigenous. Innholdet av andre komponenter var omtrent likt mellom raser. Kasein tallet, innhold av $\alpha_{s2}$-, and κ-kasein, α-laktalbumin, fett, umettede fettsyrer, Ca og Ca:P var høyere i bøffel melk enn i ku melk. Kasein miceller og størrelser av fettglobuler ble også funnet å være høyere i bøffel-melk. En lavere ratio av kasein:myseprotein, samt høyere innhold av β-Laktoglobulin og naturlig forekommende peptider ble observert i melk fra Holstein cross. Med hensyn til proteolyse og lipolyse i *ex vivo* fordøyelse viste bøffel-melk en rask og fullstendig fordøyelse av alle kaseinene og β-Laktoglobulin, videre ble det ikke observert noen effekt fra melkefett eller tilsatt torskelever olje som kilde til omega-3 fettsyrer, på nedbryting av proteinene. I motsetning til H-melk fra bøffel, Holstein cross og Indigenous cattle var β-Laktoglobulin fremdeles intakt etter 120 min. duodenal fordøyelse i H-melk fra Red Chittagong Cattle. Det antas at genotypen og konsentrasjonen av β-lactoglobulin i melken bidrar til denne variasjonen. En rask økning i lipolyse ble observert i løpet av de første 30 min av duodenal fordøyelse, deretter var det ingen/liten økning i lipolyse. Lipolyse av de totale mettede fettsyrer og de umettede fettsyrer var omtrent likt i H-melk fra ku og i melk tilsatt torskelever olje. Derimot ble det funnet 9% høyere lipolyse i de mettede fettsyrer og de umettede fettsyrer fra bøffel melk. Høyere grad av lipolyse ble også observert av kortkjedede fettsyrer etterfulgt av langkjedede og medium kjedede fettsyrer. I bøffel-melk med tilsatt torskelever olje ble monoumettede fettsyrer hydrolysert i større grad (7%) enn polyumettede fettsyrer. Eicosapenten syre (C20:5n-3) og docosaheksen syre (C22:6n-3), hadde begge 23% lipolyse.

Konklusjonen av dette studiet viser at bøffelmelk er å foretrekke på grunn av sine ernæringsmessige fordeler når det gjelder immunogene proteiner og som kilde for berikning

med omega-3 fettsyrer. Melk fra Red Chittagong viste seg å ha et høyere innhold av proteiner og kasein enn fra ku, og kan derved være et bedre valg for produktutvikling.

**List of papers**

I. **Islam, M. A.**, Alam, M. K., Islam, M. N., Khan, M. A. S., Ekeberg, D., Rukke, E. O. and Vegarud, G. E. (2014). Principal milk components in buffalo, Holstein cross, Indigenous cattle and Red Chittagong Cattle from Bangladesh. *Asian-Australasian Journal of Animal Science*, 27, 886-897.

II. **Islam, M. A.**, Ekeberg, D., Rukke, E. O. and Vegarud, G. E. (2014). *Ex vivo* digestion of proteins and fat in buffalo milk. *International Dairy Journal*, Submitted.

III. **Islam, M. A.**, Ekeberg, D., Rukke, E. O. and Vegarud, G. E. (2014). *Ex vivo* digestion of omega-3 enriched buffalo skimmed milk. *Journal of Functional Foods*, Submitted.

IV. **Islam, M. A.**, Ekeberg, D., Rukke, E. O. and Vegarud, G. E. (2014). *Ex vivo* digestion of milk from Red Chittagong Cattle focusing proteolysis and lipolysis. *Asian-Australasian Journal of Animal Science*, Submitted.

## 1. General Introduction

Milk is a complex biological fluid with diverse composition of nutrients. It contains all the components required for the newborn and adds quality to the diet of growing children and of adults/elderly. The composition of milk is influenced by the genetics, breeding, feeding, season, number and stage of lactation, number of milking per day, health status and even by a period of time (Schonfeldt et al., 2012; Abd El-Salam and El-Shibiny, 2011; Medhammar et al., 2011; Heck et al., 2009a, b). The nutritional properties of milk and suitability of milk processing to different products largely depends on milk composition (Heck et al., 2009b). In the history of mankind, a wide variety of animal species and a number of cattle breeds have been utilized for their milking capacity. To improve the milk composition and to develop more consumer friendly products, knowledge on the variation in the productivity and milk components of different species is important (Medhammar et al., 2011; Glantz et al., 2009). All over the world, a number of dairy animals including cow, buffalo, goat, sheep, camel, donkey, horse and other region specific minor dairy animals have been used for the milk production (Medhammar et al., 2011). Recently, Schonfeldt et al. (2012) compared the bovine milk from different countries and highlighted that the "international data for milk cannot be used at national level" and suggested that – the country-specific milk composition data should be used.

Health effects of isolated food components have been studied for many years without considering the inherent complexities of the digestive processes and hence, the fates of the most of the bioactive components are still not clear. Milk and milk products cover only 9% of the total digestion model studies on food (Hur et al., 2011) reflecting ample opportunities to contribute in this area of study. One of the purposes of using model digestion is to monitor the degradation pattern of the food components, like food allergen. The release of other degradation products is important to monitor as well. The buffalo milk is still missing to be tested in model digestion studies. Though cow, human, sheep, goat, donkey, horse and camel milk have been reported (Tidona et al., 2014; Salami et al., 2011; Inglingstad et al., 2010). Buffalo milk is different in its composition from those milk (Abd El-Salam and El-Shibiny, 2011; Medhammar et al., 2011) and interestingly children who has cow milk allergy can tolerate the buffalo milk (Sheehan and Phipatanakul, 2009), however, further studies have been suggested.

Often the consumers blame milk and milk products for its high content of saturated fatty acids and low content of polyunsaturated fatty acids, especially the omega-3 fatty acids. Now a day, industries are processing milk and milk products with enhanced omega-3 fatty acids content by adding omega-3 fatty acids to the milk from non-milk origin. Influence of such inclusion on the digestion of other milk components, especially on the milk allergen β-lactoglobulin is the interesting one.

If not specify, milk means the cow milk. It is the most abundant milk available to consume in the form of liquid milk or milk products. So in reporting any milk particularly in model digestion study, inclusion of cow milk will provide more common baseline to compare. The wide variability in the used digestion models makes it difficult to compare with the previous data unless the model conditions are considerably same. Nevertheless, the model digestion study provides useful information on the nutritional quality of milk and milk products or any other food products.

## 2. Aims of the study

The present study was aimed at characterizing the buffalo, Holstein cross, Indigenous cattle and Red Chittagong Cattle milk from Bangladesh in terms of their principal components. Further to investigate proteolysis and lipolysis of whole, skimmed and omega-3 fatty acids enriched milk to monitor the hydrolysis of protein (especially the milk allergen like $\alpha_{s1}$-casein and β-lactoglobulin) and lipids. These parameters are important for nutritional and technological aspects of the milk as a part of the future breeding programme.

The study was divided into the following sub-goals:

- Study of the physical and chemical parameters of principal components in milk from Bangladeshi buffalo, Holstein cross, Indigenous cattle and Red Chittagong Cattle.
- To investigate the influence of milk fat on the degradation of caseins and whey proteins in buffalo milk mainly focusing on the allergenic milk proteins.
- To assess the effect of enriching skimmed buffalo milk with omega-3 fatty acid by adding cod liver oil on the proteolysis of milk proteins.
- Study the lipolysis and proteolysis of Red Chittagong Cattle milk in *ex vivo* digestion model with particular attention to $\alpha_{s1}$-casein and β-lactoglobulin.

## 3. Theory

### 3.1 Dairy animals, milk production and dairy products of Bangladesh

Bangladesh is one of the top countries of the world regarding the density of the livestock (GED, 2010). The dairy animal population is shown in Table 1 and the distribution of the cattle throughout the country is depicted in Figure 1. The genotypes available are low productive and the average milk yield per cow is 1-2 kg/day. Poor animal feed resources and lack of preventive veterinary practices are also responsible for this low productivity. However, the productivity of the native cattle has increased by 5% during 1996-2002, which was 4-8% in crossbred cows and buffaloes and the total milk production increased by 3% (Hemme et al., 2004). Bangladesh has 80% deficiency in their milk production (Tareque and Chowdhury, 2010). The available market milk mainly comes from cattle (99%: BLRI, 2004)) and buffalo (frequently mixed with and sold as cow milk). In the total milk production, crossbred cows contribute 56.6%. It was 35.9%, 5.6% and 1.89% from the Indigenous cow, buffalo and others, respectively (Tareque and Chowdhury, 2010).

**Table 1.** Number of dairy animals in Bangladesh.

| Animal species | Population (million) |
|---|---|
| Cattle | 23.15 |
| Buffalo | 1.44 |
| Goat | 55.00 |
| Sheep | 1.89 |

Source: FAOSTAT (2012a).

Cattles are comprised of Indigenous, exotic and crossbred. In Indigenous, the types of cattle are – non-descriptive, Red Chittagong Cattle (RCC), Pabna, North Bengal Gray, Madaripur, and Munshiganj. The available exotic breeds are Jersey, Holstein-Friesian, Sahiwal, and Australian Friesian Sahiwal (AFS) that are used mainly for crossbreeding purposes. Crossbreeding of the native poor producer with a high yielding animal is in practice and some happen because of unavoidable breeding. The most common crossbreds are – Holstein x Local, Sahiwal x

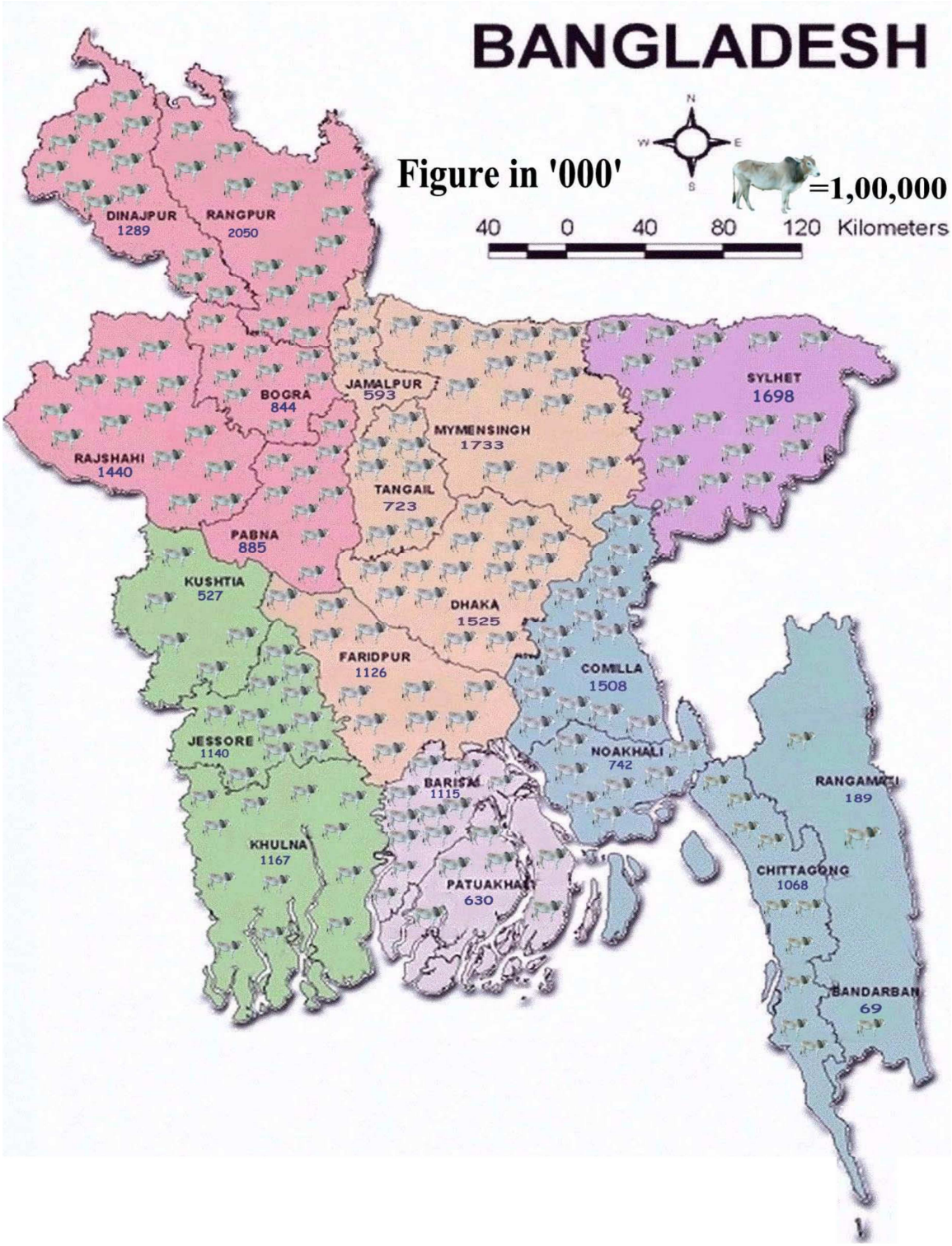



**Figure 1.** Cattle distribution throughout the Bangladesh (Bhuiyan, 2007).

Red Sindhi, Sahiwal x Holstein-Friesian, Holstein x Sahiwal, Friesian x Indigenous and Sahiwal-Sindhi x Jersey. The available buffaloes are – Indigenous (non-descriptive), River type (Nili-Ravi), Swamp type (Surti) and Crossbred (River type x Swamp type). Geographically, buffaloes are mainly concentrated in the sugar-cane belt, hilly region, coastal area and marshy land of the Bangladesh. These areas mainly belong to the Brahmaputra-Jamuna flood plain area and Meghna-Padma tidal flood plain area agro-ecological zone. Buffalo and the types of cattle used in the present study are shown in Figure 2.

**Figure 2.** Buffalo (upper left), Holstein cross (upper right), Indigenous cattle (lower left) and Red Chittagong Cattle (lower right) of Bangladesh.

The consumption of milk products is a part of Bangladeshi tradition and culture. Milk is processed to a wide variety of milk products. Cheese/paneer, yoghurt/doi, rasogolla, rasomalai, sandesh, kalojam, chomchom, ice cream, butter, ghee, buttermilk/mattha are the main dairy

products available almost all through the country. The production of butter, cheese and ghee/butter oil from cow milk was 1180, 1000 and 24100 tonnes, respectively in 2012 and in the same year the ghee produced from buffalo milk was 640 tonnes (FAOSTAT, 2012b).

### 3.2 Buffalo milk production – global context

The name of the domesticated buffalo is *Bubalus bubalis*. Factors that favor the buffalo farming are – ability to utilize efficiently the low quality high roughage diet, resistance to parasites, yielding good quality meat, rich milk and milk products (Abd El-Salam and El-Shibiny, 2011). In 2012, the world buffalo population was approximately 199 million (FAOSTAT, 2012b). They are widely distributed throughout the Asia, Europe, South America and Caribbean. India and Pakistan have the largest buffalo population, 58% and 16%, respectively of the world population and Bangladesh has less than 1% (FAOSTAT, 2012b). Buffaloes are mainly considered as multipurpose animal, but their dairy ability has been improved considerably through selection programme. Now a day, there are 22 dairy breeds of buffalo available all over the world and buffalo is the second largest milk producing dairy animal in the world. The buffalo milk production was 13% of the total world milk production in 2012 and India and Pakistan produced 67% and 24% of the total world buffalo milk, respectively. While, Bangladesh has produced only 4% of the world buffalo milk (FAOSTAT, 2012b). The annual growth rate of buffalo milk production is almost 2% more than the cow milk production (IDF, 2010).

### 3.3 Milk components

Milk contains all the required nutrients for the newborn, nutritionally regarded as one of the most complete food. It is an excellent source of macro- and micro-nutrients and plays an important role in mitigating individual's nutritional requirement. This highly diversified and complex biological fluid contain a balance of proteins, lipids, carbohydrates, vitamins, minerals and other minor components. An overview of gross milk components from different species is given in Table 2. The composition of the milk is very important for its nutritional and technological properties (Heck et al., 2009b). Cheese yield is strongly positively correlated with

the fat, protein and casein content of the milk (Pretto et al., 2013). Milk proteins have high biological value because of their essential amino acids content and are regarded as a rich source of bioactive peptides (Medhammar et al., 2012; Pihlanto-Leppala et al., 1998).

**Table 2.** Average composition of milk components (%) from various mammalian species.

| Animal Species | Total-solids | Protein | Fat | Sugar | Ash |
|---|---|---|---|---|---|
| Cow | 13.1 | 3.5 | 4.4 | 4.5 | 0.7 |
| Buffalo | 16.7 | 3.8 | 7.3 | 4.8 | 0.7 |
| Goat | 12.8 | 3.2 | 4.5 | 4.3 | 0.8 |
| Sheep | 17.5 | 4.6 | 7.2 | 4.8 | 0.9 |
| Donkey | 11.3 | 2.0 | 1.4 | 7.4 | 0.5 |
| Horse | 11.1 | 2.5 | 1.9 | 6.2 | 0.5 |
| Human | 12.1 | 1.9 | 3.5 | 6.5 | 0.2 |

Source: Heck et al., 2009a; Islam et al., 2008; Khan et al., 2007; Pandya and Khan, 2006; Belitz and Grotz, 1999.

### 3.3.1 Milk proteins

The protein content of milk is different in different species (Table 2). They are also different in their composition (Table 3). The milk proteins are generally classified into two – caseins (precipitates at pH ~4.6) and whey proteins (soluble at pH ~4.6). There are four major caseins present in milk, namely – $\alpha_{s1}$-, $\alpha_{s2}$-, β- and κ-casein. The β-lactoglobulin and α-lactalbumin are the major whey proteins. There are some other minor proteins present in the milk like serum albumin, lactoferrin, immunoglobulins, transferrin, ferritin, proteose peptone, prolactin and folate binding protein.

#### 3.3.1.1 *Caseins*

The $\alpha_{s1}$-casein constitutes up to 40% of the casein fraction in bovine milk. The reference protein consists of 199 amino acids, with 8 phosphorylated serine and no cysteinyl residues. The protein exists in several genetic variants termed A to H. This protein does not form

crystals, so its 3-D structures cannot be determined and because of the intrinsic aggregation of the protein, the nuclear magnetic resonance study is also problematic (Farrell Jr. et al., 2004). The buffalo $\alpha_{s1}$-casein has 97.2% similarity with bovine $\alpha_{s1}$-casein (9 substitutions) and showed reduced phosphorylation (D'Ambrosio et al., 2008). The $\alpha_{s1}$-casein BB genotype is related with higher milk yield, fat yield, protein yield, higher lactation cheese yield but with reduced protein concentration, however, BC genotype gives highest protein concentration (Aleandri et al., 1990; Ng-Kwai-Hang et al., 1984, 1986).

**Table 3.** Major milk protein concentration (g/L) and the respective Molecular weight (Mw) in cow and buffalo milk.

| Milk protein type | Content | | Molecular weight (Da) | |
|---|---|---|---|---|
| | Cow | Buffalo | Cow | Buffalo |
| $\alpha_{s1}$-casein | 12-15 | 8.9 | 23,615 | 22,773 |
| $\alpha_{s2}$-casein | 3-4 | 5.1 | 25,226 | 24,700 |
| β-casein | 9-11 | 21 | 23,983 | 23,582 |
| κ-casein | 2-4 | 4.1 | 19,037 | 19,247 |
| β-lactoglobulin | 2-4 | - | 18,277 | - |
| α-lactalbumin | 0.6-1.7 | - | 14,178 | - |

Source: Feligini et al. (2009); D'Ambrosio et al. (2008); Farrell Jr. et al. (2004).

The $\alpha_{s2}$-casein contributes 10% of the casein in bovine milk and shows different levels of post-translational phosphorylation. It consists of 207 amino acids with 11 phosphorylated serine and is a single chain polypeptide with an internal disulfide bond. There are four genetic variants of $\alpha_{s2}$-casein available (A to D). According to D'Ambrosio et al. (2008), ten substitutions were found between cow and buffalo $\alpha_{s2}$-casein resulting in ca. 98% homology. It is the most hydrophilic casein.

The most prevalent casein in the milk is β-casein (45% of the casein). The reference protein is a single polypeptide chain of 209 amino acids, with 5 phosphorylated serine and without cysteine. So far, 12 genetic variants of β-casein have been identified. Higher homology (95%) exists between cow and buffalo β-casein (six substitutions) and the peptides released after

enzymatic treatments are different (Di Luccia et al., 2009; D'Ambrosion et al., 2008; Abd El-Salam and El-Shibiny, 1975). Plasmin activity on β-casein leads to the formation of $\gamma_1$- (f29-209), $\gamma_2$- (106-209) and $\gamma_3$-casein (108-209). Moreover, proteose peptone components 5, 8-fast and 8-slow have been identified as f1-105/107, f1-28, and f29-105 of β-casein, respectively. The β-casein genetic variants and haplotypes are associated with the protein composition and technological properties of the milk. Compared to $A_1$ variant, β-casein $A_2$ is related with the higher concentration of β- and $\alpha_{s2}$-casein and protein yield which is reverse in case of $\alpha_{s1}$- and κ-casein. The β-κ-casein haplotype is favorable for cheese production (Heck et al., 2009a). According to Glantz et al. (2012), β-casein variant has influence on yields of milk, protein, casein, lactose, total Ca and P ($A_2$>$A_1$). Homozygous β-casein $A_1$ cows produce less milk with higher fat content compared to $A_2$ homozygous cows and $A_1B$ cow milk has a higher concentration of fat and protein (Ng-Kwai-Hang et al., 1986; Ng-Kwai-Hang et al., 1990). β-casein is the most hydrophobic casein.

Casein with a carbohydrate moiety and all the *N*-acetylneuraminic acid of the casein micelles is the κ-casein. The reference κ-casein (major part is carbohydrate free) is composed of 169 amino acids with 1 phosphorylated serine. In milk, it can also be found as a mixture of disulfide bonded dimer to octamers or more. Thirteen substitutions between cow and buffalo milk κ-casein resulting into ≈ 93% homology (D'Ambrosion et al., 2008). Buffalo has a lower proportion of κ-casein fraction with carbohydrate and contains less sialic acid than the cow's κ-casein (Addeo et al., 1977). Low concentration of κ-casein is a risk factor for non-coagulation of milk (Hallen et al., 2010). Chymosin hydrolyzes the bond between Phe 105 and Met 106 resulting in the formation of para-κ-casein (f1-105) and caseinomacropeptide (f106-169). Eleven different genetic variants have already been identified. According to Heck et al. (2009a), the different variant of κ-casein is associated with the protein percentage (B>A) and relative concentrations of $\alpha_{s2}$- (B>A), κ- (B>E>A) and $\alpha_{s1}$-casein and α-lactalbumin (A>B). Glantz et al. (2012) concluded that the κ-casein (A>B>E) influences the milk, protein, casein, lactose, total Ca and P yield. κ-casein genotype BB and AB have influence on the *de novo* synthesis of the fatty acids in the mammary gland. Genotype BB is related with the higher concentration of milk protein and fat. And milk from this cow is advantageous for cheese

production because of superior coagulation properties (Melia et al., 2009; Macheboeuf et al., 1993; Aleandri et al., 1990; Ng-Kwai-Hang et al., 1986).

Caseins after enzymatic hydrolysis produce a good number of bioactive peptides that can exert numerous physiological responses. Some of these peptides have been identified and sequenced, and their release conditions have been determined (Korhonen and Pihlanto, 2006). The bioactive peptides from milk are mainly ACE-inhibitory peptides, antithrombotic peptides, opioid peptides, casein phosphopeptides, immunomodulatory peptides, antimicrobial peptides, cytomodulatory peptides (Mills et al., 2011). Epidemiological studies revealed that low milk consumption has link with increased risk of stroke (Maghsoudi et al., 2013). Human milk does not contain $\alpha_{s1}$-casein. According to Lara-Villoslada et al. (2005), children with cow milk protein allergy synthesize antibodies against α-casein. Consumption of β-casein (variant $A_1$ and $A_1B$) was found related with the incidence of type-I diabetes (Elliott et al., 1999) but β-casein $A_2$ was found to exert an opposite effect and was also associated with a lower incidence of cardiovascular disease (Bell et al., 2006).

#### 3.3.1.2 *Whey proteins*

The major whey protein is β-lactoglobulin. It consists of 162 amino acids. Buffalo and bovine β-lactoglobulin differ only by two substitutions – one at C- and another at N-terminal acid. They have greater similarities in structure and solution conformation and shares similar epitopes (D'Ambrosio et al., 2008; Li et al., 2008; Ghosh et al., 2004). β-lactoglobulin is synthesized in the mammary gland and has one mole of cysteine and two moles of cystine per monomer (Fox and McSweeney, 1998). In the native protein, the disulfide bonds are between Cys 66 and Cys 160 and between Cys 106 and Cys 119 with Cys 121 as a free thiol. The compact globular structure of the β-lactoglobulin consists of one major α-helix, 8-stranded antiparallel β-barrel and a ninth β-strand on the outside (Sakurai et al., 2009; Kontopidis et al., 2004). A 3-D structure of bovine β-lactoglobulin is given in Figure 3. All bovine β-lactoglobulin genetic variants form dimers at pH 5.5-7.5 and octamers at pH 3.5-5.5 but dissociated to monomers at pH <3.5 and >7.5 (Fox and McSweeney, 2003; Perez and Calvo, 1995). The β-lactoglobulin B is associated with higher relative concentration of $\alpha_{s1}$-, $\alpha_{s2}$-, β-

and κ-casein and α-lactalbumin compared to the variant A but variant A gives more β-lactoglobulin than B does (Heck et al., 2009a). Glantz et al. (2012) also found that β-lactoglobulin A is associated with higher whey protein yield. Melia et al. (2009) showed the influence of β-lactoglobulin genotypes (AA, AB and BB) on the milk fatty acid composition. β-lactoglobulin BB is related with high cheese yield, higher fat and casein percentage and a lower percentage of total and whey protein. Milk from this genotype is preferable for cheese production (Aleandri et al., 1990; Ng-Kwai-Hang et al., 1986).

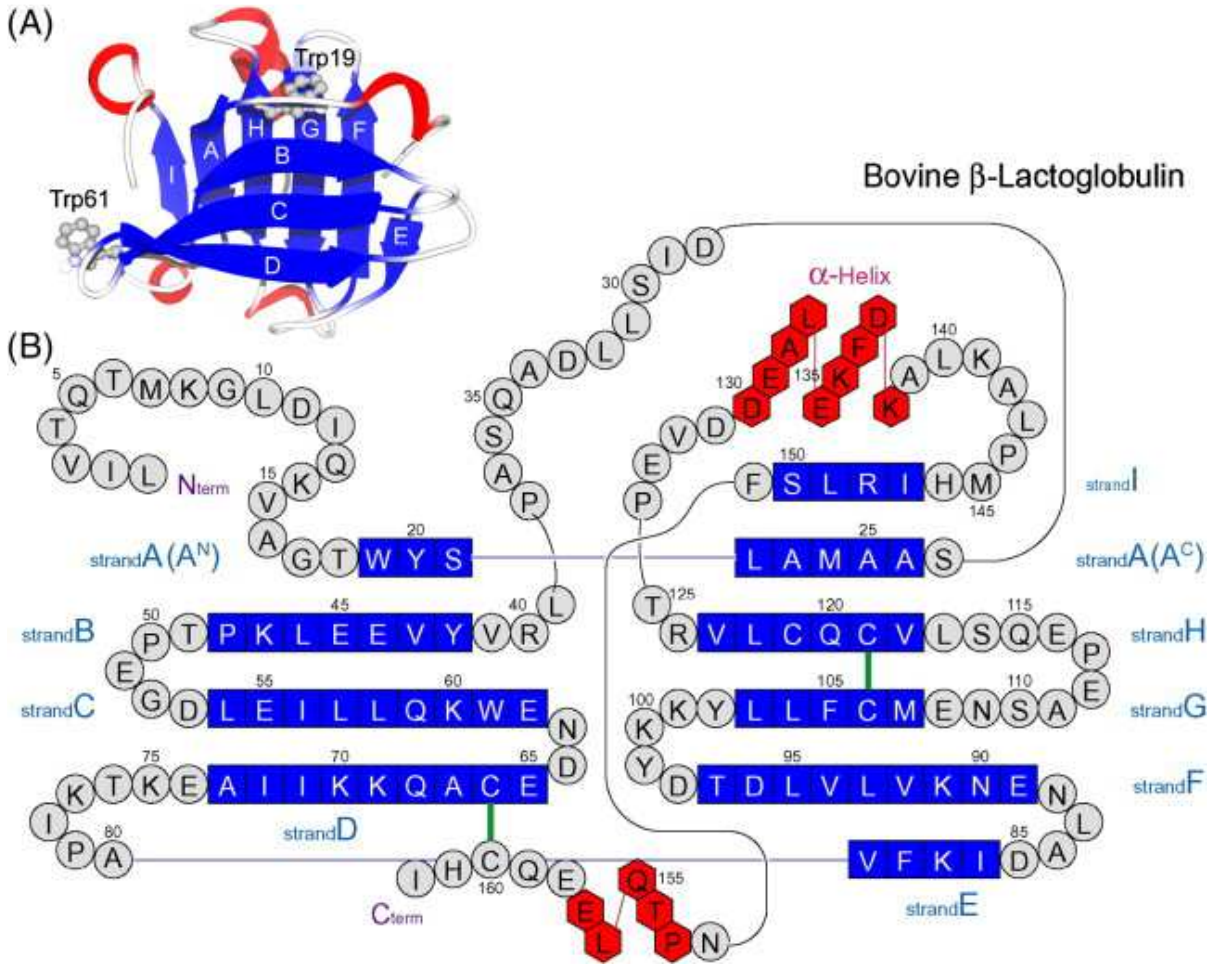



**Figure 3.** Amino acid sequence and 3D structure of bovine β-lactoglobulin. (A) β-lactoglobulin monomer. The β-strands are labelled and tryptophan (Trp) residues are presented as balls and sticks. (B) Schematic representation of β-lactoglobulin amino acid sequence. Red hexagon, residues in α-helix; blue square, residues in β-sheet; grey circles, residues in loop; green lines, disulfide bonds. B-D and $A^N$, one β-sheet; E-H and $A^C$, the other β-sheet (Sakurai et al., 2009).

The bovine α-lactalbumin is also synthesized in the mammary gland, represents 20% of the whey protein. It contains 123 amino acids, and differs with buffalo α-lactalbumin by only one substitution at position 17. Three genetic variants (A, B and C) have been identified. An X-ray structure of α-lactalbumin is given in Figure 4. Its globular structure is stabilized by four intramolecular disulfide bonds and has 63% similarity to bovine lysozyme (Farrell Jr. et al., 2004; Fox and McSweeney, 1998). α-lactalbumin is important in lactose synthesis. It forms lactose synthase complex by interacting with β-1, 4-galactosyltransferase which inhibits the binding of *N*-acetyl-glucosamine to β-1, 4-galactosyltransferase and allows the formation of lactose from glucose and UDP-galactose.

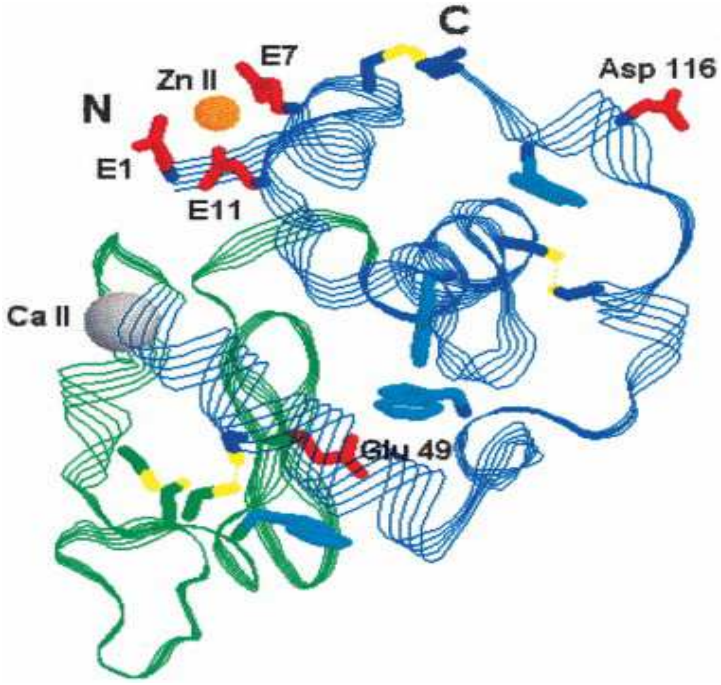



**Figure 4.** X-ray α-lactalbumin structure derived from native buffalo and recombinant bovine protein. α-domain is shown in blue while β-domain is shown in green. Trp residues are shown in blue and S–S bridges are shown in yellow. The residues which take part in coordination of $Zn^{2+}$ ions are shown in red (Permyakov & Berliner, 2000).

Whey proteins are considered as an important source of amino acids and peptides and 63% of the amino acid in the α-lactalbumin is essential for human nutrition. The peptides released after hydrolysis by digestive enzymes have ACE-inhibitory, opioid, immunomodulatory,

antimicrobial and antiviral effects (Furlund et al., 2012; Almaas et al., 2011; Mills et al., 2011; Pihlanto-Leppala, 2001; Pihlanto-Leppala et al., 2000; Meisel, 1998).

The major issue among the whey proteins is the cow milk allergenicity because of β-lactoglobulin. Human milk does not contain β-lactoglobulin and childrens with cow milk allergy synthesize antibodies predominantly against β-lactoglobulin (Barlowska et al., 2011; Lara-Villoslada et al., 2005). However, β-lactoglobulin is a lipocalin protein that can bind hydrophobic to amphiphilic molecules, e.g. palmitic acid, oleic acid, hexane, vitamin D, retinol, etc. The α-lactalbumin can bind Ca, Mg, Mn, Na, K and Zn in aspartic acid pocket (Permyakov and Berliner, 2000; Ren et al., 1993; Hiraoka et al., 1980) but at pH below 5.0 such binding cannot take place because of the protonation of aspartic acid.

#### 3.3.1.3 *Casein Micelle and coagulation of milk*

The majority of the milk protein is contained in colloidal structures/particles known as casein micelles. In normal milk, approximately 80% of the total milk protein (Dalgleish and Corredig, 2012) and 95% of caseins (Dalgleish, 1993) are present in that micelle. The aggregation of caseins to form micelles takes place in the cells of the mammary gland; in the secretory vesicles that pass between the Golgi apparatus and the apical membrane (Dalgleish, 2011). The major function of such aggregated micelles is to fluidize the casein molecules and solubilize calcium and phosphate (Farrell Jr et al., 2006). Citrate, minor ions, lipase, plasmin, and entrapped milk serums are also present in the casein micelles. Casein can aggregate and form micelles because of the amphiphilic nature of the casein peptide chains, high proline content and phosphate content. The phosphorylation of the caseins takes place at the hydroxyl group of the serine. Calcium binds to these phosphoserine residues that in turn bind the colloidal calcium phosphate. In the formation of micelles, these bonds contribute to linking the caseins, and κ-casein interacts with $\alpha_{s1}$-, $\alpha_{s2}$- and β-casein and stabilizes them, initiate the formation of micelles and a stable colloidal state.

Several theories have been proposed on the structural organization and stabilization of casein micelles in the milk and it has been a long debate. To predict better the internal structure of the

micelles and how the casein aggregates to form a stable structure, several models have been proposed but none has ever been satisfactory (Dalgleish, 2011). However, so far, it ends up with some generally established properties that are commonly accepted. The core of it is – the hydrophobic aggregation of the $\alpha_s$- and β-casein, calcium phosphate bridging and κ-casein to stabilize the surface. Dalgleish (2011) concluded that the calcium phosphate nanocluster model is more relevant from the electron microscopic and scattering experiments and proposed a modified nanocluster model including many of the known structural properties of the micelles (Figure 5). In brief, the noncovalent interactions of calcium phosphate/protein nanocluster between them and with other calcium sensitive-caseins cause their aggregation and formation of micelles. Among the calcium sensitive-caseins, β-casein is mainly found in the interior of the micelles and provides the internal stabilization to it. Because of the amphiphilic nature, β-casein can also bind to some of the hydrophobic parts of the calcium phosphate nanoclusters of $\alpha_{s1}$- and $\alpha_{s2}$-casein and as a surfactant, stabilizes the water channels inside the micelles. The calcium insensitive κ-casein acts as a mono-functional chain terminating agent, and because of lacks of phosphate centers (only one phosphoserine residue), it cannot participate in the nanocluster formation (Dalgleish and Corredig, 2012; Horne, 2009, 1998). The κ-casein via noncovalent interactions, associates with the aggregating proteins to form a surface layer and prevents the micelle aggregation. The glycosylated caseinomacropeptide (f106-169 of κ-casein) is hydrophilic, forms a hairy layer by extending from the micelle surface to the serum. The electrostatic repulsion of this hairy layer of the micelles prevents them to aggregate and flocculate and provides steric stabilization of the micelles (Figure 6) and keeps them in suspension. Destabilization can be achieved enzymatically (e.g. chymosin, removes the hairy part of the κ-casein) and chemically (e.g. acids, neutralize the negative charges and helps the drainage of calcium phosphate).

According to Abd El-Salam et al. (1978), the buffalo and cow milk casein micelles are assembled in the same way. However, the composition and size of the casein micelles are dependent on the species, breed, feeding, season, milk composition and the genetic variants (Ren et al., 2013; Glantz et al., 2010; Ahmad et al., 2009; Devold et al., 2000; Walsh et al., 1998; Abd El-Salam et al., 1978). From the particle size analyzer results, buffalo and cow milk showed normal distribution curve for their casein micelle size ranging from 30-400 nm and

similar average of about 180 nm (Ahmad et al., 2009; Ahmad et al., 2008). However, compared to cow's milk, buffalo milk has a greater percentage of larger casein micelles (Abd El-Salam et al., 1978; Ooman and Ganguli, 1973). The buffalo casein micelles also contain more $Ca^{++}$, inorganic P, $Mg^{++}$, and citric acid than the cow casein micelles (Abd El-Salam et al., 1978). Bovine casein micelles are more hydrated than the buffalo casein micelles and the dissociation of casein micelles take place at pH 8.6 and 9.7, respectively (Ahmad et al., 2009).

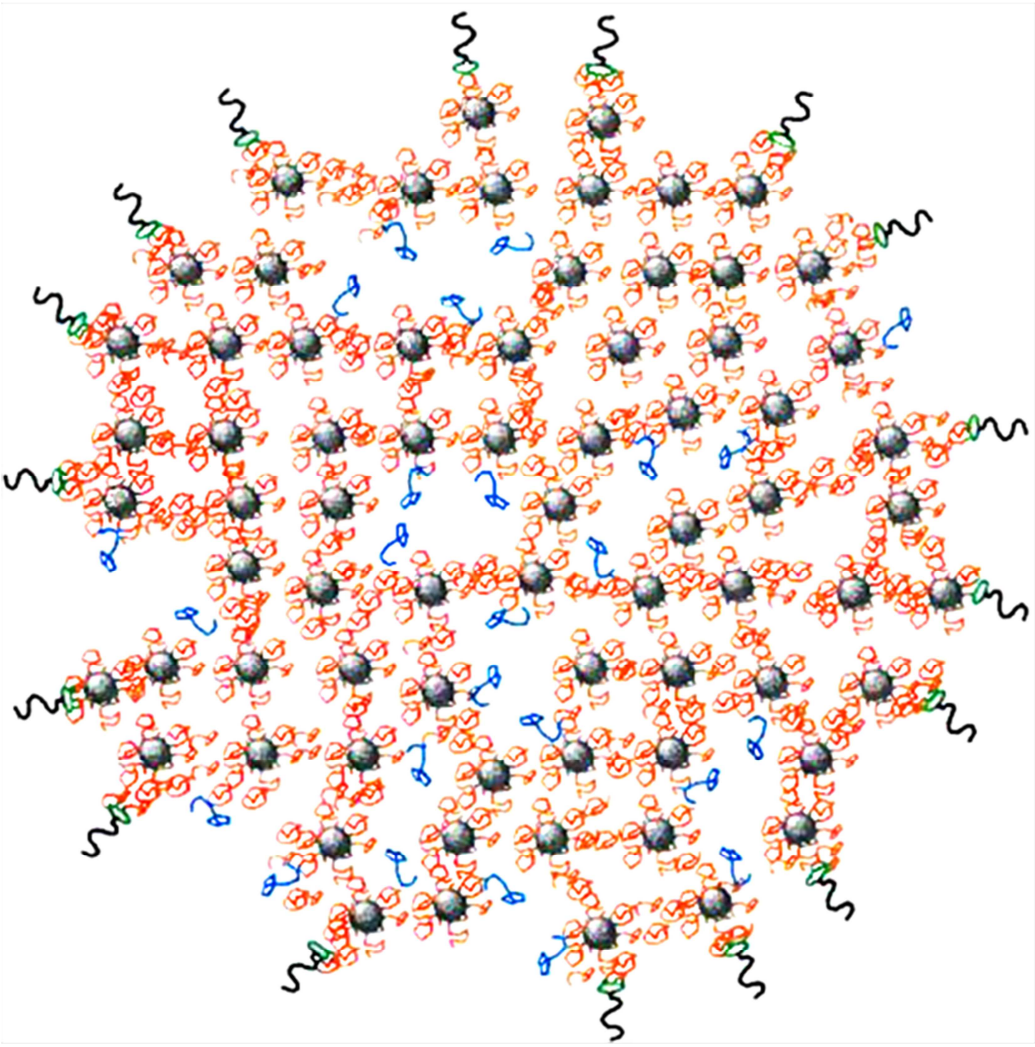


**Figure 5.** Illustration of casein micelle organization including water regions within the structure. The $\alpha_s$-casein and β-casein (orange) are attached to and link the calcium phosphate nanoclusters (grey spheres). Some β-casein (blue) hydrophobically binds to other caseins and can be removed by cooling. The para-κ-casein (green) and the caseinomacropeptide chains (black) are on the outermost parts of the surface. (Dalgleish & Corredig, 2012).

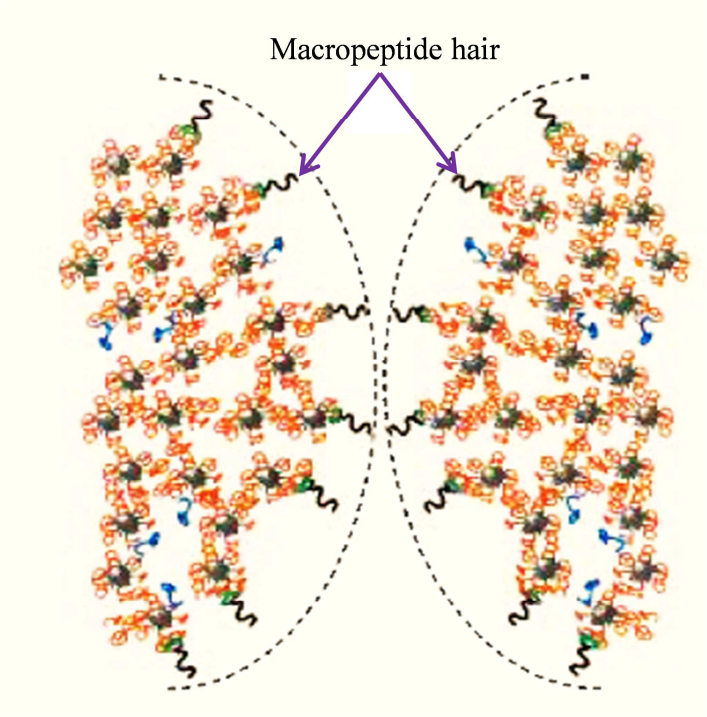



**Figure 6.** Native casein micelles in milk are sterically stabilized by macropeptide hairs. The zone of action of the steric effect is indicated by dashed lines (Dalgleish & Corredig, 2012).

At normal pH of milk, the negative charge and the hydrophilic character of C terminal end of κ-casein determines the ability of the casein micelles to stay in solution. Enzymatic action (rennet and microbial) and/or acidification can affect these two key factors, resulted in the casein aggregation and finally milk coagulation. In the enzymatic action, Chymosin (major and most active component of rennet) cleaves the $Phe_{105}$-$Met_{106}$ peptide bond of κ-casein, yielding para-κ-casein (f1-105) and caseinomacropeptide (f106-169). The hydrophilic caseinomacropeptide released into the whey and results in decreased steric stabilization and the loss of negatively charged group. The spontaneous secondary aggregation phase starts due to the enough reduction of micelles colloidal stability resulted from approximately 70% hydrolysis of the κ-casein. The decreased steric repulsion allows the micelles to approach each other to be close, the particles aggregate because of hydrophobic interaction and solidify further by the calcium cross linking and finally whey is drained out from the casein network by syneresis. The enzymatic destabilization is shown in Figure 7a.

During the acidification, with the reduction of pH (from 6.7 to 5.3), calcium phosphate, magnesium and citrates are released progressively from the micelles interior. The acidification of milk also causes the neutralization of the negative charges of the caseinomacropeptide, leads to the collapse of the κ-casein hairy layer (Figure 7b). This causes the decreased steric stabilization of the micelles, allows them to diffuse closer to each other and finally sol-gel transition occurs due to a short range attractive forces. When the pH reached 4.6 (Ip of caseins), aggregation occurs; however, the progress of the process is influenced by the physiochemical properties of the milk.

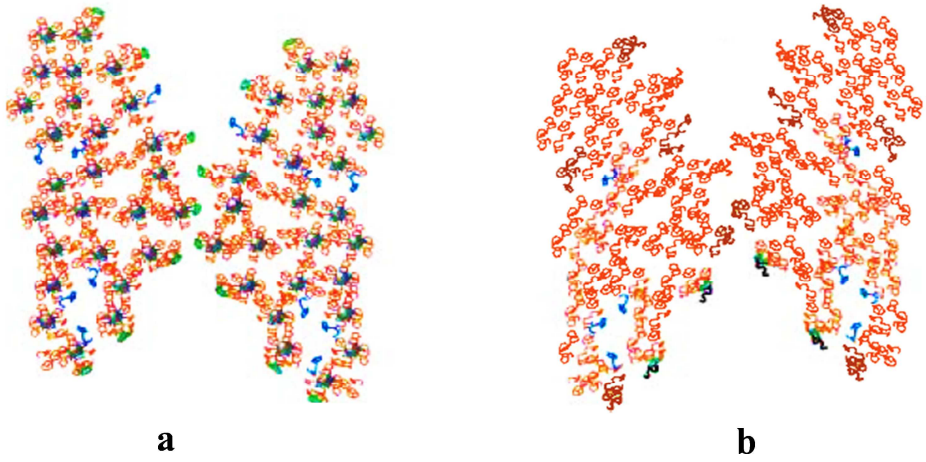



**Figure 7.** Green, para-κ-casein; orange, $\alpha_s$- and β-casein; blue, some β-casein hydrophobically binds to other caseins; grey spheres, calcium phosphate nanocluster. a) Renneted micelles where the hairs have been removed by chymosin, allowing close approach of the micellar surfaces. b) Micelles after acidification where the calcium phosphate has been dissolved and the hairs have been collapsed (Dalgleish & Corredig, 2012).

Generally the coagulation properties of milk is defined by – RCT/r (min): rennet coagulation time, when the coagulation starts after adding the rennet to the milk; $K_{20}$ (min): curd firming rate, how long does it take to reach the tail distance to 20 mm and $A_{30}$ (mm): gel strength/curd firmness, tail distance after 30 min. The coagulation properties of the milk is influenced by the milk composition and its physicochemical characteristics (Bonfatti et al., 2013; Pretto et al.,

2013; Bonfatti et al., 2012; Cecchinato et al., 2012; Glantz et al., 2010; Cassandro et al., 2008; Ariota et al., 2007; Napolano et al., 2007). Hence, the factors like breed, stage of lactation, parity, season, feeding and nutrition, somatic cell count and cow health that has impact on the milk components are also important in determining the coagulation properties of milk. Nevertheless, the most obvious important factors are pH, calcium content and temperature. A decreased coagulation time can be obtained by decreasing the pH and increasing the temperature. The increased level of bound calcium and/or $Ca^{++}$ favors the coagulation reaction.

Protein composition is also imperative in influencing the milk coagulation properties. Changes in the allelic frequency of the casein and whey protein genes and milk protein composition affect the milk coagulation properties (Bonfatti et al., 2010). In water buffalo, the A allele of $\alpha_{s1}$-casein is associated with decreased RCT, $K_{20}$ and firm curd compared to B allele. The κ-casein (variant $X_2$) also follows the same trend and the effect of B allele ($\alpha_{s1}$-casein) and $X_2$ allele (κ-casein) compensate each other. Moreover, the composite genotype of $\alpha_{s1}$-casein and κ-casein also has an influence on milk coagulation properties (Bonfatti et al., 2012). In another study, Bonfatti et al. (2013) concluded that the increased content of $\alpha_{s1}$-casein causes increased RCT and $K_{20}$ while it was reverse for β-casein, but κ-casein followed $\alpha_{s1}$-casein regarding the RCT. In cow's milk, poor or non-coagulation of milk is associated with low κ-casein concentration and its low proportion relative to total casein (Bonfatti et al., 2010; Wedholm et al., 2006). The composite genotype of β-casein and κ-casein both with at least one B allele showed best data on RCT and $A_{30}$ (Bonfatti et al., 2010; Comin et al., 2008; Ikonen et al., 1999).

Buffalo milk coagulates faster than the bovine milk. Dilution with an equal volume of water does not affect the buffalo milk rennet coagulation time but does increase the cow's milk coagulation time. The rennet coagulation time of cow's milk is less sensitive to the addition of NaCl, $H_2O_2$ and $Na_2CO_3$ and the effect of heat treatment is more pronounced than the buffalo milk. Differences in the colloidal phase of buffalo and cow milk may explain the differences between the coagulation properties of buffalo and cow milk (Abd El-Salam and El-Shibiny, 2011).

### 3.3.2 Milk lipids

Milk lipids contain 95-98% triglycerides, 0.3-1.6% diglycerides, 0.1-0.4% free fatty acids, 0.5-1.0% phospholipids and 0.2-0.5% sterols (Jensen et al., 1991; Belitz and Grotz, 1999; Walstra et al., 2006). The content of milk lipid is different in milk from different species (Table 2) and usually varies from 2-8%. It dispersed in the form of spherical droplets or globules in milk plasma. The globule is known as milk fat globule and the protective layer is known as milk fat globule membrane (MFGM) that mainly consists of phospholipids.

The triglycerides are composed of one molecule glycerol, esterified with three molecules of fatty acids. The distribution of fatty acids on the glycerol backbone is not random, however, report to report variation exists (Blasi et al., 2008; Mansson, 2008; Angers et al., 1998; Kaylegian and Lindsay, 1995; Parodi, 1975). There are reported 400-500 fatty acids present in the milk (Reklewska et al., 2002) but only 12 fatty acids are present in an amount more than 1% (Kaylegian and Lindsay, 1995). The major pathways involve with the fatty acids in milk are – directly from the diet, *de novo* synthesis in the mammary gland, biohydrogenation or microbial degradation in the rumen and release from the body reserve fat (Stoop et al., 2009). Among these, *de novo* synthesis contributes 40-50% of the fatty acids used for fat synthesis in the udder of most domestic animals (Sjaastad et al., 2003).

In general the bovine milk fat contains 70% saturated fatty acids, 25% monounsaturated fatty acids and 5% polyunsaturated fatty acids (Grummer, 1991; Lock and Shingfield, 2004). Among all the fatty acids, lauric (C12:0), myristic (C14:0) and palmitic (C16:0) acid constitutes 44% of the total milk fatty acids (Qureshi et al., 2010). For the better health effect, milk fat should contain 60% monounsaturated fatty acids, 30% saturated fatty acids and 10% polyunsaturated fatty acids (Pascal, 1996; Hayes and Khosla, 1992). There are a number of factors that could influence the fatty acid composition of the milk. e.g. species, breed, stage of lactation, mastitis, feeding and nutrition, body condition, season, supplementing fats and oils (Devle et al., 2012; Nogalski et al., 2012; Abd El-Salam and El-Shibiny, 2011; da Silva-Kazama et al., 2011; Gross et al., 2011; Falchero et al., 2010; Menard et al., 2010; Qureshi et al., 2010; Kathirvelan and

Tyagi, 2009; Talpur et al., 2008; Jensen, 2002; Randolph and Erwin, 1974). The fatty acid composition of cow and buffalo milk is given in Table 4.

**Table 4.** Cow and buffalo milk fatty acid composition (%).

| Fatty acid | Cow | Buffalo |
|---|---|---|
| C4:0 | 2.5±0.5 | 2.8±0.5 |
| C6:0 | 2.1±0.4 | 1.9±0.3 |
| C8:0 | 1.4±0.2 | 1.1±0.2 |
| C10:0 | 2.5±0.3 | 1.8±0.2 |
| C12:0 | 2.9±0.2 | 2.3±0.2 |
| C14:0 | 11.1±0.4 | 11.8±0.2 |
| C15:0 | 1.2±0.0 | 1.7±0.1 |
| C16:0 | 33.8±0.9 | 36.0±1.2 |
| C17:0 | 0.6±0.0 | 0.8±0.0 |
| C18:0 | 11.1±0.9 | 9.9±0.2 |
| C20:0 | 0.2±0.0 | 0.2±0.0 |
| C14:1n-5 | 1.1±0.2 | 0.7±0.0 |
| C15:1n-5 | 0.3±0.0 | 0.4±0.0 |
| C16:1n-7 | 1.6±0.0 | 1.9±0.0 |
| C17:1n-7t | 0.2±0.0 | 0.3±0.0 |
| C18:1n-9 | 22.1±1.7 | 20.3±0.7 |
| C18:2n-6 | 1.3±0.1 | 0.9±0.1 |
| C18:3n-3 | 0.6±0.0 | 0.7±0.2 |
| C18:1n-7t | 1.4±0.1 | 2.0±0.1 |
| C18:2 c9, t11; CLA | 0.7±0.0 | 0.9±0.0 |

Source: Menard et al. (2010).

As mentioned earlier that the triglyceride along with other lipid fractions, secreted in a globular form covered by a tri-layer (thickness 10-50 nm) biological membrane known as milk fat globule membrane (MFGM). The pathways involved in the origin, growth and secretion and the structural overview is depicted in Figure 8. Normally the milk fat globule size ranges from 0.2-

20 μm with an average of 4 μm. Buffalo milk fat globule size is larger than the cow's one and the buffalo milk showed more range of variability than that of cow (Menard et al., 2010). It is

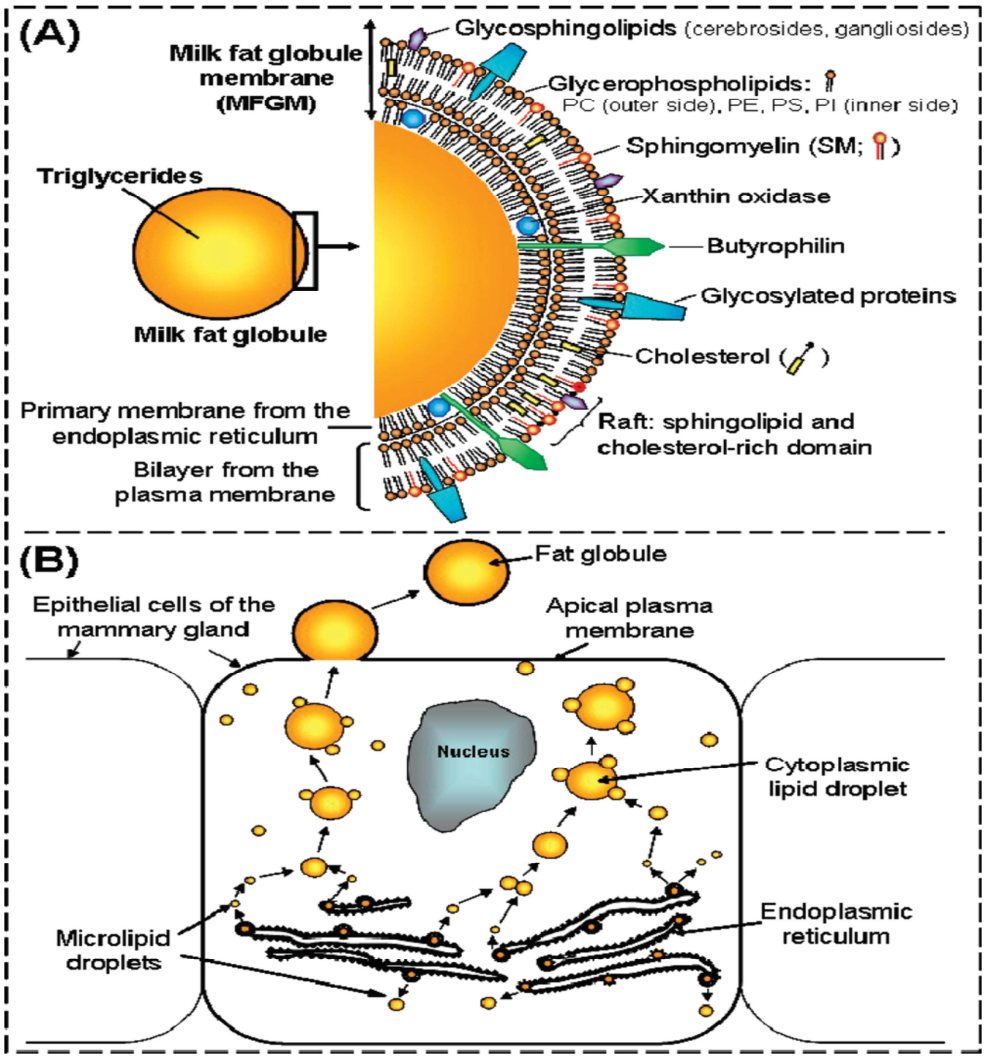



**Figure 8.** Schematic representation of (A) the structure of the milk fat globule membrane and (B) the pathways for the intracellular origin, growth, and secretion of milk fat globules (Lopez et al., 2008).

associated with the enhanced cell metabolism in the mammary gland of buffalo and its ability to produce milk fat globule (Schafberg et al., 2007). The MFGM is composed of a complex mixture of glycoproteins, triglycerides, glycerophospholipids, sphingolipids (mainly

sphingomyelin), glycolipids, cholesterol, enzymes, and other minor components (Keenan and Patton, 1995). Phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine are the main milk phospholipids and the main sphingolipid is sphingomyelin. The variation between cow and buffalo milk in the amount and composition of MFGM were reported by Menard et al. (2010) and Beri et al. (1984). The MFGM has importance in the digestion of lipids in human gastrointestinal tract (Gallier et al., 2012; Ye et al., 2011) and also has value in processing technology (Corredig et al., 2003) and in human nutrition (Spitsberg, 2005; Parodi, 1997).

A number of fatty acids of milk fat are reported to have specific function in human nutrition and health. Butyrate, the most abundant short chain fatty acid in milk is known to provide energy to the colonic epithelial cells and regulates a number of genes related with cell differentiation, proliferation and apoptosis (Hamer et al., 2008; Scheppach et al., 1992). The medium chain fatty acids (C8:0 - C12:0) may help to reduce the risk of developing features of metabolic syndrome (Pfeuffer and Schrezenmeir, 2007). In another study, Petrone et al. (1998) reported the bactericidal effect of lauric (C12:0), linoleic (C18:2n-6) and linolenic (C18:3n-3) acids and found reduced invasion of *L. monocytogenes* (food-borne pathogen) in a caco-2 enterocyte-like cell line. However, in addition to myristic and palmitic acid, lauric acid is also considered as hypercholesterolemic fatty acid (Williams, 2000) leading to cardiovascular disease. However, the presence of linolenic acid suppresses the negative effect of palmitic acid (Clandinin et al., 2000). Stearic (C18:0) acid is considered as neutral while linoleic, α-linolenic and oleic (C18:1n-9) acid are reported to be cardio-protective (Djousse et al., 2001; Bemelmans et al., 2002). Dairy products are the major dietary source (≈75%) of conjugated linoleic acid (CLA: C18:2 c9, t11). CLA and its isomers have anticarcinogenic, anti-lipogenic and anti-atherogenic effects, prevents heart diseases, improves immune function and bone health. However, most of the results were obtained in animal model research (Dilzer and Park, 2012; Bauman et al., 2006; McGuire and McGuire, 2000; Whigham et al., 2000; Williams, 2000). The long chain omega-3 polyunsaturated fatty acids, eicosapentaenoic acid (EPA: C20:5n-3) and docosahexaenoic acid (DHA: C22:6n-3) have beneficial cardiovascular and anti-inflammatory effects (Bauman et al., 2006; Williams, 2000), but the bovine and human milk contains a little of this two fatty acids.

### 3.4 Omega-3 enriched milk and milk products

The long chain omega-3 polyunsaturated fatty acids (PUFA) are considered as essential fatty acids for human because of their beneficial health effects. The omega-3 fatty acids have curative and preventive effects on cardiovascular diseases, participates in the infant's neurodevelopment, control cancers and fat glycemic, favorably affects atherosclerosis, inflammatory diseases and even the behavioral disorders and depression (Garg et al., 2006; Connor, 2000, 1997; Kinsella et al., 1990).

The omega-3 family mainly consists of α-linolenic acid (C18:3n-3), eicosapentaenoic acid (C20:5n-3) and docosahexaenoicacid (C22:6n-3). Among them, α-linolenic acid is present in plants and eicosapentaenoic and docosahexaenoic acid mainly comes from marine sources, primarily from fish and microalgae (Guil-Guerrero and Belarbi, 2001). In general, fish and fish oil provides the main dietary source of omega-3 polyunsaturated fatty acids in human nutrition (Kolanowski and Laufenberg, 2006). In particular, cod liver oil (CLO) provides an inexpensive source of omega-3 PUFA and has long been known as "nutraceuticals," widely consumed especially in Nordic countries (Mondello et al., 2006; Brustad et al., 2004; Guil-Guerrero and Belarbi, 2001). One dose (5 mL) CLO contains 1.2 g of omega-3 fatty acids and it also served as an excellent source of fat-soluble vitamins, vitamin A (500 μg), vitamin D (10 μg) and vitamin E (10 mg) (Rimestad et. al., 2001). The daily recommendations for eicosapentaenoic+docosahexaenoic acid intake ranges from 200 mg to 1000 mg or even 3000 mg, depending on the physiological condition (Garg et al., 2006; WHO, 2003).

Milk and dairy products are well known for their proteins with high biological value and the bioavailable calcium. But they are criticized for their high content of saturated fatty acids, especially lauric (C12:0), myristic (C14:0) and palmitic (C16:0) acid content and low content of PUFA. Now a day, some international dairy companies are producing "healthier" milk and some milk products by substituting the milk fat with PUFA. It exerts positive effect on health by reducing the intake of total saturated fatty acids and favorable effects from PUFA. Because of the omega-3 PUFA, the market of the fish oil enriched food products are expanding. Some of the milk and milk products that are enriched for their omega-3 PUFA content are – Omega-3

milk (Parmalat, USA), Dairy Farmers (Farmers best) milk (Australia), Lactel omega-3 milk (Lactalis Besnier Bridel, France), Omi-3 processed quark (OSM Ostrolka, Poland), Lauki omega + skim milk (Candia, Spain), Brownes (Heart Plus) milk (Australia), Especial omega-3 milk (Mimosa, Portugal), Plus omega-3-latte and omega-3 yoghurt (Parmalat, Italy) and omi-3 yoghurt (SM Siedlce, Poland) (Kolanowski and Laufenberg, 2006; Garg et al., 2006).

### 3.5 Lipid analysis

There are three steps involved in analyzing the fatty acids from biological or food samples. The steps are – extraction of total lipids, conversion of extracted lipids into volatile derivatives like fatty acid methyl esters (FAMEs) and gas chromatography of the FAMEs for the identification/quantification (Ratnayake and Galli, 2009). Depending on the objective (s), the extracted lipid may be fractionated into different lipid classes by employing appropriate methods corresponded to the lipid class.

The choice of the method to be used for the extraction of lipid depends on the sample matrix. A variety of polar and non-polar lipids together with proteins and polysaccharides are present in animal tissue. Hence, a common approach is to use a mixture of polar and non-polar solvents to obtain the quantitative recovery of the complex lipid mixture. A chloroform-methanol based extraction method was published by Folch et al. in 1957 and a modified method by Bligh and Dyer in 1959. The use of polar solvent (preferably an alcohol) in combination with a non-polar solvent is advantageous. According to Rombaut and Dewettinck (2006) use of such solvent is important for the complete release of polar lipids from a complex matrix like milk fat globule membrane. This solvent system degrades the hydrogen bonds within milk lipid-protein complexes and it also denatures and dehydrates the milk proteins. Milk lipids can also be extracted by hexane-isopropanol (Hara and Radin, 1978); and by diethyl-ether and pentane system after treating the milk with ammonium hydroxide (ISO-IDF, 2001). Microwave extraction of lipids is another technique (Pare et al., 1996; Ganzler et al., 1986). Non-solvent extraction methods are also available, using the centrifugal force (Luna et al., 2005; Feng et al., 2004).

Lipids can be separated in different classes by using liquid-liquid or liquid-solid extractions or by thin layer chromatography (Ruiz-Gutierrez and Perez-Camino, 2000). In liquid-liquid system, chloroform, diethyl ether or ethyl acetate at various proportions is used. Thin layer chromatography is another widely used method. But they are time consuming, requires large volume of solvents and loss of some lipidic fraction is evident (Striby et al., 1999; Ruiz-Gutierrez and Perez-Camino, 2000). Liquid-solid extraction for lipid fractionation has been extensively developed and widely used. In the solid phase extraction, the lipids become entrapped by the solid phase and solvents pass away. Changing the solvent environment (pH, polarity, etc.) around the solid phase or by introducing delicate changes in the solid phase, lipid classes can be separated selectively with high purity and good recovery (Kaluzny et al., 1985). Different solvents and column materials were also used to improve the solid phase extraction of lipids (Laffargue et al., 2007; Pernet et al., 2006; Ruiz et al., 2004).

Fatty acids need to be derivatized to overcome the challenges set by their polarity and limited volatility. It helps to avoid complication during gas chromatography analysis and improves the peak shape. Depending on the fatty acids and chromatographic technique, methyl, isopropyl or butyl esters can be used as derivatives to have a good selective and accurate analysis. However, methyl esters are most widely used for biological tissues and food samples as they require less temperature to change their volatility (Carrapiso and Garcia, 2000). In general, derivatization method should be simple, fast, quantitative, causes no unwanted changes or side chain reactions. There are two main reactions involved in derivitization – esterification and transesterification. Esterification reaction takes place in free fatty acids (in acidic media) and so for triglycerides, phospholipids (in acidic or basic media) or N-acyl complex lipids (in acidic media) is transesterification. In biological samples, fatty acids are mostly included in triglycerides and phospholipids. Hence, the derivatization to form FAMEs is commonly termed as transesterification. For the synthesis of FAMEs, HCl or $H_2SO_4$ or $BF_3$ in $CH_3OH$ or sodium methoxide is used.

After the formation of FAMEs, samples are applied to instrumental analysis for the identification and/or quantification of the fatty acids. For the analysis of complex fatty acid mixtures, gas chromatography (GC) coupled with Flame Ionization Detector (FID) is the most

widely used traditional method (Carrapiso and Garcia, 2000; Ratnayake and Galli, 2009). In FID (Figure 9), the sample gas is combusted by the hydrogen/air flame; organic molecules oxidized and produce electrically charged particles (ions). On a high voltage ion collector, the ions are detected by measuring the current across the collector that is proportional to the rate of ionization. The rate of ionization depends on the concentration of the hydrocarbons in the sample gas. The detection gives peak/chromatogram which by comparing with the retention time of a known standard, the FAMEs are identified. Relative concentration can be measured by using the area under the peaks taking the calibration factor in consideration and inclusion of internal standard makes possible the absolute quantification. However, compared to a typical mass spectrometer (MS), FID's sensitivity and selectivity are significantly low (Thurnhofer and Vetter, 2005). For example, the co-elution of different compound is very common in GC-FID that can be solved using MS instead of FID. Moreover, various fatty acids can be differentiated by the information on molecular mass or other structural characteristics that MS provides. The MS also provides excellent quantitative data for the lipidomics study (Ratnayake and Galli, 2009). Therefore, GC-MS has got diverse application in biological and industrial research (Quehenberger et al., 2011; Thurnhofer and Vetter, 2005).

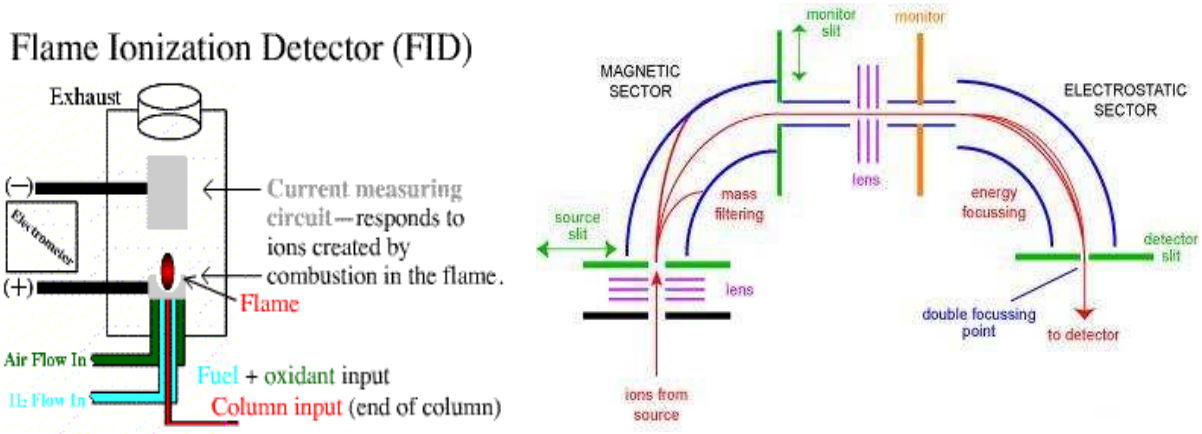



**Figure 9.** Flame ionization detector (left) and magnetic sector mass spectrometer (right) used with gas chromatography (Chasteen, 2009; Gates, 2005).

In an MS – various molecules are ionized, ions are separated according to their mass and masses of each ion are detected within a predefined range. Use of a magnetic field is one of the earliest ideas in MS to separate ions and determining their mass. The working flow of magnetic sector MS is given in Figure 9. In the magnet sector, lower mass ions deflected more than the higher mass ions. That makes possible the focusing of ions with different masses on to the monitor slit, results in the separation of ions according to their masses. To obtain a good spectral resolution (i.e. all ions with same *m/z* in one peak), ions are filtered through an electrostatic sector and then focused at the double focusing point on the detector slit.

There are several acquisition modes that can be used in GC-MS in addition to a number of chromatographic methods and ionization techniques. Within a predefined mass range, complete mass spectra can be obtained in full scan mode. During each scan cycle, all the ions reaching the detector are counted. By choosing the compound's characteristic fragments, known compounds can be detected in selected ion modes (SIM) but the analyzer need to switch quickly from one mass to another. Thus, the sensitivity can be improved and limits of detection (LOD) and potential matrix interference can be reduced. In reconstructed ion chromatogram (RIC), only the desirable masses are included in the quantification though it allows to record complete mass spectra, others being excluded. Full scan and RIC is advantageous over SIM to identify unknown compounds because they provide more information than SIM does. For the confirmation, ion ratios of the mass fragments should be compared with those from a known reference standard.

### 3.6 Protein and peptide identification

Protein identification is a four step process composed of protein separation, protein digestion, MS analysis of peptides and comparison of observed peptides with the database (O'Donnell et al., 2004). Separation can be done either by gel electrophoresis or liquid chromatography (LC) depending on the focus of interest. Tryptic digested peptides of the protein are used most widely for the protein identification purpose. The obvious choice is MS, for the analysis of resulted (separated) peptides to identify the proteins because of their higher sensitivity, sequencing speed and resolution. At present, the major MS separation principles in proteomics

are – quadrupole mass filters, time of flight (TOF) mass analyzers, linear ion traps and orbitrap analyzers (Michalski et al., 2011). Generally these are combined to make hybrid configurations.

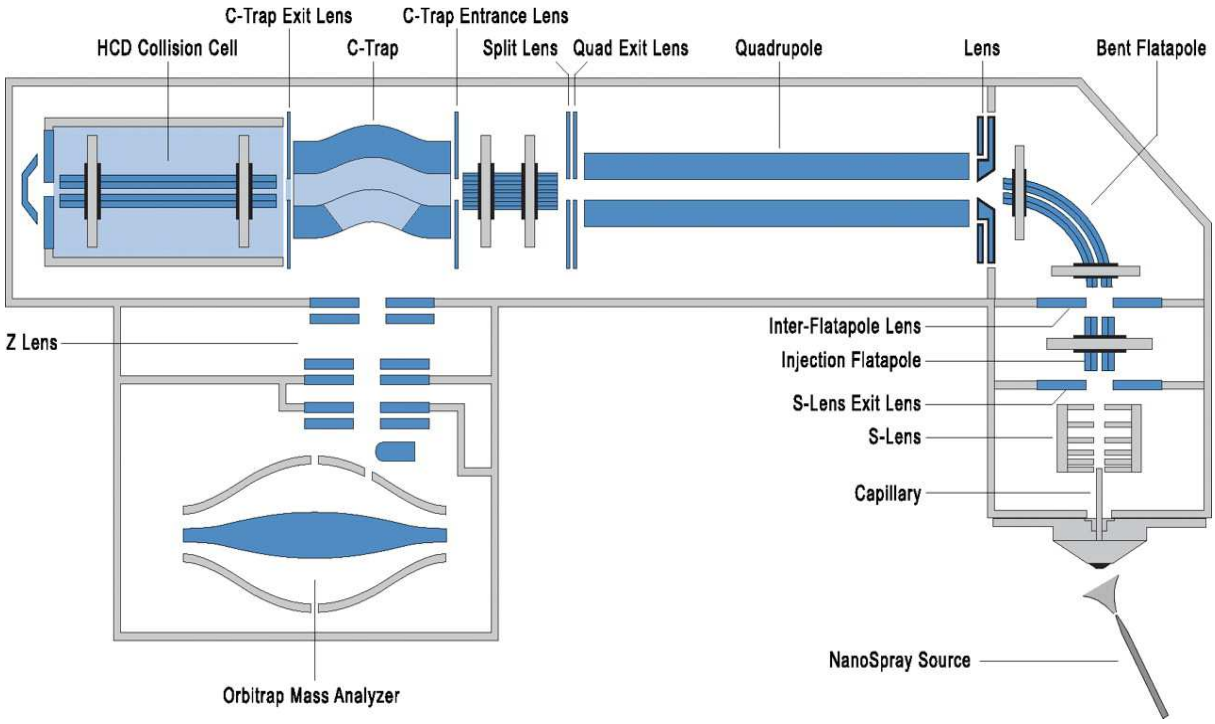



**Figure 10.** Schematic diagram of Q-exactive mass spectrometer construction (Michalski et al., 2011).

In Q-exactive MS, a quadrupole mass filter combines with an orbitrap mass analyzer (Figure 10). Because of the S-lens and parallel filling and detection modes, the Q-exactive instrument features high ion currents and fast high-energy collision-induced dissociation of peptide fragments. It has improved resolution and multiplexed operations are possible at MS and MS/MS level. Often simply the peptide mass fingerprint (PMF) is used to identify protein. However, a high number of peptides from multiple proteins obscure PMF based identification. To solve this issue, MS/MS needed which provides the sequence information and can detect modification in any individual residue.

One of the applications of MS into the food research is to study the protein hydrolysate, i.e. peptides (Panchaud et al., 2012; Alomirah et al., 2000; Leonil et al., 2000). Both the matrix-

assisted laser desorption ionization (MALDI) MS and electrospray ionization (ESI) MS can be used in peptide study (Mamone et al., 2009; Careri and Mangia, 2003). However, the ESI-MS is more desirable with online liquid chromatography detection system because it can analyze compounds directly from aqueous/organic solutions (Careri and Mangia, 2003).

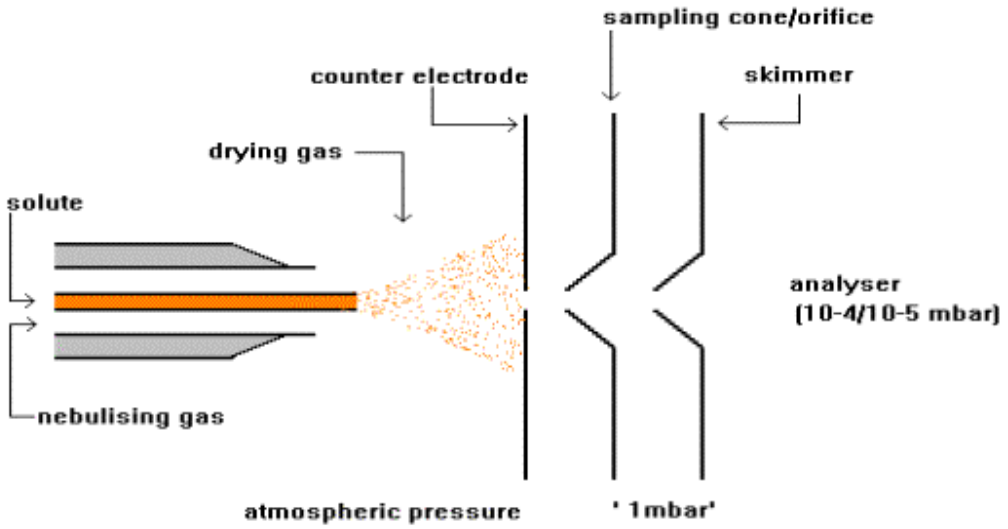



**Figure 11.** The electrospray ionization source (Ashcroft, 1997).

The ESI is the atmospheric pressure ionization (API) technique, which is an ion evaporation process that enables emission of ions from droplet into the gas phase. At the tip of the sample emitting capillary, a high voltage (3-4 kV) is applied. Nitrogen is used as a nebulizing gas, flows around the capillary. It helps to carry the sample spray towards the MS. Then passing through the warm drying gas (nitrogen), it passes the sampling cone or orifice and finally the solvent free charged sample ions are directed into the analyzer (Figure 11) (Ashcroft, 1997).

Like Q-exactive MS, Q-TOF MS is also a hybrid MS combining quadrupole mass filters and time of flight mass analyzer. There is a collision cell in between, with injected inert gas (argon/helium/xenon) for the fragmentation of samples into ions of certain masses (Ashcroft, 1997). In MS, quadrupole does not work as an analyzer but focus the ion beam into the TOF, where the ions separated as per their mass to charge ratio. In tandem MS (MS/MS), in addition

to TOF, quadrupole acts as a second analyzer. Quadrupole transmits only the ions of interest into the collision cell, where ions are bombarded into the fragments and are analyzed by the TOF (Figure 12).

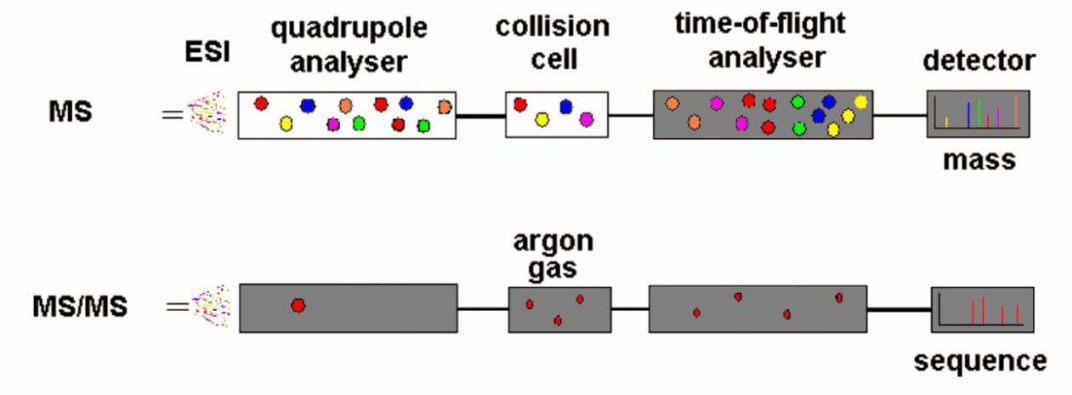



**Figure 12.** Schematic presentation of the working principles of Q-TOF in MS and MS/MS mode (source: http://www.astbury.leeds.ac.uk/facil/MStut/mstutorial.htm).

### 3.7 Human digestion

The digestive system involves food processing and degradation, extraction of nutrients from it, and elimination of the residual part. Five dynamic stages take place: i) ingestion, selective intake of food; ii) digestion, converts the food into a usable form for the body; iii) absorption, uptake of nutrients through the epithelial cells into the blood or lymph; iv) compaction, water absorption and accumulation of indigestible residue into feces; and finally v) defecation, feces elimination (Saladin, 2010). The digestion of food includes mechanical digestion and chemical digestion. In mechanical digestion, cutting and grinding action in the oral cavity and the churning contractions of the stomach and small intestine breaks the food into smaller particles to provide higher surface area for the digestive enzyme activity. In chemical digestion, a series of hydrolytic reactions takes place to break the dietary macromolecules into their monomers: proteins into amino acids, fats into monoglycerides and fatty acids, polysaccharides into monosaccharides and nucleic acids into nucleotides. An overview of the digestive system is shown in Figure 13.

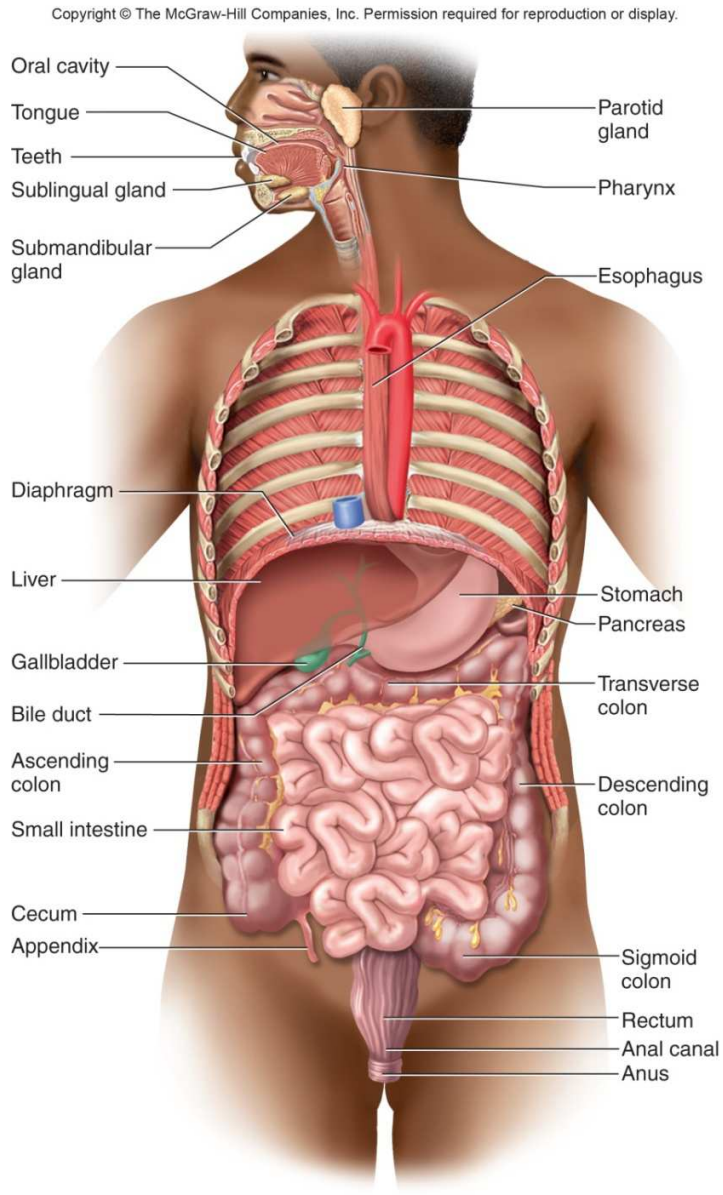



**Figure 13.** The human digestive system – an overview (Saladin, 2010).

In the oral cavity, food is transformed into bolus (saliva lubricated agglomeration of food particles), and then food is transferred to the stomach through the oesophagus by the peristaltic movement, where food stays for a certain time and further to the upper part of the duodenum through the pylorus. Then absorption of nutrients and the expulsion of the residual part take place. The whole digestion process takes ca. 6-12 hours depending upon the food matrix as liquid or solid food.

The digestion and absorption of lipids are more complicated than the protein and carbohydrate mainly because of its hydrophobic character. Lipases of different origin digest lipids (Saladin, 2010). The dietary lipids mainly consist of triacylglycerol (Mu and Porsgaard, 2005). It must to be broken down into smaller pieces (fatty acids, monoglyceride, and glycerol) to enables the absorption by the gut wall cell lining, the enterocytes. The digestion is very efficient and allows the small intestine to absorb approximately 95-98% of the ingested triglycerides (Armand, 2007; Carey et al., 1983). Lipid emulsification is important during the digestion. Emulsification affects the lipid/water interface area directly, thus affect the lipase binding onto the surface of the lipid droplet. The available surface area of the lipid droplet to the lipase is directly proportionally influence the rate of lipolysis (Bauer et al., 2005). Gastrointestinal lipid digestion takes place mainly in two steps – gastric and duodenal. However, Lipid digestion (a minor extent) may starts from the mouth by the lingual lipase from the tongue's intrinsic salivary glands. Lingual lipase showed even more activity in the acidic pH of the stomach and exerts lipolytic effect along with the gastric lipase (Saladin, 2010). However, the existence of lingual lipase has been questioned. Moreau et al. (1988) established the cellular and tissue origin of the preduodenal lipases in human and concluded the absence of lingual lipase in the human mouth. Fernandez et al. (2007) also defy the presence of lingual lipase in human's preduodenal lipase.

### 3.7.1 Gastric digestion of proteins and lipids

*Protein digestion*: As soon as the food arrives into the stomach, it causes stimulation of gastric secretion by inducing stomach stretching and natural pH (1.5 to 2) of the stomach contents increase depending on the buffering capacity of the entered food (Kaye, 2011). The chief cells of the gastric gland secrets pepsinogen, the inactive pepsin. The HCL secreted from the parietal cells, remove an N-terminal pro-peptide from the pepsinogen, exposed the active site cleft and pepsin is activated. When pepsin has been formed, it exerts autocatalytic effect by activating its zymogen (Figure 14). It has broad specificity and preferentially attacks the protein at sites with hydrophobic and aromatic residues (phenylalanine, tyrosine, leucine, valine or methionine), either in combination or at close proximity of the cleaved bond (Roberts and Taylor, 1979). Pepsin breaks the proteins into polypeptides (Figure 14 and 15) and is supposed to hydrolyze 10-15% of the dietary proteins (Saladin, 2010). The acidic environment of the stomach helps to

denature some of the proteins and thereby facilitate the protein digestion. Mucus, secreted from the mucous neck cells of the gastric glands provides the protection to the stomach against the acid and digestive enzymes. The rich content of bicarbonate and surface-active phospholipids in the mucus are the key player. The mucus prevents the acid attack (Campbell, 2012). The pepsin activity is highly pH dependent and up to pH 4.0, human gastric juice is highly effective for proteolysis (Roberts, 2006).

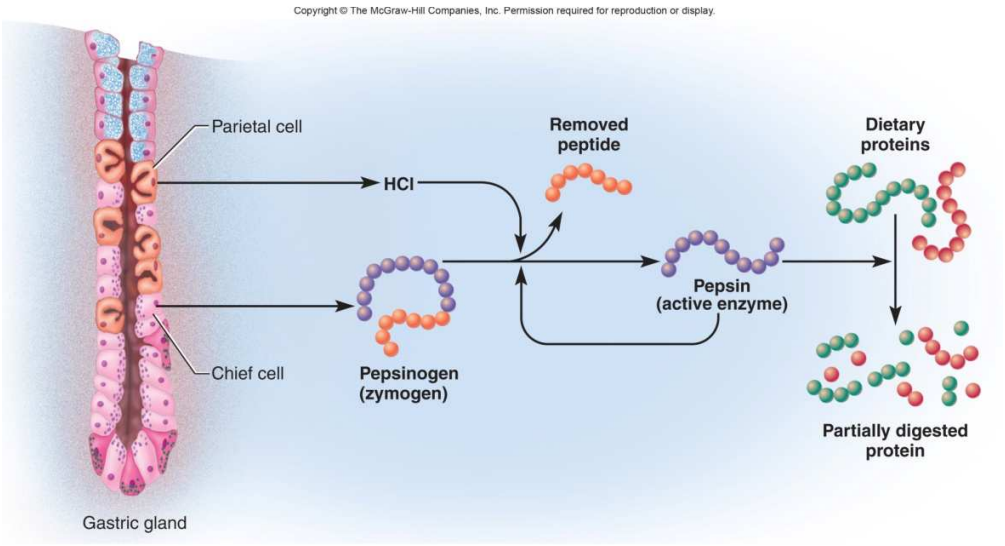



**Figure 14.** Schematic presentation of production, activation and action of pepsin (Saladin, 2010).

*Lipid digestion*: The digestion of the triglycerides starts in the stomach by the action of gastric lipase. Chief cells in the fundic region of the stomach secrets the gastric lipase. A pepsinogen secretion stimulator, pentagastrin also stimulates the gastric lipase secretion. It is an acid lipase, acid-stable (pH 3-6) and active at acidic pH but the optimum pH is 5-6 (Armand, 2007; Carriere et al., 1993; Gargouri et al., 1986a; Gargouri et al., 1986b). Gastric lipase can hydrolyze all three ester bonds on the triglyceride (Carriere et al., 1991) but has more preference to *sn-3* (Carriere et al., 1994; Carriere et al., 1993). The structure of the lipid substrate also has an influence on gastric lipase preference which was triglyceride followed by di- and monoglyceride (Fernandez et al., 2008; Fernandez et al., 2007). It also has a preference to attack the short and medium chain fatty acid at *sn-3* (Bauer et al., 2005). It can also hydrolyze the long chain fatty acids and its specificity is dependent on the pH of the incubation medium

and presence of amphiphilic components in it (Gargouri et al., 1989). The lipolytic action of gastric lipase mainly yields one free fatty acid and one diglyceride (Hayes et al., 1994; Carriere et al., 1993; Carey et al., 1983; Patton et al., 1982). Approximately 10-40% of the dietary lipids undergo lipolysis in the stomach but the exact contribution of the preduodenal lipase to the overall lipolysis and their site of action are an issue of ongoing debate (Goodman, 2010; Mu and Hoy, 2004; Armand et al., 1999; Carriere et al., 1993).

### 3.7.2 Intestinal digestion of proteins and lipids

*Protein digestion*: Periodic contractions occur to deliver the gastric residue into the small intestine (upper duodenum part). The rate of peristaltic contractions of the distal part of the stomach is 3 per min. The passage of the larger particles is slower than the smaller ones and so for solids than the liquids. Stomach emptying slows down if the lipids are present in the chyme (Campbell, 2012). The pepsin is inactivated due to mixing with alkaline pancreatic juice with pH 8 and bicarbonate-rich mucus from the duodenal gland neutralizes the stomach acid (Saladin, 2010). Duodenal digestion normally takes place at pH 6.2-6.7 (Kalantzi et al., 2006; Ekmekcioglu, 2002), however, pH 7-8 are also recorded in both *in vivo* and *in vitro* studies (Russel et al., 1993; McCloy et al., 1984). Duodenal juice is composed of duodenal epithelial cells, bile and pancreatic juices. Enzymes functioning in the small intestinal digestion are mostly found in the pancreatic juice and brush border. All the pancreatic proteases are secreted as zymogens to avoid pancreatitis. Enteropeptidase residing on the duodenal brush border cleaved the trypsinogen to form the active enzyme trypsin. The trypsin activates other zymogens, e.g. chymotrypsinogen to chymotrypsin and procarboxypeptidase to carboxypeptidase. Trypsin and chymotrypsin (pancreatic enzymes) hydrolyze the proteins and polypeptides. The entered (from stomach) hydrolysis product consists of 30% free amino acids and 70% oligopeptides (Goodman, 2010) but it is dependent on the type of food. Trypsin has preference to cleaves at the –COOH side of lysine or arginine whereas chymotrypsin cleaves at the –COOH side of tyrosine, tryptophan, phenylalanine and leucine.

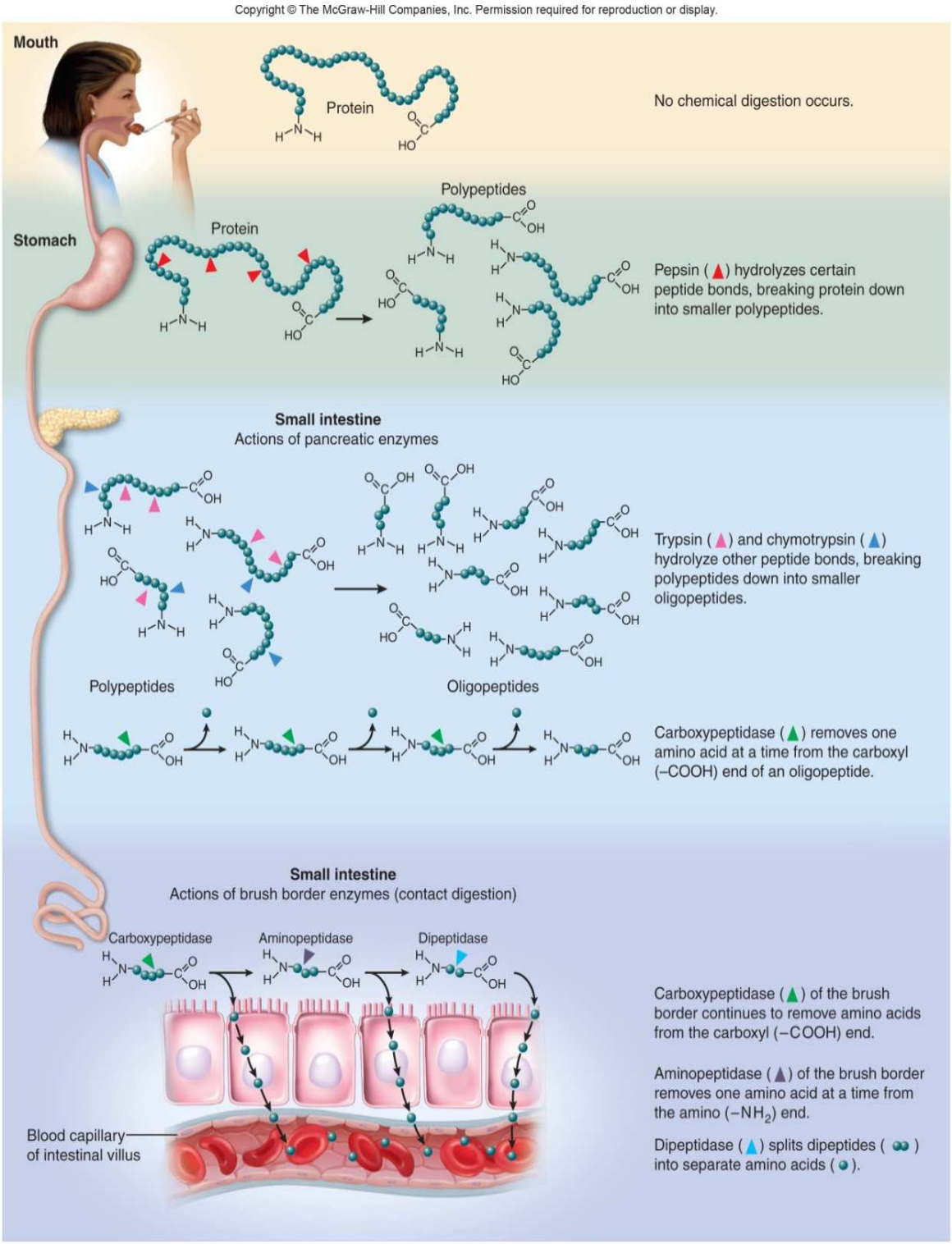



**Figure 15.** Illustration of protein digestion (gastric and intestinal) and absorption in human (Saladin, 2010).

Another pancreatic enzyme, carboxypeptidase, acts on oligopeptides and removes amino acids from the –COOH end of the peptide chain. Two brush border enzymes, aminopeptidase and dipeptidase are involved in the removal of amino acids from the $–NH_2$ end of the chain and splitting dipeptides in middle to yield the last two amino acids, respectively (Figure 15). The absorption of amino acids and small peptides takes place across the enterocytes brush-border membrane and further released into the bloodstream.

*Lipid digestion*: The complete overview of lipid digestion and absorption is shown in Figure 16. Through the action of the pancreatic lipase, most of the fat digestion takes place in the small intestine. The bile (produced by liver, stored and concentrated in the gallbladder) components – lecithin and bile acids emulsify the larger fat globules into a smaller one that provides more surface area for the lipase activity. Pancreatic lipase removes the fatty acid from *sn*-1 and *sn-3* of the triglyceride, thus producing two free fatty acids and one monoglyceride. However, this is also dependent on the fatty acid type. The absorption takes place through the incorporation into the micelles and chylomicron formation (Saladin, 2010). The released short chain and medium chain fatty acids are soluble in aqueous media. This will help to solubilize the lipid droplets efficiently into the mixed micelles and will increase the lipid droplets emulsification. But as the long chain fatty acids are more hydrophobic, they will cause somewhat inhibition of the lipase action by accumulating at oil-water interphase (McClements et al., 2008). For the optimum activity of the pancreatic lipase, in addition to bile, colipase is important. It binds the lipase and anchors to the substrate.

### 3.8 *In vitro, ex vivo* and *in vivo* digestion models

The most widely used digestion model is the *in vitro* digestion models. The purpose is to study the digestibility, structural changes, release of components, production of bioactive components and survival of drugs under conditions mimicking the *in vivo* gastrointestinal tract (Hur et al., 2011; Escudero et al., 2010; Jantratid et al., 2008; Korhonen and Pihlanto, 2006; Dressman et al., 1998). However, the results obtained from simulated *in vitro* digestion model are often different from those obtained using *in vivo* models because of the inherent complexity of the process results in difficulties in accurate simulation (Hur et al., 2011). The best approach to

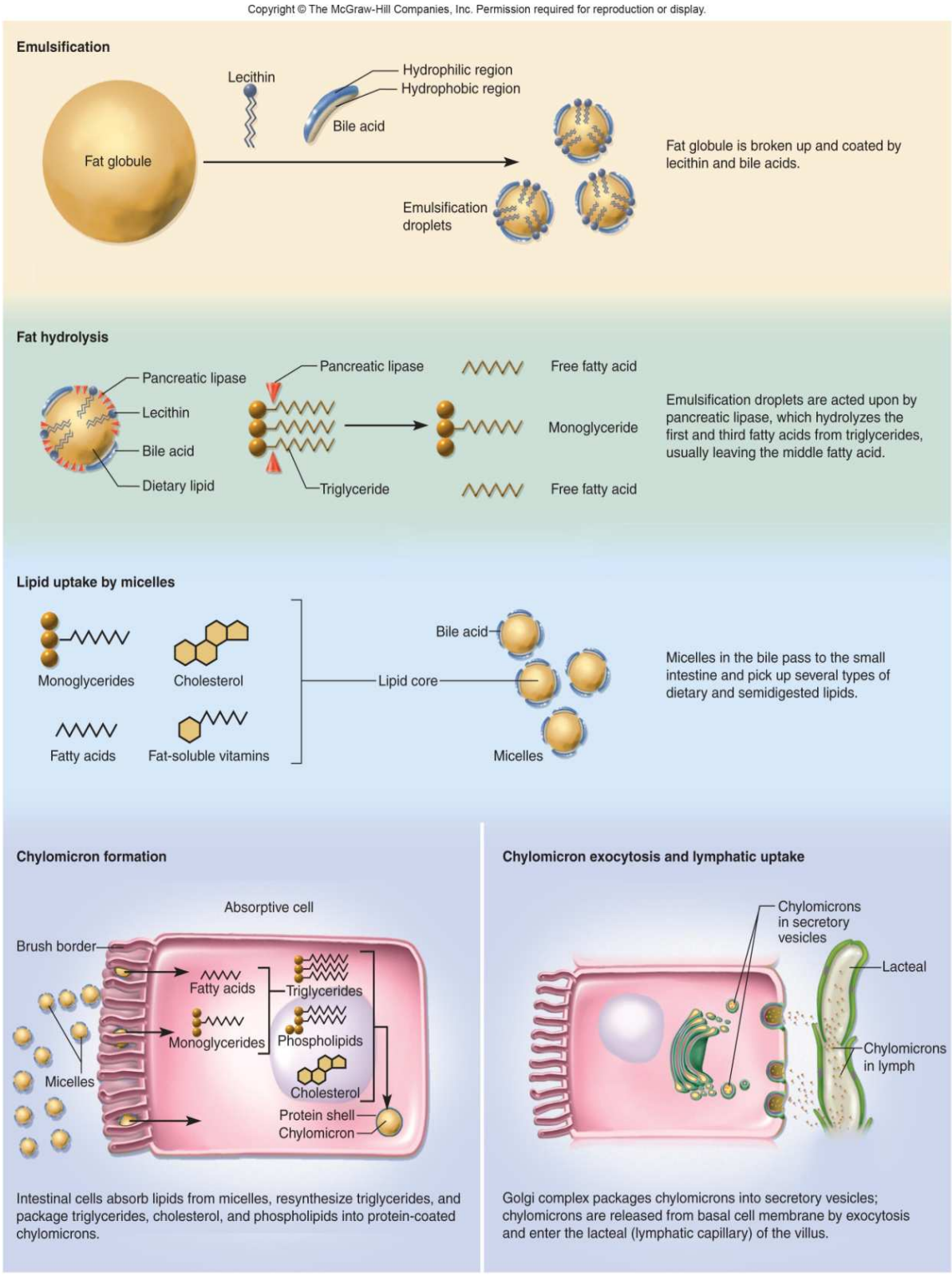



**Figure 16.** Illustration of lipid digestion and absorption in humans (Saladin, 2010).

monitor such phenomena is to use *in vivo* digestion. However, *in vivo* study has some limitations from practical application point of view involving mainly ethical, technical, time and cost related issues. In addition, a large number of subjects are needed to obtain standardized and comparable results due to the higher extent of individual variation. Considering these issues, compromise has made between accuracy and ease of utilization, and *in vitro* model is used most extensively.

There are two types of *in vitro* digestion model available – static and dynamic models. Static model is a closed system and less sophisticated. The movement of the gastrointestinal tract is simulated by using magnetic stirrer or shaker. The efficiency of such model is commonly measured by the loss of or release of macromolecule hydrolysis products. In a dynamic model, the simulation of the gastrointestinal tract is computer controlled, mechanical forces being simulated by churning and movement. Digestion products are removed after each step by dialysis and followed by analysis.

To date, in all the developed models, simulation for feedback mechanisms, resident microbiota, immune responses and hormonal control are absent. The most widely used model is static model (Guerra et al., 2012). The models are designed to simulate only one/two/four stages of the *in vivo* digestion process (Hur et al., 2011; Boison and Eggum, 1991). During the simulation, a number of variables including pH, transit time, and enzymatic conditions must be considered to make the model useful (Guerra et al., 2012). Enzyme activity is the most important factors which depend on the concentration, pH, temperature, stability, activators, inhibitors and incubation time (Boisen and Eggum, 1991). Coles et al. (2005) suggest to use single purified enzyme for *in vitro* digestion. Now a day, most of the model digestion study is conducted using the highly pure commercial non-human enzymes, like porcine or bovine. In protein digestion, commercial enzymes seem more efficient than the human gastrointestinal enzymes (Eriksen et al., 2010; Almaas et al., 2006b) and according to these studies commercial enzymes give different peptide profile. The proteolytic enzymes with their various isoforms are present in the human digestive juice (Jones et al., 1993) and these isoforms could be different from those present in other species. Depending on the pH and substrate in digestion, this could have different catalytic pattern (Fujinaga et al., 1995). Other components (e.g. bile,

phospholipids, enzyme inhibitor etc.) that are normally present in the digestive juices may also play a role in digestion and contribution of such components is neglected when purified enzymes are used. Use of human digestive juice aspirates is the best known alternative to address this issue and from this perspective, Devle et al. (2014) used the term *ex vivo* instead of *in vitro*.

### 3.9 Model digestion of milk and milk products and milk allergens

Milk and milk products have been digested in model digestion, however, wide variability prevails regarding the model used, digestion simulation, milk components interest, source of enzyme etc. *In vivo* digestion of milk and yogurt in adult human was studied to monitor the release of peptides from caseins and lactoferrin in stomach and duodenum and their absorption into the blood (Chabance et al., 1998). Whereas, Furlund et al. (2013) digested bovine lactoferrin both *in vivo* and *in vitro* involving gastric and duodenal digestion steps. The effect of fast and slow gastric reduction in pH on lactoferrin digestion was studied. Inglingstad et al. (2010) compared the *ex vivo* digestion of caseins and whey proteins of milk from different species, while Eriksen et al. (2010) focused on the caprine whey proteins and used both human and non-human enzymes. Gallier et al. (2013), Gallier et al. (2012) and Ye et al. (2011) studied the milk fat and fat globule membrane and structure during digestion, but Almaas et al. (2006a) focused only on milk proteins digestion. Devle et al. (2014) used human enzymes, while Kopf-Bolanz et al. (2012) used non-human enzyme on cow milk proteins and lipids. Because of different digestion protocols and models used, the results are difficult to compare. However, static *in vitro* models have opportunities with simulated or human gastric and duodenal fluids to achieve the digestion.

The milk protein breakdown in the gastrointestinal tract is of considerable interest because of their allergenic properties. In a general population, 1-3% people suffer from cow's milk allergy and children are more prone to it than the adults. Geographical location and ethnicity are the two key determinants of its prevalence in a population (Bahna, 2002). The α-, β-, and κ-casein, α-lactalbumin and β-lactoglobulin have been identified as major cow milk allergens (Downs et al., 2013; Chatterton et al., 2006; Kitabatake and Kinekawa, 1998; Adams et al., 1991).

In the milk of most mammals except human, β-lactoglobulin is present. Most of the patients (13-76%) were found to react on β-lactoglobulin (Caira et al., 2012). The major IgE epitopes in β-lactoglobulin are f41-60, 102-124 and 149-162. Fragment 1-8, 25-40 and 92-100 are intermediate while f9-14, f84-91, f125-135 and f78-83 are considered as minor epitopes (Selo et al., 1999). It is a globular protein present in the whey portion of milk and show resistance to digestion, particularly to the peptic digestion. Wal et al. (2001) suggested that the absence of this protein in human milk is partially responsible for its allergenicity in humans. The hydrophobic amino acids (preferential cleavage sites of pepsin) in β-lactoglobulin are present in the core of the tertiary structure that makes the peptic digestion more challenging (El-Zahar et al., 2005; Pintado and Malcata, 2000; Kitabatake and Kinekawa, 1998). The ovine β-lactoglobulin's peptic hydrolysis is faster than the bovine β-lactoglobulin (El-Zahar et al., 2005). In another study using human digestive juices, Almaas et al. (2006a) found more rapid hydrolysis of caprine β-lactoglobulin compared to the bovine one and Inglingstad et al. (2010) reported higher digestion of equine β-lactoglobulin than bovine and caprine β-lactoglobulin. Within the same species, the genetic variant of β-lactoglobulin may also show differences in their digestibility. The ovine β-lactoglobulin variant B was digested more rapidly than the variant A (El-Zahar et al., 2005). Moreover, Tidona et al. (2014) reported that the β-lactoglobulin in donkey's milk degraded rapidly when it consisted of only β-lactoglobulin I. The replacement of amino acids that could change the tertiary structure and surface hydrophobicity of the β-lactoglobulin may cause alteration in the rate of its hydrolysis (Ulleberg, 2011; El-Zahar et al., 2005). However, β-lactoglobulin can be hydrolyzed by trypsin and chymotrypsin even though the rate of proteolysis is slow (Kopf-Bolanz et al., 2012; Mota et al., 2006; Perez and Calvo, 1995).

Another whey protein, α-lactalbumin showed less resistance to commercial pepsin hydrolysis than the β-lactoglobulin and a complete hydrolysis was obtained only by the non-human pepsin (Kopf-Bolanz et al., 2012). Natale et al. (2004) found no evidence of α-lactalbumin to be allergenic and no consensus was found among the studies regarding the allergenicity of α-lactalbumin (Chatterton et al., 2006).

Even though caseins coagulate in the stomach, caseins digest rapidly and completely than the whey proteins (Devle et al., 2014; Kopf-Bolanz et al., 2012; Inglingstad et al., 2010; Almaas et al., 2006a). However, the individual casein hydrolysis may vary from one to another (Devle et al., 2014; Gallier et al., 2012). Because of this rapid and complete hydrolytic feature along with non-compact, flexible structure, caseins are often considered poorly immunogenic (Wal, 2001).

## 4. Summary of papers

### *Paper I*

### Principal milk components in buffalo, Holstein cross, Indigenous cattle and Red Chittagong Cattle from Bangladesh (2014)

In this paper milk from buffalo, Holstein cross, Indigenous cattle and Red Chittagong Cattle were analyzed for protein and casein composition and type, casein micelle size, naturally occurring peptides, free amino acids, fat, fat globule size, fatty acids, carbohydrates and minerals. This was done to obtain a total characterization of those milk and to compare them. Most of the components varied considerably among the milk types. However, the milk of Indigenous cattle and Red Chittagong Cattle were found more or less similar. The buffalo milk contained higher $\alpha_{s2}$- and κ-casein and α-lactalbumin, free amino acids, fat, unsaturated fatty acids, Ca and Ca:P but had lowest β-lactoglobulin, almost half of the cow milk. The casein number was also higher in buffalo milk and it also has largest casein micelle and milk fat globule. Higher content of protein, casein, β-casein, whey protein, lactose, total mineral and P were found in the milk of Indigenous cattle and Red Chittagong Cattle. The milk of Holstein cross was found rich in β-lactoglobulin and naturally occurring peptides but was lowest in casein to whey protein ratio. From nutritional and technological point of view, the milk of buffalo and Red Chittagong Cattle should be preferred.

### *Paper II*

### *Ex vivo* digestion of proteins and fat in buffalo milk (2014)

In this paper buffalo whole milk and skimmed milk was digested in an *ex vivo* model to investigate the effect of lipids on proteolysis, specifically the more allergenic proteins – β-lactoglobulin and $\alpha_{s1}$-casein. The lipolysis and release of fatty acids at different stage of digestion and the generation of peptides were also reported. Some intact caseins were found

after gastric digestion, however, digested completely after 5 min duodenal digestion. At the same time α-lactalbumin and β-lactoglobulin were readily and mostly completely digested though they were resistant to gastric hydrolysis. The effect of fat was not profound and only small variation in the proteolysis of whole and skimmed milk was observed. The milk types were also found more or less similar in the number of identified peptides, localization of proteolytic site and sequence alignment of the generated peptides. A rapid lipolysis (30%) was observed during 30 min duodenal digestion and then remained static until the end, 120 min duodenal digestion. The total unsaturated fatty acid and medium chain fatty acid (C10:0 - C16:0) showed 8-10% less lipolysis than the total saturated fatty acid and short (C4:0 - C8:0) and long chain (≥C17:0) fatty acid, respectively. The rapid digestion of buffalo milk $\alpha_{s1}$-casein and β-lactoglobulin may be of nutritional importance, especially for those who suffer from cow milk allergy.

***Paper III***

***Ex vivo* digestion of omega-3 enriched buffalo skimmed milk (2014)**

This study investigated whether the addition of cod liver oil, as a source of omega-3 fatty acids to the buffalo skimmed milk influence the proteolysis during *ex vivo* model digestion or not. The protein degradation and generation of peptides were compared with that of the skimmed buffalo milk. The addition of omega-3 fatty acid did not affect the protein degradation pattern and the peptides generation. The cod liver oil showed a lipolysis pattern similar to milk fat, 28% lipolysis after 30 min duodenal digestion and became more or less static till 120 min duodenal digestion. The recorded lipolysis of both the omega-3 fatty acids, eicosapentaenoic (EPA; C20:5n-3) and docosahexaenoic(DHA; C22:6n-3) acid were 23%. Skimmed buffalo milk fortified by adding cod liver oil may be regarded as a good source of omega-3 fatty acids.

***Paper IV***

## *Ex vivo* digestion of milk from Red Chittagong Cattle focusing proteolysis and lipolysis (2014)

This work describes the *ex vivo* digestion of Red Chittagong Cattle milk to obtain more insight into the degradation of milk from this breed/type in comparison to other bovine breeds reported worldwide. The proteolysis (with a focus on the allergenic $\alpha_{s1}$-casein and β-lactoglobulin) and lipolysis with subsequent generation of peptides and fatty acids were monitored. All the caseins were digested after 40 min gastric digestion while some β-lactoglobulin still remained intact after 120 min duodenal digestion. Maximum number of peptides identified was generated from β-casein followed by $\alpha_{s1}$-, κ-, and $\alpha_{s2}$-casein and β-lactoglobulin. All the peptides were rich in hydrophobic amino acids and proline was present in almost all of the peptides. The milk fat showed 48% lipolysis after 120 min duodenal digestion. Short chain fatty acids underwent more lipolysis than the medium and long chain fatty acids and the lipolysis of saturated and unsaturated fatty acids were found more or less similar. Considering the overall proteolysis and lipolysis pattern, milk from this Bangladeshi breed/type was found to stay close with the Nordic cattle (Norwegian Red Cattle).

## 5. Key results and general discussion

The milk of Bangladesh has only been evaluated for its gross content of the milk components (Khan et al., 2007; Islam et al., 2008) and recently Islam et al. (2013) reported the concentration of metabolites in Bangladeshi milk. In this milk, the protein composition and type, fatty acid composition, casein micelle and fat globule size and mineral composition have not been characterized yet. In the previous studies, protein content was estimated as crude protein and lactose content by subtracting the sum of protein, fat and ash from the total solids. Naturally occurring peptides from raw dairy milk is also a new area of interest. Therefore, in **Paper I** we characterize the buffalo (B), Holstein cross (HX), Indigenous cattle (IC) and Red Chittagong Cattle (RCC) milk from Bangladesh on the basis of aforementioned components and attributes to obtain a more country and species/breed specific knowledge on this milk as suggested by Schonfeldt et al. (2012) and Medhammar et al. (2011), respectively.

The true protein content of buffalo milk was 35 g/kg milk. Khan et al. (2007) found 37.67 g/kg milk crude protein in the milk of water buffalo which is comparable with the present study. The reported crude protein content of the HX and RCC milk was 31.10 and 40.60 g/kg milk, respectively (Islam et al., 2008) which is also comparable with the present result of HX and RCC milk true protein content, 27.15 and 37.80 g/kg milk, respectively. Coagulation properties and cheese production of milk is mainly dependent on the protein, casein content and type and casein micelle size. The casein number of Bangladeshi milk was 5-15% less than the European milk (Dutch dairy milk; Heck et al., 2009b). Casein number is positively related with the cheese yield. The B and RCC milk had a casein number of 76-77, which was higher than HX and IC. Content of κ-, and β-casein were highest in B and RCC milk, respectively. RCC milk also contained higher proportionate amount of $\alpha_{s1}$-, β- and κ-casein compared to other milk. The genotype of the animals is a key factor in the variation of the casein composition (Farrell Jr et al. 2004). Milk production trait, milk composition, protein composition and technological properties are significantly different in different genetic variants. The $\alpha_{s1}$-:$\alpha_{s2}$-:β-:κ-casein of all milk in the present study also differed from the general 4:1:4:1 ratio; which is related to the genetic polymorphism, post translational modification (s), stage of lactation and feeding regime (Barlowska et al., 2011). Higher proportion of $\alpha_{s1}$-casein in RCC milk may cause to increase

coagulation and curd firming time which may be neutralized by the higher proportion of β-casein (Bonfatti et al., 2013). Instead of casein content only, the proportion of $\alpha_s$- and β-casein is important in milk coagulation properties and milk with a higher proportion of β-casein showed poor coagulation properties (St-Gelais and Hache, 2005). Though in general κ-casein is positively related to improved coagulation properties of milk, Bonfatti et al. (2013) showed the reverse and concluded that the extent of glycosylation of the κ-casein is the determinant of its effect in coagulation properties. Buffalo milk has the advantage over cow milk as they have 40% of κ-casein without carbohydrate, which is 20% in cow milk (Addeo et al., 1977). The presence of more κ-casein is associated with smaller casein micelle size which was not shown in the present study. The buffalo milk had the largest casein micelle of 188.73 nm and smallest was 157.1 nm in IC milk and HX differed non-significantly from IC. Glantz et al. (2010) reported that smaller casein micelles are more likely to give stronger gels. This was partially reflected in a preliminary study using Formagraph (**Appendix 1**). That preliminary study was also ended with better coagulation properties of B milk followed by RCC, IC and HX which is more or less consistent with the results on protein presented above.

The milk protein is also known for its nutritional importance. Higher proportion of whey protein is immunogenically unfavorable, particularly with regard to β-lactoglobulin, however, whey proteins are nutritionally favorable. Persons with milk allergy can prefer the B milk as it has β-lactoglobulin, almost half of the cow's milk and has higher α-lactalbumin content than the cow milk. Buffalo milk was also rich in free amino acids. The present study has identified 45, 79, 19 and 12 naturally occurring peptides from B, HX, IC and RCC milk, respectively. Maximum number of peptides has angiotensin converting enzyme inhibitory activity while some has opioid and immunomodulatory effects and activity in mineral nutrition. All the bioactive peptides appeared as a part of larger peptide and according to Hayes et al. (2007) it will improve the availability of the bioactive fragment after the gastrointestinal digestion of the larger peptides.

The fat content varied significantly among the animals. In the fatty acid composition of saturated fatty acid in neutral lipid, only C20:0 showed non-significant variation among the milk types. Buffalo milk was rich in C4:0, C15:0, C17:0 and C18:0 while IC and RCC milk

were higher in C12:0, C14:0 and C16:0 content. Feeding, genotype, rumen microbial activity and health status of the animal are the main contributors in the variation of fatty acid composition. The concentration of C4:0 to C10:0 obtained in all milk of the present study reveals that all the animals were not at positive energy balance (Van Knegsel et al., 2005). The variation in the C15:0 and C17:0 is mainly because of the variation in the ruminal activity and feeding and health status may also contribute to it. The quality of the feed and body condition score is also important in C16:0 and C18:0. Buffalo milk was rich in total unsaturated fatty acids and had lowest saturated fatty acids compared to cow's milk and all the cow's milk were found similar in this respect.

Except C10:1n-6 cis and C14:1n-5 cis, all the unsaturated fatty acids including conjugated linoleic acid isomer (C18:2n-7 trans) and precursors (C18:1n-7 trans and C18:2n-6 cis) were found significantly higher in the buffalo milk than the cow's milk. The C18:3n-3 cis was only detected in the B milk. Being of different genotype, IC and RCC showed greater similarities in their fatty acid composition, reflecting the importance of feeding in this regard as they received the same diet. However, HX cattle were fed differently, but showed similarities with IC and RCC (e.g. C4:0 - C8:0) and Menard et al. (2005) found differences in the fatty acid composition of cow and buffalo maintained under same feeding and management. So the genotype of the animal seems to affect fatty acid composition and Devle et al. (2012) also reported the species variation of fatty acid composition. Though RCC and B animals belonged to different genotype and fed differently, the milk showed similarities in C16:1n-7 cis and C18:2n-7 trans content and HX milk was found similar to B milk in C17:0 content.

Generally the milk fat globule size ranges from 0.2-20 μm in diameter. The milk fat globule size is corresponding well to the total fat content of B, HX, IC and RCC milk. Supporting that, the fat globule size need to grow larger with the increased fat content as the mammary gland has a limited capacity to synthesize the globule materials. The higher range of variability in buffalo milk fat globule size compared to the cow milk is in agreement with Menard et al. (2005) and Akhundov (1959).

The significantly higher lactose content was 52.65 g/kg milk in RCC milk followed by IC, B and HX milk. Total mineral content varied significantly among the different milk and RCC and IC had the highest content, 8.5 and 8.4 g/kg milk, respectively. The Ca content was higher in B milk (1.48 g/kg milk). The highest P content was 1.14 g/kg milk in RCC milk. Genotype has an influence on the Ca and P content of milk. The β-casein $A_2$ variant is associated with more Ca and P in milk than the milk with β-casein $A_1$ variant and the effect of κ-casein is like A>B>E. In the present study, Ca in all milk was more than the P which is in line with Ariota et al. (2007). Patino et al. (2007) reported higher P than Ca in different buffalo breeds. Ariota et al. (2007) also reported a positive relationship between Ca and P content and fresh cheese yield, between gel strength ($A_{30}$) and soluble Ca and P content and a negative relationship between rennet coagulation time and ratio of Ca and P.

In addition to the results obtained in **Paper I**, increased interest for alternative milk due to cow milk allergy; possible variability of milk digestibility because of their differences in composition and structure (Almaas et al., 2006a); and species differences in the milk protein digestibility (Inglingstad et al., 2010); set the buffalo milk in focus for further nutritional details regarding their digestibility especially the main two milk allergen, $\alpha_{s1}$-casein and β-lactoglobulin. With this respect, **Paper II** investigated the effect of milk fat on milk protein hydrolysis and peptides generation in an *ex vivo* model. Considering the results obtained there, **Paper III** describes the effect of adding the external lipid (cod liver oil) as a source of omega-3 fatty acid to overcome the limitations of long chain polyunsaturated fatty acids (mainly omega-3) content of milk on the milk protein degradation. While **Paper IV** describes the proteolysis and lipolysis of whole Red Chittagong Cattle milk that provides a basis to compare the buffalo milk results with the cow's milk and also a comparison with other regional breeds (Asia and Europe). The Norwegian full fat cow milk (**Appendix 2**), omega-3 enriched Norwegian skimmed cow milk (**Appendix 3**), Full fat milk of Bangladeshi Holstein cross (**Appendix 4**) and Indigenous cattle (**Appendix 5**) were also digested in the same model to furnish more conclusive data.

The protein degradation pattern was visualized by SDS-PAGE. Majority of the caseins in whole buffalo and skimmed buffalo milk (**Paper II**), omega-3 enriched buffalo skimmed milk (**Paper**

**III**) and in whole cow milk (**Paper IV**) were digested after 20 min of gastric digestion. After 40 min gastric digestion, a tiny trace of casein was present in whole, skimmed and omega-3 enriched buffalo milk though it was more prominent in the whole and skimmed buffalo milk. However, in cow's milk it was digested completely. In buffalo milk, $\alpha_s$-caseins appeared to be more resistant than the β- and κ-casein which is consistent with Gallier et al. (2012). In digesting bovine milk by using non-human enzymes, Kopf-Bolanz et al. (2012) reported a complete digestion of all the bovine caseins after 30 min gastric digestion and Gallier et al. (2012) reported 45 min gastric digestion in their study. Devle et al. (2014) showed a complete digestion of caseins after 40 min gastric digestion using human digestive juices. So the caseins digestion obtained in Red Chittagong Cattle (**Paper IV**), Holstein cross (**Appendix 4**) and Indigenous cattle (**Appendix 5**) milk are in line with those studies. The residual caseins in whole, skimmed (**Paper II**) and omega-3 enriched (**Paper III**) buffalo milk were digested completely after 5 min duodenal digestion. The protein composition of buffalo and cow milk is different (**Paper I**) and according to Almaas et al. (2006a) this could lead to differences in their digestion pattern. Inglingstad et al. (2010) reported a rapid digestion of caseins in equine milk after 30 min gastric digestion compared to human, bovine and caprine milk. So the differences observed between the buffalo and cow's milk regarding caseins digestion pattern is attributed to the species variation.

Among the whey proteins serum albumin was digested completely after 40 min gastric digestion in whole, skimmed (**Paper II**) and omega-3 enriched (**Paper III**) buffalo milk and in full fat cow milk (**Paper IV, Appendix 4** and **5**). However, α-lactalbumin and β-lactoglobulin were resistant to gastric digestion. In buffalo milk, they were digested readily during the duodenal digestion after 5 min. By UPLC-MS, traces of β-lactoglobulin as a single band was identified in whole buffalo milk but was not observed in the skimmed buffalo milk (**Paper II**) and omega-3 enriched skimmed buffalo milk (**Paper III**). After 5 min duodenal digestion in whole, skimmed and omega-3 enriched buffalo milk, traces of α-lactalbumin and degradation product of β-lactoglobulin was also observed by UPLC-MS, however, and degraded completely during duodenal digestion for 30 min. In the full fat Red Chittagong Cattle milk digestion (**Paper IV**), β-lactoglobulin remained intact after 120 min duodenal digestion and the results of UPLC-MS indicate the degradation of α-lactalbumin. Devel et al. (2014) reported that the β-

lactoglobulin digestion was affected by milk fat and obtained more or less complete digestion of β-lactoglobulin in skimmed bovine milk after 30 min of duodenal digestion while it remained intact in full fat milk after 120 min of duodenal digestion.

Gass et al. (2007) reported that the presence of oleic acid and monoolein reduced the positive effect of bile salts in the digestion of β-lactoglobulin. But, Le Maux et al. (2013) showed that the addition of linoleic acid increased the digestion of purified β-lactoglobulin and Kopf-Bolanz et al. (2012) observed a complete digestion of β-lactoglobulin after 30 min duodenal digestion using commercial gastric and duodenal enzymes. The bile salt concentration in the present study was 2.4 mM while it was 1.0 mM in the study of Devle et al. (2014). The Norwegian full fat cow milk was digested with the same digestive juices and bile salt concentration as in the present study (**Appendix 2**) and this also resulted in intact β-lactoglobulin after 120 min duodenal digestion. A digestion study on the Norwegian cow skimmed milk with added cod liver oil was also conducted (**Appendix 3**) and showed a complete digestion of β-lactoglobulin after 5 min of duodenal digestion. Bile salts can destabilize the β-lactoglobulin and thereby enhance its digestibility (Gass et al. 2007). Kopf-Bolanz et al. (2012) also showed the effect of bile acids on the digestion of β-lactoglobulin. Gass et al. (2007) reported that 2.0 mM bile salts can improve the β-lactoglobulin digestion, but the effect is more pronounced above the critical micelle concentration (3.5 mM).

The other two Bangladeshi cow milk, Holstein cross and Indigenous cattle milk showed no intact β-lactoglobulin after 120 min duodenal digestion (**Appendix 4** and **5**). The genotypic variation of the animal may cause changes in the amino acid sequence and thereby the structure of β-lactoglobulin leading to the differences in the digestibility. Because, Tidona et al. (2014) found more rapid degradation of β-lactoglobulin in donkey's milk when β-lactoglobulin II fraction was absent. In addition, El-Zahar et al. (2005) reported more rapid hydrolysis of ovine β-lactoglobulin than that of bovine milk and also showed the variation in the digestibility between the genetic variants of β-lactoglobulin. Inglingstad et al. (2010) showed higher digestibility of equine β-lactoglobulin compared to that of bovine and caprine. In the present study, the used bile salt concentration was similar for buffalo and cows' milk. So, the genotype of the animal seems to exert more prominent effect to the variation in the β-lactoglobulin

digestion between buffalo and cow milk and within the cows' milk. Along with genotype and bile salts, the concentration of β-lactoglobulin in milk may also contribute to the variation in β-lactoglobulin digestion. The concentration of β-lactoglobulin was different in buffalo and cows' milk (**Paper 1**).

After digestion, the identified peptides were aligned with the complete sequence of the protein for determining the site of proteolysis. In all type of buffalo and cow milk, extensive proteolysis was observed in β-casein followed by $\alpha_{s1}$-, κ- and $\alpha_{s2}$-casein and β-lactoglobulin. The number of identified peptides also followed the same order. The buffalo whole, skimmed and omega-3 enriched milk showed very little variation in the peptide pattern regarding the proteolytic cutting site – same site with different residue or very few new cutting sites (**Paper II and III**). In **Paper II, III and IV**, almost all the κ-casein peptides were identified from the gastric phase and β-lactoglobulin peptides from duodenal phase. This corresponded well with the degradation pattern visualized by SDS-PAGE. However, the number of peptides identified was not in line with the extent of protein degradation observed on SDS-PAGE. The detection limit of UPLC/Q-TOF MS might be a factor as this instrument can detect peptide size of 0.80 kDa to 4.5 kDa. The low molecular size peptides (e.g. di-, tri- and tetra-peptides) and the generated free amino acids were not detected. In digestion model, half of the milk proteins degraded into di- and tripeptides and as much as 10% of the proteins could be degraded to their component residues as free amino acid (Kopf-Bolanz et al., 2012). They also reported that the size distribution of the proteins and peptides ranging from 5 kDa to tripeptide remains unclear due to the detection limits.

Almost all the identified peptides contained proline and/or hydrophobic amino acids like leucine, isoleucine, valine, phenylalanine, alanine, glycine. Proline is a helix breaker and the proteolytic enzymes may have less access to the hydrophobic sequence for further proteolysis. From the digested caprine milk, Almaas et al. (2011) have also identified peptides with these type of residues from β- and κ-casein, β-lactoglobulin and glycomacropeptide. Proline could restrict proteases like trypsin for further proteolytic processing (Jornvall and Persson, 1983). Some peptides showed to be fully resistant to further degradation (e.g. f84-108 and f96-108 from β-casein in **Paper III**).

Milk fat mainly contains triglyceride (95%) (Haug et al., 2007) and according to Mu and Hoy (2004), 95% of the milk fat can be absorbed. There is no consensus about the exact contribution of pre-duodenal lipase (gastric and lingual). In the present study, there was no gastric lipolysis of milk fat observed (**Paper II, III and IV**). Devle et al. (2014) reported the same on Norwegian bovine milk digested under similar *ex vivo* condition like the present study and speculated that the pH 2.5 is far from the optimum pH of gastric lipase activity which is 5 to 6 (Carriere et al., 1993). Another speculation is that the volunteers were not stimulated for lipid digestion and leads to insufficient secretion of gastric lipase. The effect of the second issue seems more prominent because milk was digested at pH 5 for 20 min before it set for digestion at pH 2.5. However, gastric digestion is reported to be important for further duodenal digestion (Gallier et al., 2012 and Ye et al., 2011).

A rapid rise in free fatty acids after 30 min duodenal digestion was observed, which was 30% in buffalo milk fat (**Paper II**), 28% in cod liver oil enriched buffalo milk (**Paper III**) and 33% in cow milk fat (**Paper IV**). Then after, till 120 min duodenal digestion, buffalo milk fat and cod liver oil's lipolysis remained more or less static and ended up with 35% and 32% lipolysis, respectively. Whereas, the cow milk fat lipolysis became static first after 60 min duodenal digestion and finally resulted in 48% lipolysis after 120 min duodenal digestion. The static conditions were mainly because of product inhibition (Devle et al. 2014 and Gallier et al., 2012). The differences between the buffalo milk fat and cow milk fat lipolysis may be attributed to the differences in their fat globule size. The mean diameter of buffalo milk fat globule was almost four times of Red Chittagong Cattle milk fat globule and buffalo milk also had a greater percentage of larger fat globules (**Paper I**). The catalytic efficiency of human pancreatic lipase is higher on small fat globule than large globule (Berton et al., 2012).

The average lipolysis of short chain (C4:0 - C8:0) and long chain (>C17:0) fatty acid in Red Chittagong Cattle milk fat were 11% and 4% higher than that of buffalo milk fat, respectively. The average lipolysis of medium chain (C10:0 - C16:0) fatty acids was low and similar in buffalo milk fat, cod liver oil and cow milk fat. Lipase has preference to attack the *sn*-1 and *sn*-3 position of the triglyceride (Armand, 2007 and Carriere et al., 1993). Short and long chain fatty acids prevail at *sn*-1 and *sn*-3 position of the triglyceride (Blasi et al., 2008 and Angers et

al., 1998) as compared to the medium chain fatty acids and this could explain the low lipolysis. Total saturated fatty acid in cow milk fat and cod liver oil showed 1-2% higher lipolysis than the total unsaturated fatty acid while it was 9% higher in buffalo milk fat. Devle et al. (2014) reported 10% more lipolysis of unsaturated fatty acids than the saturated fatty acids in Norwegian bovine milk. The omega-3 fatty acids in cod liver oil, C20:5 n-3 *cis* and C22:6 n-3 *cis* showed 23% lipolysis though their presence is different in lipase preferred position (Zeng et al., 2010). The phospholipid fraction was not considered as Devle et al. (2014) found non-significant digestion of phospholipids. However, it is difficult to be conclusive on the lipolytic variation observed both at group or individual level of fatty acid because the distribution of fatty acids in the triglyceride shows considerable variation (Blasi et al., 2008, Maanson et al., 2008, Angers et al., 1998 and Parodi, 1979).

## 6. Conclusions and future perspectives

Milk of buffalo, Holstein cross, Indigenous cattle and Red Chittagong Cattle were characterized and compared based on their principal components. This was followed by the monitoring of proteolysis and lipolysis in an *ex vivo* digestion model to gather further nutritional details on aforementioned milk. Particular attention was paid to the two major milk allergens, β-lactoglobulin and $\alpha_{s1}$-casein. Protein degradation pattern, peptides generated and release of fatty acids were also recorded.

Considering the protein composition and casein types, fat content, Ca and P; buffalo and Red Chittagong Cattle milk should be preferred from the technological stand-point. From the nutritional perspective, milk from these two types of animal also showed better data than others with regards to protein content and type, peptides and amino acids, fat content and unsaturated fatty acids including conjugated linoleic acid precursors and isomers, lactose, minerals, Ca, P, Mg, Mn and Zn and the two major milk allergens, $\alpha_{s1}$-casein and β-lactoglobulin. However, additional data on large population with controlled feeding, genotype and lactation would be an advantage.

The *ex vivo* digestion showed a rapid and complete degradation of caseins. The β-lactoglobulin in buffalo milk also underwent more rapid and complete digestion and showed no effect of milk fat compared to that of cow. Within the cow, Holstein cross and Indigenous cattle milk showed advantageous digestion pattern of β-lactoglobulin. Therefore, buffalo milk could be an alternative to the cow milk for individuals with cow milk allergy. However, cow milk from specific genotype still could have opportunities in this regard. It seems that the genotype and/or β-lactoglobulin concentration in milk is involved in this variation of β-lactoglobulin digestion. Further studies on the allergenic epitopes of buffalo milk caseins and whey proteins are needed and their presence/state after the digestion needs to be monitored. Another interesting study to perform in the future is genotyping of these cows. The addition of cod liver oil as a source of omega-3 fatty acids to the milk did not affect the milk protein digestion including the allergens and the generation of peptides. It could be a good nutritional way to supply the omega-3 fatty acids. However, rheological study along with processing effect (s) is needed. The identified

peptides were rich in hydrophobic residues and contained proline. For tracing well the path and final fate of protein digestion products, smaller peptides and free amino acids need to be analyzed.

Medium chain fatty acids showed lowest lipolysis and so were the omega-3 fatty acids. The lipolysis pattern of buffalo milk fat and cod liver oil in buffalo skimmed milk were similar. However, continuous removal of lipid digestion product during the digestion process is needed. Use of dynamic *ex vivo* model or *in vivo* study could provide that provision. To monitor better, in addition to phospholipids, mono-, di- and triglyceride should be separated and their fatty acid composition should be investigated. All the data obtained during *ex vivo* study need to be validated by *in vivo* studies including a considerable number of individuals to minimize the effect of individual variation.

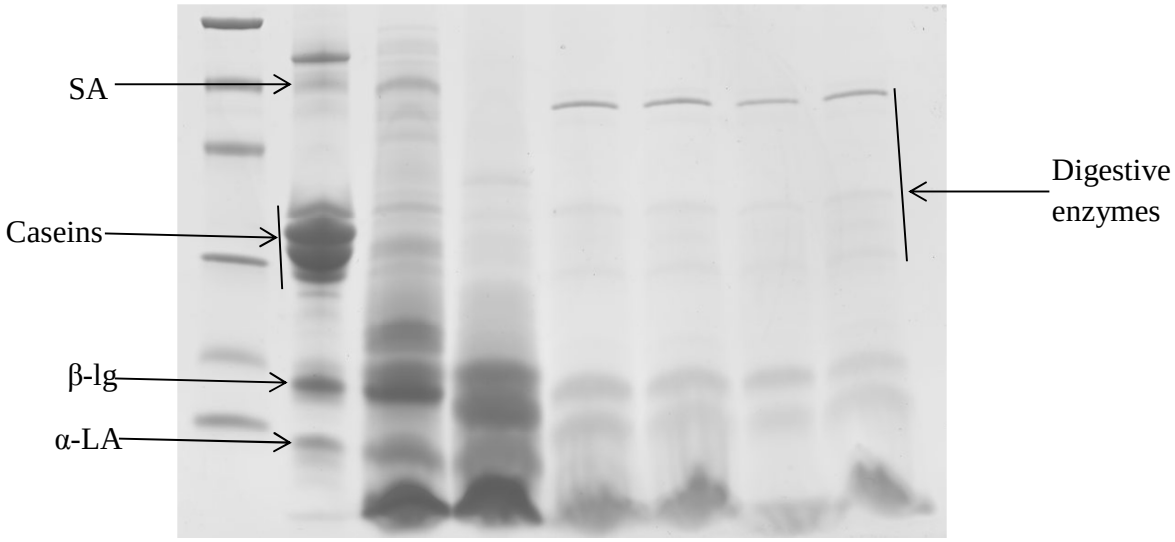
The data obtained on Bangladeshi cow and buffalo milk is important for the future breeding programme and to select milk for product development. It also reflects the nutritional quality of those milk as well.

## 7. Appendices

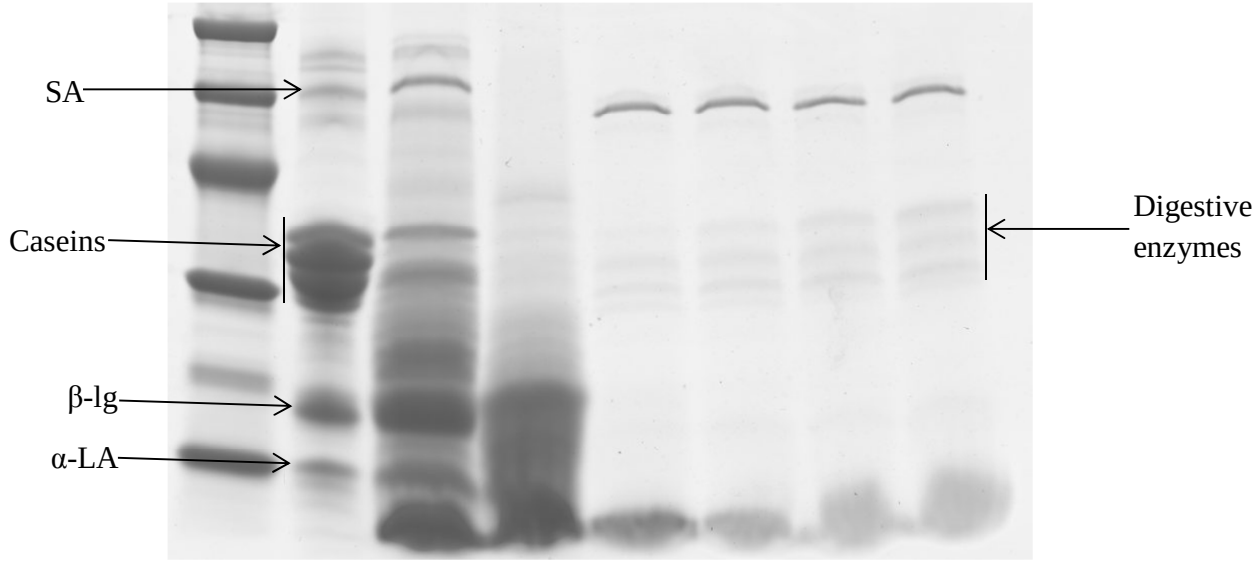
***Appendix 1*:** Rennet coagulation time (r), curd firming rate ($K_{20}$) and gel strength ($A_{30}$) of buffalo (B), Holstein cross (HX), Indigenous cattle (IC) and Red Chittagong Cattle (RCC) milk from Bangladesh

| | B | HX | IC | RCC |
|---|---|---|---|---|
| r (min) | 9:25 | 17:55 | 17:25 | 15:00 |
| $K_{20}$ (min) | 7:20 | 28:15 | 10:55 | 10:25 |
| $A_{30}$ (mm) | 24.21 | 12.10 | 22.11 | 24.06 |

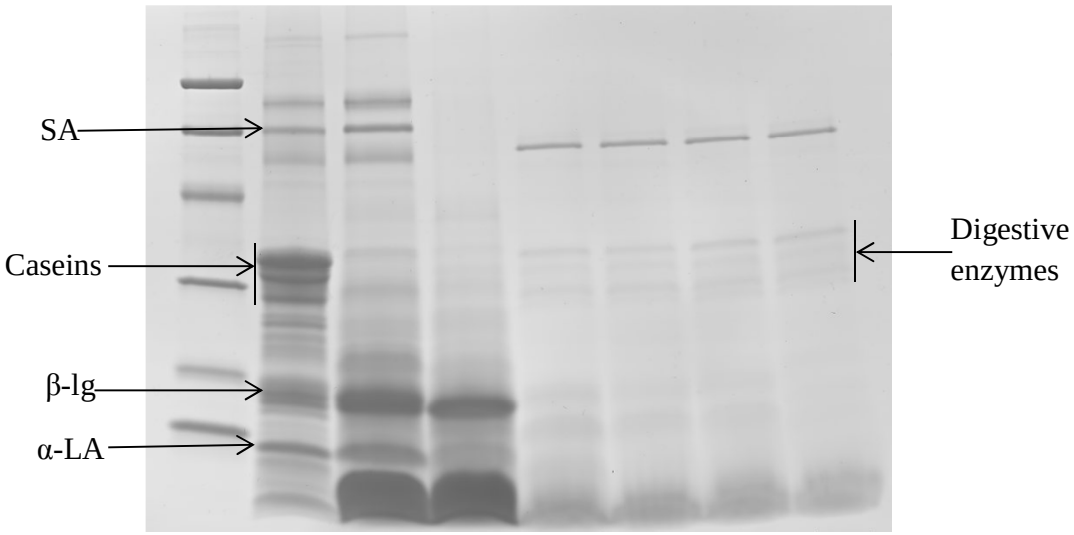
***Appendix 2*:** Protein hydrolysis during the *ex vivo* digestion of full fat Norwegian cow milk. From left: Lane 1, low molecular weight marker; Lane 2, undigested milk sample, Lane 3, gastric digested samples for 20 min at pH 5.0; Lane 4, gastric digested samples for 20 min at pH 2.5; Lane 5-8, duodenal digested samples for 5, 30, 60 and 120 min, respectively at pH 7.0. Digestive enzymes are present only in the duodenal samples (Devle et al., 2014). SA, Serum albumin; β-lg, β-lactoglobulin; α-LA, α-lactalbumin.



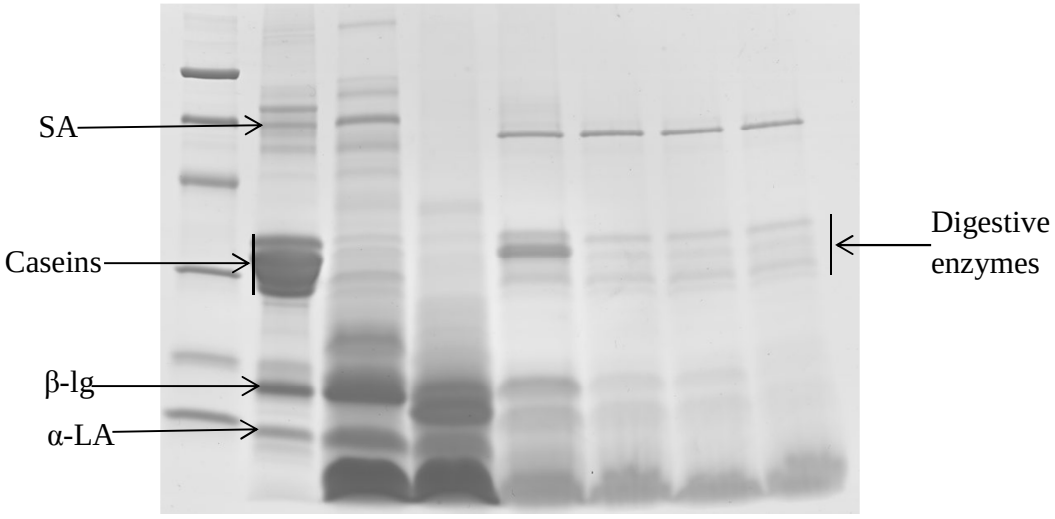
***Appendix 3*:** Pattern of protein degradation during the *ex vivo* digestion of skimmed Norwegian cow milk with added cod liver oil. From left: Lane 1, low molecular weight marker; Lane 2, undigested milk sample, Lane 3, gastric digested samples for 20 min at pH 5.0; Lane 4, gastric digested samples for 20 min at pH 2.5; Lane 5-8, duodenal digested samples for 5, 30, 60 and 120 min, respectively at pH 7.0. Digestive enzymes are present only in the duodenal samples (Devle et al., 2014). SA, Serum albumin; β-lg, β-lactoglobulin; α-LA, α-lactalbumin.



***Appendix 4*:** Degradation pattern of Holstein cross milk proteins during *ex vivo* digestion. From left: Lane 1, low molecular weight marker; Lane 2, undigested milk sample, Lane 3, gastric digested samples for 20 min at pH 5.0; Lane 4, gastric digested samples for 20 min at pH 2.5; Lane 5-8, duodenal digested samples for 5, 30, 60 and 120 min, respectively at pH 7.0. Digestive enzymes are present only in the duodenal samples (Devle et al., 2014). SA, Serum albumin; β-lg, β-lactoglobulin; α-LA, α-lactalbumin.



***Appendix 5*:** The Bangladeshi Indigenous cattle milk during the *ex vivo* digestion featuring the degradation pattern of milk proteins. From left: Lane 1, low molecular weight marker; Lane 2, undigested milk sample, Lane 3, gastric digested samples for 20 min at pH 5.0; Lane 4, gastric digested samples for 20 min at pH 2.5; Lane 5-8, duodenal digested samples for 5, 30, 60 and 120 min, respectively at pH 7.0. Digestive enzymes are present only in the duodenal samples (Devle et al., 2014). SA, Serum albumin; β-lg, β-lactoglobulin; α-LA, α-lactalbumin.



## 8. References


Abd El-Salam, M. H. and El-Shibiny, S. (2011). A comprehensive review on the composition and properties of buffalo milk. *Dairy Science & Technology*, 91: 663-699.

Abd El-Salam, M. H. and El-Shibiny S. (1975). Preparation and properties of β-casein from buffalo's milk. *Journal of Dairy Research*, 42:163–167.

Abd El-Salam, M. H., Osman Y. M., and Nagmoush M. R. (1978). Comparative study of the composition of casein micelles from buffalo's and cow's milk. *Egyptian Journal of Dairy Science*, 6:1–8.

Addeo, F., Mercier, J.-C. and Ribadeau-Dumas, B. (1977). The caseins of buffalo milk. *Journal of Dairy Research*, 44: 455-468.

Adams, S. L., Barnett, D., Walsh, B. J., Pearce, R. J., Hill, D. J. and Howden, M. E. H. (1991). Human IgE-binding synthetic peptides of bovine [beta]-lactoglobulin and [alpha]-lactalbumin. In vitro cross-reactivity of the allergens. *Immunology & Cell Biology*, 69: 191-197.

Ahmad, S., Piot, M., Rousseau, F., Grongnet, J. F. and Gaucheron, F. (2009). Physico-chemical changes in casein micelles of buffalo and cow milks as a function of alkalinisation. *Dairy Science & Technology*, 89: 387-403.

Ahmad, S., Gaucher, I., Rousseau, F., Beaucher, E., Piot, M., Grongnet, J. F. and Gaucheron, F. (2008). Effects of acidification on physico-chemical characteristics of buffalo milk: A comparison with cow's milk. *Food Chemistry*, 106: 11-17.

Akhundov, D. (1959). Some data on Buffaloes milk. *Dairy Science Abstract*, 22:211.

Aleandri, R., Buttazzoni, L. G., Schneider, J. C., Caroli, A. and Davoli, R. (1990). The Effects of Milk Protein Polymorphisms on Milk Components and Cheese-Producing Ability. *Journal of Dairy Science*, 73: 241-255.

Almaas, H., Eriksen, E., Sekse, C., Comi, I., Flengsrud, R., Holm, H., Jensen, E., Jacobsen, M., Langsrud, T. and Vegarud, G. E. (2011). Antibacterial peptides derived from caprine whey proteins, by digestion with human gastrointestinal juice. *British Journal of Nutrition*, 106: 896-905.

Almaas, H., Cases, A.-L., Devold, T. G., Holm, H., Langsrud, T., Aabakken, L., Aadnoey, T. and Vegarud, G. E. (2006a). In vitro digestion of bovine and caprine milk by human gastric and duodenal enzymes. *International Dairy Journal*, 16: 961-968.

Almaas, H., Holm, H., Langsrud, T., Flengsrud, R. and Vegarud, G. E. (2006b). In vitro studies of the digestion of caprine whey proteins by human gastric and duodenal juice and the effects on selected microorganisms. *British Journal of Nutrition*, 96: 562-569.

Alomirah, H., Alli, I. and Konishi, Y. (2000). Applications of mass spectrometry to food proteins and peptides. *Journal of chromatography A*, 893: 1-21.

Angers, P., Tousignant, E., Boudreau, A. and Arul, J. (1998). Regiospecific analysis of fractions of bovine milk fat triacylglycerols with the same partition number. *Lipids*, 33: 1195-1201.

Ariota, B., Campanile, G., Potena, A., Napolano, R., Gasparrini, B., Neglia, G. and Di Palo, R. (2007). Ca and P in buffalo milk: curd yield and milk clotting parameters. *Italian Journal of Animal Science*, 6: 497-499.

Armand, M. (2007). Lipases and lipolysis in the human digestive tract: where do we stand? *Current Opinion in Clinical Nutrition and Metabolic Care*, 10: 156-164.

Armand, M., Pasquier, B., André, M., Borel, P., Senft, M., Peyrot, J., Salducci, J., Portugal, H., Jaussan, V. and Lairon, D. (1999). Digestion and absorption of 2 fat emulsions with different droplet sizes in the human digestive tract. *The American journal of clinical nutrition*, 70: 1096-1106.

Ashcroft, A. E. (1997). Ionization methods in organic mass spectrometry. Barnett, N. W. (Ed.), The Royal Society of Chemistry, UK. pp. 27-29.

Bahna, S. L. (2002). Cow's milk allergy versus cow milk intolerance. *Annals of Allergy, Asthma & Immunology*, 89: 56-60.

Barlowska, J., Szwajkowska, M., Litwińczuk, Z. and Król, J. (2011). Nutritional Value and Technological Suitability of Milk from Various Animal Species Used for Dairy Production. *Comprehensive Reviews in Food Science and Food Safety*, 10: 291-302.

Bauer, E., Jakob, S. and Mosenthin, R. (2005). Principles of physiology of lipid digestion. *Asian-Australasian Journal of Animal Sciences*, 18: 282-295.

Bauman, D. E., Mather, I. H., Wall, R. J. and Lock, A. L. (2006). Major Advances Associated with the Biosynthesis of Milk. *Journal of Dairy Science*, 89: 1235-1243.

Belitz, H. –D. and Grotz, W. 1999. Food Chemistry, 2. ed. Springer-Verlag, Berlin, Germany.

Bell, S. J., Grochoski, G. T. and Clarke, A. J. (2006). Health implications of milk containing β-casein with the $A_2$ genetic variant. *Critical Reviews in Food Science and Nutrition*, 46: 93-100.

Bemelmans, W. J., Broer, J., Feskens, E. J., Smit, A. J., Muskiet, F. A., Lefrandt, J. D., Bom, V. J., May, J. F. and Meyboom-de Jong, B. (2002). Effect of an increased intake of α-linolenic acid and group nutritional education on cardiovascular risk factors: the Mediterranean Alpha-linolenic Enriched Groningen Dietary Intervention (MARGARIN) study. *The American Journal of Clinical Nutrition*, 75: 221-227.

Beri R., Sharma K., C, Singh, S. (1984). Lipid composition of fat globule membrane from buffalo and cow's milk. *New Zealand Journal of Dairy Science and Technology*, 19: 31–35.

Berton, A., Rouvellac, S., Robert, B., Rousseau, F., Lopez, C. and Crenon, I. (2012). Effect of the size and interface composition of milk fat globules on their in vitro digestion by the human pancreatic lipase: Native versus homogenized milk fat globules. *Food Hydrocolloids*, 29: 123-134.

Bhuiyan, A. K. F. H. (2007). Cattle and livelihood in Bangladesh. Vishwa Gou Sammelana - An international conference and festival on Indian breeds of cattle. Hosanagara, Shimoga district, Karnataka, India. 21st April to 29th April, 2007.

Blasi, F., Montesano, D., De Angelis, M., Maurizi, A., Ventura, F., Cossignani, L., Simonetti, M. S. and Damiani, P. (2008). Results of stereospecific analysis of triacylglycerol

fraction from donkey, cow, ewe, goat and buffalo milk. *Journal of Food Composition and Analysis*, 21: 1-7.

Bligh, E. G. and Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*, 37: 911-917.

BLRI, Bangladesh Livestock Research Institute. (2004). Animal genetic resources of Bangladesh. First report on the state of the world's animal genetic resources (AnGR). Bangladesh Livestock Research Institute, MoFL, GoB, Savar, Dhaka, Bangladesh.

Boisen, S. and Eggum, B. O. (1991). Critical Evaluation of in Vitro Methods for Estimating Digestibility in Simple-Stomach Animals. *Nutrition Research Reviews*, 4: 141-162.

Bonfatti, V., Gervaso, M., Rostellato, R., Coletta, A. and Carnier, P. (2013). Protein composition affects variation in coagulation properties of buffalo milk. *Journal of Dairy Science*, 96: 4182-4190.

Bonfatti, V., Giantin, M., Gervaso, M., Coletta, A., Dacasto, M. and Carnier, P. (2012). Effect of CSN1S1-CSN3 (αS1-κ-casein) composite genotype on milk production traits and milk coagulation properties in Mediterranean water buffalo. *Journal of Dairy Science*, 95: 3435-3443.

Bonfatti, V., Di Martino, G., Cecchinato, A., Degano, L. and Carnier, P. (2010). Effects of β-κ-casein (CSN2-CSN3) haplotypes, β-lactoglobulin (BLG) genotypes, and detailed protein composition on coagulation properties of individual milk of Simmental cows. *Journal of Dairy Science*, 93: 3809-3817.

Brustad, M., Braaten, T. and Lund, E. (2004). Predictors for cod-liver oil supplement use—the Norwegian Women and Cancer Study. *European Journal of Clinical Nutrition*, 58: 128-136.

Caira, S., Pizzano, R., Picariello, G., Pinto, G., Cuollo, M., Chianese, L., Addeo, F. and Hurley, W. (2012). Allergenicity of Milk Proteins. In: *Milk Protein* (Ed. W. L. Hurley). InTech, Rijeka, Croatia. pp. 173-214.

Campbell, I. (2012). The mouth, stomach and intestines. *Anaesthesia and Intensive Care Medicine*, 13: 56-58.

Careri, M. and Mangia, A. (2003). Analysis of food proteins and peptides by chromatography and mass spectrometry. *Journal of Chromatography A*, 1000: 609-635.

Carey, M. C., Small, D. M. and Bliss, C. M. (1983). Lipid digestion and absorption. *Annual Review of Physiology*, 45: 651-677.

Carrapiso, A. I. and Garcia, C. (2000). Development in lipid analysis: some new extraction techniques and in situ transesterification. *Lipids*, 35: 1167-1177.

Carriere, F., Gargouri, Y., Moreau, H., Ransac, S., Rogalska, E. and Verger, R. (1994). Gastric lipases: cellular, biochemical and kinetic aspects. In: *Lipases: Their Structure, Biochemistry and Application* (Ed. P. Woolley and S. B. Petersen). Cmbridge University Press. pp. 181-205.

Carriere, F., Barrowman, J., Verger, R. and Laugier, R. (1993). Secretion and contribution to lipolysis of gastric and pancreatic lipases during a test meal in humans. *Gastroenterology*, 105: 876-888.

Carriere, F., Moreau, H., Raphel, V., Laugier, R., Benicourt, C., Junien, J.-L. and Verger, R. (1991). Purification and biochemical characterization of dog gastric lipase. *European Journal of Biochemistry*, 202: 75-83.

Cassandro, M., Comin, A., Ojala, M., Zotto, R. D., De Marchi, M., Gallo, L., Carnier, P. and Bittante, G. (2008). Genetic parameters of milk coagulation properties and their relationships with milk yield and quality traits in Italian Holstein cows. *Journal of Dairy Science*, 91: 371-376.

Cecchinato, A., Penasa, M., Cipolat Gotet, C., De Marchi, M. and Bittante, G. (2012). Short communication: Factors affecting coagulation properties of Mediterranean buffalo milk. *Journal of Dairy Science*, 95: 1709-1713.

Chabance, B., Marteau, P., Rambaud, J. C., Migliore-Samour, D., Boynard, M., Perrotin, P., Guillet, R., Jollès, P. and Fiat, A. M. (1998). Casein peptide release and passage to the blood in humans during digestion of milk or yogurt. *Biochimie*, 80: 155-165.

Chasteen, T. G. (2009). The flame ionization detector. Department of Chemistry, Sam Houston State University, Huntsville, Texas. http://www.shsu.edu/chm_tgc/primers/FID.html. Accessed on 13.04.2014.

Chatterton, D. E. W., Smithers, G., Roupas, P. and Brodkorb, A. (2006). Bioactivity of β-lactoglobulin and α-lactalbumin—Technological implications for processing. *International Dairy Journal*, 16: 1229-1240.

Clandinin, M., Cook, S., Konard, S. and French, M. (2000). The effect of palmitic acid on lipoprotein cholesterol levels. *International Journal of Food Sciences and Nutrition*, 51: s61-s71.

Coles, L., Moughan, P. and Darragh, A. (2005). In vitro digestion and fermentation methods, including gas production techniques, as applied to nutritive evaluation of foods in the hindgut of humans and other simple-stomached animals. *Animal Feed Science and Technology*, 123: 421-444.

Comin, A., Cassandro, M., Chessa, S., Ojala, M., Dal Zotto, R., De Marchi, M., Carnier, P., Gallo, L., Pagnacco, G. and Bittante, G. (2008). Effects of composite β- and κ-casein genotypes on milk coagulation, quality, and yield traits in Italian Holstein cows. *Journal of Dairy Science*, 91: 4022-4027.

Connor, W. E. (2000). Importance of n− 3 fatty acids in health and disease. *The American Journal of Clinical Nutrition*, 71: 171S-175S.

Connor, W. E. (1997). The beneficial effects of omega-3 fatty acids: cardiovascular disease and neurodevelopment. *Current Opinion in Lipidology*, 8: 1-3.

Corredig, M., Roesch, R. and Dalgleish, D. (2003). Production of a novel ingredient from buttermilk. *Journal of Dairy Science*, 86: 2744-2750.

da Silva-Kazama, D. C., Cortes, C., Kazama, R., Benchaar, C., Santos, G. T. D., Zeoula, L. M. and Petit, H. V. (2011). Ruminal fermentation characteristics and fatty acid profile of ruminal fluid and milk of dairy cows fed flaxseed hulls supplemented with monensin. *Journal of Dairy Research*, 78: 56-62.

Dalgleish, D. G. and Corredig, M. (2012). The structure of the casein micelle of milk and its changes during processing. *Annual Review of Food Science and Technology*, 3: 449-467.

Dalgleish, D. G. (2011). On the structural models of bovine casein micelles-review and possible improvements. *Soft Matter*, 7: 2265-2272.

Dalgleish, D. G. (1993). Bovine milk protein properties and the manufacturing quality of milk. *Livestock Production Science*, 35: 75-93.

D'Ambrosio, C., Arena, S., Salzano, A. M., Renzone, G., Ledda, L. and Scaloni, A. (2008). A proteomic characterization of water buffalo milk fractions describing PTM of major species and the identification of minor components involved in nutrient delivery and defense against pathogens. *Proteomics*, 8: 3657-3666.

Devle, H., Ulleberg, E. K., Naess-Andresen, C. F., Rukke, E.-O., Vegarud, G. and Ekeberg, D. (2014). Reciprocal interacting effects of proteins and lipids during ex vivo digestion of bovine milk. *International Dairy Journal*, 36: 6-13.

Devle, H., Vetti, I., Naess-Andresen, C. F., Rukke, E.-O., Vegarud, G. and Ekeberg, D. (2012). A comparative study of fatty acid profiles in ruminant and non-ruminant milk. *European Journal of Lipid Science and Technology*, 114: 1036-1043.

Devold, T. G., Brovold, M. J., Langsrud, T. and Vegarud, G. E. (2000). Size of native and heated casein micelles, content of protein and minerals in milk from Norwegian Red Cattle—effect of milk protein polymorphism and different feeding regimes. *International Dairy Journal*, 10: 313-323.

Di Luccia, A., Picariello, G., Trani, A., Alviti, G., Loizzo, P., Faccia, M. and Addeo, F. (2009). Occurrence of β-casein fragments in cold-stored and curdled river buffalo (Bubalus bubalis L.) milk. *Journal of Dairy Science*, 92: 1319-1329.

Dilzer, A. and Park, Y. (2012). Implication of conjugated linoleic acid (CLA) in human health. *Critical Reviews in Food Science and Nutrition*, 52: 488-513.

Djousse, L., Pankow, J. S., Eckfeldt, J. H., Folsom, A. R., Hopkins, P. N., Province, M. A., Hong, Y. and Ellison, R. C. (2001). Relation between dietary linolenic acid and coronary artery disease in the National Heart, Lung, and Blood Institute Family Heart Study. *The American Journal of Clinical Nutrition*, 74: 612-619.

Downs, M. L., Kabourek, J. L., Baumert, J. L. and Taylor, S. L. (2013). Milk Protein Allergy. In: *Milk and Dairy Products in Human Nutrition: Production, Composition and Health* (Ed. Y. W. Park and G. F. W. Haenlein), John Wiley & Sons, Oxford. pp. 111-128.

Dressman, J., Amidon, G., Reppas, C. and Shah, V. (1998). Dissolution Testing as a Prognostic Tool for Oral Drug Absorption: Immediate Release Dosage Forms. *Pharmaceutical Research*, 15: 11-22.

Ekmekcioglu, C. (2002). A physiological approach for preparing and conducting intestinal bioavailability studies using experimental systems. *Food Chemistry*, 76: 225-230.

Elliott, R. B., Harris, D. P., Hill, J. P., Bibby, N. J. and Wasmuth, H. E. (1999). Type I (insulin-dependent) diabetes mellitus and cow milk: casein variant consumption. *Diabetologia*, 42: 292-296.

El-Zahar, K., Sitohy, M., Choiset, Y., Métro, F., Haertlé, T. and Chobert, J.-M. (2005). Peptic hydrolysis of ovine β-lactoglobulin and α-lactalbumin Exceptional susceptibility of native ovine β-lactoglobulin to pepsinolysis. *International Dairy Journal*, 15: 17-27.

Eriksen, E. K., Holm, H., Jensen, E., Aaboe, R., Devold, T. G., Jacobsen, M. and Vegarud, G. E. (2010). Different digestion of caprine whey proteins by human and porcine gastrointestinal enzymes. *British Journal of Nutrition*, 104: 374-381.

Escudero, E., Sentandreu, M. A. n. and Toldra, F. (2010). Characterization of peptides released by in vitro digestion of pork meat. *Journal of Agricultural and Food Chemistry*, 58: 5160-5165.

Falchero, L., Lombardi, G., Gorlier, A., Lonati, M., Odoardi, M. and Cavallero, A. (2010). Variation in fatty acid composition of milk and cheese from cows grazed on two alpine pastures. *Dairy Science & Technology*, 90: 657-672.

FAOSTAT, The Statistics Division of the Food and Agriculture Organization. 2012a. http://faostat3.fao.org/faostat-gateway/go/to/home/E. Accessed on 26.03.2014.

FAOSTAT, The Statistics Division of the Food and Agriculture Organization. 2012b. http://faostat.fao.org/site/339/default.aspx. Accessed on 14.04.2014.

Farrell Jr, H., Malin, E., Brown, E. and Qi, P. (2006). Casein micelle structure: What can be learned from milk synthesis and structural biology? *Current Opinion in Colloid & Interface Science*, 11: 135-147.

Farrell Jr, H., Jimenez-Flores, R., Bleck, G., Brown, E., Butler, J., Creamer, L., Hicks, C., Hollar, C., Ng-Kwai-Hang, K. and Swaisgood, H. (2004). Nomenclature of the proteins of cows' milk—sixth revision. *Journal of dairy science*, 87: 1641-1674.

Feligini, M., Bonizzi, I., Buffoni, J. N., Cosenza, G. and Ramunno, L. (2009). Identification and Quantification of αS1, αS2, β, and κ-Caseins in Water Buffalo Milk by Reverse Phase-High Performance Liquid Chromatography and Mass Spectrometry. *Journal of Agricultural and Food Chemistry*, 57: 2988-2992.

Feng, S., Lock, A. L. and Garnsworthy, P. C. (2004). Technical Note: A Rapid Lipid Separation Method for Determining Fatty Acid Composition of Milk. *Journal of Dairy Science*, 87: 3785-3788.

Fernandez, S., Rodier, J.-D., Ritter, N., Mahler, B., Demarne, F., Carrière, F. and Jannin, V. (2008). Lipolysis of the semi-solid self-emulsifying excipient Gelucire® 44/14 by digestive lipases. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, 1781 (8): 367-375.

Fernandez, S., Jannin, V., Rodier, J.-D., Ritter, N., Mahler, B. and Carriere, F. (2007). Comparative study on digestive lipase activities on the self emulsifying excipient Labrasol®, medium chain glycerides and PEG esters. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, 1771: 633-640.

Folch, J., Lees, M. and Sloane-Stanley, G. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *Journal of biological Chemistry*, 226: 497-509.

Fox, P. F. and McSweeney, P. L. H. 2003. Advanced Dairy Chemistry. Volume 1 proteins. 3rd Edition, NY, USA: Kluwer Academic/Plenum Publishers.

Fox, P. F. and McSweeney, P. L. H. 1998. Dairy Chemistry and Biochemistry. Blackie Academic and Professional, London, UK.

Furlund, C. B., Ulleberg, E. K., Devold, T. G., Flengsrud, R., Jacobsen, M., Sekse, C., Holm, H. and Vegarud, G. E. (2013). Identification of lactoferrin peptides generated by digestion with human gastrointestinal enzymes. *Journal of Dairy Science*, 96: 75-88.

Furlund, C. B., Kristoffersen, A. B., Devold, T. G., Vegarud, G. E. and Jonassen, C. M. (2012). Bovine lactoferrin digested with human gastrointestinal enzymes inhibits replication of human echovirus 5 in cell culture. *Nutrition Research*, 32: 503-513.

Fujinaga, M., Chernaia, M. M., Mosimann, S. C., James, M. N. G. and Tarasova, N. I. (1995). Crystal structure of human pepsin and its complex with pepstatin. *Protein Science*, 4: 960-972.

Gallier, S., Ye, A. and Singh, H. (2012). Structural changes of bovine milk fat globules during in vitro digestion. *Journal of Dairy Science*, 95: 3579-3592.

Gallier, S., Cui, J., Olson, T. D., Rutherfurd, S. M., Ye, A., Moughan, P. J. and Singh, H. (2013). In vivo digestion of bovine milk fat globules: Effect of processing and interfacial structural changes. I. Gastric digestion. *Food Chemistry*, 141: 3273-3281.

Ganzler, K., Salgo, A. and Valko, K. (1986). Microwave extraction : A novel sample preparation method for chromatography. *Journal of Chromatography A*, 371: 299-306.

Garg, M., Wood, L., Singh, H. and Moughan, P. (2006). Means of Delivering recommended levels of long chain n-3 polyunsaturated fatty acids in human diets. *Journal of Food Science*, 71: R66-R71.

Gargouri, Y., Moreau, H. and Verger, R. (1989). Gastric lipases: biochemical and physiological studies. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism*, 1006: 255-271.

Gargouri, Y., Pieroni, G., Lowe, P. A., Sarda, L. and Verger, R. (1986a). Human gastric lipase. *European Journal of Biochemistry*, 156: 305-310.

Gargouri, Y., Pieroni, G., Riviere, C., Sauniere, J.-F., Lowe, P. A., Sarda, L. and Verger, R. (1986b). Kinetic assay of human gastric lipase on short- and long-chain triacylglycerol emulsions. *Gastroenterology*, 91: 919-925.

Gass, J., Vora, H., Hofmann, A. F., Gray, G. M. and Khosla, C. (2007). Enhancement of dietary protein digestion by conjugated bile acids. *Gastroenterology*, 133: 16-23.

Gates, P. (2005). Mass spectrometry resource – sector mass analysis. School of chemistry, The University of Bristol. http://www.chm.bris.ac.uk/ms/theory/sector-massspec.html. Accessed on 12.04.2014.

GED, General Economics Division, Planning Commission, GoB, 2010. Livestock and poultry. In: Outline perspective of Bangladesh 2010-2021. pp. 51.

Ghosh, C. P., Nagpaul, P. K., Prasad, S. (2004). Sub-clinical mastitis in cattle and buffaloes and its impact on somatic cell count and milk composition. *Indian Journal of Dairy Science*, 57:329–333.

Glantz, M., Lindmark Mansson, H., Stalhammar, H. and Paulsson, M. (2012). Effect of polymorphisms in the leptin, leptin receptor and acyl-CoA:diacylglycerol acyltransferase 1 (DGAT1) genes and genetic polymorphism of milk proteins on bovine milk composition. *Journal of Dairy Research*, 79: 110-118.

Glantz, M., Devold, T., Vegarud, G., Lindmark Mansson, H., Stalhammar, H. and Paulsson, M. (2010). Importance of casein micelle size and milk composition for milk gelation. *Journal of Dairy Science*, 93: 1444-1451.

Glantz, M., Lindmark Mansson, H., Stalhammar, H., Barstrom, L. O., Frojelin, M., Knutsson, A., Teluk, C. and Paulsson, M. (2009). Effects of animal selection on milk composition and processability. *Journal of Dairy Science*, 92: 4589-4603.

Goodman, B. E. (2010). Insights into digestion and absorption of major nutrients in humans. *Advances in Physiology Education*, 34: 44-53.

Gross, J., van Dorland, H. A., Bruckmaier, R. M. and Schwarz, F. J. (2011). Milk fatty acid profile related to energy balance in dairy cows. *Journal of Dairy Research*, 78: 479-488.

Grummer, R. R. (1991). Effect of feed on the composition of milk fat. *Journal of Dairy Science*, 74: 3244-3257.

Guerra, A., Etienne-Mesmin, L., Livrelli, V., Denis, S., Blanquet-Diot, S. and Alric, M. (2012). Relevance and challenges in modeling human gastric and small intestinal digestion. *Trends in Biotechnology*, 30: 591-600.

Guil-Guerrero, J. L. and Belarbi, E.-H. (2001). Purification process for cod liver oil polyunsaturated fatty acids. *Journal of the American Oil Chemists' Society*, 78: 477-484.

Hallen, E., Lunden, A., Tyriseva, A.-M., Westerlind, M. and Andren, A. (2010). Composition of poorly and non-coagulating bovine milk and effect of calcium addition. *Journal of Dairy Research*, 77: 398-403.

Hamer, H. M., Jonkers, D., Venema, K., Vanhoutvin, S., Troost, F. J., and Brummer, R. J. (2008). Review article: the role of butyrate on colonic function. *Alimentary Pharmacology and Therapeutics*, 104:104-119.

Hara, A. and Radin, N. S. (1978). Lipid extraction of tissues with a low-toxicity solvent. *Analytical biochemistry*, 90: 420-426.

Haug, A., Hostmark, A. T. and Harstad, O. M. (2007). Bovine milk in human nutrition–a review. *Lipids in Health and Disease*, DOI: 10.1186/1476-511X-6-25.

Hayes, M., Stanton, C., Fitzgerald, G. F. and Ross, R. P. (2007). Putting microbes to work: Dairy fermentation, cell factories and bioactive peptides. Part II: Bioactive peptide functions. *Biotechnology Journal*, 2 (4): 435-449.

Hayes, K. and Khosla, P. (1992). Dietary fatty acid thresholds and cholesterolemia. *The FASEB Journal*, 6: 2600-2607.

Hayes, J. R., Pence, D. H., Scheinbach, S., D'Amelia, R. P., Klemann, L. P., Wilson, N. H. and Finley, J. W. (1994). Review of triacylglycerol digestion, absorption, and metabolism with respect to SALATRIM triacylglycerols. *Journal of Agricultural and Food Chemistry*, 42: 474-483.

Heck, J., Schennink, A., Van Valenberg, H., Bovenhuis, H., Visker, M., Van Arendonk, J. and Van Hooijdonk, A. (2009a). Effects of milk protein variants on the protein composition of bovine milk. *Journal of Dairy Science*, 92: 1192-1202.

Heck, J., Van Valenberg, H., Dijkstra, J. and Van Hooijdonk, A. (2009b). Seasonal variation in the Dutch bovine raw milk composition. *Journal of Dairy Science*, 92: 4745-4755.

Hemme, T., Garcia, O. and Khan, A. (2004). Overview – Milk production in Bangladsh. In: A review of milk production in Bangladesh with particular emphasis on small-scale producers. PPLPI working paper no. 7. pp. 4-7.

Hiraoka, Y., Segawa, T., Kuwajima, K., Sugai, S. and Murai, N. (1980). α-lactalbumin: a calcium metalloprotein. *Biochemical and Biophysical Research Communications*, 95: 1098-1104.

Horne, D. S. (2009). Casein micelle structure and stability. In: *Milk proteins: From expression to food* (Ed. Thompson, A., Boland, M. and Singh, H.), Elsevier Inc. pp: 133-162.

Horne, D. S. (1998). Casein Interactions: Casting Light on the Black Boxes, the Structure in Dairy Products. *International Dairy Journal*, 8: 171-177.

Hur, S. J., Lim, B. O., Decker, E. A. and McClements, D. J. (2011). In vitro human digestion models for food applications. *Food Chemistry*, 125: 1-12.

IDF. (2010). The world dairy situation 2010. *Bulletin of the IDF*, 446/2010. International Dairy Federation, Brussels, Belgium.

Ikonen, T., Ahlfors, K., Kempe, R., Ojala, M. and Ruottinen, O. (1999). Genetic Parameters for the Milk Coagulation Properties and Prevalence of Noncoagulating Milk in Finnish Dairy Cows. *Journal of Dairy Science*, 82: 205-214.

Inglingstad, R. A., Devold, T. G., Eriksen, E. K., Holm, H., Jacobsen, M., Liland, K. H., Rukke, E. O. and Vegarud, G. E. (2010). Comparison of the digestion of caseins and whey proteins in equine, bovine, caprine and human milks by human gastrointestinal enzymes. *Dairy Science & Technology*, 90: 549-563.

Islam, M. R., Hassan, M., Siddiki, M., Khan, M. and Islam, M. A. (2008). Determination of quality of milk from different genotype of dairy cows. *Bangladesh Journal of Animal Science*, 37: 52-56.

Islam, M. A., Uddin, M., Jahan, R., Wadud, A. and Sarkar, M. (2013). Metabolites in the milk of buffalo, Holstein cross, indigenous and Red Chittagong Cattle of Bangladesh. *Bangladesh Journal of Animal Science*, 42 (2): 152-157.

ISO-IDF. (2001). Milk and milk products—Extraction methods for lipids and liposoluble compound. International Standard ISO 14156-IDF 172, International Dairy Federation, Brussels, Belgium.

Jantratid, E., Janssen, N., Reppas, C. and Dressman, J. (2008). Dissolution media simulating conditions in the proximal human gastrointestinal tract: An update. *Pharmaceutical Research*, 25: 1663-1676.

Jensen, R. G., Ferris, A. M. and Lammi-Keefe, C. J. (1991). The composition of milk fat. *Journal of Dairy Science*, 74: 3228-3243.

Jensen, R. G. (2002). The composition of bovine milk lipids: January 1995 to December 2000. *Journal of Dairy Science*, 85: 295-350.

Jones, A., Balan, K., Jenkins, S., Sutton, R., Critchley, M. and Roberts, N. (1993). Assay of gastricsin and individual pepsins in human gastric juice. *Journal of Clinical Pathology*, 46: 254-258.

Jornvall, H. and Persson, B. (1983). Amino acid sequence restriction in relation to proteolysis. *Bioscience Reports*, 3: 225-232.

Kalantzi, L., Goumas, K., Kalioras, V., Abrahamsson, B., Dressman, J. B. and Reppas, C. (2006). Characterization of the human upper gastrointestinal contents under conditions simulating bioavailability/bioequivalence studies. *Pharmaceutical Research*, 23: 165-176.

Kaluzny, M. A., Duncan, L. A., Merritt, M. V. and Epps, D. E. (1985). Rapid separation of lipid classes in high yield and purity using bonded phase columns. *Journal of Lipid Research*, 26: 135-140.

Kathirvelan, C. and Tyagi, A. K. (2009). Conjugated linoleic acid content of milk from buffaloes fed a mustard oil-based diet. *International Journal of Dairy Technology*, 62: 141-146.

Kaye, J. L. (2011). Review of paediatric gastrointestinal physiology data relevant to oral drug delivery. *International Journal of Clinical Pharmacy*, 33: 20-24.

Kaylegian, K. E., and Lindsay, R. C. 1995. Milk fat usage and modification. In: Handbook of milk fat fractionation technology and application. Am. Oil Chem. Soc. Press, Champaign, IL, USA. pp. 1-18

Keenan, T. W., Patton, S. (1995). The Milk Lipid Globule Membrane. In: *Handbook of Milk Composition*, (Ed. R. G. Jensen), Academic Press, San Diego, CA, USA. pp 5-50.

Khan, M., Islam, M. and Siddiki, M. (2007). Physical and chemical composition of swamp and water buffalo milk: a comparative study. *Italian Journal of Animal Science*, 6 (2s): 1067-1070.

Kinsella, J. E., Lokesh, B. and Stone, R. A. (1990). Dietary omega-3 polyunsaturated fatty acids and amelioration of cardiovascular disease: possible mechanisms. *The American Journal of Clinical Nutrition*, 52: 1-28.

Kitabatake, N. and Kinekawa, Y.-I. (1998). Digestibility of bovine milk whey protein and β-lactoglobulin in vitro and in vivo. *Journal of Agricultural and Food Chemistry*, 46: 4917-4923.

Kolanowski, W. and Laufenberg, G. (2006). Enrichment of food products with polyunsaturated fatty acids by fish oil addition. *European Food Research and Technology*, 222: 472-477.

Kontopidis, G., Holt, C. and Sawyer, L. (2004). Invited Review: β-lactoglobulin: Binding Properties, Structure, and Function. *Journal of Dairy Science*, 87: 785-796.

Kopf-Bolanz, K. A., Schwander, F., Gijs, M., Vergeres, G., Portmann, R. and Egger, L. (2012). Validation of an in vitro digestive system for studying macronutrient decomposition in humans. *The Journal of Nutrition*, 142: 245-250.

Korhonen, H. and Pihlanto, A. (2006). Bioactive peptides: production and functionality. *International Dairy Journal*, 16: 945-960.

Laffargue, A., de Kochko, A. and Dussert, S. (2007). Development of solid-phase extraction and methylation procedures to analyse free fatty acids in lipid-rich seeds. *Plant Physiology and Biochemistry*, 45: 250-257.

Lara-Villoslada, F., Olivares, M. and Xaus, J. (2005). The Balance Between Caseins and Whey Proteins in Cow's Milk Determines its Allergenicity. *Journal of Dairy Science*, 88: 1654-1660.

Le Maux, S., Brodkorb, A., Croguennec, T., Hennessy, A. A., Bouhallab, S. and Giblin, L. (2013). β-lactoglobulin-linoleate complexes: In vitro digestion and the role of protein in fatty acid uptake. *Journal of Dairy Science*, 96: 4258-4268.

Leonil, J., Gagnaire, V., Molle, D., Pezennec, S. and Bouhallab, S. d. (2000). Application of chromatography and mass spectrometry to the characterization of food proteins and derived peptides. *Journal of Chromatography A*, 881: 1-21.

Li, X., Luo, Z., Chen, H. and Cao, Y. (2008). Isolation and antigenicity evaluation of β-lactoglobulin from buffalo milk. *African Journal of Biotechnology*, 7: 2258-2264.

Lock, A. L. and K. J. Shingfield. 2004. Optimising milk composition. In: Dairying-Using Science to Meet Consumers' Needs (Ed. E. Kebreab, J. Mills and D. E. Beever). Occ. Pub. No. 29, Brit. Soc. Anim. Sci., Nottingham University Press, Loughborough, UK. pp. 107-188.

Lopez, C., Briard-Bion, V., Menard, O., Rousseau, F., Pradel, P. and Besle, J. M. (2008). Phospholipid, sphingolipid, and fatty acid compositions of the milk fat globule membrane are modified by diet. *Journal of Agricultural and Food Chemistry*, 56: 5226-5236.

Luna, P., Juárez, M. and de la Fuente, M. A. (2005). Validation of a rapid milk fat separation method to determine the fatty acid profile by Gas Chromatography. *Journal of Dairy Science*, 88: 3377-3381.

Macheboeuf, D., Coulon, J.-B. and D'Hour, P. (1993). Effect of breed, protein genetic variants and feeding on cows' milk coagulation properties. *Journal of Dairy Research*, 60: 43-54.

Maghsoudi, Z., Askari, G., Ghiasvand, R., Khorvash, F., Iraj, B., Shokri, N. and Darvishi, L. (2013). Dairy Consumption and Stroke Risk. *International Journal of Preventive Medicine*, 4: S294-S299.

Mamone, G., Picariello, G., Caira, S., Addeo, F. and Ferranti, P. (2009). Analysis of food proteins and peptides by mass spectrometry-based techniques. *Journal of Chromatography A*, 1216: 7130-7142.

Mansson, H. L. (2008). Fatty acids in bovine milk fat. *Food & Nutrition Research*, 52, DOI: 10.3402/fnr.v52i0.1821.

McGuire, M. and McGuire, M. (2000). Conjugated linoleic acid (CLA): A ruminant fatty acid with beneficial effects on human health. *Journal of Animal Science*, 77: 1-8.

McClements, D., Decker, E., Park, Y. and Weiss, J. (2008). Designing Food Structure to Control Stability, Digestion, Release and Absorption of Lipophilic Food Components. *Food Biophysics*, 3: 219-228.

McCloy, R., Greenberg, G. and Baron, J. (1984). Duodenal pH in health and duodenal ulcer disease: effect of a meal, Coca-Cola, smoking, and cimetidine. *Gut*, 25: 386-392.

Medhammar, E., Wijesinha-Bettoni, R., Stadlmayr, B., Nilsson, E., Charrondiere, U. R. and Burlingame, B. (2012). Composition of milk from minor dairy animals and buffalo breeds: a biodiversity perspective. *Journal of the Science of Food and Agriculture*, 92: 445-474.

Meisel, H. (1998). Overview on milk protein-derived peptides. *International Dairy Journal*, 8: 363-373.

Melia, S., Losi, G. and Castagnetti, G. B. (2009). The influence of milk κ-casein and β-lactoglobulin phenotypes on fatty acid composition of milk from Reggiana cows. *Dairy Science & Technology*, 89: 115-122.

Menard, O., Ahmad, S., Rousseau, F., Briard-Bion, V., Gaucheron, F. and Lopez, C. (2010). Buffalo vs. cow milk fat globules: Size distribution, zeta-potential, compositions in total fatty acids and in polar lipids from the milk fat globule membrane. *Food Chemistry*, 120: 544-551.

Michalski, A., Damoc, E., Hauschild, J.-P., Lange, O., Wieghaus, A., Makarov, A., Nagaraj, N., Cox, J., Mann, M. and Horning, S. (2011). Mass spectrometry-based proteomics using Q Exactive, a high-performance benchtop quadrupole orbitrap mass spectrometer. *Molecular and Cellular Proteomics*, DOI:10.1074/mcp.M111.011015.

Mills, S., Ross, R., Hill, C., Fitzgerald, G. and Stanton, C. (2011). Milk intelligence: Mining milk for bioactive substances associated with human health. *International Dairy Journal*, 21: 377-401.

Mondello, L., Tranchida, P. Q., Dugo, P. and Dugo, G. (2006). Rapid, micro-scale preparation and very fast gas chromatographic separation of cod liver oil fatty acid methyl esters. *Journal of Pharmaceutical and Biomedical Analysis*, 41: 1566-1570.

Moreau, H., Gargouri, Y., Lecat, D., Junien, J. L. and Verger, R. (1988). Screening of preduodenal lipases in several mammals. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism*, 959: 247-252.

Mota, M. V. T., Ferreira, I. M. P. L. V. O., Oliveira, M. B. P., Rocha, C., Teixeira, J. A., Torres, D. and Gonçalves, M. P. (2006). Trypsin hydrolysis of whey protein concentrates: Characterization using multivariate data analysis. *Food Chemistry*, 94: 278-286.

Mu, H. and Porsgaard, T. (2005). The metabolism of structured triacylglycerols. *Progress in Lipid Research*, 44 (6): 430-448.

Mu, H. and Hoy, C.-E. (2004). The digestion of dietary triacylglycerols. *Progress in Lipid Research*, 43: 105-133.

Murphy, R. C. and Axelsen, P. H. (2011). Mass spectrometric analysis of long-chain lipids. *Mass Spectrometry Reviews*, 30: 579-599.

Napolano, R., Potena, A., Fusco, G., Caso, C., Campanile, G., Di Palo, R. and Zicarelli, F. (2007). Relationships between buffalo milk components and curd characteristics. *Italian Journal of Animal Science*, 6: 1092-1095.

Natale, M., Bisson, C., Monti, G., Peltran, A., Perono Garoffo, L., Valentini, S., Fabris, C., Bertino, E., Coscia, A. and Conti, A. (2004). Cow's milk allergens identification by two-dimensional immunoblotting and mass spectrometry. *Molecular Nutrition & Food Research*, 48: 363-369.

Ng-Kwai-Hang, K. F., Monardes, H. G. and Hayes, J. F. (1990). Association between genetic polymorphism of milk proteins and production traits during three lactations. *Journal of Dairy Science*, 73: 3414-3420.

Ng-Kwai-Hang, K., Hayes, J., Moxley, J. and Monardes, H. (1986). Relationships between milk protein polymorphisms and major milk constituents in Holstein-Friesian cows. *Journal of Dairy Science*, 69: 22-26.

Ng-Kwai-Hang, K., Hayes, J., Moxley, J. and Monardes, H. (1984). Association of genetic variants of casein and milk serum proteins with milk, fat, and protein production by dairy cattle. *Journal of Dairy Science*, 67: 835-840.

Nogalski, Z., Wronski, M., Sobczuk-Szul, M., Mochol, M. and Pogorzelska, P. (2012). The Effect of Body Energy Reserve Mobilization on the Fatty Acid Profile of Milk in High-yielding Cows. *Asian-Australasian Journal of Animal Sciences*, 25: 1712-1720.

O'Donnell, R., Holland, J., Deeth, H. and Alewood, P. (2004). Milk proteomics. *International Dairy Journal*, 14: 1013-1023.

Ooman, S. and Ganguli, N. C. (1973). Changes in the casein micelles of buffalo milk during the transition from colostrum to normal milk. *Indian Journal of Dairy Science*, 26:200–206.

Panchaud, A., Affolter, M. and Kussmann, M. (2012). Mass spectrometry for nutritional peptidomics: how to analyze food bioactives and their health effects. *Journal of Proteomics*, 75: 3546-3559.

Pandya, A. J. and Khan, M. M. H. 2006. Buffalo milk utilization for dairy prodcuts. In: Handbook of milk of non-bovine mammals (Ed. By Park, Y. W. and Haenlein, F. W.). Blackwell Publishing, Iowa, USA. pp. 215-256.

Pare, J., Matni, G., Belanger, J., Li, K., Rule, C., Thibert, B., Yaylayan, V., Liu, Z., Mathe, D. and Jacquault, P. (1996). Use of the microwave-assisted process in extraction of fat from meat, dairy, and egg products under atmospheric pressure conditions. *Journal of AOAC International*, 80: 928-933.

Parodi, P. W. (1975). Detection of acetodiacylglycerols in milkfat lipids by thin-layer chromatography. *Journal of Chromatography A*, 111: 223-226.

Parodi, P. W. (1979). Stereospecific distribution of fatty acids in bovine milk fat triglycerides. *Journal of Dairy Research*, 46: 75-81.

Parodi, P. W. (1997). Cows' milk fat components as potential anticarcinogenic agents. *Journal of Nutrition*, 127: 1055-1060.

Pascal, G. (1996). Les apports quotidiens recommandés en lipides et en acides gras: Lipides et santé. *OCL. Oléagineux, corps gras, lipides*, 3: 205-210.

Patino, E., Pochon, D., Faisal, E., Cedres, J., Mendez, F., Stefani, C. and Crudeli, G. (2007). Influence of breed, year season and lactation stage on the buffalo milk mineral content. *Italian Journal of Animal Science*, 6: 1046-1049.

Patton, J. S., Rigler, M. W., Liao, T. H., Hamosh, P. and Hamosh, M. (1982). Hydrolysis of triacylglycerol emulsions by lingual lipase a microscopic study. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism*, 712: 400-407.

Perez, M. D. and Calvo, M. (1995). Interaction of β-lactoglobulin with Retinol and Fatty Acids and Its Role as a Possible Biological Function for This Protein: A Review. *Journal of Dairy Science*, 78: 978-988.

Permyakov, E. A. and Berliner, L. J. (2000). α-lactalbumin: structure and function. *FEBS Letters*, 473: 269-274.

Pernet, F., Pelletier, C. J. and Milley, J. (2006). Comparison of three solid-phase extraction methods for fatty acid analysis of lipid fractions in tissues of marine bivalves. *Journal of Chromatography A*, 1137: 127-137.

Petrone, G., Conte, M. P., Longhi, C., di Santo, S., Superti, F., Ammendolia, M. G., et al. (1998). Natural milk fatty acids affect survival and invasiveness of *Listeria monocytogenes*. *Letters in Applied Microbiology*, 27: 362-368.

Pfeuffer, M., and Schrezenmeir, J. (2007). Milk and the metabolic syndrome. *Obesity Reviews*, 8: 109-118.

Pihlanto-Leppala, A. (2001). Bioactive peptides derived from bovine whey proteins: opioid and ace-inhibitory peptides. *Trends in Food Science and Technology*, 11: 347-356.

Pihlanto-Leppala, A., Koskinen, P., Piilola, K., Tupasela, T. and Korhonen, H. (2000). Angiotensin I-converting enzyme inhibitory properties of whey protein digests: concentration and characterization of active peptides. *Journal of Dairy Research*, 67: 53-64.

Pihlanto-Leppala, A., Rokka, T. and Korhonen, H. (1998). Angiotensin I converting enzyme inhibitory peptides derived from bovine milk proteins. *International Dairy Journal*, 8: 325-331.

Pintado, M. E. and Malcata, F. X. (2000). Characterization of whey cheese packaged under vacuum. *Journal of Food Protection*, 63: 216-221.

Pretto, D., De Marchi, M., Penasa, M. and Cassandro, M. (2013). Effect of milk composition and coagulation traits on Grana Padano cheese yield under field conditions. *Journal of Dairy Research*, 80: 1-5.

Quehenberger, O., Armando, A. M. and Dennis, E. A. (2011). High sensitivity quantitative lipidomics analysis of fatty acids in biological samples by gas chromatography–mass spectrometry. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, 1811: 648-656.

Qureshi, M. S., Mushtaq, A., Khan, S., Habib, G. and Swati, Z. A. (2010). Variation in milk fatty acids composition with body condition in dairy buffaloes (Bubalus bubalis). *Asian-Australasian Journal of Animal Sciences*, 23: 340-345.

Randolph, H. E. and Erwin, R. E. (1974). Influence of mastitis on properties of milk. X. Fatty acid composition. *Journal of Dairy Science*, 57: 865-868.

Ratnayake, W. N. and Galli, C. (2009). Fat and fatty acid terminology, methods of analysis and fat digestion and metabolism: a background review paper. *Annals of Nutrition and Metabolism*, 55: 8-43.

Reklewska, B., Oprzadek, A., Reklewski, Z., Panicke, L., Kuczynska, B. and Oprzadek, J. (2002). Alternative for modifying the fatty acid composition and decreasing the cholesterol level in the milk of cows. *Livestock Production Science*, 76: 235-243.

Ren, D., Chen, B., Chen, Y., Miao, S. and Liu, J. (2013). The effects of κ-casein polymorphism on the texture and functional properties of mozzarella cheese. *International Dairy Journal*, 31: 65-69.

Ren, J., Stuart, D. I. and Acharya, K. R. (1993). Alpha-lactalbumin possesses a distinct zinc binding site. *Journal of Biological Chemistry*, 268: 19292-19298.

Rimestad, A., Borgejordet, A., Verterhus, K., Sygnestveit, K., Loken, E., Trygg, K., Pollestad, M., Lund-Larsen, K., Omholt-Jensen, G. and Nordbotten, A. (2001). *Den Store Matvaretabellen Gyldendal Norsk forlag ASA*: Oslo.

Roberts, N. B. (2006). Review article: Human pepsins – their multiplicity, function and role in reflux disease. *Alimentary Pharmacology and Therapeutics*, 24: 2-9.

Roberts, N. and Taylor, W. (1979). Action of human pepsins 1, 2, 3 and 5 on the oxidized B-chain of insulin. *Biochemical Journal*, 179: 183-189.

Rombaut, R. and Dewettinck, K. (2006). Properties, analysis and purification of milk polar lipids. *International Dairy Journal*, 16: 1362-1373.

Ruiz-Gutierrez, V. and Perez-Camino, M. C. (2000). Update on solid-phase extraction for the analysis of lipid classes and related compounds. *Journal of Chromatography A*, 885: 321-341.

Ruiz, J., Antequera, T., Andres, A., Petron, M. and Muriel, E. (2004). Improvement of a solid phase extraction method for analysis of lipid fractions in muscle foods. *Analytica Chimica Acta*, 520: 201-205.

Sakurai, K., Konuma, T., Yagi, M. and Goto, Y. (2009). Structural dynamics and folding of β-lactoglobulin probed by heteronuclear NMR. *Biochimica et Biophysica Acta (BBA) - General Subjects*, 1790: 527-537.

Saladin, K. S. 2010. The digestive system. In: *Anatomy and Physiology: The Unity of Form and Function*, 5th Ed. McGraw-Hill, NY-USA. pp. 965-1012.

Salami, M., Moosavi-Movahedi, A. A., Moosavi-Movahedi, F., Ehsani, M. R., Yousefi, R., Farhadi, M., Niasari-Naslaji, A., Saboury, A. A., Chobert, J.-M. and Haertle, T. (2011). Biological activity of camel milk casein following enzymatic digestion. *Journal of Dairy Research*, 78: 471-478.

Schafberg, R., Schmidt, R., Thiele, M. and Swalve, H. (2007). Fat globule size distribution in milk of a German buffalo herd. *Italian Journal of Animal Science*, 6: 1080-1083.

Scheppach,W., Bartram, P., Richter, A., Richter, F., Liepold, H., Dusel, G., et al. (1992). Effect of short chain fatty acids on human colonic mucosa in vitro. *Journal of Parenteral and Enteral Nutrition*, 16: 43-48.

Schonfeldt, H. C., Hall, N. G. and Smit, L. E. (2012). The need for country specific composition data on milk. *Food Research International*, 47: 207-209.

Selo, Clement, Bernard, Chatel, Creminon, Peltre and Wal. (1999). Allergy to bovine β-lactoglobulin: specificity of human IgE to tryptic peptides. *Clinical & Experimental Allergy*, 29: 1055-1063.

Sheehan, W. J. and Phipatanakul, W. (2009). Tolerance to water buffalo milk in a child with cow milk allergy. *Annals of Allergy, Asthma & Immunology*, 102: 349.

Sjaastad, O. V., Hove, K. and Sand, O. (2003). *Physiology of Domestic Animals*, Scandanavian Veterinary Press, Oslo, Norway.

Spitsberg, V. (2005). Invited Review: Bovine milk fat globule membrane as a potential nutraceutical. *Journal of Dairy Science*, 88: 2289-2294.

St-Gelais, D. and Hache, S. (2005). Effect of β-casein concentration in cheese milk on rennet coagulation properties, cheese composition and cheese ripening. *Food Research International*, 38 (5): 523-531.

Stoop, W. M., Bovenhuis, H., Heck, J. M. L. and van Arendonk, J. A. M. (2009). Effect of lactation stage and energy status on milk fat composition of Holstein-Friesian cows. *Journal of Dairy Science*, 92: 1469-1478.

Talpur, F. N., Bhanger, M. I., Khooharo, A. A. and Memon, G. Z. (2008). Seasonal variation in fatty acid composition of milk from ruminants reared under the traditional feeding system of Sindh, Pakistan. *Livestock Science*, 118: 166-172.

Tareque, A. M. M. and Chowdhury, S. M. Z. H. (2010). Agricultural research priority: vision-2030 and beyond – sub sector: Livestock. Bangladesh Agricultural Research Council, Dhaka, Bangladesh.

Thurnhofer, S. and Vetter, W. (2005). A Gas chromatography/electron ionization−mass spectrometry−selected ion monitoring method for determining the fatty acid pattern in food after formation of fatty acid methyl esters. *Journal of Agricultural and Food Chemistry*, 53: 8896-8903.

Tidona, F., Criscione, A., Devold, T. G., Bordonaro, S., Marletta, D. and Vegarud, G. E. (2014). Protein composition and micelle size of donkey milk with different protein patterns: Effects on digestibility. *International Dairy Journal*, 35: 57-62.

Ulleberg, E. K. (2011). *In Vitro* Digestion of Caprine Whey Proteins by Human Gastrointestinal Juices: Effect of Whey Hydrolysates and Peptides on in vitro Cell Responses. PhD Thesis. UMB, Aas, Norway.

Van Knegsel, A. T. M., van den Brand, H., Dijkstra, J., Tamminga, S. and Kemp, B. (2005). Effect of dietary energy source on energy balance, production, metabolic disorders and reproduction in lactating dairy cattle. *Reproduction Nutrition Development*, 45: 665-688.

Wal, J. M. (2001). Structure and function of milk allergens. *Allergy*, 56: 35-38.

Walsh, C. D., Guinee, T. P., Reville, W. D., Harrington, D., Murphy, J. J., O'Kennedy, B. T. and FitzGerald, R. J. (1998). Influence of κ-Casein Genetic Variant on Rennet Gel Microstructure, Cheddar Cheesemaking Properties and Casein Micelle Size. *International Dairy Journal*, 8: 707-714.

Walstra, P., Wouters, J. T. M. and Geurts, T. J. (2006). *Dairy Science and Technology*, 2.ed. CRC press. Taylor and Francis Group, London, England.

Wedholm, A., Larsen, L. B., Lindmark-Månsson, H., Karlsson, A. H. and Andrén, A. (2006). Effect of Protein Composition on the Cheese-Making Properties of Milk from Individual Dairy Cows. *Journal of Dairy Science*, 89: 3296-3305.

Whigham, L. D., Cook, M. E. and Atkinson, R. L. (2000). Conjugated linoleic acid: implications for human health. *Pharmacological Research*, 42: 503-510.

WHO, The World Health Organisation. 2003. Diet nutrition and the prevention of chronic diseases. Report of the WHO/FAO Joint Expert Consultation, WHO, Technical Report Series 916.

Williams, C. M. (2000). Dietary fatty acids and human health. Annales de Zootechnie: Paris: Institut national de la recherche agronomique, 1960-2000. pp. 165-180.

Ye, A., Cui, J. and Singh, H. (2011). Proteolysis of milk fat globule membrane proteins during in vitro gastric digestion of milk. *Journal of Dairy Science*, 94: 2762-2770.

Zeng, Y.-X., Araujo, P., Du, Z.-Y., Nguyen, T.-T., Froyland, L. and Grung, B. (2010). Elucidation of triacylglycerols in cod liver oil by liquid chromatography electrospray tandem ion-trap mass spectrometry. *Talanta*, 82: 1261-1270.

## 9. Enclosed papers I-IV

# PAPER I

Open Access
Asian Australas. J. Anim. Sci.
Vol. 27, No. 6 : 886-897 June 2014
http://dx.doi.org/10.5713/ajas.2013.13586
www.ajas.info
pISSN 1011-2367 eISSN 1976-5517

# Principal Milk Components in Buffalo, Holstein Cross, Indigenous Cattle and Red Chittagong Cattle from Bangladesh

**M. A. Islam*, M. K. Alam[1], M. N. Islam[2], M. A. S. Khan[2], D. Ekeberg, E. O. Rukke, and G. E. Vegarud**
Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences,
P.O. Box 5003, NO-1432 Aas, Norway

**ABSTRACT:** The aim of the present study was to get a total physical and chemical characterization and comparison of the principal components in Bangladeshi buffalo (B), Holstein cross (HX), Indigenous cattle (IC) and Red Chittagong Cattle (RCC) milk. Protein and casein (CN) composition and type, casein micellar size (CMS), naturally occurring peptides, free amino acids, fat, milk fat globule size (MFGS), fatty acid composition, carbohydrates, total and individual minerals were analyzed. These components are related to technological and nutritional properties of milk. Consequently, they are important for the dairy industry and in the animal feeding and breeding strategies. Considerable variation in most of the principal components of milk were observed among the animals. The milk of RCC and IC contained higher protein, CN, β-CN, whey protein, lactose, total mineral and P. They were more or less similar in most of the all other components. The B milk was found higher in CN number, in the content of $\alpha_{s2}$-, κ-CN and α-lactalbumin, free amino acids, unsaturated fatty acids, Ca and Ca:P. The B milk was also lower in β-lactoglobulin content and had the largest CMS and MFGS. Proportion of CN to whey protein was lower in HX milk and this milk was found higher in β-lactoglobulin and naturally occuring peptides. Considering the results obtained including the ratio of $\alpha_{s1}$-, $\alpha_{s2}$-, β- and κ-CN, B and RCC milk showed best data both from nutritional and technological aspects. (**Key Words:** $\alpha_{s1}$-, $\alpha_{s2}$-, β- and κ-casein, α-lactalbumin, β-lactoglobulin, Casein Micellar Size, Naturally Occurring Peptides, Free Amino Acids, Milk Fat Globule Size, Fatty Acids, Minerals, Carbohydrates)

## INTRODUCTION

Milk has a diverse composition of nutrients. The milk composition is subjected to change in response of genetics, breeding, feeding, number and stage of lactation and health status of the animal. Misra et al. (2008) compared four breeds of buffalo and showed breed variation in fat, total solids and solids-not-fat content. Islam et al. (2008) reported significantly more fat, protein and total solids in Red Chittagong Cattle (RCC) milk compared to crossbred (Holstein cross [HX], Jersey cross and Sahiwal cross) cow's milk.

The composition of milk may also change over a period of time and may vary from country to country as a result of interaction effects of several factors like breeding program and feeding strategy. Lindmark-Mansson et al. (2003) reported that casein (CN) content in raw milk decreased from 2.61% (in the year 1970) to 2.56% (in the year 1995 through 1996) and whey protein (WP) content increased from 0.73% to 0.81% during the same period of time. Similar results in buffalo milk were also reported by Zicarelli (2004). Furthermore, Schonfeldt et al. (2012) called for more country specific milk composition data. Therefore, a more detailed overview of milk composition in terms of principal components is needed to set a standard on technological properties for processing the milk. The dairy industry in each country has challenges regarding

* Corresponding Author: M. A. Islam. Tel: +47-64966235, Fax: +47-64965901, E-mail: mohammad.islam0@nmbu.no
[1] Bangladesh Livestock Research Institute, Savar, Dhaka-1341, Bangladesh.
[2] Department of Dairy Science, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh.
Submitted Sept. 18, 2013; Revised Dec. 10, 2013; Accepted Feb. 2, 2014

breeding and nutritional strategy to produce milk with optimum quality for different purposes.

The composition of milk largely indicates its nutritional value and technological properties (Heck et al., 2009). The individual CNs, especially $\alpha_{s1}$-CN, α-lactalbumin (α-LA) and β-lactoglobulin (β-Lg) are considered as major milk allergens (El-Agamy, 2007; Downs et al., 2013). And more controversially, β-CN (variant $A_1$, $A_1$+B) is claimed to be more associated with type-I diabetes (Elliott et al., 1999), whereas, Bell et al. (2006) reported a lower incidence of type-I diabetes and cardiovascular diseases in a population consuming milk with high levels of β-CN variant $A_2$. Butter from the milk with more unsaturated fatty acid (USFA) is suggested to have lower atherogenic index. In a technological perspective, such butter will be more spreadable, softer and less adhesive (Bobe et al., 2003). Milk protein, CN and fat content were found strongly positively correlated with the cheese yield (Pretto et al., 2013). Hallen et al. (2010) concluded that low concentration of κ-CN in milk is a risk factor for non-coagulation of milk. In addition to the individual CNs and casein micellar size (CMS) that influence the milk coagulation properties, milk fat globule size (MFGS) may also be important in cheese manufacturing. Michalski et al. (2004) found more firm and flexible ripened Emmental cheese from milk with larger MFGS than the milk with smaller ones.

The breeding strategy presently followed in Bangladesh takes into account only the volume of milk. Though milk fat and cchana (mainly protein curd) based milk pricing are also in practice. The information on milk composition can also be used to adjust the breeding practices to optimize milk quality (Glantz et al., 2009). Therefore, details on milk composition are important.

Buffalo milk is less studied than the cow's milk. To our knowledge, naturally occurring peptides (NOP) from raw dairy milk has not been reported yet. Details on the true protein (TP), individual CN types and content, CN number, WP content and composition, non-protein nitrogen (NPN), CMS, free amino acids (FAA), fatty acid (FA) composition, MFGS, lactose, glucose, galactose, and individual minerals are important for technological and nutritional properties of milk. Some of this information can also be used to select animals in a breeding program to produce desirable quality milk.

The aim of the present study was to characterize and compare the principal components in buffalo (B), HX, Indigenous cattle (IC) and RCC milk from Bangladesh according to the protein content and types, CMS, naturally occurring peptides, free amino acids, fat content, FA composition and type, MFGS, carbohydrate and mineral content.

## MATERIALS AND METHODS

### Milk samples

Raw milk from B, IC and RCC were collected from the Bangladesh Livestock Research Institute and HX milk was collected from Central Cattle Breeding Station and Dairy Farm, Savar, Dhaka-1341, Bangladesh. The B cows were fed on ca. 4 kg straw with 0.250 kg of molasses/head/d, ca. 4 kg of concentrate mixture (wheat bran:khesari bran:wheat crust:oilcake:fishmeal:salt:premix = 3:0.75:0.30:0.20:0.04: 0.125:0.075)/head/d. The RCC and IC were supplied with ca. 3 kg of concentrate mixture (wheat bran:khesari bran:sesame oil cake:soybean oil cake:oyster shell crust:corn crust:common salt = 140:25:15:20:5:7.5:1)/head/d. The B, RCC, and IC were also supplied with german (*Echinochloa crus-galli*) and napier (*Pennisetum purpureum*) grass. They were also allowed grazing on road side grass (of various types, non-descriptive). The HX cows were fed on german (*Echinochloa crus-galli*), napier (*Pennisetum purpureum*), oat (*Avena sativa*) and para (*Brachiaria mutica*) grass. In addition, they were supplied with ca. 2.75 kg concentrate mixture (wheat bran:broken maize:khesari:soyabean:DCP:salt = 50:15:15:17:2:1)/head/d for first 3 L of milk. The HX cows also got additional 0.5 kg of that concentrate mixture for the production of every additional liter of milk. The milk production during the sampling time was 1.5 to 3.0 L, 2.5 to 8.5 L, 2.5 to 5.8 L and 1.3 to 5.0 L in B, HX, IC, and RCC, respectively. Different individuals of each group of animals were at different number and stage of lactation. All the milk samples were taken from the morning milking. Pooled milk sample of B was obtained from nine buffalo cows and for HX, IC and RCC, milk from twenty five, fifteen and nineteen cows, respectively were obtained. The collected pooled milk was then divided into several cellstar tubes (Greiner Bio-One, Maybachstrasse, Frickenhausen, Germany), approximately 40 mL/tube. The content of each tube was preserved with 1 bronopol tablet (D & F control systems, Inc. Boston, MA, USA). Samples for protein, CMS and MFGS analyses were kept at 4°C and samples for other analyses were frozen at −20°C.

### Protein composition and types, casein micelle size, naturally occurring peptides and free amino acids

The TP, CN, WP, and NPN analyses of the milk were done by micro-Kjeldahl method according to Devold et al. (2011). In brief, the samples were digested by one digestion tablet (Kjeltabs Auto, Thompson and Capper Ltd., Runcorn WA7 1PH, UK) and 3 mL $H_2SO_4$ (96 to 97%; Merck, Darmstadt, Germany) in a Kjeldahl tube on an auto-digester (Foss Teactor, Foss analytical lab, Hoganas, Sweden) for 80 to 90 min at 420°C. Distillation and titration was carried-out on a Foss Kjeltec 8400 analyzer unit (Software version

1.5.18, Foss analytical lab, Hoganas, Sweden). The ratio of CN and WP was calculated and so was the CN number (CN ×100/true protein).

The individual CNs ($\alpha_{s1}$-, $\alpha_{s2}$-, β- and κ-CN) and WPs (α-LA and β-Lg) were quantified by capillary zone electrophoresis (Agilent Technologies No. DE01603565, Santa Clara, CA, USA) and Agilent Chemstation software (Rev. B.03.02 [341]). A method described by Mestawet et al. (2013) was used. Briefly, skimmed milk was mixed with the sample buffer at a ratio of 1:1.5. Then it was vortexed, incubated for an hour at room temperature and centrifuged at 5,000 rpm at room temperature for 5 min. Both the samples and run buffer were filtered through 0.45 μm filter unit (Millipore Corp. Carrigtwohill, County Cork, Ireland). For the separation, a positive polarity at 45°C with a linear voltage gradient from 0 to 25 kV in 3 min, followed by a constant voltage of 25 kV for 45 min with 20 mM sodium acetate (Merck, Darmstadt, Germany) buffer at pH (3±0.1) were employed. The individual CN ratio ($\alpha_{s1}$:$\alpha_{s2}$:β:κ) was calculated from their relative concentration.

The CMS was measured by Zeta Sizer 3000 HS (Malvern Instruments Ltd. Malvern, Worcestershire, UK), a method according to Devold et al. (2011). Ten microliter milk was diluted into 8 mL simulated milk ultrafiltrate filtered through a 0.22 μm filter (Millipore Corp. Carrigtwohill, County Cork, Ireland). Then the diluted samples were filtered through a 0.8 μm filter (Millipore Corp. Carrigtwohill, County Cork, Ireland) and the temperature was adjusted to 25°C before measurement.

For the NOP analysis, a method by Qureshi et al. (2012) with some modifications was used. The molecular weight cut-off spin column membrane (Molecular weight 10 kDa, regenerated cellulose, VWR, Cork, Ireland) was prepared by loading 400 μL milli-Q water and centrifuged at 1,000 rpm, at room temperature for 30 min. Then 330 μL of skimmed milk was loaded onto the column and centrifuged at 13,000 rpm, at 4°C for 45 min. The milk filtrate was then desalted. Desalted samples were kept at −20°C until analyzed. Before analysis, 10 μL of 0.5% formic acid was added to the samples and loaded on a nano-ACQUITY Ultra Performance Liquid Chromatograph (UPLC, Waters, Milford, USA). The UPLC was coupled with a Quadrapole-Time of Flight (Q-TOF) Ultima Mass Spectrometer (MS; Micromass, Manchester, UK). The resulted peak list files were searched against the National Center for Biotechnology Information (NCBI) protein sequence databases. An in-house Mascot server (version 2.3; Matrix Sciences, London, UK) was used. Analyses were done for both single and multiple charged peptides.

A modified method described by Qureshi et al. (2012) was used to measure the content of FAA in milk. Milk was mixed at a ratio of 1:1 with 0.1 M HCL (Merck, Darmstadt, Germany). One milliliter of this HCL contained 0.4 μmol L-norvalin (Sigma, St. Louis, USA) and 0.4 μmol piperidine-4-carboxylic acid (Fluka, St. Louis, USA) as internal standards.

**Fat, milk fat globule size and fatty acid composition in neutral lipids, free fatty acids and polar lipids**

Milk fat content was estimated by using an auto milk analyzer (Lactostar, Funke-Gerber, Berlin, Germany). The MFGS was measured by the method of Jones (2003), using Master Sizer 2000 (Malvern Instrument, Uppsala, Sweden). Milk was diluted by 35 mM EDTA (VWR International, Radnor, PA, USA) at a ratio of 1:1 and incubated for 30 min at room temperature followed by vortex. For the measurement, ca. 1.5 mL sample was used.

For the FA composition, total lipid was extracted by a modified method according to Folch et al. (1957). Twenty milliliter of chloroform (VWR International, Radnor, PA, USA) and methanol (Merck, Darmstadt, Germany) mixture (2:1) was added to 1 mL milk followed by adding internal standards (IS). Tri-nonadecanoin (10 mg/mL in chloroform) and nonadecanoic acid (1 mg/mL in chloroform) were used as IS, both from Larodan AB, Malmo, Sweden. The content was then incubated on a shaker at 150 rpm, at room temperature for 15 min. After incubation, 4 mL NaCl (0.9% in water) was added. Then it was vortexed and centrifuged at 2,000 rpm, at room temperature, for 10 min. The organic phase was collected and dried under $N_2$ gas. The dried lipids were dissolved in 1 mL chloroform and were fractionated into neutral lipid (NL), free fatty acids (FFA) and polar lipid (PL) by a modified method according to Ruiz et al. (2004). To elute the PL, only methanol was used. These fractions were also dried under the $N_2$ gas. Two milliliter hexane (Merck, Darmstadt, Germany) was added to the tubes containing dried NL and PL. Methylation of FFA was done by adding 1 mL boron tri-fluoride – methanol complex (14% $BF_3$ in $CH_3OH$, Sigma Aldrich, Seelze, Germany) and incubated at 70°C for 5 min. Two milliliter hexane was added to it and the upper phase was transferred in to GC-vials. Methylation of NL and PL and analysis of NL, FFA, and PL were performed according to Devle et al. (2012). In brief, metallic sodium (Merck, Darmstadt, Germany) was dissolved in methanol (3 mg/mL) to make the sodium methanolate solution. For the methylation of NL and PL, sodium methanolate (1.5 mL/sample) was added and the content was incubated on a horizontal shaker set at 350 rpm, at room temperature for 30 min. Then the sample tubes were left at a vertical position for 10 min and hexane phase was transferred in to GC-vials. An Agilent 6890 Series gas chromatograph (GC; Agilent Technology, Wilmington, DE, USA) coupled with an

Autospec Ultima MS (Micromass Ltd., Manchester, England) was used for the FA composition analysis.

**Content of carbohydrates**

The carbohydrate analysis was done by a modified method according to Narvhus et al. (1998) using High Performance Liquid Chromatography technique. One gram milk was diluted by 2.5 mL milli-Q water. The analysis was carried out with an Aminex HPX-87H column (Bio-Rad laboratories, Hercules, CA, USA) at 30°C connected to a – Perkin Elmer Series 200 pump (Perkin Elmer, Waltham, MA, USA), Perkin Elmer series 200 auto sampler (Perkin Elmer, Shelton, USA) and Perkin Elmer LC oven 101 (Perkin Elmer, Shelton, USA). Five milli molar $H_2SO_4$ (Merck, Darmstadt, Germany) was used as the mobile phase with a flow rate of 0.4 mL/min. Lactose, glucose and galactose were identified according to the standards (lactose, glucose and galactose, all from Merck, Darmstadt, Germany) using Perkin Elmer series 200 refractive index detector (Perkin Elmer, Norwalk, USA).

**Minerals content**

Total mineral content was measured by using the Lactostar auto milk analyzer (Funke-Gerber, Berlin, Germany). The quantity of the individual minerals was measured by inductively coupled plasma optical emission spectroscopy (ICP-OES; Optima 5300DV, Perkin Elmer, Shelton, USA). Five milliliter sub-boiled $HNO_3$ (65 to 70%; VWR International, Radnor, PA, USA) were added to 1 to 2.5 mL of milk. Then it was heated at 250°C for 15 min in an Ultraclave (Milestone microwave Ultraclave III, Sorisole, Italy). After heating, milli-Q water was added to make a final volume of 50 mL, and analyzed. A standard curve of each mineral was used for quantification.

**Statistical analysis**

All the analyses were run in triplicate. The one way analysis of variance (ANOVA) and mean separation (Tukey's test) were done by Minitab 16 (Minitab Ltd., Brandon Court, Coventry, UK).

## RESULTS

**Protein composition and types, casein micelle size, naturally occurring peptides and free amino acids**

The results on protein composition and types and CMS are given in Table 1. The content of TP and CN were 37.80 and 28.83 g/kg milk, respectively in RCC milk and this was higher than others milk. The highest CN number was 77.12 in B milk and in RCC milk it was 76.28. The lowest CN:WP ratio was 2.1 in HX and highest in B milk was 3.37. The $\alpha_{s1}$-CN content did not vary among the different milk. However, the content of $\alpha_{s2}$-CN in B milk was 2.3 times of RCC milk. And the κ-CN content in B milk was very high, – almost double of the cow's milk. Milk of IC and RCC were found much higher in β-CN content compared to B and HX milk. Proportionate amount of $\alpha_{s1}$-, β- and κ-CN were found higher in RCC milk. In WP, highest content of α-LA was found in B milk but the β-Lg content was almost half of all the cow's milk. The largest CMS was 188.73 nm and smallest was 157.07 nm, found in B and IC milk, respectively.

The present study has identified many NOP – 45, 79, 19 and 12 in B, HX, IC and RCC milk, respectively. Four peptides – FPIIV, GPVRGPFPII, EPVLGPVRGPFP and

**Table 1.** Protein composition and casein micelle size (CMS) of buffalo (B), Holstein cross (HX), Indigenous cattle (IC) and Red Chittagong Cattle (RCC) milk

| | B | HX | IC | RCC | p-value |
|---|---|---|---|---|---|
| TP (g/kg milk) | $35.01^{c}$±0.63 | $27.15^{d}$±0.47 | $36.40^{b}$±0.33 | $37.80^{a}$±0.38 | <0.001 |
| CN (g/kg milk) | $27.00^{b}$±0.67 | $18.38^{c}$±0.50 | $27.14^{b}$±0.43 | $28.83^{a}$±0.30 | <0.001 |
| CN number[1] | $77.12^{a}$±0.54 | $67.70^{c}$±0.65 | $74.55^{b}$±0.50 | $76.28^{a}$±0.04 | <0.001 |
| WP (g/kg milk) | $8.01^{d}$±0.06 | $8.77^{c}$±0.03 | $9.26^{a}$±0.10 | $8.96^{b}$±0.08 | <0.001 |
| CN:WP | 3.37:1 | 2.1:1 | 2.94:1 | 3.22:1 | - |
| NPN (g/kg milk) | 0.35±0.00 | 0.36±0.00 | 0.35±0.00 | 0.36±0.00 | >0.05 |
| $\alpha_{s1}$-CN (g/kg milk protein) | 315.28±14.35 | 348.41±9.21 | 312.31±29.66 | 321.90±17.02 | >0.05 |
| $\alpha_{s2}$-CN (g/kg milk protein) | $112.33^{a}$±13.78 | $90.20^{ab}$±7.48 | $71.44^{ab}$±34.21 | $48.75^{b}$±1.41 | <0.05 |
| β-CN (g/kg milk protein) | $351.82^{b}$±26.07 | $366.57^{b}$±11.91 | $432.69^{a}$±38.34 | $457.14^{a}$±14.12 | <0.01 |
| κ-CN (g/kg milk protein) | $138.97^{a}$±2.06 | $65.49^{b}$±1.77 | $76.50^{b}$±9.20 | $69.54^{b}$±3.57 | <0.001 |
| $\alpha_{s1}$:$\alpha_{s2}$:β:κ-CN | 2.80:1:3.13:1.24 | 3.86:1:4.06:0.73 | 4.37:1:6.06:1.07 | 6.61:1:9.4:1.43 | - |
| α-LA (g/kg milk protein) | $38.27^{a}$±2.68 | $31.37^{b}$±1.60 | $27.48^{bc}$±0.61 | $24.20^{c}$±1.21 | <0.001 |
| β-Lg (g/kg milk protein) | $43.35^{c}$±5.18 | $97.94^{a}$±5.48 | $80.78^{b}$±7.04 | $79.67^{b}$±6.71 | <0.001 |
| CMS (nanometer) | $188.73^{a}$±1.25 | $159.37^{c}$±1.60 | $157.07^{c}$±3.04 | $164.57^{b}$±1.20 | <0.001 |

TP, true protein; CN, casein; WP, whey protein; NPN, non protein nitrogen; LA, lactalbumin; Lg, lactoglobulin.

[1] CN number = (CN/true protein)×100. [a,b,c] Mean with different superscript(s) in a row differs significantly.

EPVLGPVRGPFPII from β-CN were identified in common to all the four types of milk. Only those peptides that have been previously reported bioactive sequence (s) (matched with peptides reviewed by Meisel, 1998; Clare and Swaisgood, 2000; Silva and Malcata, 2005) are listed in Table 2. Some of the bioactive fragments like TTMPLW, GPVRGPFPIIV, KVLPVPQ, and YQEPVL were detected as a part of several different larger peptides.

**Table 2.** Naturally occurring peptides (NOP) from buffalo (B), Holstein cross (HX), Indigenous cattle (IC) and Red Chittagong Cattle (RCC) milk

| EMW | Peptide | Reported bioactivity[1] | Protein | B | HX | IC | RCC |
|---|---|---|---|---|---|---|---|
| 1379.80 | YLGYLEQLLRL | Opoid activity | $\alpha_{s1}$-CN | + | + | - | - |
| 1740.00 | FFVAPFPEVFGKEKV | ACE inhibitory | $\alpha_{s1}$-CN | - | + | - | - |
| 1896.12 | RFFVAPFPEVFGKEKV | ACE inhibitory | $\alpha_{s1}$-CN | - | + | - | - |
| 1957.06 | IPNPIGSENSGKTTMPLW | ACE inhibitory and Immunomodulating | $\alpha_{s1}$-CN | - | + | - | - |
| 2012.98 | IPNPIGSENSEKTTMPLW | ACE inhibitory and Immunomodulating | $\alpha_{s1}$-CN | - | + | - | - |
| 2048.03 | VPQLEIVPNSAEERLHSM | CPP; Mineral nutrition | $\alpha_{s1}$-CN | - | + | - | - |
| 2055.99 | DIPNPIGSENSGKTTMPLW | ACE inhibitory and Immunomodulating | $\alpha_{s1}$-CN | - | + | - | - |
| 2064.09 | VPQLEIVPNSAEERLHSM | CPP; Mineral nutrition | $\alpha_{s1}$-CN | - | - | + | - |
| 2143.12 | SDIPNPIGSENSGKTTMPLW | ACE inhibitory and Immunomodulating | $\alpha_{s1}$-CN | - | + | - | - |
| 2215.04 | SDIPNPIGSENSEKTTMPLW | ACE inhibitory and Immunomodulating | $\alpha_{s1}$-CN | - | + | - | - |
| 2826.64 | HIQKEDVPSERYLGYLEQLLRLK | Opoid activity | $\alpha_{s1}$-CN | - | + | - | - |
| 1250.74 | TKVIPYVRYL | ACE inhibitory | $\alpha_{s2}$-CN | - | + | - | - |
| 1385.67 | TVDMESTEVFTK | CPP; Mineral nutrition | $\alpha_{s2}$-CN | - | + | - | + |
| 2064.10 | VPQLEIVPNSAEERLHSM | CPP; Mineral nutrition | $\alpha_{s2}$-CN | - | + | - | - |
| 2159.03 | SDIPNPIGSENSGKTTMPLW | ACE inhibitory and Immunomodulating | $\alpha_{s2}$-CN | - | + | - | - |
| 2231.04 | SDIPNPIGSENSEKTTMPLW | ACE inhibitory and Immunomodulating | $\alpha_{s2}$-CN | - | + | - | - |
| 1313.74 | SLVYPFPGPIPK[2] | Opoid activity | β-CN | + | - | - | - |
| 1362.82 | VLGPVRGPFPIIV | ACE inhibitory | β-CN | + | - | - | - |
| 1378.83 | PVPQKAVPYPQR | ACE inhibitory | β-CN | - | + | - | - |
| 1409.90 | SLSQSKVLPVPQK | Antihypertensive | β-CN | - | + | - | - |
| 1441.79 | QSLVYPFPGPIPK[2] | Opoid activity | β-CN | + | - | - | - |
| 1484.75 | AVPYPQRDMPIQA | ACE inhibitory | β-CN | + | - | - | - |
| 1493.96 | VLSLSQSKVLPVPQ | Antihypertensive | β-CN | - | + | - | - |
| 1542.83 | TQSLVYPFPGPIPK[2] | Opoid activity | β-CN | + | - | - | - |
| 1554.87 | YQEPVLGPVRGPFP | ACE inhibitory | β-CN | - | + | - | - |
| 1588.92 | EPVLGPVRGPFPIIV | ACE inhibitory | β-CN | + | - | + | + |
| 1716.93 | QEPVLGPVRGPFPIIV | ACE inhibitory | β-CN | + | - | - | + |
| 1717.00 | EQPVLGPVRGPFPIIV | ACE inhibitory | β-CN | - | + | + | + |
| 1719.10 | KVLPVPQKAVPYPQR | ACE inhibitory | β-CN | - | + | - | - |
| 1741.98 | AQTQSLVYPFPGPIPK[2] | Opoid activity | β-CN | + | - | - | - |
| 1780.95 | YQEPVL*GPVRGPFPI*I | ACE inhibitory | β-CN | + | + | - | - |
| 1880.00 | YQEPVL*GPVRGPFPI*IV* | ACE inhibitory | β-CN | + | - | - | - |
| 1888.98 | FAQTQSLVYPFPGPIPK[2] | Opoid activity | β-CN | + | - | - | - |
| 1993.14 | L YQEPVL*GPVRGPFPII*V* | ACE inhibitory | β-CN | + | - | - | - |
| 2125.08 | EMPFPKYPVEPFTESQSL | ACE inhibitory | β-CN | - | + | - | - |
| 2141.04 | EMPFPKYPVEPFTESQSL | ACE inhibitory | β-CN | - | + | - | - |
| 2236.20 | IHPFAQTQSLVYPFPGPIPK[2] | Opoid activity | β-CN | + | - | - | - |
| 2374.40 | KVLPVPQKAVPYPQRDMPIQA | ACE inhibitory | β-CN | - | + | - | - |
| 2406.28 | HKEMPFPKYPVEPFTESQSL | ACE inhibitory | β-CN | - | + | - | - |
| 2537.50 | KVLPVPQKAVPYPQRDMPIQAF | ACE inhibitory | β-CN | - | + | - | - |
| 3720.21 | AVPYPQRDMPIQAF*LLY*EQPVLGPVRGPFPIIV | ACE inhibitory and *Immunomodulating* | β-CN | - | + | - | - |

[1] Clare and Swaisgood, 2000; Meisel, 1998; Silva and Malcata, 2005.

[2] Marked fragment in these peptides are also known as β-Casomorphin-5; EMW, experimental molecular weight; +, present; -, absent; ACE, angiotensin converting enzyme; CPP, caseinophospho peptide; CN, casein; Grey/italic and underlined/* marked segments are the reported bioactive fragments.

Table 3 summarizes the content of FAA in the milk. All the amino acids varied significantly except glutamic acid and asparagine. Highest concentration of all the amino acids was found in B milk. The milk of HX did not differ from B in leucine, valine, lysine, methionine, phenylalanine, arginine, and tyrosine content. The IC milk shared the higher content of threonine, alanine and aspartic acid with B milk, whereas, RCC and B milk contained approximately the same amount of valine, threonine, alanine, aspartic, and serine.

**Fat, milk fat globule size and fatty acid composition in neutral lipids, free fatty acids and polar lipids**

The results on fat (g/kg milk) and FA composition (% of total FA in each lipid class) are presented in Table 4. The fat content varied significantly among all the milk. The highest fat content was 58.40 g/kg in B and lowest was 37.13 g/kg in HX milk.

In NL, among all the saturated FA (SFA) only C20:0 showed non-significant variation. The B milk had the highest C4:0 (1.43%), C15:0 (2.04%), C17:0 (1.54%), and C18:0 (16.72%) content. All the cow's milk was found similar in their C6:0 and C8:0 content and more than in B milk. The HX and B milk did not differ in their C17:0 content. Milk of IC had the highest C12:0, C14:0, and C16:0. In respect of all the SFA, IC, and RCC milk were found similar. In addition to C6:0 and C8:0, HX milk was also found similar to RCC milk in C4:0, C14:0 and C16:0 content. And finally all the cow's milk was found similar in their total SFA content, more than the B milk. In USFA, only C10:1*n*-6 *cis* did not differ significantly. Milk from B showed the maximum content of all USFA (except C14:1*n*-5 *cis*) and of total USFA. The content of C18:1*n*-9 *cis*, C18:2*n*-6 *cis*, C18:1*n*-7 *trans*, C18:2*n*-7 *trans*, and total USFA in B milk was 31.52%, 0.90%, 1.22%, 0.48%, and 36.48%, respectively. Similarities in all the cow's milk were found in their C18:1*n*-9 *cis*, C18:2*n*-6 *cis*, C18:1*n*-7 *trans* and total USFA content.

All three cow's milk FFA fraction did not vary in C10:0, C12:0 and C14:0 content, however, higher than B milk. The content of C14:1*n*-5 *cis* was low in B and HX milk compared to IC and RCC.

In the PL fraction, B milk had the lowest C14:0 and C16:0 and the highest C18:0. As compared to the cows' milk, B milk also had lower total SFA (40.86%) and higher total USFA (59.13%).

The results on MFGS (μm) are summarized in Table 5. The mean MFGS ($d_{4,3}$) was largest in B (12.29 μm) and smallest in RCC ( 3.41 μm) milk. Compared to the cows' milk, all through the distribution ($d_{0.1...0.9}$), B milk had significantly larger MFGS (except at $d_{0.1}$).

**Content of carbohydrates and minerals**

Table 6 represents the results of the carbohydrates and

**Table 3.** Free amino acids (FAA) in buffalo (B), Holstein cross (HX), Indigenous cattle (IC) and Red Chittagong Cattle (RCC) milk; concentration (mg/kg milk)

| | B | HX | IC | RCC | p-value |
|---|---|---|---|---|---|
| Leucine | $6.12^{a}$±2.59 | $3.56^{ab}$±0.843 | $1.57^{b}$±0.53 | $1.31^{b}$±0.45 | <0.05 |
| Isoleucine | $3.32^{a}$±1.29 | $1.31^{b}$±0.00 | $0.78^{b}$±0.26 | $0.78^{b}$±0.26 | <0.01 |
| Valine | $8.28^{a}$±2.50 | $4.69^{ab}$±0.62 | $3.59^{b}$±0.75 | $4.22^{ab}$±1.69 | <0.05 |
| Lysine | $9.84^{ab}$±4.26 | $12.38^{a}$±3.39 | $3.90^{b}$±1.22 | $4.19^{b}$±1.95 | <0.05 |
| Methionine | $1.99^{a}$±0.96 | $1.49^{ab}$±0.52 | $0.70^{b}$±0.17 | $0.60^{b}$±0.00 | <0.05 |
| Phenylalanine | $3.97^{a}$±2.01 | $2.09^{ab}$±0.38 | $0.44^{b}$±0.19 | $0.33^{b}$±0.00 | <0.01 |
| Threonine | $1.99^{a}$±0.50 | $0.87^{b}$±0.14 | $1.27^{ab}$±0.14 | $1.91^{a}$±0.48 | <0.05 |
| Tryptophan | 1.23±0.41 | Trace | n.d. | n.d. | - |
| Arginine | $6.85^{a}$±2.32 | $6.50^{ab}$±1.12 | $3.25^{c}$±0.80 | $3.72^{bc}$±1.22 | <0.05 |
| Tyrosine | $3.26^{a}$±0.96 | $3.02^{ab}$±0.75 | $1.21^{c}$±0.21 | $1.33^{bc}$±0.55 | <0.05 |
| Alanine | $6.47^{a}$±1.15 | $3.98^{b}$±0.37 | $4.28^{ab}$±0.71 | $5.17^{ab}$±1.17 | <0.05 |
| Aspartic | $2.93^{a}$±0.27 | $1.95^{b}$±0.15 | $2.40^{ab}$±0.53 | $2.13^{ab}$±0.27 | <0.05 |
| Glutamic | 64.44±9.68 | 43.55±1.77 | 62.29±11.74 | 55.71±12.75 | >0.05 |
| Glutamine | 0.49±0.17 | Trace | Trace | n.d. | - |
| Glycine | $20.32^{a}$±2.48 | $9.51^{b}$±0.38 | $6.01^{b}$±0.84 | $8.41^{b}$±1.67 | <0.001 |
| Serine | $5.89^{a}$±1.09 | $2.38^{c}$±0.24 | $3.50^{bc}$±0.44 | $4.34^{ab}$±0.74 | <0.01 |
| GABA | $25.78^{a}$±3.32 | $3.85^{b}$±0.24 | $2.41^{b}$±0.52 | $6.60^{b}$±1.49 | <0.001 |
| Citrulline | $1.29^{a}$±0.20 | $0.70^{b}$±0.00 | $0.47^{b}$±0.20 | $0.58^{b}$±0.20 | <0.01 |
| Asparagine | 0.44±0.15 | 0.26±0.00 | 0.44±0.30 | 0.62±0.15 | >0.05 |

Trace, not quantified in all replications; n.d., not detected; GABA, γ-amino butyric acid.
[a,b,c] Mean with different superscript(s) in a row differs significantly.

**Table 4.** Fat content (g/kg milk) and fatty acid composition (% of total fatty acid in each class of lipid) in neutral lipid (NL), free fatty acids (FFA) and polar lipid (PL) of milk from buffalo (B), Holstein cross (HX), Indigenous cattle (IC) and Red Chittagong Cattle (RCC)

| Fatty acid | B | HX | IC | RCC | p-value |
|---|---|---|---|---|---|
| Fat | 58.40$^{a}$±0.35 | 37.13$^{d}$±0.06 | 43.40$^{b}$±0.35 | 42.33$^{c}$±0.23 | <0.001 |
| NL | | | | | |
| C4:0 | 1.43$^{a}$±0.12 | 1.14$^{b}$±0.02 | 1.03$^{b}$±0.04 | 1.04$^{b}$±0.08 | <0.01 |
| C6:0 | 0.87$^{b}$±0.10 | 1.07$^{a}$±0.05 | 1.14$^{a}$±0.03 | 1.18$^{a}$±0.06 | <0.01 |
| C8:0 | 0.37$^{b}$±0.06 | 0.78$^{a}$±0.05 | 0.84$^{a}$±0.02 | 0.91$^{a}$±0.07 | <0.001 |
| C10:0 | 0.85$^{c}$±0.03 | 1.95$^{b}$±0.08 | 2.30$^{a}$±0.05 | 2.47$^{a}$±0.18 | <0.001 |
| C12:0 | 1.36$^{c}$±0.05 | 2.56$^{b}$±0.03 | 3.02$^{a}$±0.07 | 3.13$^{a}$±0.19 | <0.001 |
| C14:0 | 8.75$^{c}$±0.27 | 10.75$^{b}$±0.14 | 11.76$^{a}$±0.43 | 11.24$^{ab}$±0.33 | <0.001 |
| C15:0 | 2.04$^{a}$±0.02 | 1.85$^{b}$±0.06 | 1.51$^{c}$±0.00 | 1.49$^{c}$±0.03 | <0.001 |
| C16:0 | 29.46$^{c}$±0.66 | 33.19$^{b}$±0.57 | 35.45$^{a}$±1.08 | 34.75$^{ab}$±0.08 | <0.001 |
| C17:0 | 1.54$^{a}$±0.08 | 1.32$^{a}$±0.06 | 1.03$^{b}$±0.14 | 1.03$^{b}$±0.09 | <0.001 |
| C18:0 | 16.72$^{a}$±0.49 | 15.09$^{b}$±0.26 | 11.60$^{c}$±0.45 | 10.70$^{c}$±0.21 | <0.001 |
| C20:0 | 0.11±0.01 | 0.10±0.02 | n.d. | n.d. | >0.05 |
| Total SFA | 63.52$^{b}$±0.66 | 69.81$^{a}$±0.36 | 69.68$^{a}$±1.07 | 67.95$^{a}$±0.70 | <0.001 |
| C10:1*n*-6 *cis* | n.d. | n.d. | 0.30±0.02 | 0.33±0.04 | >0.05 |
| C14:1*n*-5 *cis* | 0.50$^{d}$±0.03 | 0.70$^{c}$±0.01 | 1.24$^{b}$±0.01 | 1.50$^{a}$±0.07 | <0.001 |
| C16:1*n*-7 *cis* | 1.80$^{a}$±0.05 | 1.00$^{c}$±0.03 | 1.44$^{b}$±0.08 | 1.85$^{a}$±0.05 | <0.001 |
| C18:1*n*-9 *cis* | 31.52$^{a}$±0.53 | 26.78$^{b}$±0.36 | 25.47$^{b}$±0.82 | 26.46$^{b}$±0.60 | <0.001 |
| C18:1*n*-7 *trans* | 1.22$^{a}$±0.05 | 0.84$^{b}$±0.04 | 0.76$^{b}$±0.10 | 0.73$^{b}$±0.01 | <0.001 |
| C18:2*n*-6 *cis* | 0.90$^{a}$±0.10 | 0.65$^{b}$±0.05 | 0.70$^{b}$±0.05 | 0.67$^{b}$±0.04 | <0.01 |
| C18:2*n*-7 *trans* | 0.48$^{a}$±0.03 | 0.22$^{c}$±0.06 | 0.33$^{b}$±0.01 | 0.38$^{ab}$±0.01 | <0.001 |
| C18:3*n*-3 *cis* | 0.05±0.00 | n.d. | n.d. | n.d. | - |
| Total USFA | 36.48$^{a}$±0.66 | 30.19$^{b}$±0.36 | 30.24$^{b}$±1.07 | 31.91$^{b}$±0.58 | <0.001 |
| FFA | | | | | |
| C6:0 | 0.58±0.17 | 0.66±0.25 | 0.69±0.10 | 0.66±0.10 | >0.05 |
| C8:0 | 0.38±0.08 | n.d. | n.d. | n.d. | - |
| C10:0 | 0.56$^{b}$±0.13 | 1.73$^{a}$±0.22 | 1.87$^{a}$±0.29 | 1.75$^{a}$±0.22 | <0.001 |
| C12:0 | 1.25$^{b}$±0.17 | 3.10$^{a}$±1.22 | 3.13$^{a}$±0.19 | 3.09$^{a}$±0.16 | <0.05 |
| C14:0 | 7.00$^{b}$±1.14 | 10.67$^{a}$±2.53 | 10.67$^{a}$±0.57 | 9.25$^{ab}$±0.13 | <0.05 |
| C15:0 | 1.46±0.26 | 1.60±0.58 | 1.39±0.06 | 1.29±0.06 | >0.05 |
| C16:0 | 38.60±0.25 | 37.52±5.03 | 41.75±0.45 | 41.70±0.77 | >0.05 |
| C17:0 | 0.71±0.13 | 0.71±0.08 | 0.65±0.10 | 0.59±0.02 | >0.05 |
| C18:0 | 19.47±0.62 | 13.54±5.82 | 13.44±1.80 | 14.88±0.74 | >0.05 |
| Total SFA | 70.00±1.34 | 69.51±6.08 | 73.57±1.18 | 73.21±0.35 | >0.05 |
| C14:1*n*-5 *cis* | 0.28$^{b}$±0.01 | 0.41$^{b}$±0.05 | 0.86$^{a}$±0.02 | 0.85$^{a}$±0.12 | <0.001 |
| C16:1*n*-7 *cis* | 1.23±0.37 | 0.77±0.12 | 1.26±0.08 | 1.26±0.22 | >0.05 |
| C18:1*n*-9 *cis* | 18.08±2.68 | 15.90±0.89 | 17.17±1.07 | 14.96±2.33 | >0.05 |
| Total USFA | 30.00±1.34 | 30.49±6.08 | 26.69±0.86 | 26.79±0.35 | >0.05 |
| PL | | | | | |
| C14 | 3.00$^{b}$±0.22 | 4.97$^{ab}$±0.69 | 6.14$^{a}$±2.17 | 4.15$^{ab}$±0.43 | <0.05 |
| C16 | 23.60$^{b}$±1.38 | 28.97$^{a}$±0.21 | 31.41$^{a}$±1.60 | 30.39$^{a}$±0.37 | <0.001 |
| C18 | 14.27$^{a}$±1.45 | 12.43$^{ab}$±1.92 | 10.12$^{b}$±1.90 | 10.55$^{ab}$±0.78 | <0.05 |
| Total SFA | 40.86$^{b}$±2.89 | 46.37$^{a}$±2.73 | 47.68$^{a}$±1.23 | 45.08$^{ab}$±0.02 | <0.05 |
| C18:1 1*n*-9 *cis* | 52.77±3.81 | 49.04±3.10 | 48.30±2.43 | 50.07±0.31 | >0.05 |
| C18:2*n*-6 *cis* | 6.36±0.92 | 4.59±0.43 | 4.03±1.50 | 4.85±0.28 | >0.05 |
| Total USFA | 59.13$^{a}$±2.89 | 53.63$^{b}$±2.73 | 52.32$^{b}$±1.23 | 54.92$^{ab}$±0.02 | <0.05 |

SFA, saturated fatty acid; n.d., not detected; USFA, unsaturated fatty acid.
$^{a,b,c}$ Mean with different superscript(s) in a row differs significantly.

**Table 5.** Milk fat globule size (MFGS; μm) distribution in buffalo (B), Holstein cross (HX), Indigenous cattle (IC) and Red Chittagong Cattle (RCC) milk

| | B | HX | IC | RCC | p-value |
|---|---|---|---|---|---|
| $d_{4,3}$ | $12.29^{a}$±0.32 | $3.76^{bc}$±0.08 | $4.20^{b}$±0.16 | $3.41^{c}$±0.00 | <0.001 |
| $d_{0.1}$ | 1.84±0.12 | 1.71±0.03 | 1.73±0.03 | 1.77±0.00 | >0.05 |
| $d_{0.2}$ | $3.04^{a}$±0.14 | $2.15^{b}$±0.13 | $1.86^{b}$±0.60 | $2.17^{b}$±0.00 | <0.01 |
| $d_{0.5}$ | $8.10^{a}$±0.09 | $3.47^{b}$±0.05 | $3.35^{b}$±0.06 | $3.16^{c}$±0.00 | <0.001 |
| $d_{0.8}$ | $20.91^{a}$±0.63 | $5.15^{b}$±0.16 | $5.10^{b}$±0.09 | $4.53^{b}$±0.00 | <0.001 |
| $d_{0.9}$ | $28.66^{a}$±1.22 | $6.22^{b}$±0.23 | $5.42^{b}$±1.82 | $5.40^{b}$±0.00 | <0.001 |

$d_{4,3}$ = Volume weighted mean diameter; $d_{0.1...0.9}$ = 10% ... 90% fat globules are less than mean±standard deviation μm in diameter.
[a,b,c] Mean with different superscript(s) in a row differs significantly.

mineral composition. All the four different milk types showed significant difference in their lactose content. The order of lactose content was RCC>IC>B>HX. Glucose content was found similar in B, IC, and RCC milk and significantly more than in HX milk. The IC and RCC milk were found similar and intermediate in their galactose content while B had the maximum galactose and minimum was in HX milk.

Total mineral and all individual mineral content also varied significantly. The highest total mineral content was 8.53 g/kg milk found in RCC milk and IC milk was similar to it. The B milk was found rich in Ca content (1.48 g/kg milk) and RCC in P content (1.14 g/kg milk). But the Ca:P ratio was higher in B milk followed by HX>RCC>IC. Compared to others, B milk was also found rich in Mg and Mn content.

## DISCUSSION

### Protein composition and types, casein micelle size, naturally occurring peptides and free amino acids

The proteins, CN content and type and CMS in milk are very important for coagulation properties and cheese production. The TP content in B milk was comparable to the observation of Khan et al. (2007) who reported 37.67 g/kg crude protein in water buffalo milk. Islam et al. (2008) studied the quality of milk from different genotypes and reported 3.11% and 4.06% crude protein in HX and RCC milk, respectively, which is comparabale with the present study. The CN number in the present study is about 5 to 15% less than the results on Dutch dairy milk obtained by Heck et al. (2009). Higher CN number is favorable for cheese yield. Milk of B and RCC had higher CN number than HX and IC, where HX was exceptional low.

The content of individual CNs was also varied, except $\alpha_{s1}$-CN. The differences in CN composition is mainly due to the genotypic differences of the animals as described by Farrell Jr et al. (2004). Different genetic variants showed significant differences in milk production trait, milk composition, protein composition and technological properties. The average $\alpha_{s1}$:$\alpha_{s2}$:β:κ-CN is generally recorded as 4:1:4:1 and according to Barlowska et al. (2011), this ratio is diverse. The diversity is related to genetic polymorphism, post translational modification,

**Table 6.** Content of carbohydrates and minerals in buffalo (B), Holstein cross (HX), Indigenous cattle (IC) and Red Chittagong Cattle (RCC) milk

| | B | HX | IC | RCC | p-value |
|---|---|---|---|---|---|
| Lactose (g/kg milk) | $47.48^{c}$±1.01 | $45.95^{d}$±0.31 | $50.55^{b}$±0.31 | $52.65^{a}$±0.06 | <0.001 |
| Glucose (mg/kg milk) | $16.25^{a}$±4.80 | $7.55^{b}$±1.30 | $20.45^{a}$±3.37 | $20.62^{a}$±0.63 | <0.01 |
| Galactose (mg/kg milk) | $74.24^{a}$±9.46 | $42.69^{c}$±0.63 | $58.43^{b}$±4.63 | $57.35^{b}$±2.51 | <0.01 |
| Total mineral (g/kg milk) | $7.90^{b}$±0.26 | $7.17^{c}$±0.06 | $8.43^{a}$±0.15 | $8.53^{a}$±0.11 | <0.001 |
| Ca (g/kg milk) | $1.48^{a}$±0.01 | $1.09^{d}$±0.01 | $1.32^{c}$±0.01 | $1.40^{b}$±0.01 | <0.001 |
| P (g/kg milk) | $1.07^{c}$±0.00 | $0.81^{d}$±0.00 | $1.08^{b}$±0.00 | $1.14^{a}$±0.00 | <0.001 |
| Ca:P | 1.38 | 1.35 | 1.22 | 1.23 | - |
| Mg (g/kg milk) | $0.14^{a}$±0.00 | $0.09^{d}$±0.00 | $0.12^{b}$±0.00 | $0.12^{c}$±0.00 | <0.001 |
| Na (g/kg milk) | $0.37^{c}$±0.00 | $0.48^{a}$±0.00 | $0.40^{b}$±0.00 | $0.40^{b}$±0.00 | <0.001 |
| K (g/kg milk) | $0.86^{c}$±0.01 | $1.52^{b}$±0.00 | $1.61^{a}$±0.00 | $1.52^{b}$±0.01 | <0.001 |
| Mn (mg/kg milk) | $0.07^{a}$±0.00 | $0.06^{b}$±0.00 | $0.06^{b}$±0.00 | $0.06^{b}$±0.00 | <0.01 |
| Zn (mg/kg milk) | $4.58^{c}$±0.03 | $3.49^{d}$±0.02 | $4.78^{b}$±0.06 | $5.03^{a}$±0.00 | <0.001 |
| Cu (mg/kg milk) | <0.05 | <0.05 | <0.05 | 0.07±0.01 | - |

[a,b,c] Mean with different superscript(s) in a row differs significantly.

stage of lactation and feeding regime. The ratio reveals that RCC milk is exceptionally high in $\alpha_{s1}$- and β-CN content. Higher $\alpha_{s1}$-CN is related with delayed milk coagulation time and increased curd firming time. Whereas, increased β-CN content decreases the milk coagulation time and curd firming time (Bonfatti et al., 2013). However, according to St-Gelais and Hache (2005), rennet coagulation properties are related not only to CN content but also to the proportion of β-CN and $\alpha_s$-CN present in milk. They conclude that milk with relatively high proportion of β-CN showed poorer coagulation properties. On the other hand, the presence of more κ-CN is generally related with improved coagulation properties. But Bonfatti et al. (2013) found low rennet coagulation time (RCT) in milk with low κ-CN and the glycosylated κ-CN had unfavorable effects on RCT. According to Addeo et al. (1977), 40% of B milk κ-CN is carbohydrate free while it is only 25% in cow's milk. So, considering all the CNs, B milk should have better coagulation properties than the cows' milk. And this was confirmed in a preliminary study using formagraph (results not shown).

In our study the CMS was smallest in IC milk and largest in B milk. According to Glantz et al. (2010) smaller CMS gives stronger gels. Milk that contains more κ-CN is reported to have smaller CMS, however, this was not shown in our study.

Milk protein is also well known for its nutritional importance. In the context of TP and CN content, RCC and IC milk were higher than B and HX milk. The general ratio of CN and WP in cow's milk is 4:1 (Lara-Villoslada et al., 2005). In our results the CN:WP ratio varied greatly, showing the highest ratio in B and lowest in HX milk. As compared to Heck et al. (2009) and Farrell Jr et al. (2004), it seems to be a genotypic variation. A higher proportion of WP is nutritionally favorable, however, immunogenically unfavorable, especially with regard to β-Lg. The lowest content of β-Lg in B milk, almost half of the others milk, could makes it more preferable for the individuals with milk allergy. In addition, HX milk is preferable for those who needs a proportionately low CN milk, like babies, since the content is more human like.

The present study has identified many NOP with previously reported bioactive sequence (s) for ACE-inhibitory, opoid, immunomodulatory and mineral binding effects from *in vitro* studies. Milk from HX was high in ACE-inhibitory peptides that prevent the formation of angiotensin-II (vasoconstrictor) and hydrolysis of bradykinin (vasodilator). Opoid peptide has similarities to opium and acts as opoid receptor ligands with agonistic activity. We found B milk rich in opoid peptide sequences. Immunomodulatory peptides stimulate the immunity and HX contained more of these peptides than others. Mineral binding peptides are caseinophospho-peptides that act as the biocarriers of divalent cations; in this study HX milk was rich in this. The peptide chain of identified NOP is longer compared to previously reported bioactive fragments. It may enhance the delivery of bioactive peptides to the target organs, suggested by Hayes et al. (2007). Accordingly larger peptides may be digested in the gastrointestinal tract and release a smaller part that may act as a bioactive peptide.

The concentration of all of the FAA, which can easily be absorbed, was higher in B milk than others milk. Beside participating in the protein syntheis, they also exert some important physiological roles e.g. γ-amino butyric acid (GABA) acts in neurotransmission. Moreover, they may also have importance in the sensory quality of milk as Drake et al. (2007) reported the contribution of glutamic acid to the umami taste in cheese.

**Fat, milk fat globule size and fatty acid composition in neutral lipids, free fatty acids and polar lipids**

Even though the B milk fat content was less than 72.67 g/kg as reported by Khan et al. (2007) in the water buffalo, our results are within the range of 3.37 to 14.42% reported by Meena et al. (2007). And compared to others milk, HX had 5 to 21 g less fat in milk. It's higher milk production than B, IC, and RCC may contribute to this variation.

Feed and the microbial activity are two main sources of the milk FA variation. In addition, there are a number of factors like – animal status, feeding regime etc. that may influence the FA composition. The animals under the present study are of different genotype and only RCC and IC received the same feeding. In the NL, FFA, and PL fractions of all the milk of present study, C16:0 was the dominating FA followed by C18:0 and C14:0. We observed a lower concentration of C4:0 in all milk. Timmen and Patton (1988) reported that the underfeeding of animals depressed the production of C4:0 and C16:0 compensated by increased C18:1*n*-9 *cis*. Compared to Menard et al. (2010), our results showed lower amounts of C4:0 and C16:0 (only in B milk) and higher C18:1*n*-9 *cis*. Lower concentration of C4:0-C10:0 indicates the animals were underfed or in negative energy balance (Van Knegsel et al., 2005). However, Devle et al. (2012) and Menard et al. (2010) also reported a lower concentration of C4:0-C8:0 in Norwegian cow and Mediterranean buffalo milk, respectively.

The variation in C15:0 and C17:0 indicates the variation in ruminal activity of buffalo and cow as they are synthesized by the rumen bacterial flora. But Heck et al. (2012) suggested that a part of these two FAs derives from the blood, meaning that the influence of animal's diet also is of importance. Similarities between IC and RCC also agree

with this as they were on the same diet. But HX milk had similarities with B milk in C17:0 though on different diets. Half of the C16:0 derives from *de novo* synthesis in the mammary gland and the rest half along with C18:0 originates from dietary lipids and mobilization of adipose tissue. So, the quality of the feed and the body condition score of the lactating animal is important regarding these two FAs.

Both the conjugated linoleic acid (CLA) isomer (C18:2*n*-7 *trans*) and precursor (C18:1*n*-7 *trans*) were higher in B milk. Similarly the $\omega_6$ (C18:2*n*-6 *cis*, also a precursor of CLA) FA was also higher in B and we were able to detect $\omega_3$ (C18:3*n*-3 *cis*) only in B milk. In FFA content, B was found poorer than the cow's milk. And to best of our knowledge, we did not find any published article that describes the individual FFA of buffalo and cow's milk. Unlike Menard et al. (2010), B milk contained more total USFA and less total SFA in NL and PL compared to cow's milk and no such differences were observed in FFA. All through the FA composition, the similarities between IC and RCC indicate the importance of feed in FA composition as they are of different genotype. And their similarities with HX might be indicative for the genotypic importance in FA composition. Menard et al. (2010) maintained cow and buffalo on same diet and management. But they found significant differences in fatty acid composition (except C4:0, C6:0, C18:1 t10 and C18:1 t12); revealing the importance of genotype and ruminal activity in fatty acid composition. Being of different genotype and fed differently, RCC milk was found similar to B milk in C16:1*n*-7 *cis* and C18:2*n*-7 *trans* content in NL fraction and few others in FFA and PL fractions.

In general the MFGS varies from 1 to 20 μm in diameter. Along with the fat content, season, number and stage of lactation are the main factors influencing the MFGS. The B milk showed the largest MFGS in the present study. This may be because of higher fat content as the mammary gland has a limited capacity of producing fat globule membrane material. So the fat droplets seem to grow and become larger before they are covered with the membrane in the secretory apical membrane. However, Menard et al. (2010) observed a smaller diameter (5.18 μm $d_{4,3}$) in buffalo milk with 73.4 g/kg fat compared to our results – 12.29 μm $d_{4,3}$ in milk with 58.40 g/kg fat. Schafberg et al. (2007) suggested a difference between buffalo and *Bos Taurus* with respect to the milk fat globule synthesis in their mammary gland. The MFGS of B milk also showed more variation throughout the size distribution and this is in agreement with Akhundov (1959). Menard et al. (2010) also reported more variation in buffalo MFGS than in the cow's milk. And the cows' MFGS $d_{4,3}$ obtained from the present study is comparable with their results.

**Carbohydrates and minerals content**

The lactose content was 47.48 g/kg in B milk. This is similar to the report of Khan et al. (2007) who found 47.55 g/kg lactose in water buffalo milk. Hossain and Dev (2013) found 4.59% lactose in cow's milk which is comparable with IC and HX milk in our study. However, RCC was a bit higher – containing 52.65 g/kg lactose. It is interesting to observe the differences between the concentration of glucose and galactose within the milk type. Because, to synthesize lactose, 1:1 glucose and galactose is required. The source of galactose is glucose and part of the galactose also participates in the biosynthesis of glycoproteins and glycolipids (Fox, 2009). This may be one of the possible reason of the presence of more galactose compared to glucose. In addition, α-LA is important in the regulation of the lactose synthesis; RCC was lowest in α-LA but highest in lactose content. The positive correlation between α-LA and lactose is at the end of the lactation (Farrell Jr et al., 2004). We used pooled samples having milk from animals of different stage of lactation. Among the sampled nineteen RCC cows, most of the cows were at early or mid lactation. There might be a threshhold of α-LA to influence the lactose synthesis and RCC may have above of the threshold.

The minerals content in all milk from the different animals is comparable with the report of Medhammar et al. (2012). In B milk, the amount of Ca was higher than the P content. However, Patino et al. (2007) reported more P than Ca in different buffalo breeds. Genotypic variation may be a matter of concern as Glantz et al. (2012) found more Ca and P in milk with β-CN $A_2$ than β-CN $A_1$, and the influence of κ-CN was A>B>E. Ariota et al. (2007) observed a strong correlation between fresh cheese yield and Ca and P content. They also found a negative relationship between Ca:P and rennet coagulation time and positive relationship between $A_{30}$ and soluble Ca and P content. So, the B milk seems to have advantages in this respect compared to others. In B milk, Mg, Na, and K are comparabale with Patino et al. (2007) but Zn content was higher while Cu and Mn were lower. In HX milk, all the individual minerals were comparable with Medhammar et al. (2012). And IC and RCC milk were also found similar to that report except for Ca and P that were higher. Feeding and nutritional status of the animals may also strongly contribute to the variation among the animals. The variation can also be explained by breed differences and the differences in stage of lactation as reported by Patino et al. (2007).

## CONCLUSIONS

All the animals showed considerable variation regarding the principal components in milk. In earlier studies, buffalo milk showed advantages compared to cow's milk with

regard to milk components. However, this study showed that Red Chittagong Cattle and Indigenous cattle milk were more favorable because of their higher true protein, casein, β-casein, lactose and total mineral content. Nevertheless, to select quality milk for curd based milk products (cheese and sweetmeats) depending on highest protein, casein, casein number, casein:whey protein, ratio of $\alpha_{s1}$-, $\alpha_{s2}$- β- and κ-casein and fat content, Red Chittagong Cattle and buffalo milk showed the best data. All the cow's milk contained small casein micelles. But they had lower κ- casein and Ca:P than the buffalo milk, meaning that other factors may play a role in the micellar formation as well.

Buffalo and Red Chittagong Cattle milk should also be preferred from a nutritional point of view because of their high protein content and type, free amino acids, naturally occurring peptides, fat content, conjugated linoleic acid precursors and isomers, total unsaturated fatty acid, lactose, minerals, Ca, P, Mg, Mn and Zn. They were also low in $\alpha_{s1}$-casein and β-lactoglobulin, the two major milk allergen.

The Indigenous cattle milk was more or less similar to Red Chittagong Cattle milk regarding all most all the milk components. Milk of Holstein cross cows was found high in β-lactoglobulin, naturally occuring peptides and total saturated fatty acid.

This work may be regarded as a benchmark for future work on the Bangladeshi dairy cattle for milk and milk products. And the animal breeder should take into consideration the information on milk components before desgining a breeding policy to get higher milk production with desirable quality.

## ACKNOWLEDGMENTS

The authors thank the Food and Health Programme, IKBM , NMBU, Norway for financing the laboratory work and the States Education Loan Fund (Lanekassen), Norway for financing the PhD student M. A. Islam. We are gratefull to the Department of Livestock Services (DLS), GOB, Farmgate, Dhaka, Bangladesh for allowing to take the milk samples from Bangladesh and Veterinary control in Norway to take those samples in Norway. We also extend our gratitude to Mr. Moznu Sarker, PKSF, Dhaka, Bangladesh for his kind help during the sampling in Bangladesh. Our thanks also to Ragnhild Aaboe Inglingstad and Hanne Marie Devle, IKBM, NMBU, Norway for their help in laboratory.

## REFERENCES

Akhundov, D. 1959. Some data on Buffaloes milk. Dairy Sci. (Abstr.) 22:211.

Addeo, F., J. C. Mercier, and B. Ribadeau-Dumas. 1977. The caseins of buffalo milk. J. Dairy Res. 44:455-468.

Ariota, B., G. Campanile, A. Potena, R. Napolano, B. Gasparrini, G. Neglia, and R. Di Palo. 2007. Ca and P in buffalo milk: curd yield and milk clotting parameters. Ital. J. Anim. Sci. 6:497-499.

Barlowska, J., M. Szwajkowska, Z. Litwińczuk, and J. Krol. 2011. Nutritional value and technological suitability of milk from various animal species used for dairy production. Compr. Rev. Food Sci. Food Saf. 10:291-302.

Bell, S. J., G. T. Grochoski, and A. J. Clarke. 2006. Health implications of milk containing β-casein with the $A^2$ genetic variant. Crit. Rev. Food Sci. Nutr. 46:93-100.

Bobe, G., E. G. Hammond, A. E. Freeman, G. L. Lindberg, and D. C. Beitz. 2003. Texture of butter from cows with different milk fatty acid compositions. J. Dairy Sci. 86:3122-3127.

Bonfatti, V., M. Gervaso, R. Rostellato, A. Coletta, and P. Carnier. 2013. Protein composition affects variation in coagulation properties of buffalo milk. J. Dairy Sci. 96:4182-4190.

Clare, D. A. and H. E. Swaisgood. 2000. Bioactive milk peptides: a prospectus. J. Dairy Sci. 83:1187-1195.

Devle, H., I. Vetti, C. F. Naess-Andresen, E. O. Rukke, G. E. Vegarud, and D. Ekeberg. 2012. A comparative study of fatty acid profiles in ruminant and non-ruminant milk. Eur. J. Lipid Sci. Technol. 114:1036-1043.

Devold, T. G., R. Nordbo, T. Langsrud, C. Svenning, M. Jansen Brovold, E. S. Sorensen, B. Christensen, T. Adnoy, and G. E. Vegarud. 2011. Extreme frequencies of the $\alpha_{s1}$-casein "null" variant in milk from Norwegian dairy goats–Implications for milk composition, micellar size and renneting properties. Dairy Sci. Technol. 91:39-51.

Downs, M. L., J. L. Kabourek, J. L. Baumert, and S. L. Taylor. 2013. Milk protein allergy. In: Milk and Dairy Products in Human Nutrition: Production, Composition and Health (Ed. Y. W. Park and G. F.W. Haenlein). Wiley-Blackwell, John Wiley and Sons Ltd. West Sussex, UK. pp. 111-128.

Drake, S. L., M. E. Carunchia Whetstine, M. A. Drake, P. Courtney, K. Fligner, J. Jenkins, and C. Pruitt. 2007. Sources of umami taste in Cheddar and Swiss cheeses. J. Food Sci. 72:S360-S366.

El-Agamy, E. 2007. The challenge of cow milk protein allergy. Small Rumin. Res. 68:64-72.

Elliott, R. B., D. P. Harris, J. P. Hill, N. J. Bibby, and H. E. Wasmuth. 1999. Type I (insulin-dependent) diabetes mellitus and cow milk: casein variant consumption. Diabetologia 42:292-296.

Farrell Jr, H. M., R. Jimenez-Flores, G. T. Bleck, E. M. Brown, J. E. Butler, L. K. Creamer, C. L. Hicks, C. M. Hollar, K. F. Ng-Kwai-Hang, and H. E. Swaisgood. 2004. Nomenclature of the proteins of cows' milk—sixth revision. J. Dairy Sci. 87:1641-1674.

Folch, J., M. Lees, and G. H. Sloane-Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226:497-509.

Fox, P. F. 2009. Lactose: chemistry and properties. In: Advanced Dairy Chemistry. Vol. 3 lactose, water, salts and minor constituents, 3rd Ed. (Ed. P. L. H. McSweeney and P. F. Fox). Springer, NY, USA. pp. 1-15.

Glantz, M., T. G. Devold, G. E. Vegarud, H. Lindmark Mansson, H.

Stalhammar, and M. Paulsson. 2010. Importance of casein micelle size and milk composition for milk gelation. J. Dairy Sci. 93:1444-1451.

Glantz, M., H. L. Mansson, H. Stalhammar, L. O. Barstrom, M. Frojelin, A. Knutsson, C. Teluk, and M. Paulsson. 2009. Effects of animal selection on milk composition and processability. J. Dairy Sci. 92:4589-4603.

Glantz, M., H. L. Mansson, H. Stalhammar, and M. Paulsson. 2012. Effect of polymorphisms in the *leptin*, *leptin receptor* and *acyl-CoA:diacylglycerol acyltransferase 1* (*DGAT1*) genes and genetic polymorphism of milk proteins on bovine milk composition. J. Dairy Res. 79:110-118.

Hallen, E., A. Lunden, A. M. Tyriseva, M. Westerlind, and A. Andren. 2010. Composition of poorly and non-coagulating bovine milk and effect of calcium addition. J. Dairy Res. 77:398-403.

Hayes, M., C. Stanton, G. F. Fitzgerald, and R. P. Ross. 2007. Putting microbes to work: dairy fermentation, cell factories and bioactive peptides. Part II: bioactive peptide functions. Biotechnol. J. 2:435-449.

Heck, J. M. L., H. J. F. Van Valenberg, H. Bovenhuis, J. Dijkstra, and T. C. M. Van Hooijdonk. 2012. Characterization of milk fatty acids based on genetic and herd parameters. J. Dairy Res. 79:39-46.

Heck, J. M. L., H. J. F. Van Valenberg, J. Dijkstra, and A. C. M. Van Hooijdonk. 2009. Seasonal variation in the Dutch bovine raw milk composition. J. Dairy Sci. 92:4745-4755.

Hossain, M. B. and S. R. Dev. 2013. Physiochemical characteristics of various raw milk samples in a selected dairy plant of Bangladesh. Int. J. Eng. Appl. Sci. 1:91-96.

Islam, M. R., M. N. Hassan, M. S. R. Siddiki, M. A. S. Khan, and M. A. Islam. 2008. Determination of quality of milk from different genotype of dairy cows. Bangladesh J. Anim. Sci. 37:52-56.

Jones, R. M. 2003. Particle size analysis by laser diffraction: ISO 13320, standard operating procedures, and Mie theory. Am. Lab. (USA) 35:44-47.

Khan, M. A. S., M. N. Islam, and M. S. R. Siddiki. 2007. Physical and chemical composition of swamp and water buffalo milk: A comparative study. Ital. J. Anim. Sci. 6(Suppl. 2):1067-1070.

Lara-Villoslada, F., M. Olivares, and J. Xaus. 2005. The balance between caseins and whey proteins in cow's milk determines its allergenicity. J. Dairy Sci. 88:1654-1660.

Lindmark-Mansson, H., R. Fonden, and H. E. Pettersson. 2003. Composition of Swedish dairy milk. Int. Dairy J. 13:409-425.

Medhammar, E., R. Wijesinha-Bettoni, B. Stadlmayr, E. Nilsson, U. R. Charrondiere, and B. Burlingame. 2012. Composition of milk from minor dairy animals and buffalo breeds: A biodiversity perspective. J. Sci. Food Agric. 92:445-474.

Meena, H. R., H. Ram, and T. Rasool. 2007. Milk constituents in non-descript buffaloes reared at high altitudes in the Kumaon hills of the central Himalayas. Buffalo Bull. 26:72-76.

Meisel, H. 1998. Overview on milk protein-derived peptides. Int. Dairy J. 8:363-373.

Mestawet, T. A., A. Girma, T. Adnoy, T. G. Devold, and G. E. Vegarud. 2013. Newly identified mutations at the *CSN1S1* gene in Ethiopian goats affect casein content and coagulation properties of their milk. J. Dairy Sci. 96:4857-4869.

Menard, O., S. Ahmad, F. Rousseau, V. Briard-Bion, F. Gaucheron, and C. Lopez. 2010. Buffalo vs. cow milk fat globules: Size distribution, zeta-potential, compositions in total fatty acids and in polar lipids from the milk fat globule membrane. Food Chem. 120:544-551.

Michalski, M. C., B. Camier, V. Briard, N. Leconte, J. Y. Gassi, H. Goudedranche, F. Michel, and J. Fauquant. 2004. The size of native milk fat globules affects physico-chemical and functional properties of Emmental cheese. Lait 84:343-358.

Misra, S. S., A. Sharma, T. K. Bhattacharya, P. Kumar, and S. S. Roy. 2008. Association of breed and polymorphism of $\alpha_{s1}$- and $\alpha_{s2}$-casein genes with milk quality and daily milk and constituent yield traits of buffaloes (*Bubalus bubalis*). Buffalo Bull. 27:294-301.

Narvhus, J. A., K. Osteraas, T. Mutukumira, and R. K. Abrahamsen. 1998. Production of fermented milk using a malty compound-producing strain of *Lactococcus lactis* subsp. lactis biovar. *diacetylactis*, isolated from Zimbabwean naturally fermented milk. Int. J. Food Microbiol. 41:73-80.

Patino, E. M., D. O. Pochon, E. L. Faisal, J. F. Cedres, F. I. Mendez, C. G. Stefani, and G. Crudeli. 2007. Influence of breed, year season and lactation stage on the buffalo milk mineral content. Ital. J. Anim. Sci. 6:1046-1049.

Pretto, D., M. De Marchi, M. Penasa, and M. Cassandro. 2013. Effect of milk composition and coagulation traits on Grana Padano cheese yield under field conditions. J. Dairy Res. 80:1-5.

Qureshi, T. M., G. E. Vegarud, R. K. Abrahamsen, and S. Skeie. 2012. Characterization of the Norwegian autochthonous cheese Gamalost and its angiotensin I-converting enzyme (ACE) inhibitory activity during ripening. Dairy Sci. Technol. 92:613-625.

Ruiz, J., T. Antequera, A. I. Andres, M. J. Petron, and E. Muriel. 2004. Improvement of a solid phase extraction method for analysis of lipid fractions in muscle foods. Anal. Chim. Acta. 520:201-205.

Schafberg, R., R. Schmidt, M. Thiele, and H. H. Swalve. 2007. Fat globule size distribution in milk of a German buffalo herd. Ital. J. Anim. Sci. 6:1080-1083.

Schonfeldt, H. C., N. G. Hall, and L. E. Smit. 2012. The need for country specific composition data on milk. Food Res. Int. 47: 207-209.

Silva, S. V. and F. X. Malcata. 2005. Caseins as source of bioactive peptides. Int. Dairy J. 15:1-15.

St-Gelais, D. and S. Hache. 2005. Effect of β-casein concentration in cheese milk on rennet coagulation properties, cheese composition and cheese ripening. Food Res. Int. 38:523-531.

Timmen, H. and S. Patton. 1988. Milk fat globules: fatty acid composition, size and *in vivo* regulation of fat liquidity. Lipids 23:685-689.

Van Knegsel, A. T. M., H. van den Brand, H. J. Dijkstra, S. Tamminga, and B. Kemp. 2005. Effect of dietary energy source on energy balance, production, metabolic disorders and reproduction in lactating dairy cattle. Reprod. Nutr. Dev. 45:665-688.

Zicarelli, L. 2004. Buffalo milk: Its properties, dairy yield and Mozzarella production. Vet. Res. Commun. 28:127-135.

# PAPER II

# *Ex vivo* digestion of proteins and fat in buffalo milk

**Mohammad Ashiqul Islam[*1], Dag Ekeberg, Elling-Olav Rukke, Gerd Elisabeth Vegarud**

Department of Chemistry, Biotechnology and Food Science,

Norwegian University of Life Sciences, P. O. Box 5003, NO-1432 Aas, Norway

*E-mail addresses*:

mohammad.islam0@nmbu.no

dag.ekeberg@nmbu.no

elling-olav.rukke@nmbu.no

gerd.vegarud@nmbu.no

[*]Corresponding author: Tel.: +47-64965900, Fax: +47-64965901

*Email address*: mohammad.islam0@ nmbu.no or m.a.islam@bau.edu.bd

Department of Chemistry, Biotechnology and Food Science,

Norwegian University of Life Sciences, P. O. Box 5003, NO-1432 Aas, Norway

[1]Permanent Address: Department of Dairy Science, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh.

## Abstract

*Ex vivo* digestion of proteins and fat in whole and skimmed buffalo milk was studied. The aim of the study was to investigate whether the lipids in milk could have an effect on the digestion of the caseins and whey proteins, more specifically the immunogenic proteins, $\alpha_{s1}$-casein and β-lactoglobulin (β-Lg). Human digestion was simulated using human gastric and duodenal juices in a two phase model. All the caseins were fast digested during gastric digestion (20 and 40 min), while α-lactalbumin (α-LA) and (β-Lg) were resistant. However, a residual trace of $\alpha_s$-casein was detected but completely digested after 5 min duodenal digestion. During the duodenal digestion, α-LA and β-Lg were readily digested and after 5 min most of the proteins were hydrolyzed. Very little differences in the degradation profile was shown between whole and skimmed milk, however, in whole buffalo milk small rests of intact α-LA and β-Lg were identified. Only small variation between whole and skimmed buffalo milk was found regarding number of peptides identified, localization of proteolytic site and alignment of the generated peptides. Maximum number of peptides was identified from β-casein and $\alpha_{s1}$-casein followed by κ-casein, $\alpha_{s2}$-casein and β-Lg. Lipid digestion was also fast and after 30 min duodenal digestion, 30% total lipolysis was observed in neutral lipids. Total saturated fatty acids showed 9% more lipolysis than the total unsaturated fatty acids. The short (C4:0 to C8:0) and long chain (≥C17:0) fatty acid showed 8-10% higher lipolysis than the medium chain (C10:0 to C16:0) fatty acids. With regard to the fast digestion of the immunogenic proteins, $\alpha_{s1}$-casein and β-Lg, the results obtained in buffalo milk may be of nutritional importance.


## 1. Introduction

Milk is regarded as nutritionally the most complete food for the offspring. The milk constituents - water, protein, lipids, carbohydrate, minerals, vitamins and other minor

components are present proportionately in milk, required for the neonate of the respective mammals. The composition of milk varies in response of species, breed/type, number and stage of lactation, nutrition and other animal production factors. Medhammar et al. (2012) reviewed the milk composition of several different milk producing animals and considerable variation among the species and even within the species was reported.

Cow, buffalo, sheep and goat holds the major share of world milk production. According to FAOSTAT (2011), buffalo produced 12.96% of the total world milk production, placed next to the cow's milk. But buffalo milk is less studied compare to cow's milk (Abd El-Salam & El-Shibiny, 2011).

Buffalo milk is reported to contain between 2.7 to 4.6% proteins (Medhammar et al., 2012). Caseins (CNs; $\alpha_{s1}$-, $\alpha_{s2}$-, β- and κ-CN) and whey proteins (α-lactalbumin: α-LA and β-lactoglobulin: β-Lg) are the major protein types in buffalo milk and comparable to cow milk. However, the CNs ratio in buffalo and bovine milk may be somewhat different, with a ratio of 2.8:1.0:3.1:1.2 of $\alpha_{s1}$-, $\alpha_{s2}$-, β- and κ-CN in buffalo milk (Islam et al., 2014) and 2.7:1.0:2.7:0.9 in bovine milk (Miranda, Mahe, Leroux & Martin, 2004). The most prominent result was found in the β-Lg content of buffalo milk, which was almost half of the cow's milk (Islam et al., 2014). The differences in protein content may affect the digestibility of the proteins (Almaas et al., 2006). The four CNs exist as micelles, that have an average pI of 4.6, coagulates at low pH like the stomach pH (Dalgleish & Corredig, 2012), whereas the whey proteins remain in solution under physiologic condition. The homolgy of buffalo and cow CNs ranges from ~93% to ~98% (Abd El-Salam & El-Shibiny, 2011). This variation may also lead to the variability in the formation and content of peptides after proteolysis (Ulleberg, 2011).

Milk is a natural emulsion where the lipids are present in the form of colloidal assemblies, the milk fat globule. Meena, Ram & Rasool (2007) reported that, the total fat content of buffalo milk ranges from 3.37 to14.42%. Milk fat contains mainly triglyceride (98%) with small fractions of 0.3% di-, and 0.03% monoglycerides, 0.1% free fatty acids (FFA) , 0.8% phospholipids and 0.32% sterols (Walstra, Wouters & Geurts, 2006). The understanding of the fate and kinetics of dietary lipid digestion is important because of lipid's implication on the human health and in the development of new products. Gastrointestinal (GI) digestion of dietary lipid is influenced by the characteristics of lipid droplet – size, fatty acids distribution of the triglyceride and the surface organization and composition (Berton et al. 2012). The milk fat globule size of buffalo is larger than the cow's milk. Buffalo milk fat globule has been reported to have less membrane materials than that of the cow's milk (Abd El-Salam & El-Shibiny, 2011). The fatty acid (FA) composition of bovine milk fat is highly complex (Reklewska et al. 2002), however, C4-C24 FAs are more common and dominant in the form of saturated (S) or unsaturated (US) FA. Considerable variations in the FA composition of buffalo milk fat and cow milk fat have been reported (Abd El-Salam & El-Shibiny 2011; Islam et al. 2014; Medhammar et al. 2012; Menard et al., 2010). The variation in the cow and buffalo milk fatty acid composition may results in variation in the fatty acid distribution in the triglyceride and thus, also contributes to the variation of the molecular size of the triglycerides. Arumughan &Narayanan (1982) found all most all the C4:0 of buffalo milk fat in the low molecular weight triacylglycerol. Buffalo milk fat had a higher percentage of medium chain triglycerides than the cow's milk (El-Shibiny, Fontecha, Juarez & Abd Rabou, 2005). All these are related with the physiological fate of the dietary lipids including digestion, absorption and subsequent metabolism.

A complex combination of mechanical, physiochemical and physiological processes are involved in the GI digestion of proteins and lipids in human. A number of factors like food composition, buffering capacity of the food, pH, concentration and activities of the enzyme secreted, peristaltic movements, emptying of the stomach and duration of the digestion may influence the digestion. To understand better, how food is being digested, *in vivo* studies provide the most accurate data but are time consuming, costly and have ethical aspects. Compromising between accuracy and ease of utilization, interests in *ex vivo* digestion model is increasing. However, still simulation of human digestion is challenging because of the inherent complexity of the process.

In a review, Hur, Lim, Decker & McClements (2011) has shown wide variability in the existing *in vitro* and *ex vivo* models, especially regarding enzyme used, pH, duration time and steps of digestion. They also emphasize the importance of using physiologically relevant enzymes and other gut-relevant components (bile acids etc.) while designing digestive fluids. So, by using aspirates and GI juices from human volunteers in the *ex vivo* model digestion may be considered as a good approach to mimic the *in vivo* physiological condition.

There are two main phases during protein and lipid digestion in the GI tract. *Firstly*, gastric digestion at pH 1.5 to 5.0 together with pepsin and gastric and lingual lipase. *Secondly*, duodenal digestion at pH 6.5-8.0 by pancreatic and brush border enzymes. Among the pancreatic enzymes, trypsin and chymotrypsin are strongly proteolytic; while lipase along with bile acids (from gall bladder) is strongly lipolytic. Carboxypeptidase and aminopeptidase of brush border origin acts on smaller peptides.

Almaas et al. (2006) developed a model digestion using human GI enzymes and observed that caprine milk proteins were digested faster than the bovine milk proteins. In

111 another study, Inglingstad et al. (2010) reported species variability in the *in vitro* digestion of
112 equine, bovine, caprine and human milk protein by using human GI enzymes. Devle et al.
113 (2014) have showed the reciprocal interacting effects of proteins and lipids during *ex vivo*
114 digestion of bovine milk. To best of our knowledge, no such *ex vivo* study has been done on
115 buffalo milk proteins and lipids digestion. In addition, the data on milk peptides derived from
116 whole and skimmed buffalo milk by using human GI enzymes are also very scanty.

117 The aim of the present study was to investigate the fate of digestion of buffalo milk
118 proteins and lipids by using human gastric and duodenal juices related to the degradation of the
119 immunogenic proteins, $\alpha_{s1}$-casein and β-Lactoglobulin, generation of peptides and free fatty
120 acids and the effect of lipids on the proteolysis.

121 **2. Materials and Methods**

122 *2.1 Milk samples*

123 Pooled, whole, raw fluid milk was collected from nine lactating buffaloes of Bangladesh
124 Livestock Research Institute (BLRI) buffalo farm, Savar, Dhaka-1341, Bangladesh. The
125 sampling was done from the morning milk. The milk samples were preserved by adding
126 bronopol tablet (1 tablet/40 mL milk; D & F control systems, Inc. USA) immediately after
127 milking of the animals, followed by freezing (-20 °C), transported to the Department of
128 Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, P. O.
129 Box 5003, N-1432 Aas, Norway. The milk was kept at -20 °C until used. The milk was thawed
130 in ice water overnight, and then tempered to 37 °C in a water bath. Skimmed milk was
131 prepared by removing lipids by centrifugation at 850 x *g*, at 04 °C for 20 min (Beckman
132 Coulter, Allegra 25R Centrifuge, TS-5.1-500 rotor head, Brea, CA, USA). The true protein
133 (3.5%) and fat (5.84) content were measured as reported earlier (Islam et al. 2014).

134 *2.2 Human gastric and duodenal juices*

135 The human gastric juice (HGJ) and duodenal juice (HDJ) were collected by following
136 the methods described by Holm, Hanssen, Krogdahl & Florholmen (1988) and Ulleberg et al.
137 (2011). The aspiration of juices were performed on six adults (20 to 37 years old), healthy,
138 fasted (for ≥8 hrs) volunteers at Lovisenberg Diakonale Hospital, Oslo, Norway. Aspiration
139 was approved by the Norwegian Research Ethics Committee. In brief, the aspirates were
140 collected in a tube placed on ice by using a triple lumen tube (Maxters catheters, Marceille,
141 France). After collection, the cell debris and mucus were removed by centrifugation and then
142 aspirates were stored at -20 °C until used. The volume, pH, and enzyme activities of HGJ and
143 HDJ were measured. Then a pooled batch of HGJ and HDJ were made to use in *ex vivo*
144 digestion model. A method described by Ulleberg et al. (2011) was used to assay the pepsin
145 activity in HGJ, lipase and total proteolytic activity and total bile salts in HDJ. The pooled
146 batch of HGJ and HDJ were stored at -80 °C until used. Before use, the juices were thawed by
147 keeping them overnight in ice (at 4-6 °C) and then in ice water at room temperature.

148 *2.3 Ex vivo digestion*

149 A method described by Devle et al. (2014) with some modifications was used for the
150 digestion of whole and skimmed buffalo milk. A continuous two phase digestion was
151 performed; first a gastric phase (by HGJ), then a duodenal phase (by HDJ). In the gastric phase,
152 1 mL milk sample – pH was first reduced to 5.0 by 2M HCL and digested for 20 min (G20)
153 then pH was further reduced to 2.5 and digestion continued for another 20 min (G40).
154 Thereafter, pH was adjusted to 7.0 by 2M NaOH, HDJ added and sampling was performed at
155 05 min (D05), 30 min (D30), 60 min (D60) and 120 min (D120). The volume of HGJ and HDJ
156 added to 1 mL of milk was 800 μL (711 unit pepsin activity/g milk protein) and 1150 μL (558

157 unit total proteolytic activity/g milk protein), respectively. The HDJ contained 889 unit lipase
158 activity and 2.4 mM bile acids as well. For protein and peptide analyses, samples (1 mL) were
159 placed on ice immediately after digestion and stored at -20 °C with minimum delay. The lipid
160 samples (1 mL), 20 mL chloroform:methanol (2:1) was added immediately after the digestion
161 and stored at -20 °C. The digestion was performed 3 times.

162 *2.4 Protein and peptide analysis*

163 *2.4.1 Milk protein degradation profiles by SDS-PAGE*

164 To visualize the protein degradation in whole and skimmed buffalo milk at different
165 gastric and duodenal steps of digestion, SDS-PAGE was performed by a method according to
166 Devle et al. (2014) with some modifications. The gastric samples (1 mL) were homogenized by
167 using Ultra Turrax (Yellow Line DI 18 basic, IKA®-Werke GmbH & Co. KG, staufen,
168 Germany) at speed 3 for few seconds. The sample was mixed with SDS-PAGE sample buffer at
169 a ratio of 1:2. Ten microliter of prepared samples were apllied to the respective wells of Any
170 kD™ polyacrylamide separating gels (6.5 – 200 kDa; mini PROTEAN® TGX™ precast gels,
171 Tris Glycine extended, Bio-Rad laboratories, Inc. Made in United States). A low molecular
172 weight marker (LMW-SDS Marker Kit; GE Healthcare, Little Chalfont, Bucks, UK) was used.
173 The gel was run for 35 min at 200 V. Then the gels were fixed, Comassie Briliant Blue stained
174 and destained according to original procedure.

175 *2.4.2 In gel digestion of protein band and identification by Ultra Performance Liquid*
176 *Chromatography (UPLC) and Q-Exactive Mass Spectrometry (MS)*

177 For protein identity, the bands on the SDS-PAGE gels, were cut and in-gel digested
178 according Devle et al. (2014). Loading solution (0.05 %TFA, 2% ACN in water) was added to

179 the dried peptides and the samples were loaded onto a nano-UPLC
180 (RSLC3000, Dionex/Thermo Fisher Scientific, Bremen, Germany) equipped with a trap
181 column (Acclaim PepMap100, C18, 5 µm, 100 Å, 300 µm i.d. x 5 mm, Thermo Fisher
182 Scientific, Bremen, Germany), back flushed onto a 50 cm x 75 µm analytical column (Acclaim
183 PepMap RSLC C18, 2 µm, 100 Å, 75 µm i.d. x 50 cm, nanoViper, Bremen, Germany). For the
184 separation of the peptides, a 45 min gradient from 4 to 40 % solution B (80 % ACN, 0.1%
185 formic acid) at a flow rate of 300 nL $min^{-1}$ was used. The set up of the Q-Exactive MS (Thermo
186 Fisher Scientific, Bremen, Germany) was – full scan (300-1600 m/z) at R=70.000, followed by
187 (up to) 5 MS2 scans at R=35000, using a NCE setting of 28. For MS/MS, singly charged
188 precursors were excluded as were precursors with z>5. And dynamic exclusion was set to 30
189 seconds. The Masconvert module of ProteoWizard (http://proteowizard.sourceforge.net/) was
190 used to convert the raw files to mgf format. And submitted to database search (Swissprot,
191 taxonomy - other mammals) on an in-house Mascot (v.2.4) server. Mass tolerance was 10 ppm
192 and 20 mamu for MS and MS/MS, respectively and allowing for up to 2 missed cleavages. The
193 selected fixed and variable modifications were carbamidomethylated cysteine and oxidized
194 methionine, respectively.
195 *2.4.3 Peptide identification by UPLC and Quadrapole-Time Of Flight (Q-TOF) MS*
196 Peptides from the G20, G40, D05 and D120 samples were desalted and concentrated
197 accroding to a method described by Furlund et al. (2013). Identification of peptides were done
198 by the method of Qureshi, Vegarud, Abrahamsen & Skeie (2012) with some modifications. In
199 brief, peptide mixtures (containing 0.5% formic acid) were applied to a nanoACQUITY™
200 UPLC® (Waters, Milford, USA), equiped with 5-µm symmetry C18 trap column (180 µm × 20
201 mm; Waters, Milford, USA) in front of a 1.7-µm BEH C18 analytical column (75 µm × 100

mm; Waters, Milford, USA). Each sample was run in MS and data dependent tandem MS mode into a Q-TOF Ultima MS (Micromass Ltd., Manchester, UK). The used non-redundant protein sequence database version was NCBInr 20130131 (22749596 sequences; 7819872540 residues).

*2.4.4 Multiple sequence alignment (MSA) of peptides*

A method described by Furlund et al. (2013) with some modifications was used to perform the sequence alignment of peptides. First peptides were selected with minimal operlap for every samples. Then a pool of G peptides from G20 and G40 samples and so for D peptides from D05 and D120 samples were made. The whole and skimmed buffalo milk were compared for their G and D peptides by using Clustal omega (version: CLUSTAL O (1.2.0); http://www.ebi.ac.uk/Tools/msa/clustalo/) and for consensus sequence, MS editor Jalview 2.8 (http://www.jalview.org/) was used. The whole sequence of $\alpha_{s1}$-, $\alpha_{s2}$-, β- and κ-CN and β-Lg and other relevant information were obtained from http://www.ncbi.nlm.nih.gov/ and http://www.uniprot.org/. The MSA technique was also used to localize the proteolytic sites of a particular protein by aligning the peptides from the specific protein on its whole sequence.

*2.5 Lipid analysis*

Total lipid extraction, solid phase extraction (SPE) of neutral lipids (NL) and free fatty acids (FFA), formation of fatty acid methy esters (FAMEs) and Gass Chromatography-Mass Spectrometry (GC-MS) analysis of FAMEs were done by following the method described by Devle et al. (2014) with few modifications.

Briefly, for total lipid extraction, 20 mL chloroform:methanol (2:1, added immediately after digestion) and internal standards in chloroform were added. Then the tubes were allowed for horizontal shaking for 20 min at 350 rpm. Four milliliter NaCl (0.9% in water) was added

to it and vortexed followed by centrifugation at 850 x *g* for 10 min at 20 °C. The organic phase was collected, dried under $N_2$ gas at 37 °C and re-dissolved in 2 mL chloroform.

A liquid handling robot (Gilson, GX-274 ASPEC, Middleton, USA) was used for SPE of 1 mL re-dissolved lipids. The NL and FFA fraction were eluted with 5 mL chloroform and 5 mL diethyl ether:acetic acid (98:2), respectively. Both the fraction were again dried under $N_2$ gas at 37 °C. The NL fraction was re-dissolved by adding 2 mL hexane and methylated by adding 1.5 mL sodium methanolate (3.3 mg $mL^{-1}$) followed by horizontal shaking for 30 min at 350 rpm. Then it was left for 10 min in a vertical position and the hexane phase was transferred in to the GC-vials. The FFA fraction were added with 1 mL boron trifluoride-methanol complex (14% $BF_3$ in $CH_3OH$, Sigma-Aldrich, steinheim, Germany), heated at 70 °C for 5 min. Then 1 mL hexane was added and the hexane phase was transferred in to the GC-vials. Both the NL and FFA FAMEs were then stored at -20 °C until analysed by GC-MS.

An Autospec Ultima MS (Micromass Ltd. Manchester, England) equiped with electron ionization ion source (mass ranze *m/z* 40-600), coupled with gas chromatograph (Agilent 6890 series, Agilent Technology, Wilmington, DE, USA) was used for FAMEs analysis. The type of column used was 50 m CP-Sil 88 capillary column with ID 0.25 and 0.20 μm thickness (Varian, Middelburgh, The Netherlands).

## 3. Results and discussion

### *3.1 Protein digestion*

The protein degradation profiles of buffalo whole and skimmed milk are presented in Figure 1. In both milk, most of the CNs, β- and κ-CN, were degraded after 20 min gastric digestion at pH 5.0 and further degraded after 40 min, $\alpha_s$-CNs. The pattern of CNs degradation appeared similar in both whole and skimmed milk.

248 The α-LA and β-Lg were resistant to gastric digestion, while serum albumin (SA) was
249 degraded. However, after 5 min duodenal digesetion both α-LA and β-Lg were readily
250 digested. In whole milk, traces of β-Lg as a single band was identified by UPLC-MS that was
251 not observed in skimmed milk (Figure 1A, D05, band 1). Traces of α-LA with the degradation
252 product of β-Lg was also observed both in whole milk (Figure 1A, D05, band 2) and skimmed
253 milk (Figure 1B, D05, band 1 and 2) after 5min duodenal digesstion, however, and completely
254 degraded after 30 min (D30). The α-LA was of bovine origin as per database suggestion. The
255 buffalo and cow milk α-LA differs only by a single amino acid, buffalo (Asp17) → cow
256 (Gly17) (D'Ambrosio et al., 2008) reflecting the greater homology between them.
257 The clear protein bands observed in the region 60-30 kDa after duodenal digestion were
258 identified as the digestive enzymes in the HDJ (Devle et al., 2014). Some differences in the
259 gastric digestion after 40 min was observed between buffalo and cow's milk (Devle et al.,
260 2014). The $\alpha_s$-CNs appeared more resistant than the β- and κ-CN in buffalo milk and these
261 results seems to be more consistent with the results of Gallier, Ye & Singh (2012). Almaas et
262 al. (2006) reported that the protein composition in different species may have an influence on
263 the protein digestibility, as higher dgradation of caprine milk CN than the bovine milk CN was
264 observed. Theese results were confirmed by Inglingstad et al. (2010) who reported high CN
265 degradation variability among the milk from bovine, caprine, equine and human. Buffalo milk
266 CN degradability seems to differ from other species in respect to differences in the relative
267 ratios of $\alpha_{s1}$-, $\alpha_{s2}$-, β- and κ-CN (Islam et al., 2014; Miranda, Mahe, Leroux & Martin, 2004).
268 Devle et al. (2014) reported complete resistance of β-Lg in full fat cow's milk after 120
269 min duodenal digestion. In whole buffalo milk, β-Lg was readily digested after 5 min duodenal
270 digestion and fully digested after 120 min (Fig 1A, D05 and D120). The fat in buffalo milk did

not affect the digestion of β-Lg as it did in cow's milk. In the present study the concentration of bile acid was 2.4 mM in the HDJ aspirate used while in the study reported by Devle et al. (2014) the concentration of bile acid was 1.0 mM. According to Gass, Vora, Hofmann, Gray & Khosla (2007), a concentration of 2 mM bile acid may accelerate the β-Lg digestion. The variation in the bile acid concentration may explain some of the variation between the present study and the results obtained by Devle et al. (2014). Another explanation may be that the β-Lg in buffalo milk was almost half of the cow's milk concentration (Islam et al. 2014). However, the genetic variant may also be a factor in β-Lg degradation variability as reported by Tidona et al. (2014).

*3.2 Site of proteolysis and peptides*

The peptides identified were matched with the whole sequence of the protein to localize the proteolytic cutting sites (Figure 2). Among the proteins, $\alpha_{s1}$-CN and β-CN showed extensive proteolysis. The whole and skimmed buffalo milk showed minimum variability in the peptide pattern only by a few residues at the same region of proteolysis or by very few new regions of proteolysis. All most all the κ-CN peptides were identified from the gastric digestion, while most peptides from β-Lg were observed from the duodenal digestion. These results are consistent with the results observed by the protein degradation visualized by SDS-PAGE. The variability of peptide regions among different phase and steps of digestion reveals the subsequent degradation of peptides and release of amino acids or cleaves at a new site due to different specificity of the GI enzymes. The relative low amount of peptides observed may be due the limitations of the UPLC/Q-TOF MS with an identification range of peptides of 800-4500 Da and the amount of amino acids was not analysed.

293 Maximum number of peptides were identified from β-CN and $\alpha_{s1}$-CN followed by κ-
294 CN, $\alpha_{s2}$-CN and β-Lg (Table 1). Some peptides were identified both in the gastric and duodenal
295 phase. The multiple sequence alignments of the peptides generated from the gastric and
296 duodenal digestion showed very little variation between buffalo whole and skimmed milk
297 (Figure 3). Most of the the peptides contained proline neighbouring hydrophobic residue (s) –
298 leucine, isoleucine, valine, phenylalanine, alanine, glycine that could provide a motif for
299 prefered uncleaved peptide bonds. The consensus sequence obtained from Jalview 2.8 also
300 reflects so. This is in agreement with results discussed by Almaas et al. (2011) on peptides
301 generated from β-CN, κ-CN, β-Lg and glycomacropeptide by the digestion of goats milk.
302 Jornvall & Persson (1983) reported that proline restricts further proteolytic processing
303 especially toward protease with trypsin type specificity.

304 *3.3 lipid analysis*

305 The pattern of lipolysis of neutral lipids during gastric and duodenal digestion of whole
306 buffalo milk is shown in Figure 4. No lipolysis was observed after 40 min gastric digestion
307 (G40). A fast lipolysis was shown during the first 30 min duodenal digestion. Thereafter, only
308 very little lipolysis was observed. These results are in agreement with Devle et al. (2014) on
309 cow's milk. According to Pafumi et al. (2002), 10-30% triacylglycerol can be hydrolyzed by
310 the gastric lipase in the stomach. The lack of lipolysis in our study may be due to the low pH
311 2.5 that is far from gastric lipase activity (optimum pH 5 to 6, Carriere, Barrowman, Verger &
312 Laugier, 1993) or insufficient secretion during aspiration of the fasted volunteers. Gastric
313 digestion is reported to be important for further duodenal lipolysis (Gallier, Ye & Singh, 2012;
314 Ye, Cui & Singh, 2011).

315 The concentration of FFAs during the digestion are summerized in Table 2 including
316 the lipolysis. The amount of each fatty acid in the FFA increased sharply after 30 min duodenal
317 digestion and thenafter showed very little changes, as shown in Figure 4. The average lipolysis
318 of saturated short (C4:0 to C8:0), medium (C10:0 to C16:0) and long (≥C17) chain FA were
319 41%, 33% and 43%, respectivley. Among the individual SFA, C4:0 showed the highest
320 lipolysis which was 48% and C6:0 was only 1.5% behind of it. In the USFA, only C18:1
321 Σothers and C16:1 n-7 *cis* showed more than 30% lipolysis. The lowest lipolysis was observed
322 in C12:0 (27%) and C8:0 (28%) among the SFAs and so for C18:2 n-6 *cis* (14%) and C18:2 n-
323 7 *trans* (17%) among the USFAs. The lipolysis of total SFA was 9% higher than the total
324 USFA.

325 The pancreatic lipase has a preference to attack the *sn*-1 and *sn*-3 position of the
326 triglyceride (Armand, 2007; Rogalska, Ransac & Verger, 1990). This could explain the low
327 digestibility of C8:0 and C12:0 as reported to have a preference in *sn*-2 position in bovine milk
328 (Angers, Tousignant, Boudreau & Arul, 1998; Maansson, 2008). However, Blasi et al. (2008)
329 reported that C8:0 is in *sn*-3 position in buffalo milk and C12:0 at *sn*-1 and 3 position.
330 Compared to medium chain FA, the prevalence of short and long chain fatty acids in *sn*-1 and 3
331 positions are reported (Angers, Tousignant, Boudreau & Arul, 1998; Blasi et al., 2008). This
332 explains why the average lipolysis of medium chain FA was less than the long and short chain
333 FA.

334 The overall results showed some variability with cow's milk (Devle et al., 2014). The
335 possible reasons are – *firstly*, the buffalo milk fat globule is larger than the cow's milk and
336 moreover, in the cow's milk study homogenized whole milk was used. Human pancreatic
337 lipase's catalytic efficiency is 4.6-fold higher on smaller than the larger native milk fat globule

(Berton et al., 2012). *Secondly*, the reported variability in the FA distribution in the triacylglycerol of cow's milk and buffalo milk (Angers, Tousignant, Boudreau & Arul, 1998; Blasi et al., 2008 & Maansson, 2008).

## Conclusions

*Ex vivo* digestion of whole and skimmed buffalo milk were performed to study – protein and lipid degradation and the effect of lipid on protein digestion, specifically the immunogenic $\alpha_{s1}$-casein and β-Lactoglobulin . Except for $\alpha_s$-caseins, all the caseins were digested fast during the gastric phase and completely after 40 min. During the duodenal phase, $\alpha_s$-caseins were digested completely after 5 min. In addition, β-lactoglobulin was readily digested after 5 min duodenal digestion in both skimmed and whole buffalo milk. Minimal variations in the peptide pattern was observed between the whole and skimmed milk.

A rapid lipolysis of neutral lipids was observed after 30 min duodenal digestion and the lipolysis was 30%. The short (C4:0-C8:0) and long (≥C17:0) chain fatty acids showed 8-10% more lipolysis than the medium (C10:0-C16:0) chain fatty acids. The lipolysis of total unsaturated fatty acids was 9% less than the total saturated fatty acids.

## Acknowledgements

The authors extend their thanks to Irene Comi for aspiration of human digestive juices with the cooperation of Lovisenberg Diakonale Hospital, Oslo, Norway and making the juices ready to use.

## References


Abd El-Salam, M. H. & El-Shibiny, S. (2011). A comprehensive review on the composition and properties of buffalo milk. *Dairy Science & Technology*, 91, 663-699.

Almaas, H., Cases, A.-L., Devold, T. G., Holm, H., Langsrud, T., Aabakken, L., Aadnoey, T. & Vegarud, G. E. (2006). In vitro digestion of bovine and caprine milk by human gastric and duodenal enzymes. *International Dairy Journal*, 16, 961-968.

Almaas, H., Eriksen, E., Sekse, C., Comi, I., Flengsrud, R., Holm, H., Jensen, E., Jacobsen, M., Langsrud, T. & Vegarud, G. E. (2011). Antibacterial peptides derived from caprine whey proteins, by digestion with human gastrointestinal juice. *British Journal of Nutrition*, 106, 896-905.

Angers, P., Tousignant, É., Boudreau, A. & Arul, J. (1998). Regiospecific analysis of fractions of bovine milk fat triacylglycerols with the same partition number. *Lipids*, 33, 1195-1201.

Armand, M. (2007). Lipases and lipolysis in the human digestive tract: where do we stand? *Current Opinion in Clinical Nutrition & Metabolic Care*, 10, 156-164.

Arumughan, C. & Narayanan, K. (1982). Triacylglycerol composition of buffalo milk fat. *Journal of Dairy Research*, 49, 81-85.

Blasi, F., Montesano, D., De Angelis, M., Maurizi, A., Ventura, F., Cossignani, L., Simonetti, M. S. & Damiani, P. (2008). Results of stereospecific analysis of triacylglycerol fraction from donkey, cow, ewe, goat and buffalo milk. *Journal of Food Composition and Analysis*, 21, 1-7.

Berton, A., Rouvellac, S., Robert, B., Rousseau, F., Lopez, C. & Crenon, I. (2012). Effect of the size and interface composition of milk fat globules on their in vitro digestion by the

human pancreatic lipase: Native versus homogenized milk fat globules. *Food Hydrocolloids*, 29, 123-134.

Carriere, F., Barrowman, J., Verger, R. & Laugier, R. (1993). Secretion and contribution to lipolysis of gastric and pancreatic lipases during a test meal in humans. *Gastroenterology*, 105 (3): 876-888.

Dalgleish, D. G. & Corredig, M. (2012). The structure of the casein micelle of milk and its changes during processing. *Annual Review of Food Science and Technology*, 3, 449-467.

D'Ambrosio, C., Arena, S., Salzano, A. M., Renzone, G., Ledda, L. & Scaloni, A. (2008). A proteomic characterization of water buffalo milk fractions describing PTM of major species and the identification of minor components involved in nutrient delivery and defense against pathogens. *Proteomics*, 8, 3657-3666.

Devle, H., Ulleberg, E. K., Naess-Andresen, C. F., Rukke, E.-O., Vegarud, G. & Ekeberg, D. (2014). Reciprocal interacting effects of proteins and lipids during ex vivo digestion of bovine milk. *International Dairy Journal*, 36, 6-13.

El-Shibiny, S., Fontecha, J., Juarez, M. & Abd Rabou, N. (2005). Triglycerides of buffalo's and cow's samna. *Egyptian Journal of Dairy Science*, 33, 115-133.

FAOSTAT (2011). The statistics division of the Food and Agriculture Organization of the United Nations. Online, available, http://faostat3.fao.org/faostat-gateway/go/to/home/E (accessed on 03 December 2013).

Furlund, C. B., Ulleberg, E. K., Devold, T. G., Flengsrud, R., Jacobsen, M., Sekse, C., Holm, H. & Vegarud, G. E. (2013). Identification of lactoferrin peptides generated by digestion with human gastrointestinal enzymes. *Journal of Dairy Science*, 96, 75-88.

404 Gallier, S., Ye, A. & Singh, H. (2012). Structural changes of bovine milk fat globules during in
405 vitro digestion. *Journal of Dairy Science*, 95, 3579-3592.
406 Gass, J., Vora, H., Hofmann, A. F., Gray, G. M. & Khosla, C. (2007). Enhancement of dietary
407 protein digestion by conjugated bile acids. *Gastroenterology*, 133, 16-23.
408 Holm, H., Hanssen, L. E., Krogdahl, Å. & Florholmen, J. (1988). High and low inhibitor
409 soybean meals affect human duodenal proteinase activity differently: in vivo
410 comparison with bovine serum albumin. *The Journal of Nutrition*, 118, 515-520.
411 Hur, S. J., Lim, B. O., Decker, E. A. & McClements, D. J. (2011). In vitro human digestion
412 models for food applications. *Food Chemistry*, 125, 1-12.
413 Inglingstad, R. A., Devold, T. G., Eriksen, E. K., Holm, H., Jacobsen, M., Liland, K. H., Rukke,
414 E. O. & Vegarud, G. E. (2010). Comparison of the digestion of caseins and whey
415 proteins in equine, bovine, caprine and human milks by human gastrointestinal
416 enzymes. *Dairy Science & Technology*, 90, 549-563.
417 Islam, M. A., Alam, M. K., Islam, M. N., Khan, M. A. S., Ekeberg, D., Rukke, E. O. &
418 Vegarud, G. E. (2014). Principal milk components in buffalo, Holstein cross, indigenous
419 cattle and Red Chittagong Cattle from Bangladesh. *Asian-Australasian Journal of*
420 *Animal Sciences*, Accepted manuscript.
421 Jornvall, H. & Persson, B. (1983). Amino acid sequence restriction in relation to proteolysis.
422 *Bioscience reports*, 3, 225-232.
423 Maansson, H. L. (2008). Fatty acids in bovine milk fat. *Food & nutrition research*, 52, DOI:
424 10.3402/fnr.v52i0.1821.
425 Medhammar, E., Wijesinha-Bettoni, R., Stadlmayr, B., Nilsson, E., Charrondiere, U. R. &
426 Burlingame, B. (2012). Composition of milk from minor dairy animals and buffalo

breeds: a biodiversity perspective. *Journal of the Science of Food and Agriculture,* 92, 445-474.

Meena, H., Ram, H. & Rasool, T. (2007). Milk constituents in non-descript buffaloes reared at high altitudes in the Kumaon hills of the central Himalayas. *Buffalo Bulletin*, 26, 72-76.

Menard, O., Ahmad, S., Rousseau, F., Briard-Bion, V., Gaucheron, F. & Lopez, C. (2010). Buffalo vs. cow milk fat globules: Size distribution, zeta-potential, compositions in total fatty acids and in polar lipids from the milk fat globule membrane. *Food chemistry*, 120, 544-551.

Miranda, G., Mahé, M.-F., Leroux, C. & Martin, P. (2004). Proteomic tools to characterize the protein fraction of Equidae milk. *Proteomics,* 4, 2496-2509.

Pafumi, Y., Lairon, D., de la Porte, P. L., Juhel, C., Storch, J., Hamosh, M. & Armand, M. (2002). Mechanisms of Inhibition of Triacylglycerol Hydrolysis by Human Gastric Lipase. *Journal of Biological Chemistry*, 277, 28070-28079.

Qureshi, T. M., Vegarud, G. E., Abrahamsen, R. K. & Skeie, S. (2012). Characterization of the Norwegian autochthonous cheese Gamalost and its angiotensin I-converting enzyme (ACE) inhibitory activity during ripening. *Dairy Science & Technology*, 92, 613-625.

Reklewska, B., Oprządek, A., Reklewski, Z., Panicke, L., Kuczyńska, B. & Oprządek, J. (2002). Alternative for modifying the fatty acid composition and decreasing the cholesterol level in the milk of cows. *Livestock Production Science*, 76, 235-243.

Rogalska, E., Ransac, S. & Verger, R. (1990). Stereoselectivity of lipases. II. Stereoselective hydrolysis of triglycerides by gastric and pancreatic lipases. *Journal of Biological Chemistry*, 265 (33): 20271-20276.

449 Tidona, F., Criscione, A., Devold, T. G., Bordonaro, S., Marletta, D. & Vegarud, G. E. (2014).
450 Protein composition and micelle size of donkey milk with different protein patterns:
451 Effects on digestibility. *International Dairy Journal*, 35, 57-62.
452 Ulleberg, E. K. (2011). In vitro digestion of caprine whey proteins by human gastrointestinal
453 juices: Effect of whey hydrolysates and peptides on in vitro cell responses. PhD thesis.
454 IKBM, UMB, Ås-1432, Norway.
455 Ulleberg, E. K., Comi, I., Holm, H., Herud, E. B., Jacobsen, M. & Vegarud, G. E. (2011).
456 Human gastrointestinal juices intended for use in in vitro digestion models. *Food*
457 *digestion*, 2, 52-61.
458 Walstra, P., Wouters, J. T. M. & Geurts, T. J. (2006). *Dairy Science and Technology*. (2nd ed.).
459 London: CRC Press. Taylor & Frances Group, (Chapter 2).
460 Ye, A., Cui, J. & Singh, H. (2011). Proteolysis of milk fat globule membrane proteins during in
461 vitro gastric digestion of milk. *Journal of Dairy Science*, 94, 2762-2770.
462
463

464 Table 1. Number of peptides identified from whole (W) and skimmed (S) buffalo milk after
465 gastric (G) and duodenal (D) digestion.

| Protein | G 20 | | G 40 | | D 05 | | D 120 | |
|---|---|---|---|---|---|---|---|---|
| | W | S | W | S | W | S | W | S |
| $\alpha_{s1}$-CN | 18 | 18 | 17 | 19 | 11 | 10 | 02 | 03 |
| $\alpha_{s2}$-CN | - | 03 | 03 | 04 | 03 | 04 | 04 | - |
| β-CN | 28 | 29 | 25 | 26 | 37 | 39 | 37 | 21 |
| κ-CN | 09 | 11 | 05 | 08 | 02 | - | - | - |
| β-Lg | - | - | 01 | - | 02 | 05 | 04 | 02 |

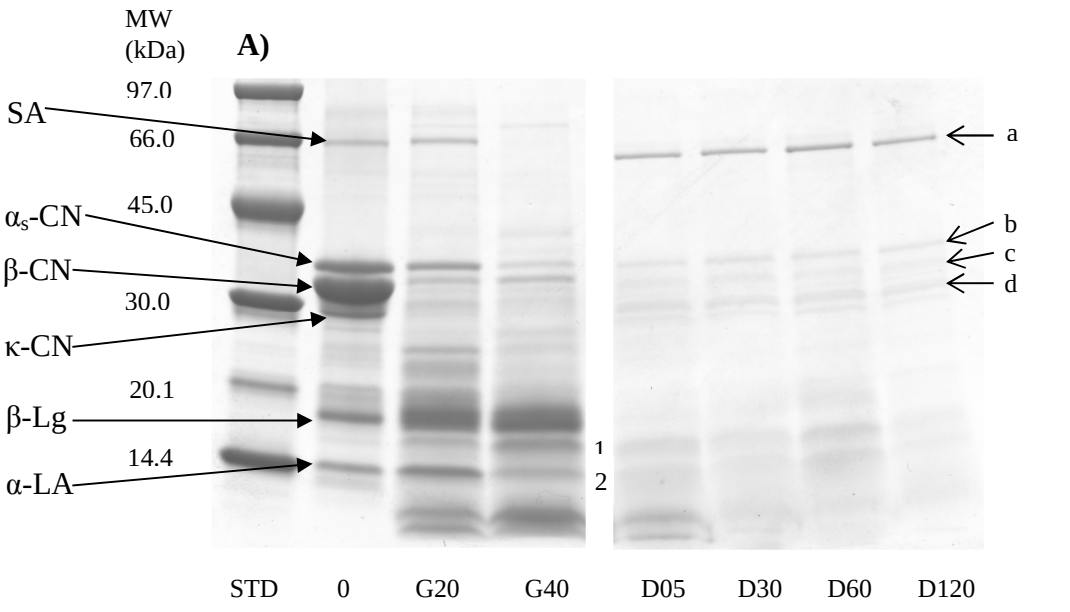
466 The pH of all the duodenal digestion was 7.0. Abbreviations: CN, casein; β-Lg, β-lactoglobulin;
467 G20, gastric digestion at pH 5.0 for 20 min; G40, gastric digestion at pH 2.5 for 20 min after 20
468 min gastric digestion at pH 5.0; D05 and D120, duodenal digestion for 05 and 120 min, respectively.
469

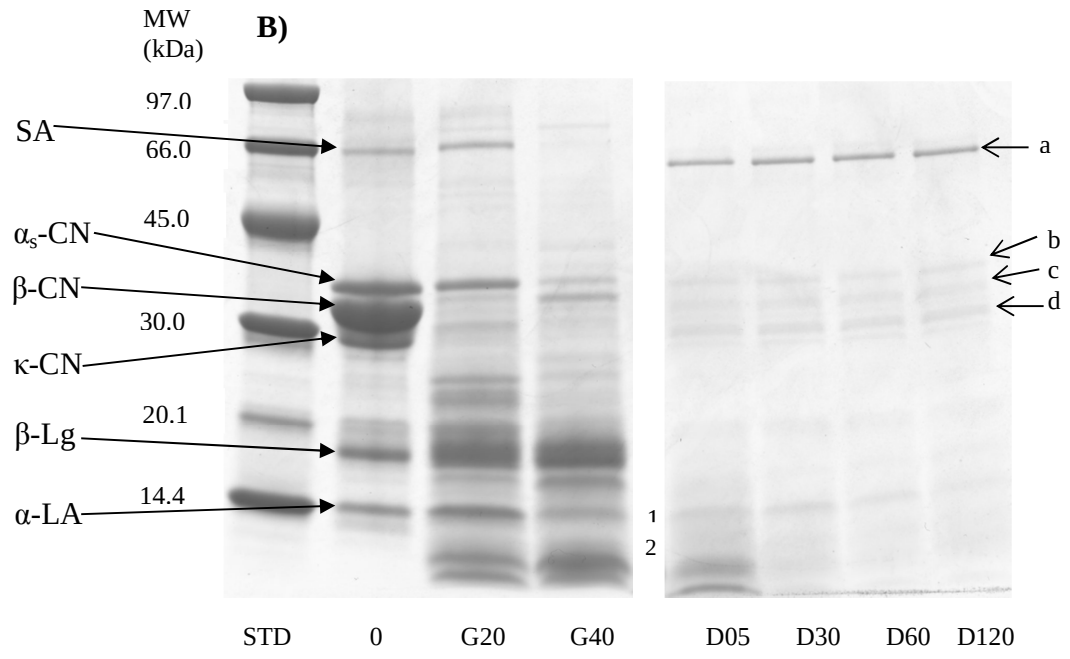
**Table 2.** Fatty acid (FA) concentrations (μg $mL^{-1}$ milk ± standard deviation) in the free fatty acid fraction of whole buffalo milk during digestion with human gastric (G) and duodenal (D) juices. It also includes the lipolysis (%) of individual FA in neutral lipids (NL) fraction after 120 min duodenal digestion.

| FA | G40 | D30 | D60 | D120 | Lipolysis (%) |
|---|---|---|---|---|---|
| C4:0 | nd | nd | nd | nd | 48.0 |
| C6:0 | 1.7±0.0 | 32.6±3.3 | 20.7±1.3 | 21.6±4.9 | 46.5 |
| C8:0 | 1.4±0.5 | 44.7±6.4 | 34.9±4.0 | 29.7±9.2 | 28.4 |
| C10:0 | 2.4±0.4 | 110.4±10.1 | 118.3±5.0 | 114.3±3.5 | 34.1 |
| C12:0 | 17.1±10.8 | 176.8±17.0 | 191.2±5.6 | 186.14±6.2 | 27.5 |
| C14:0 | 28.8±2.5 | 1111.0±68.0 | 1258.2±30.3 | 1226.9±53.1 | 33.6 |
| C15:0 | 3.6±0.7 | 258.90±11.4 | 332.6±6.4 | 335.8±7.7 | 33.0 |
| C16:0 | 159.2±2.8 | 3999.0±268.0 | 4709.7±138.8 | 4660.3±168.2 | 36.5 |
| C17:0 | 3.3±0.7 | 110.2±5.3 | 152.0±3.9 | 162.3±2.7 | 43.3 |
| C18:0 | 163.2±4.3 | 2012.9±131.5 | 2804.9±120.5 | 2899.7±82.2 | 42.8 |
| C20:0 | nd | 22.9±4.0 | 34.0±2.4 | 35.0±2.0 | 42.2 |
| ΣSFA | 380.8±7.1 | 7879.0±501.0 | 9656.0±315.0 | 9672.0±286.0 | 37.6 |
| C14:1 n-5 *cis* | 2.2±0.4 | 66.4±3.5 | 82.5±0.9 | 78.4±2.6 | 24.8 |
| C16:1 n-7 *cis* | 4.3±0.9 | 312.1±13.0 | 392.6±6.1 | 382.1±8.5 | 31.9 |
| C18:1 n-9 *cis* | 79.1±10.8 | 3167.0±287.0 | 4269.0±191.0 | 4037.0±226.0 | 27.9 |
| C18:1 Σothers | nd | 236.8±18.8 | 351.8±6.0 | 359.3±5.2 | 38.3 |
| C18:2 n-6 *cis* | 7.6±2.1 | 186.0±12.3 | 225.2±1.2 | 215.7±6.8 | 14.1 |
| C18:2 n-7 *trans* | nd | 38.9±3.9 | 54.6±2.0 | 49.8±2.0 | 17.0 |
| C18:3 n-3 *cis* | nd | 11.3±1.4 | 15.3±3.1 | 15.4±3.1 | - |
| C20:4 n-6 *cis* | nd | 15.9±3.1 | 15.9±2.1 | 16.4±1.6 | - |
| ΣUSFA | 93.1±11.3 | 4034.0±329.0 | 5407.0±198.0 | 5154.0±243.0 | 28.3 |

Lipolysis: {(FA in undigested NL – FA in D120 NL)/FA in undigested NL} x 100. The pH of all the duodenal digestion was 7.0. Abbreviations: G40, gastric digestion at pH 2.5 for 20 min after 20 min gastric digestion at pH 5.0; D30, D60 and D120, duodenal digestion for 30, 60 and 120 min, respectively; nd, not detected; ΣSFA, total saturated fatty acids; ΣUSFA, total unsaturated fatty acids.

**Figure 1**


A)
MW
(kDa)
97.0
66.0
45.0
30.0
20.1
14.4
SA
αs-CN
β-CN
κ-CN
β-Lg
α-LA
1
2
a
b
c
d
STD
0
G20
G40
D05
D30
D60
D120


B)
MW
(kDa)
97.0
66.0
45.0
30.0
20.1
14.4
SA
αs-CN
β-CN
κ-CN
β-Lg
α-LA
1
2
a
b
c
d
STD
0
G20
G40
D05
D30
D60
D120

503 **Figure 2**

504

$\alpha_{s1}$-Casein (Accession number: O62823)

```
G20   1  MKLLILTCLVAVALARPKQPIKHQGLPQGVLNENLLRFFVAPFPEVFGKEKVNELSTDIG    60
G40   1  MKLLILTCLVAVALARPKQPIKHQGLPQGVLNENLLRFFVAPFPEVFGKEKVNELSTDIG    60
D05   1  MKLLILTCLVAVALARPKQPIKHQGLPQGVLNENLLRFFVAPFPEVFGKEKVNELSTDIG    60
D120  1  MKLLILTCLVAVALARPKQPIKHQGLPQGVLNENLLRFFVAPFPEVFGKEKVNELSTDIG    60

G20   61  SESTEDQAMEDIKQMEAESISSSEEIVPISVEQKHIQKEDVPSERYLGYLEQLLRLKKYN   120
G40   61  SESTEDQAMEDIKQMEAESISSSEEIVPISVEQKHIQKEDVPSERYLGYLEQLLRLKKYN   120
D05   61  SESTEDQAMEDIKQMEAESISSSEEIVPISVEQKHIQKEDVPSERYLGYLEQLLRLKKYN   120
D120  61  SESTEDQAMEDIKQMEAESISSSEEIVPISVEQKHIQKEDVPSERYLGYLEQLLRLKKYN   120

G20   121  VPQLEIVPNLAEEQLHSMKEGIHAQQKEPMIGVNQELAYFYPQLFRQFYQLDAYPSGAWY  180
G40   121  VPQLEIVPNLAEEQLHSMKEGIHAQQKEPMIGVNQELAYFYPQLFRQFYQLDAYPSGAWY  180
D05   121  VPQLEIVPNLAEEQLHSMKEGIHAQQKEPMIGVNQELAYFYPQLFRQFYQLDAYPSGAWY  180
D120  121  VPQLEIVPNLAEEQLHSMKEGIHAQQKEPMIGVNQELAYFYPQLFRQFYQLDAYPSGAWY  180

G20   181  YVPLGTQYPDAPSFSDIPNPIGSENSGKTTMPLW 214
G40   181  YVPLGTQYPDAPSFSDIPNPIGSENSGKTTMPLW 214
D05   181  YVPLGTQYPDAPSFSDIPNPIGSENSGKTTMPLW 214
D120  181  YVPLGTQYPDAPSFSDIPNPIGSENSGKTTMPLW 214
```

$\alpha_{s2}$-Casein (Accession number: **Q3Y443**)

```
G20    1  MKFFIFTCLLAVALAKHTMEHVSSSEESIISQETYKQEKNMAIHPSKENLCSTFCKEVIR   60
G40    1  MKFFIFTCLLAVALAKHTMEHVSSSEESIISQETYKQEKNMAIHPSKENLCSTFCKEVIR   60
D05    1  MKFFIFTCLLAVALAKHTMEHVSSSEESIISQETYKQEKNMAIHPSKENLCSTFCKEVIR   60
D120   1  MKFFIFTCLLAVALAKHTMEHVSSSEESIISQETYKQEKNMAIHPSKENLCSTFCKEVIR   60

G20    61  NANEEEYSIGSSSEESAEVATEEVKITVDDKHYQKALNEINQFYQKFPQYLQYLYQGPIV  120
G40    61  NANEEEYSIGSSSEESAEVATEEVKITVDDKHYQKALNEINQFYQKFPQYLQYLYQGPIV  120
D05    61  NANEEEYSIGSSSEESAEVATEEVKITVDDKHYQKALNEINQFYQKFPQYLQYLYQGPIV  120
D120   61  NANEEEYSIGSSSEESAEVATEEVKITVDDKHYQKALNEINQFYQKFPQYLQYLYQGPIV  120

G20    121  LNPWDQVKRNAVPITPTLNREQLSTSEENSKKTVDMESTEVITKKTKLTEEDKNRLNFLK 180
G40    121  LNPWDQVKRNAVPITPTLNREQLSTSEENSKKTVDMESTEVITKKTKLTEEDKNRLNFLK 180
D05    121  LNPWDQVKRNAVPITPTLNREQLSTSEENSKKTVDMESTEVITKKTKLTEEDKNRLNFLK 180
D120   121  LNPWDQVKRNAVPITPTLNREQLSTSEENSKKTVDMESTEVITKKTKLTEEDKNRLNFLK 180

G20    181  KISQHYQKFTWPQYLKTVYQYQKAMKPWTQPKTKVIPYVRYL 222
G40    181  KISQHYQKFTWPQYLKTVYQYQKAMKPWTQPKTKVIPYVRYL 222
G05    181  KISQHYQKFTWPQYLKTVYQYQKAMKPWTQPKTKVIPYVRYL 222
D120   181  KISQHYQKFTWPQYLKTVYQYQKAMKPWTQPKTKVIPYVRYL 222
```

505

β-Casein (Accession number**: Q9TSI0**)

506

```
G20  1   MKVLILACLVALALARELEELNVPGEIVESLSSSEESITHINKKIEKFQSEEQQQMEDEL   60
G40  1   MKVLILACLVALALARELEELNVPGEIVESLSSSEESITHINKKIEKFQSEEQQQMEDEL   60
D05  1   MKVLILACLVALALARELEELNVPGEIVESLSSSEESITHINKKIEKFQSEEQQQMEDEL   60
D120 1   MKVLILACLVALALARELEELNVPGEIVESLSSSEESITHINKKIEKFQSEEQQQMEDEL   60

G20  61    QDKIHPFAQTQSLVYPFPGPIPKSLPQNIPPLTQTPVVVPPFLQPEIMGVSKVKEAMAPK  120
G40  61    QDKIHPFAQTQSLVYPFPGPIPKSLPQNIPPLTQTPVVVPPFLQPEIMGVSKVKEAMAPK  120
D05  61    QDKIHPFAQTQSLVYPFPGPIPKSLPQNIPPLTQTPVVVPPFLQPEIMGVSKVKEAMAPK  120
D120 61    QDKIHPFAQTQSLVYPFPGPIPKSLPQNIPPLTQTPVVVPPFLQPEIMGVSKVKEAMAPK  120

G20  121   HKEMPFPKYPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQPPQPLPPTVMFPPQSVLSL  180
G40  121   HKEMPFPKYPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQPPQPLPPTVMFPPQSVLSL  180
D05  121   HKEMPFPKYPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQPPQPLPPTVMFPPQSVLSL  180
D120 121   HKEMPFPKYPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQPPQPLPPTVMFPPQSVLSL  180

G20  181   SQSKVLPVPQKAVPYPQRDMPIQAFLLYQEPVLGPVRGPFPIIV 224
G40  181   SQSKVLPVPQKAVPYPQRDMPIQAFLLYQEPVLGPVRGPFPIIV 224
D05  181   SQSKVLPVPQKAVPYPQRDMPIQAFLLYQEPVLGPVRGPFPIIV 224
D120 181   SQSKVLPVPQKAVPYPQRDMPIQAFLLYQEPVLGPVRGPFPIIV 224
```

κ-Casein (Accession number**: A8KRP5**)

```
G20    1   MMKSFFLVVTILALTLPFLGAQEQNQEQPIRCEKEERFFNDKIAKYIPIQYVLSRYPSYG   60
G40    1   MMKSFFLVVTILALTLPFLGAQEQNQEQPIRCEKEERFFNDKIAKYIPIQYVLSRYPSYG   60
D05    1   MMKSFFLVVTILALTLPFLGAQEQNQEQPIRCEKEERFFNDKIAKYIPIQYVLSRYPSYG   60
D120   1   MMKSFFLVVTILALTLPFLGAQEQNQEQPIRCEKEERFFNDKIAKYIPIQYVLSRYPSYG   60

G20    61   LNYYQQKPVALINNQFLPYPYYAKPAAVRSPAQILQWQVLPNTVPAKSCQAQPTTMTRHP  120
G40    61   LNYYQQKPVALINNQFLPYPYYAKPAAVRSPAQILQWQVLPNTVPAKSCQAQPTTMTRHP  120
D05    61   LNYYQQKPVALINNQFLPYPYYAKPAAVRSPAQILQWQVLPNTVPAKSCQAQPTTMTRHP  120
D120   61   LNYYQQKPVALINNQFLPYPYYAKPAAVRSPAQILQWQVLPNTVPAKSCQAQPTTMTRHP  120

G20    121  HPHLSFMAIPPKKNQDKTEIPTINTIVSVEPTSTPITEAIENTVATLEASSEVIESVPET 180
G40    121  HPHLSFMAIPPKKNQDKTEIPTINTIVSVEPTSTPITEAIENTVATLEASSEVIESVPET 180
D05    121  HPHLSFMAIPPKKNQDKTEIPTINTIVSVEPTSTPITEAIENTVATLEASSEVIESVPET 180
D120   121  HPHLSFMAIPPKKNQDKTEIPTINTIVSVEPTSTPITEAIENTVATLEASSEVIESVPET 180

G20    181  NTAQVTSTVV 190
G40    181  NTAQVTSTVV 190
D05    181  NTAQVTSTVV 190
D120   181  NTAQVTSTVV 190
```

β-Lactoglobulin (Accession number: **C3W955**)

```
G20    1  MKCLLLALGLALACGAQAIIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVY   60
G40    1  MKCLLLALGLALACGAQAIIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVY   60
D05    1  MKCLLLALGLALACGAQAIIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVY   60
D120   1  MKCLLLALGLALACGAQAIIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVY   60

G20   61  VEELKPTPEGDLEILLQKWENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKY  120
G40   61  VEELKPTPEGDLEILLQKWENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKY  120
D05   61  VEELKPTPEGDLEILLQKWENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKY  120
D120  61  VEELKPTPEGDLEILLQKWENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKY  120

G20   121  LLFCMENSAEPEQSLACQCLVRTPEVDDEALEKFDKALKALPMHIRLSFNPTQLEEQCHV 180
G40   121  LLFCMENSAEPEQSLACQCLVRTPEVDDEALEKFDKALKALPMHIRLSFNPTQLEEQCHV 180
D05   121  LLFCMENSAEPEQSLACQCLVRTPEVDDEALEKFDKALKALPMHIRLSFNPTQLEEQCHV 180
D120  121  LLFCMENSAEPEQSLACQCLVRTPEVDDEALEKFDKALKALPMHIRLSFNPTQLEEQCHV 180
```

**Figure 3**

A) Peptides from $\alpha_{s1}$-casein (gastric phase)

```
BWG-05      ----------------------------RPKQPI-KHQGLPQGVLNENL 20
BSG-01      ----------------------------RPKQPI-KHQGLPQGVLNENL 20
BWG-02      ------------FVAPFPEVFGKEKVNELS------------------ 18
BSG-02      ------------FVAPFPEVFGKEKVNELS------------------ 18
BWG-08      ---------------------AEEQLHSMKEGIHAQQKEPMIGVNQEL 27
BSG-05      ---------------------AEEQLHSMKEGIHAQQKEPMIGVNQEL 27
BWG-09      --------------EIVP-NLAEEQLHSMKE----------------- 16
BSG-06      --------------EIVP-NLAEEQLHSMKE----------------- 16
BWG-06      ----------------------------LRLKKYNV-----PQL------ 11
BSG-03      ----------------------LEQLLRLKKYNV-----PQL------ 15
BWG-07      ----------------------FRQFYQL------------------- 7
BSG-04      ----------------------FRQFYQL------------------- 7
BWG-01      --------------SDIPNPIGSENSGKTTM----------------- 17
BSG-07      --------------SDIPNPIGSENSGKTTM----------------- 17
BSG-08      ------------IQKEDVPSERY------------------------- 11
BSG-09      ---PLGTQYPDAPLFSDIPN---------------------------- 17
BWG-03      YYVPLGTQYPDAPLF--------------------------------- 15
BSG-11      YYVPLGTQYPDAPLF--------------------------------- 15
BWG-04      YYVPLGTQYPDAPSF--------------------------------- 15
BSG-10      YYVPLGTQYPDAPSF--------------------------------- 15
BSG-12      ------DAYPSGAW---------------------------------- 8
Consensus   YYVPLGTQYPDAPLFEDIPNNFAEEQLHRLKEGIHAHQGEPQGGLNEEL
```

B) Peptides from $\alpha_{s1}$-casein (duodenal phase)

```
BWD-03      ----SDIPNPIGSENSGK------  14
BSD-01      ----SDIPNPIGSENSGK------  14
BWD-07      ------------EIVPNLAEEQLH  12
BSD-04      ------------EIVPNLAEEQLH  12
BWD-01      ---------YLGYLEQLL------  9
BWD-05      ------YYVPLGTQYPDAPL----  14
BWD-02      QPIK-HQGLPQGVLNENL------  17
BSD-03      QPIK-HQGLPQGVLNENL------  17
BSD-06      QPIK-HQGLPQGVLNENL------  17
BSD-02      ------YFYPQ------------  5
BWD-04      EGIHAQQKEPMIGVNQEL------  18
BSD-05      EGIHAQQKEPMIGVNQEL------  18
BWD-06      ---HIQKED-VP---SE-------  10
Consensus   QPIHAHQGLPQGVLNENLAEEQLH
```

519 C) Peptides from $\alpha_{s2}$-casein (gastric phase)

```
BSG-01      VYQYQKAMKPWTQPKTNVIPYVRYL 25
BSG-02      LYQGPIVLNPWDQVKRNAVPITPTL 25
BWG-01      LYQGPIVLNPWDQVKRNAVPITPTL 25
            :**   .::** * * *.:* .  *
Consensus   LYQGPIVLNPWDQVKRNAVPITPTL
```

D) Peptides from $\alpha_{s2}$-casein (duodenal phase)

```
BWD-02      ------ALNEINQFYQKFPQ      14
BWD-03      LYQGPIVLNPWDQVKRN---      17
BWD-01      -------LTEEDKNRLN---      10
BSD-02      -----TKLTEEDKNRLNFL-      14
BSD-01      -------ITVDDKHYQ----      9
                   :.  ::
Consensus:  -------LTEEDKNRLNF-
```

E) Peptides from β-casein (gastric phase)

```
BWG-08      -------GVSKVKE--AMAPKHKEMPFPK--YPVEPFTESQ------------------- 30
BSG-01      -------GVSKVKE--AMAPKHKEMPFPK--YPVEPFTESQSLTL--------------- 34
BWG-04      --------------------------------PVEPFTESQSLTLTDVENLHLPLPLL--- 26
BSG-02      ---------------AMAPKHKEMPFPK--YPVEPFTESQSLTLTDVENLHLPLPLL--- 40
BWG-06      ----------------------------------------------TDVENLHLPLPLLQSW 15
BWG-05      ---------------------------------------------LTLTDVENLHLPLPLLQS- 18
BSG-03      ---------------------------------------------LTLTDVENLHLPLPLLQSW 18
BWG-02      -----QSWMHQPPQ-----------PLPPTVM----------------------------- 16
BSG-06      -----QSWMHQPPQ-----------PLPPTVM----------------------------- 16
BWG-03      --------MHQPPQ-----------PLPPTVMFPPQ----SVL------------------ 20
BSG-05      --------MHQPPQ-----------PLPPTVMFPPQ----SVL------------------ 20
BWG-07      FLLYQEPVLGPVRGPFPIIV------------------------------------------ 20
BSG-04      --LYQEPVLGPVRGPFPIIV------------------------------------------ 18
BWG-09      -------------------LQDKIHPFAQTQS----------------------------- 13
BSG-08      -------------------LQDKIHPFAQTQS----------------------------- 13
BWG-01      --SLSQSKVLPVP--------QKAVPYPQRDMPIQA------------------------- 26
BSG-09      --SLSQSKVLPVP--------QKAVPYPQRDMPIQAFL----------------------- 28
BWG-10      -------------------------------PVVVPPFLQPEIMGVSKVKE----------- 20
BSG-07      -------------------------------PVVVPPFLQPEIMGVSKVKE----------- 20
BWG-11      -------LVYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEIM------------------ 36
BSG-11      -------LVYPFPGPIPNSLPQNIPPLTQT------------------------------- 23
BSG-10      -----QSLVYPFPGPIPK------------------------------------------- 13
Consensus:  --LLQQSLVHPVPGPIPMALKQKIMPLPQTVMPVPPFLESQILTLTDVENLHLPLPLLQSW
```

F) Peptides from β-casein (duodenal phase)

```
BWD-01      --RELEELNVPGEIVE---------------------------     14
BWD-03      SWM----HQPPQPLPPTV-------------------------     14
BWD-04      -WM----HQPPQPLPPTVM--FPPQS------------V----     20
BSD-04      -WM----HQPPQPLPPTVM--FPPQS------------V----     20
BWD-05      --M----HQPPQPLPPTVM--FPPQS------------VL---     20
BSD-05      --M----HQPPQPLPPTVM--FPPQS------------VLS--     21
BWD-06      SLTLTDVENLHLPLPL---------------------------     16
BSD-01      SLTLTDVENLHLPLPL---------------------------     16
BWD-07      -LTLTDVENLHLPLPLL--------------------------     16
BSD-02      -LTLTDVENLHLPLPLL--------------------------     16
BWD-10      AQTQSLVYPFPGPIPK---------------------------     16
BSD-06      AQTQSLVYPFPGPIPK---------------------------     16
BWD-11      -----LVYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEIM---     36
BSD-08      -----LVYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEIM---     36
BWD-12      -------------------------PVVVPPFLQPEIMG--      14
BSD-07      ---------------SLPQNIPPLTQTPVVVPPFLQPEIMGVS    28
BWD-08      LYQEPVLGPVRGPFPIIV-------------------------     18
BSD-09      LYQEPVLGPVRGPFPIIV-------------------------     18
BWD-02      -QMEDELQDKIHPF-----------------------------     13
BSD-03      -QMEDELQDKIHPF-----------------------------     13
BWD-09      --------HKEMPFPK---------------------------     8
BSD-10      --------HKEMPFPK---------------------------     8
Consensus   SLMETDVHPPPGPLPPTVMQNFPPQSQTPVVVPPFLQPEIMG-
```

543

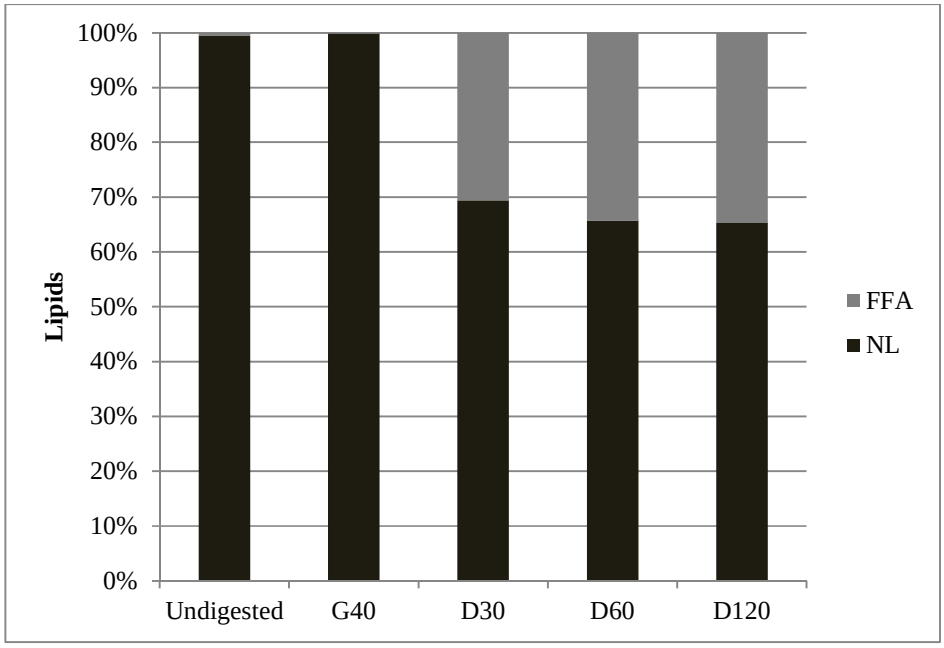
G) Peptides from κ-casein (gastric phase)

```
BWG-01      --------------------MAIPPKKNQDKTEIPTINT  19
BSG-02      --------------------MAIPPKKNQDKTEIPTINT  19
BWG-06      ----------------GLNYYQQKPVAL-----------  12
BSG-07      ------------------YYQQKPVAL-----------  9
BWG-07      ----------INNQFLPYPYYA-KPAAVRSPAQILQ---  25
BSG-06      ---------LINNQFLPYPYYA-KPAAVRSPAQILQ---  26
BWG-02      FNDKIAKYIPIQY-------------------------  13
BSG-01      FNDKIAKYIPIQYVL-----------------------  15
BWG-03      ----------TRHPHPHLSF------------------  10
BSG-03      ----------TRHPHPHLSF------------------  10
BWG-04      -------YVLSRYPSY----------------------  9
BSG-05      -------YVLSRYPSY----------------------  9
BWG-05      --------VLSRYPSYGLN--------------------  11
BSG-04      --------VLSRYPSYGLNY------------------  12
Consensus:  FNDKIAKYVLIRYPSYGLNYYAIKPAALQDKAEILQINT
```

H) Peptides from β-lactoglobulin (duodenal phase)

```
BWD-02      ----KIDALNENK--VL------   11
BSD-01      ----KIDALNENK--VLV-----   12
BWD-01      LYQGPI---------VLNPWDQV   14
BWD-03      VYVEELKPTPEGDLEILLQ----   19
BSD-03      VYVEELKPTPEGDLEILLQ----   19
BSD-04      VYVEELKPTPEGDLE--------   15
BWD-04      --------TPEVDDEALEKFDK-   14
BSD-02      --------TPEVDDEALEKFDKA   15
BWD-05      --------LTEEDKNRLN-----   10
Consensus   VYVEEIKPTPEGDLEVLEKFDK-
```

544 **Figure 4**




545

546 **Figure Legend**

547 **Figure 1.** Protein degradation profile in whole buffalo milk (A) and skimmed buffalo milk (B)
548 after human gastric (G) and duodenal (D) digestion. The pH at G20 and G40 was 5.0 and 2.5,
549 respectively and at D05, D30, D60 and D120 was 7.0. Abbreviations: MW, molecular weight; kDa,
550 kilo dalton; SA, serum albumin; CN, casein; β-Lg, β-lactoglobulin; α-LA, α-lactalbumin. STD, low
551 molecular weight marker; 0, undigested sample; G20 and G40, gastric digestion for 20 and 40 min,
552 respectively; D05, D30, D60 and D120, duodenal digestion for 05, 30, 60 and 120 min, respectively; A)
553 D05: 1, β-lactoglobulin; 2, α-lactalbumin (?) with β-lactoglobulin; B) D05: 1and 2, α-lactalbumin (?)
554 with β-lactoglobulin; ?, database search suggests, this protein is of bovine origin; a, amylase; b,
555 carboxypeptidase, chymotrypsin , elastase, lipase, gastricsin and amylase; c, carboxypeptidase, elastase,
556 lipase, trypsin and amylase; d, elastase, carboxypeptidase, chymotrypsin, amylase, lipase and trypsin.

557 **Figure 2.** Comparative peptide regions derived from the different milk proteins of buffalo
558 whole (grey) and skimmed milk (underlined) after *ex vivo* gastric (G) and duodenal (D)
559 digestion. Single letter amino acid code used. bold and Italic residues, signal peptide. The pH at
560 G20 and G40 was 5.0 and 2.5, respectively and at D05 and D120 was 7.0. Abbreviations: G20 and
561 G40, gastric digestion for 20 and 40 min, respectively; D05 and D120, duodenal digestion for 05and 120
562 min, respectively.

563 **Figure 3.** Multiple sequence alignment (software: CLUSTAL O (1.2.0)) of peptides generated
564 from different proteins of whole (BW) and skimmed (BS) buffalo milk by gastric (G) and
565 duodenal (D) digestion. Numbers on the left is the serial number of the minimal overlapped
566 peptides. Numbers on the right indicates the number of amino acid residues in that peptide.
567 Consensus obtained from Jalview 2.8. Abbreviations: ., residues with weakly similar properties

568 and conserved; :, residues with strongly similar properties and conserved; *, residue which is
569 fully conserved.

570 **Figure 4.** Proportion (%) of lipolysis of neutral lipids (NL) of whole buffalo milk and
571 subsequent formation of free fatty acids (FFA) during digestion with human gastric (G) and
572 duodenal (D) juices. Abbreviations: G40, gastric digestion at pH 2.5 for 20 min after 20 min
573 gastric digestion at pH 5.0; D30, D60 and D120, duodenal digestion for 30, 60 and 120 min,
574 respectively at pH 7.0.

# PAPER III

# *Ex vivo* digestion of omega-3 enriched buffalo skimmed milk

**Mohammad Ashiqul Islam[*1], Dag Ekeberg, Elling-Olav Rukke, Gerd Elisabeth Vegarud**

Department of Chemistry, Biotechnology and Food Science,

Norwegian University of Life Sciences, P. O. Box 5003, NO-1432 Aas, Norway

*E-mail addresses*:

mohammad.islam0@nmbu.no

dag.ekeberg@nmbu.no

elling-olav.rukke@nmbu.no

gerd.vegarud@nmbu.no

[*]Corresponding author: Tel.: +47-64965900, Fax: +47-64965901

*Email address*: mohammad.islam0@ nmbu.no or m.a.islam@bau.edu.bd

Department of Chemistry, Biotechnology and Food Science,

Norwegian University of Life Sciences, P. O. Box 5003, NO-1432 Aas, Norway

[1]Permanent Address: Department of Dairy Science, Bangladesh Agricultural University,

Mymensingh-2202, Bangladesh.

19 **Abstract**

20 The aim of the present study was to investigate whether the protein digestion in skimmed
21 buffalo milk was affected by adding cod liver oil as a source of omega-3 fatty acid to the milk.
22 Human gastrointestinal enzymes, gastric and duodenal juices were used in a two phase model
23 digestion. After gastric and duodenal digestion, 32% of the neutral lipid underwent lipolysis.
24 Both the omega-3 fatty acids, eicosapentanoic (EPA; C20:5 n-3) and docosahexanoic (DHA;
25 C22:6 n-3) acid showed 23% lipolysis. The α-lactalbumin and β-lactoglobulin were resistant to
26 gastric digestion, however, readily digested after 5 min duodenal digestion. The addition of cod
27 liver oil as a source of omega-3 fatty acids did not affect the milk protein digestion and
28 subsequent peptide generation after digestion of the fortified skimmed buffalo milk. Thereby,
29 enrichment of skimmed milk by cod liver oil could be a good nutritional source of omega-3
30 fatty acids.

31 ***Key words***: Omega-3 fatty acids, Fortification, *Ex vivo* digestion, Casein, β-lactoglobulin,
32 Peptides

33 **1. Introduction**

34 Milk is regarded as the nutritionally most complete food but is criticized for its low content of
35 unsaturated fatty acids, especially polyunsaturated fatty acids (PUFA). The fat content in
36 bovine milk varies from 3.0-6.0% (MacGibbon & Taylor, 2006) and in buffalo milk it is 3.37 to
37 14.42% (Meena, Ram & Rasool, 2007). In general, full fat cow's milk fat contains 70%
38 saturated fatty acids (SFA), 25% mono-unsaturated fatty acids (MUFA) and 5% PUFA (Lock
39 & Shingfield, 2004; Grummer, 1991). Related to a positive health effect, milk fat should
40 contain 60% MUFA (Pascal, 1996), 30% SFA and 10% PUFA (Hayes & Khosla, 1992), even

though according to O'Donnell (1989) milk fat should contain 82%, 8% and 10% MUFA, SFA and PUFA, respectively.

Among the saturated fatty acids, C12:0, C14:0 and C16:0 constitutes 44% of the total milk fatty acids (Qureshi, Mushtaq, Khan, Habib & Swati, 2010). They are considered as hypercholesterolemic fatty acids leading to cardiovascular diseases (Williams, 2000). However, sufficient availability of C18:2 n-6 seems to inhibit the negative effect of C16:0 (Clandinin, Cook, Konard & French, 2000). Connor (2000) reported potential health benefits from the omega-6 and particularly from the omega-3 fatty acids both *in vitro* and *in vivo*. These polyunsaturated fatty acids reduce the risk of cardiovascular diseases, hypertension and type-2 diabetes (Siddiqui, Harvey & Zaloga, 2008; Wijendran & Hayes, 2004; Bemelmans et al., 2002). Intake of eicosapentaenoic (EPA; 20:5 n-3) and docosahexaenoic (DHA; 22:6 n-3) acid may reduce the risk of prostate cancer (Leitzmann et al., 2004), improves the brain development and function (Kolanowski & Laufenberg 2006) and prevent inflammatory diseases, dyslexia and depression (Garg, Wood, Singh & Moughan, 2006).

Fish is generally considered as an important source of essentials fatty acids (Mondello, Tranchida, Dugo & Dugo, 2006), more specifically the main dietary source of omega-3 polyunsaturated fatty acids (Kolanowski & Laufenberg, 2006). Among this source, cod liver oil (CLO) contains a balance of saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids (includes α-linolenic; ALA, EPA and DHA) in a ratio of 1.0:2.6:1.4 (Zeng et al., 2010). Because of higher EPA and DHA (31% and 35% of the total PUFA, respectively; Zeng et al., 2010), cod liver oil has been used as a nutritional supplement and recommended for the relief of arthritis, depression and high blood pressure (Ayorinde, Keith Jr, & Wan, 1999). As a source of PUFA and vitamin-D, CLO has a long historical tradition as an

nutritional supplement in Nordic countries (Mondello, Tranchida, Dugo & Dugo, 2006). In Norway, dietary supplement of vitamin-D is recommended from the infancy and CLO is specifically recommended for that purpose because of its higher EPA and DHA content (Stene, Ulriksen, Magnus & Joner, 2000). According to Rimestad et al. (2001), one dose (5 mL) CLO contains 1.2 g of omega-3 fatty acids. The European Academy of Nutritional Science recommended a daily dose of 200 mg of omega-3 fatty acids but it can be more, upto 3 g depending on the physiological condition (Garg, Wood, Singh & Moughan, 2006).

A complex physico-chemical and enzymatic processes are involved in the digestion of lipids and proteins and the subsequent bioavailability of these components. Lipid and protein digestion comprise mainly two phase, gastric and duodenal digestion. In the first phase, digestion takes place in the stomach at pH 1-5 by the action of pepsin and gastric lipase. Followed by digestion in the small intestine (mainly at duodenum) at pH 6-7.5 by pancreatic and brush border enzymes in the presence of bile acids from the gall bladder (Guerra et al., 2012). Trypsin, chymotrypsin and carboxypeptidase are the main proteases attacks the proteins and peptides and pancreatic lipases acts on the lipids.

To predict the nutritional quality of food products and their constituents, bettter knowledge on the digestibility of the individual components needed (Devle et al., 2014). *In vitro* or *ex vivo* model digestion is widely used for this purpose, considering the accuracy, ease of utilization, time and cost involvement. During *in vitro* digestion, food characteristics, type and concetration of the enzymes are the key controlling factors (Hur, Lim, Decker & McClements, 2011). The simulation of the human physiological condition is important to correlate better the *in vitro* studies with the *in vivo* data. For better simulation, use of physiologically relevant enzymes and

other gut-relevant components like HCL, bile salts are important. This can be better achieved by using the human gastrointestinal aspirates.

Studies showed the influence of milk fat on the *ex vivo* digestion of milk protein, especially β-lactoglobulin. The presence of milk fat considerably influence the hydrolysis of β-lactoglobulin in cow milk (Devle et al., 2014) and such effect was much less in buffalo milk (Islam, Ekeberg, Rukke & Vegarud, 2014b). Binding of fatty acids to β-lactoglobulin and phospholipids (especially phosphotidylcholine) to both α-lactalbumin and β-lactoglobulin alters their hydrolysis (Mandalari, Mackie, Rigby, Wickham & Mills, 2009; Moreno, Mackie & Mills, 2005; Puyol, Perez, Mata, Ena & Calvo, 1993). But Le Maux et al. (2013) reported the positive effect of linoleate on the β-lactoglobulin digestibility.

The aim of the present study was to investigate the effects of fortifying skimmed buffalo milk with cod liver oil on the protein degradation during *ex vivo* digestion. The protein profile and generation of peptides in the milk were assessed, in addition to lipolysis and release of fatty acids from cod liver. Especially, the digestion of the immunogenic proteins, $\alpha_{s1}$-casein and β-lactoglobulin and the peptides generated were recorded.

## 2. Materials and Methods

### 2.1 Milk samples and cod liver oil

Pooled, whole, raw fluid buffalo milk was obtained from Bangladesh Livestock Research Institute (BLRI) buffalo farm, Savar, Dhaka-1341, Bangladesh. The sampling was done from the pooled morning milk of nine buffaloes. Immediately after milking of the animals, the well mixed pooled milk samples were divided into 40 mL volume in cellstar® tubes (Greiner Bio-One, Maybachstrasse, Frickenhausen, Germany) and preserved by adding bronopol tablet (1 tablet/40 mL milk; D & F control systems, Inc. USA). After freezing (-20 °C), the samples

were transported to the Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, P. O. Box 5003, NO-1432 Aas, Norway. Skimming of the milk was done after thawing of the milk. Thawing was done by keeping the milk tubes in ice water for overnight and tempered gradually to 37 °C in a water bath. Skimming of the milk was done by removing the milk lipids by centrifugation at 850×*g*, at 4 °C for 20 min (Beckman Coulter, Allegra 25R Centrifuge, TS-5.1-500 rotor head, Brea, CA, USA). The true protein content of the milk was 3.5% (Islam, Ekeberg, Rukke & Vegarud, 2014a).

The cod liver oil (Möller's tran, 500 mL, Axellus As, Oslo) was purchased from the local super market. It was kept in the refrigerator (4-6 ºC). The CLO was mixed with the milk at 37 ºC by magnetic stirring (500 rpm). The concentration of CLO in the fortified milk was 2%, considering the daily requirement of milk for an adult (250 mL).

**2.2 Human gastrointestinal juices**

The human gastrointestinal (GI) juices, human gastric juice (HGJ) and human duodenal juice (HDJ) were collected from six healthy, fasted (for ≥8 hrs), 20 to 37 years old volunteers at Lovisenberg Diakonale Hospital, Oslo, Norway. A method according to Holm, Hansen, Krogdahl & Florholmen (1988) and Ulleberg et al. (2011) was used. The complete protocol used was approved by the Norwegian Ethical Committee, and all the volunteers were well briefed about what is going to happen with them and has to sign an agreement before participating in the aspiration. Simultaneous instillation of a stimulating solution and aspiration of HGJ and HDJ were done by a three-lumen silicone tube (Maxter Catheters, Marseille, France) especially developed for this purpose. Both the aspirates, from stomach and duodenum, were collected in tubes and placed on ice. Centrifugation was done at 4500×*g* for 10 min at 4 ºC to remove the mucus and cell debris, aliquots were kept at -20 ºC. Pooled batches of HGJ and

of HDJ from six donors were used in the *ex vivo* digestion study. The pepsin activity in HGJ, total proteolytic and lipase activity and total bile salts in HDJ were measured according to a method narated by Ulleberg et al. (2011).

### 2.3 Ex vivo digestion

To simulate the gastric and intestinal digestion, a two-phase *ex vivo* static digestion model was used using HGJ and HDJ. A method according to Devle et al. (2014) was used with some modifications. In Brief, the protein samples (1 mL fortified milk) were digested at gastric phase by HGJ for 20 minutes (G20) at pH 5.0 and 20 minutes at pH 2.5 (G40; it also pass G20 step). Following the gastric phase, the duodenal samples were digested at pH 7.0 for 5 min (D5), 30 min (D30), 60 min (D60) and 120 min (D120). The digested lipid samples (1 mL fortified milk) were G40, D30, D60 and D120. The gastric and duodenal pH was adjusted by using 2M HCL and 2M NaOH, respectively. In the gastric phase, 711 U pepsin activity/g milk protein was used. Total proteolytic and lipase activity and total bile salts used in the duodenal phase were 558 U/g milk protein, 1022 U/mL milk and 2.4 mM/mL HDJ, respectively. The digestion was carried out in a water bath at 37 ºC with continuous magnetic stirring at 200-300 rpm. After digestion, samples were placed in -20 ºC with minimum delay. In case of lipid samples, 20 mL chloroform and methanol (2:1) mixture was added before they placed in -20 ºC. Each step of the digestion in every phase was repeated for 3 times.

### 2.4 Milk protein degradation profiles by SDS-PAGE

Protein dgradation in fortified buffalo milk at different stages of digestion was visualized by SDS-PAGE by following the method of Devle et al. (2014) with some modifications. Ultra Turrax (Yellow Line DI 18 basic, IKA®-Werke GmbH & Co. KG, staufen, Germany) at speed 3 for few seconds was used to homogenize the gastric samples. The mixing ratio of sample and

sample buffer was 1:2. From this mixture, 10 µL was apllied to the respective wells of polyacrylamide separating gels (Any kD™, 6.5 – 200 kDa, mini PROTEAN® TGX™ precast gels, Tris Glycine extended, Bio-Rad laboratories, Inc. Made in United States). Five µL of low molecular weight marker (LMW-SDS Marker Kit; GE Healthcare, Little Chalfont, Bucks, UK) and undigested milk samples were also applied to two other different wells. The gel was run at 200 V for 35 min. According to the original protocol, it was followed by fixation, comassie briliant blue staining and destaining and finally it was placed into the preservation solution. The gels were run five times.

**2.5 In gel digestion and Ultra Performance Liquid Chromatography (UPLC) and Q-Exactive Mass Spectrometry (MS) for protein band identification**

For the identification of the proteins on the SDS-PAGE gels, a method described by Devle et al. (2014) was used for cutting the bands, in-gel reduction, alkylation, and trypsin digestion and elution of peptides. Dried peptides were dissolved in loading solution (0.05 %TFA, 2% ACN in water) before it was loaded onto a nano-UPLC (RSLC3000, -Dionex/Thermo Fisher Scientific, Bremen, Germany) equipped with a trap column (Acclaim PepMap100, C18, 5 µm, 100 Å, 300 µm i.d. x 5 mm, Thermo Fisher Scientific, Bremen, Germany); followed by back flushing onto a 50 cm x 75 µm analytical column (Acclaim PepMap RSLC C18, 2 µm, 100 Å, 75 µm i.d. x 50 cm, nanoViper, Bremen, Germany). The separation of the peptides was attained by a 45 min gradient from 4 to 40 % solution B (80 % ACN, 0.1% formic acid) at a flow rate of 300 nL/min. The Q-Exactive MS (Thermo Fisher Scientific, Bremen, Germany) was adjusted to scan (300-1600 m/z) mode at resolution (R) 70.000, and subsequent (up to) 5 MS2 scans at R=35000 and 28 neutral collision energy. In MS/MS, singly charged precursors and precursors with z>5 were excluded with a dynamic exclusion at 30 seconds. The raw data files were

converted to mg format by using the masconvert module of ProteoWizard (http://proteowizard.sourceforge.net/). An in-house Mascot (v.2.4) server was used for database search (Swissprot, taxonomy - other mammals). Mass tolerance for MS and MS/MS was 10 ppm and 20 mamu, respectively and 2 missed cleavages were allowed. Carbamidomethylated cysteine and oxidized methionine were the selected fixed and variable modifications, respectively.

**2.6 Peptide identification by UPLC and Quadrapole-Time Of Flight (Q-TOF) MS**

A method described by Furlund et al. (2013) was used to desalt and concentrate the peptides from the G20, G40, D5 and D120 samples. The method described by Qureshi, Vegarud, Abrahamsen & Skeie (2012) with some modifications was followed for the identification of peptides. In brief, a nanoACQUITY$^{TM}$ UPLC$^{®}$ (Waters, Milford, USA) coupled with a Q-TOF Ultima MS (Micromass Ltd., Manchester, UK) was used for this purpose. The UPLC was equiped with 5-µm symmetry C18 trap column (180 µm × 20 mm; Waters, Milford, USA) and 1.7-µm BEH C18 analytical column (75 µm × 100 mm; Waters, Milford, USA). Peptide mixtures (containing 0.5% formic acid) were applied to the UPLC and each sample was run in MS and data dependent tandem MS mode. Non-redundant protein sequence database version NCBInr 20130131 (22749596 sequences; 7819872540 residues) was used to search the resulted peak list.

**2.7 Multiple sequence alignment (MSA) of peptides**

Minimal overlap peptides of every samples were slected. Then the pool of gastric (G) and duodenal (D) peptides were made from G20 and G40 and from D5 and D120, respectively. Clustal omega (version: CLUSTAL O (1.2.0); http://www.ebi.ac.uk/Tools/msa/clustalo/) was used to compare the fortifed buffalo milk with buffalo skimmed milk (data obtained from

201 Islam, Ekeberg, Rukke & Vegarud, 2014b) for their G and D peptides. The consensus sequence
202 was obtained from MS editor Jalview 2.8 (http://www.jalview.org/). The amino acid sequence
203 of $\alpha_{s1}$-, $\alpha_{s2}$-, β- and κ-casein and β-Lg were taken from http://www.ncbi.nlm.nih.gov/ and
204 http://www.uniprot.org/. The minimal overlapped peptides were also aligned on to the
205 respective protein's sequence to localize the proteolytic site and a comparison with skimmed
206 buffalo milk (data obtained from Islam, Ekeberg, Rukke & Vegarud, 2014b) was also made.
207 This was done by a modified method according to Furlund et al. (2013).

208 **2.8 Lipid analysis**

209 The fatty acid composition of neutral lipid (NL) and free fatty acid (FFA) was done by a
210 method described by Devle et al. (2014) with some modifications. The procedure involves total
211 lipid extraction, solid phase extraction (SPE) of NL and FFA, formation of fatty acid methyl
212 esters (FAMEs) and finally the Gass Chromatography-Mass Spectrometry (GC-MS) analysis of
213 FAMEs to obtain the fatty acid composition of the respective fraction. The extracted total lipids
214 were dried under nitrogen gas at 37 ºC. Two mL chlorofrom was used to redissolve the dried
215 total lipids and 1 mL of it was applied on to the liquid handling robot (Gilson, GX-274 ASPEC,
216 Middleton, USA) for the SPE purpose. Again the NL and FFA in chloroform and diethyl
217 ether:acetic acid (98:2), respectively were dried at 37 ºC under the stream of nitrogen gas. All
218 the samples were run in triplicate.

219 **3. Results**

220 **3.1 Protein digestion**

221 The degradation pattern of the milk proteins in skimmed and cod liver oil fortified buffalo milk
222 are shown in Figure 1A and 1B, respectively. The caseins showed considerable gastric

degradation after 20 min and were completely degraded after 40 min gastric digestion, leaving behind only traces of casein bands.

Both the α-lactalbumin and β-lactoglobulin seemed to be resistant to gastric digestion. However, in the duodenal phase, they were degraded very fast and completely after 5 min. Protein bands (D5) in the SDS-gels were identified by UPLC-MS. The results revealed that the origin of this bands were bovine α-lactalbumin. No difference in the protein degradation pattern of skimmed and omega-3 enriched buffalo milk was observed (Figure 1A and B).

### 3.2 Site of proteolysis and peptides

Localization of the identified peptides in the whole sequence of the respective protein is shown in Figure 2. The fortified buffalo milk showed very little difference in peptide pattern as compared to the skimmed buffalo milk. Some proteolytic regions vary, however, only by a few residues. The major differences detected (>4 residues) were in the following fragments: f165-180, f159-163 and f181-193 of $\alpha_{s1}$-casein, f114-120, f101-109, f86-94, f121-138, f166-179 and f189-193 of $\alpha_{s2}$-casein. Variations in the fragments of other proteins were f60-95, f109-115, f172-178, f185-190 and f192-197 of β-casein; f39-51, f63-71 and f127-145 of κ-casein and f19-29, f50-75 and f143-155 of β-Lg. The $\alpha_{s1}$- and β-casein were degraded more extensively than the other proteins and κ-casein and β-Lg were degraded mostly in gastric and duodenal digestion, respectively.

Total numbers of identified peptides from each protein are given in Table 1. The peptides f84-108 and f96-108 from β-casein were identified in all the gastric and duodenal samples. Few other peptides from all the proteins also showed resistance in one or two subsequent steps of digestion. The comparison between fortified and skimmed buffalo milk regarding the individual minimal overlapped peptides ends up with greater similarities (Figure 3).

### 3.3 Lipolysis of neutral lipid and fatty acid composition of free fatty acids

The lipolysis of neutral lipid and subsequent release of free fatty acids at different phase and steps of digestion are given in Figure 4. Lipolysis was very low during gastric phase. However, a rapid lipolysis was shown within the first 30 min of duodenal digestion, thereafter lipid digestion seemed to level off. In gastric 40 min and duodenal 30, 60 and 120 min digested samples, the proportion of the neutral lipid (%) was 99.13, 72.32, 72.38 and 68.08, respectively. Changes in the free fatty acid concentration with the lipolysis of respective individual fatty acid are given in Table 2. The concentration in the duodenal digested samples showed a little variation among them corresponded to Figure 4. Among the saturated fatty acids, the highest lipolysis was observed in C18:0 and lowest was in C14:0. The observed lipolysis of C16:0 was 4% more than the C14:0 and C12:0 showed higher lipolysis than both of them.

In monounsaturated fatty acids, C20:1 n-9 *cis* showed 12% and 5% more lipolysis than C16:1 n-7 *cis* and C18:1 n-9 *cis*, respectively. The observed lipolysis of C22:1 n-9 *cis* was 7% more than C18:1 n-7*cis*. Nevertheless, they showed 1-15% higher lipolysis than the C16:1 n-7 *cis* and C18:1 n-9 *cis*. In omega-6 polyunsaturated fatty acids, C18:2 n-6 *cis* showed 17% less lipolysis than C20:4 n-6 *cis*. Both the omega-3 polyunsaturated fatty acids, C20:5 n-3 *cis* and C22:6 n-3 *cis* showed similar amount of lipolysis. Total amount of monounsaturated fatty acids showed 2 and 7% higher lipolysis than the total saturated fatty acids and polyunsaturated fatty acids, respectively.

## 4. Discussion

### 4.1 Protein digestion and generation of peptides

Pepsin is the main proteolytic enzyme in the gastric compartment. In the upper part of the duodenum, trypsin, chymotrypsin, lipase and amylase are the main enzymes. In the duodenal

digested samples, many bands appeared in 60-30 kDa region (Figure 1A & B. a, b, c and d), that have been identified as digestive enzymes in the HDJ (Devle et al., 2014). All the caseins were more or less completely digested in the gastric phase, while α-lactalbumin and β-lactoglobulin were resistant. The denser appearance of bands in the region of α-lactalbumin and β-lactoglobulin may be because of accumulation of protein degradation products with overlapping molecular weight.

During early (5 min) duodenal digestion, both α-lactalbumin and β-lactoglobulin were digested. No differences in the digestion pattern of caseins, α-lactalbumin or β-lactoglobulin were observed between skimmed and fortified milk with cod liver oil. This is in line with another study where no interference of milk fat in the degradation of the proteins in full fat buffalo milk was observed (Islam, Ekeberg, Rukke & Vegarud, 2014b). However, this is contrary to a study on bovine milk where β-lactoglobulin was found resistant to duodenal digestion in full fat cow's milk (Devle et al., 2014). In another study, purified β-lactoglobulin showed increased digestion when adding linoleic acid (Le Maux et al., 2013). However, the negative effect of fatty acids was also evident in a study where the presence of oleic acid and monoolein reduced the positive effect of bile acid in β-lactoglobulin digestion (Gass, Vora, Hofmann, Gray & Khosla, 2007). The possible reasons for the contradictory results may be the differences in the amino acid sequence and thereby structure or in the concentration of β-lactoglobulin. Buffalo milk contains less β-lactoglobulin (4.3% of total protein) than cow's milk (8-10% of total protein) (Islam, Ekeberg, Rukke & Vegarud, 2014a), and may thereby be more easily degradable. Native β-lactoglobulin has a 3-dimensional structure that forms a hydrophobic barrel, which could bind lipids that may affect the degradation of the molecule. Bile salt concentration in the duodenal juice may be another limiting factor affecting the degradation of

β-lactoglobulin (Devle et al., 2014 and Gass, Vora, Hofmann, Gray & Khosla, 2007). The binding of fatty acid could stabilize β-lactoglobulin during digestion, but if the bile acid concentration is higher than 2.0 mM, as it was in our study, it should be enough to bind to β-lactoglobulin leading to destabilization and further duodenal digestion.

With regard to the peptide pattern, relatively few peptides from the proteins were observed, even though all the proteins were highly digested. This may be due to the fact that amino acids were not analyzed in this study. In addition, a detection of di-, tri-, and tetra-peptides (<500-800 Da) was not possible due to the limitation of the UPLC-Q-TOF/MS, that only detect peptides in the range of 800-4500 Da. Low variation in the proteolytic site preference between the fortified buffalo milk and skimmed buffalo milk was observed and consistent with the findings of Islam, Ekeberg, Rukke & Vegarud, (2014b). The presence of proline in all most all the peptides along with leucine and/or isoleucine and/or valine are in line with other reports (Islam, Ekeberg, Rukke & Vegarud, 2014b; Furlund et al., 2013 and Chabance et al., 1998). Proline residues in the amino acid sequence seems to be very resistant to further proteolysis. Only peptides identified as fragments, f64-71 from $\alpha_{s1}$-casein during gastric digestion, f86-94 from $\alpha_{s2}$-casein during duodenal digestion and f101-112 from β-lactoglobulin during duodenal digestion did not contain any proline residue but still contain either leucine or isoleucine or valine. Chabance et al. (1998) identified similar peptide from β-casein (f29-41 and f30-41) and $\alpha_{s1}$-casein (f35-49 and f120-131) during digestion of milk in the stomach of infants; and further from lactoferrin (f382-389; f442-447) and $\alpha_{s1}$-casein (f14-18) during duodenal digestion. So, it seems that some hydrophobic amino acids in a protein sequence also contribute to its resistance towards further proteolysis.

## 4.2 Lipolysis

Lipases are enzymes that hydrolyze triacylglycerols to free fatty acids and monoacylglycerols. There are two main lipases in the gastrointestinal tract, gastric lipase and pancreatic lipase. Gastric lipase is reported to hydrolyze up to 30% of dietary lipids (Pafumi et al., 2002; Armand et al., 1999), though the exact contribution of preduodenal lipases to the hydrolysis of lipids is still debated (Gallier et al., 2013; Mu & Hoy, 2004; Armand et al., 1999; Carriere, Barrowman, Verger & Laugier, 1993). The optimum pH for the gastric lipase is 5.0 - 6.0 and highly stereo-specific to *sn*-1 and *sn*-3 position of the triacylglycerol (Carriere et al., 1994; Carriere, Barrowman, Verger & Laugier, 1993; Tiruppathi & Balasubramania, 1982). According to Armand (2007), pancreatic lipase attacks the ester bonds at *sn*-1 and *sn*-3 position of the triacylglycerol.

The minor extent of gastric lipolysis observed in the present study can be explained with insufficient secretion of gastric lipases, since the volunteers were in fasted/semi-fasted condition (Devle et al., 2014). They were not stimulated for lipid digestion and had a low pH 2.5, far away from the optimum for the activity of the gastric lipase. In both cow's milk (Devle et al., 2014) and buffalo milk (Islam, Ekeberg, Rukke & Vegarud, 2014b), only minor lipolysis during gastric digestion was observed. However, a fast increases in the lipolysis of the milk fat during the first 30 min of the duodenal digestion was observed. Thereafter the lipolysis leveled off (60-120 min). The results in the present study showed the similar trend of lipolysis of the cod liver oil. A maximum of about 30-35% free fatty acid was released in all the studies indicating a product inhibition due to the static model used (Devle et al., 2014; Gallier, Ye & Singh, 2012). Little variation in the fatty acid concentration of the free fatty acids among the

duodenal digested samples is also in agreement with Devle et al. (2014) and Islam, Ekeberg, Rukke & Vegarud, (2014b).

Zeng et al. (2010) reported a stereo-specific positional distribution of fatty acids in the triglycerides of the cod liver oil. This distribution is important in lipid digestion as the pancreatic lipase attacks the *sn*-1 and *sn*-3 ester bond of the triglycerides. Approximately 99% of the C18:0 in cod liver oil was found in that position, while only ~69% of C14:0 was shown, and this supports the variation observed between the lipolysis of C18:0 and C14:0 in the present study. The higher lipolysis in C16:0 than the C14:0 can also be explained by ~10% more presence of C16:0 at *sn*-1 and *sn*-3 than the C14:0. Zeng et al. (2010) did not include C12:0 in their study, but the present results suggest that the pancreatic lipase preferred position have more C12:0 than C14:0 and C16:0.

The stereo-specific distribution of fatty acids of cod liver oil (Zeng et al., 2010) partially supports the lipolysis of individual monounsaturated fatty acid. The presence of C20:1 n-9 *cis* at *sn*-1 and *sn*-3 is 2 and 9% less than C16:1 n-7 *cis* and C18:1 n-9 *cis*, respectively but showed more lipolysis than them. Being present in same proportion (~89%) at lipase preferred position, lipolysis of C22:1 n-9 *cis* and C18:1 n-7 *cis* was different. But higher lipolysis in these two fatty acids compared to C16:1 n-7 *cis* and C18:1 n-9 *cis* can be explained by their more presence at *sn*-1 and *sn*-3 (3 to 10 %).

The lipolysis of individual polyunsaturated fatty acid was not in line with the stereo-specific distribution of the fatty acids of cod liver. Approximately 81% of the C18:2 n-6 *cis* is present at *sn*-1 and *sn*-3 position, which is 51% more than C20:4 n-6 *cis* but not reflected so in the observed lipolysis. Both the C20:5 n-3 *cis* and C22:6 n-3 *cis* showed similar lipolysis though their presence at *sn*-1 and *sn*-3 is different. Zeng et al. (2010) reported the fatty acid distribution

359 in total lipids, without considering the fraction of lipids (like neutral lipid, polar lipid etc.). But
360 here we only discussed about the lipolysis in neutral lipid and fish oil phospholipids contain a
361 considerable part of the polyunsaturated fatty acids (Gruger, 1967). This may be the reason of
362 inconsistency between the present results and the reported fatty acid distribution of cod liver
363 oil. Phospholipid fraction was not included in the present study as Devle et al. (2014) observed
364 non-significant digestion of phospholipids in *ex vivo* condition because of lack of
365 phospholipase in human duodenal juices or insufficient activation of the enzymes during the
366 digestion.
367 However, higher lipolysis in total monounsaturated fatty acids followed by total saturated fatty
368 acids and total polyunsaturated fatty acids, is in agreement with their proportionate presence at
369 lipase preferred position, *sn*-1 and *sn*-3. It may be noteworthy that, from the molecular structure
370 point, the fatty acid distribution pattern of fish oils are very complex at any given time for each
371 species (Gruger, 1967). But literature on stereo-specific distribution of cod liver oil fatty acid is
372 very scanty and only found the report of Zeng et al. (2010). So the variation in the distribution
373 pattern is not conclusively evident yet which is important in interpreting the digestibility data.
374 For example, variation in the stereo-specific distribution of the milk fatty acid in the
375 triacylglycerol is evident from different reports of Blasi et al. (2008), Maansson (2008), Angers,
376 Tousignant, Boudreau & Arul (1998) & Jensen and Newburg (1995).

377 5. ***Conclusions***

378 Fortification of cod liver oil as a source of omega-3 fatty acids to skimmed buffalo milk did
379 not affect the milk protein digestion and subsequent peptide generation. No lipolysis was
380 observed in the gastric phase, thereafter fast lipolysis occurred during the first 30 min duodenal
381 digestion and remained more or less stable for 120 min digestion. Higher lipolysis of the total

monounsaturated fatty acid was observed compared to the total saturated fatty acid and the total polyunsaturated fatty acid. Among all the unsaturated fatty acid, eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) showed lowest lipolysis. The results showed that, skimmed buffalo milk fortified with cod liver oil may be an effective carrier of omega-3 fatty acids and the oil enrichment did not affect the degradation of the immunogenic proteins, β-lactoglobulin and $\alpha_{s1}$-casein.

**References**


Angers, P., Tousignant, E., Boudreau, A. & Arul, J. (1998). Regiospecific analysis of fractions of bovine milk fat triacylglycerols with the same partition number. *Lipids*, 33 (12), 1195-1201.

Armand, M., Pasquier, B., Andre, M., Borel, P., Senft, M., Peyrot, J., Salducci, J., Portugal, H., Jaussan, V. & Lairon, D. (1999). Digestion and absorption of 2 fat emulsions with different droplet sizes in the human digestive tract. *The American Journal of Clinical Nutrition*, 70 (6), 1096-1106.

Armand, M. (2007). Lipases and lipolysis in human digestive tract: where do we stand? *Current Opinion in Clinical Nutrition and Metabolic Care*, 10, 156-164.

Ayorinde, F. O., Keith Jr, Q. L. & Wan, L. W. (1999). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of cod liver oil and the effect of analyte/matrix concentration on signal intensities. *Rapid Communications in Mass Spectrometry*, 13 (17), 1762-1769.

Bemelmans, W. J., Broer, J., Feskens, E. J., Smit, A. J., Muskiet, F. A., Lefrandt, J. D., Bom, V. J., May, J. F. & Meyboom-de Jong, B. (2002). Effect of an increased intake of α-linolenic acid and group nutritional education on cardiovascular risk factors: the

405 Mediterranean Alpha-linolenic Enriched Groningen Dietary Intervention
406 (MARGARIN) study. *The American Journal of Clinical Nutrition*, 75 (2), 221-227.
407 Blasi, F., Montesano, D., De Angelis, M., Maurizi, A., Ventura, F., Cossignani, L., Simonetti,
408 M. S. & Damiani, P. (2008). Results of stereospecific analysis of triacylglycerol fraction
409 from donkey, cow, ewe, goat and buffalo milk. *Journal of Food Composition and*
410 *Analysis*, 21 (1), 1-7.
411 Carriere, F., Barrowman, J., Verger, R. & Laugier, R. (1993). Secretion and contribution to
412 lipolysis of gastric and pancreatic lipases during a test meal in humans.
413 *Gastroenterology*, 105 (3), 876-888.
414 Carriere, F., Gargouri, Y., Moreau, H., Ransac, S., Rogalska, E. & Verger, R. (1994). Gastric
415 lipases: cellular, biochemical and kinetic aspects. in *Lipases: Their Structure,*
416 *Biochemistry and Application*, ed. P. Woolley, Cambridge University Press, New York.
417 pp.181-205.
418 Chabance, B., Marteau, P., Rambaud, J. C., Migliore-Samour, D., Boynard, M., Perrotin, P.,
419 Guillet, R., Jolles, P. & Fiat, A. M. (1998). Casein peptide release and passage to the
420 blood in humans during digestion of milk or yogurt. *Biochimie*, 80 (2), 155-165.
421 Clandinin, M., Cook, S., Konard, S. & French, M. (2000). The effect of palmitic acid on
422 lipoprotein cholesterol levels. *International Journal of Food Sciences and Nutrition*, 51
423 (s1), s61-s71.
424 Connor, W. E. (2000). Importance of n-3 fatty acids in health and disease. *The American*
425 *Journal of Clinical Nutrition*, 71 (1), 171S-175S.

426 Devle, H., Ulleberg, E. K., Naess-Andresen, C. F., Rukke, E.-O., Vegarud, G. & Ekeberg, D.
427 (2014). Reciprocal interacting effects of proteins and lipids during ex vivo digestion of
428 bovine milk. *International Dairy Journal*, 36 (1), 6-13.
429 Furlund, C. B., Ulleberg, E. K., Devold, T. G., Flengsrud, R., Jacobsen, M., Sekse, C., Holm,
430 H. & Vegarud, G. E. (2013). Identification of lactoferrin peptides generated by digestion
431 with human gastrointestinal enzymes. *Journal of Dairy Science*, 96, 75-88.
432 Gallier, S., Ye, A. & Singh, H. (2012). Structural changes of bovine milk fat globules during in
433 vitro digestion. *Journal of Dairy Science*, 95 (7), 3579-3592.
434 Gallier, S., Cui, J., Olson, T. D., Rutherfurd, S. M., Ye, A., Moughan, P. J. & Singh, H. (2013).
435 In vivo digestion of bovine milk fat globules: Effect of processing and interfacial
436 structural changes. I. Gastric digestion. *Food Chemistry*, 141 (3), 3273-3281.
437 Garg, M., Wood, L., Singh, H. & Moughan, P. (2006). Means of Delivering Recommended
438 Levels of Long Chain n-3 Polyunsaturated Fatty Acids in Human Diets. *Journal of*
439 *Food Science*, 71 (5), R66-R71.
440 Gass, J., Vora, H., Hofmann, A. F., Gray, G. M. & Khosla, C. (2007). Enhancement of dietary
441 protein digestion by conjugated bile acids. *Gastroenterology*, 133, 16-23.
442 Gruger, E. H. (1967). Fatty acid composition of fish oils. *United States. Bureau of commercial*
443 *fisheries. Circular 276.* Available on: http://spo.nmfs.noaa.gov/Circulars/CIRC276.pdf
444 accessed on 11.03.2014.
445 Grummer, R. R. (1991). Effect of feed on the composition of milk fat. *Journal of Dairy*
446 *Science*, 74 (9), 3244-3257.

447 Guerra, A., Etienne-Mesmin, L., Livrelli, V., Denis, S., Blanquet-Diot, S. & Alric, M. (2012).
448 Relevance and challenges in modeling human gastric and small intestinal digestion.
449 *Trends in Biotechnology*, 30 (11), 591-600.
450 Hayes, K. & Khosla, P. (1992). Dietary fatty acid thresholds and cholesterolemia. *The FASEB*
451 *journal*, 6 (8), 2600-2607.
452 Holm, H., Hanssen, L. E., Krogdahl, A. & Florholmen, J. (1988). High and low inhibitor
453 soybean meals affect human duodenal proteinase activity differently: in vivo
454 comparison with bovine serum albumin. *The Journal of Nutrition*, 118, 515-520.
455 Hur, S. J., Lim, B. O., Decker, E. A. & McClements, D. J. (2011). In vitro human digestion
456 models for food applications. *Food Chemistry*, 125, 1-12.
457 Islam, M. A., Alam, M. K., Islam, M. N., Khan, M. A. S., Ekeberg, D., Rukke, E. O., Vegarud,
458 G.E. (2014a). Principal Milk Components in Buffalo, Holstein cross, Indigenous Cattle
459 and Red Chittagong Cattle from Bangladesh. *Asian-Australasian Journal of Animal*
460 *Sciences*. http://dx.doi.org/10.5713/ajas.2013.13586.
461 Islam, M. A., Ekeberg, D., Rukke, E. O., Vegarud, G.E. (2014b). *Ex vivo* digestion of proteins
462 and fat in buffalo milk. *International Dairy Journal*. Submitted manuscript.
463 Jensen, R. G., and D. S. Newburg. 1995. Bovine Milk Lipids. *In Handbook of Milk*
464 *Composition*. R. G. Jensen, ed. Aca-demic Press, San Diego, CA. pp. 543–575.
465 Kolanowski, W. & Laufenberg, G. (2006). Enrichment of food products with polyunsaturated
466 fatty acids by fish oil addition. *European Food Research and Technology*, 222 (3-4),
467 472-477.

468 Leitzmann, M. F., Stampfer, M. J., Michaud, D. S., Augustsson, K., Colditz, G. C., Willett, W.
469 C. & Giovannucci, E. L. (2004). Dietary intake of n-3 and n-6 fatty acids and the risk of
470 prostate cancer. *The American Journal of Clinical Nutrition*, 80 (1), 204-216.
471 Le Maux, S., Brodkorb, A., Croguennec, T., Hennessy, A. A., Bouhallab, S. & Giblin, L.
472 (2013). β-Lactoglobulin-linoleate complexes: In vitro digestion and the role of protein
473 in fatty acid uptake. *Journal of Dairy Science*, 96 (7), 4258-4268.
474 Lock, A. L. and K. J. Shingfield. 2004. Optimising milk composition. In: *UK Dairying: Using*
475 *Science to Meet Consumers' Needs* (Edited by Kebreab, E., Mills, J., and Beever, D.E.),
476 Nottingham University Press, Nottingham, United Kingdom, pp. 107-188.
477 Maansson, H. L. (2008). Fatty acids in bovine milk fat. *Food & Nutrition Research*, 52, DOI:
478 10.3402/fnr.v52i0.1821.
479 MacGibbon, A. K. H. & Taylor, M. W. (2006). Composition and structure of bovine milk
480 lipids. In *Advanced Dairy Chemistry* (Edited by P. F. Fox and P. L. H. McSweeney)
481 volume 2: Lipids, 3rd edition. Springer, New York. pp.1-42.
482 Mandalari, G., Mackie, A. M., Rigby, N. M., Wickham, M. S. & Mills, E. (2009). Physiological
483 phosphatidylcholine protects bovine β-lactoglobulin from simulated gastrointestinal
484 proteolysis. *Molecular Nutrition & Food Research*, 53 (S1), S131-S139.
485 Meena, H., Ram, H. & Rasool, T. (2007). Milk constituents in non-descript buffaloes reared at
486 high altitudes in the Kumaon hills of the central Himalayas. *Buffalo Bulletin*, 26, 72-76.
487 Mondello, L., Tranchida, P. Q., Dugo, P. & Dugo, G. (2006). Rapid, micro-scale preparation
488 and very fast gas chromatographic separation of cod liver oil fatty acid methyl esters.
489 *Journal of Pharmaceutical and Biomedical Analysis*, 41 (5), 1566-1570.

490 Moreno, F. J., Mackie, A. R. & Mills, E. N. C. (2005). Phospholipid Interactions Protect the
491 Milk Allergen α-lactalbumin from Proteolysis during in Vitro Digestion. *Journal of*
492 *Agricultural and Food Chemistry*, 53 (25), 9810-9816.
493 Mu, H., & Hoy, C.-E. (2004). The digestion of dietary triacylglycerols. *Progress in Lipid*
494 *Research*, 43, 105-133.
495 O'Donnell, J. A. (1989). Milk fat technologies and markets: a summary of the Wisconsin Milk
496 Marketing Board 1988 Milk Fat Roundtable. *Journal of Dairy Science*, 72 (11), 3109-
497 3115.
498 Pafumi, Y., Lairon, D., de la Porte, P. L., Juhel, C., Storch, J., Hamosh, M. & Armand, M.
499 (2002). Mechanisms of Inhibition of Triacylglycerol Hydrolysis by Human Gastric
500 Lipase. *Journal of Biological Chemistry*, 277 (31), 28070-28079.
501 Pascal, G. (1996). Les apports quotidiens recommandés en lipides et en acides gras: Lipides et
502 santé. *OCL. Oléagineux, Corps Gras, Lipides*, 3 (3), 205-210.
503 Puyol, P., Perez, M. D., Mata, L., Ena, J. & Calvo, M. (1993). Effect of retinol and fatty acid
504 binding by bovine β-lactoglobulin on its resistance to trypsin digestion. *International*
505 *Dairy Journal*, 3 (7), 589-597.
506 Qureshi, M. S., Mushtaq, A., Khan, S., Habib, G. & Swati, Z. A. (2010). Variation in milk fatty
507 acids composition with body condition in dairy buffaloes (*Bubalus bubalis*). *Asian-*
508 *Australasian Journal of Animal Sciences*, 23, 340-345.
509 Qureshi, T. M., Vegarud, G. E., Abrahamsen, R. K. & Skeie, S. (2012). Characterization of the
510 Norwegian autochthonous cheese Gamalost and its angiotensin I-converting enzyme
511 (ACE) inhibitory activity during ripening. *Dairy Science & Technology*, 92, 613-625.

512 Rimestad, A., Borgejordet, A., Verterhus, K., Sygnestveit, K., Loken, E., Trygg, K., Pollestad,
513 M., Lund-Larsen, K., Omholt-Jensen, G. & Nordbotten, A. (2001). Den store
514 matvaretabellen. *Gyldendal Norsk forlag ASA*: Oslo (2001).
515 Siddiqui, R. A., Harvey, K. A. & Zaloga, G. P. (2008). Modulation of enzymatic activities by n-
516 3 polyunsaturated fatty acids to support cardiovascular health. *The Journal of*
517 *Nutritional Biochemistry*, 19 (7), 417-437.
518 Stene, L. C., Ulriksen, J., Magnus, P. & Joner, G. (2000). Use of cod liver oil during pregnancy
519 associated with lower risk of Type I diabetes in the offspring. *Diabetologia*, 43 (9),
520 1093-1098.
521 Tiruppathi, C. & Balasubramania, K. A. (1982). Purification and properties of an acid lipase
522 from human gastric juice. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid*
523 *Metabolism*, 712 (3), 692-697.
524 Ulleberg, E. K., Comi, I., Holm, H., Herud, E. B., Jacobsen, M. & Vegarud, G. E. (2011).
525 Human gastrointestinal juices intended for use in in vitro digestion models. *Food*
526 *Digestion*, 2, 52-61.
527 Williams, C. M. (2000). Dietary fatty acids and human health. *Annales de Zootechnie*, 49(3),
528 165-180.

529 Wijendran, V. & Hayes, K. (2004). Dietary n-6 and n-3 fatty acid balance and cardiovascular
530 health. *Annual Review of Nutrition*, 24, 597-615.

531 Zeng, Y.-X., Araujo, P., Du, Z.-Y., Nguyen, T.-T., Froyland, L. & Grung, B. (2010).
532 Elucidation of triacylglycerols in cod liver oil by liquid chromatography electrospray
533 tandem ion-trap mass spectrometry. *Talanta*, 82 (4), 1261-1270.

534 Table 1: Number of peptides identified from omega-3 enriched buffalo skimmed milk at
535 different phases and steps of *ex vivo* digestion from different milk protein.

| Protein | G20 | G40 | D5 | D120 |
|---|---|---|---|---|
| $\alpha_{s1}$-CN | 18 | 19 | 08 | 02 |
| $\alpha_{s2}$-CN | 03 | - | 03 | 05 |
| β-CN | 25 | 21 | 39 | 27 |
| κ-CN | 14 | 03 | 01 | 01 |
| β-Lg | - | - | 05 | 04 |

536 All the identified peptides counted excluding the peptides from the undigested control milk
537 samples. G20, gastric digestion at pH 5.0 for 20 min; G40, gastric digestion at pH 2.5 for 20
538 min after 20 min gastric digestion at pH 5.0; D5 and D120, duodenal digestion for 5 and 120
539 min at pH 7.0, respectively.
540

541 Table 2. Fatty acid (FA) concentrations (mean±STD μg $mL^{-1}$ milk) in the free fatty acid
542 fraction of omega-3 enriched buffalo skimmed milk. Numbers given during digestion with
543 human gastric (G) and duodenal (D) juices which also includes the lipolysis (%) of individual
544 FA in the neutral lipid (NL) fraction.

| FA | G40 min | D30 min | D60 min | D120 min | Lipolysis (%) |
|---|---|---|---|---|---|
| C12:0 | 6.81±4.5 | 61.33±17.0 | 47.2±21.7 | 54.6±7.3 | 43.7 |
| C14:0 | 9.9±1.2 | 513.1±59.5 | 432.5±104.1 | 550.7±60.9 | 24.2 |
| C16:0 | 50.3±3.2 | 1683.0±219 | 1404.0±351.0 | 1669±155.6 | 28.5 |
| C18:0 | 47.6±2.7 | 635.9±142.5 | 524.0±167.4 | 603.5±91.3 | 47.6 |
| ΣSFA | 107.9±9.0 | 2832.0±420.0 | 2361.0±623.0 | 2823.0±308 | 30.4 |
| C14:1 n-5 *cis* | nd | 22.7±5.1 | 17.7±6.2 | 21.97±2.8 | 100.0 |
| C16:1 n-7 *cis* | nd | 345.8±55.1 | 327.7±28.1 | 606.2±98.5 | 24.7 |
| C18:1 n-9 *cis* | 13.7±1.1 | 1539.0±145.2 | 1249.0±342.0 | 1782.0±264.0 | 31.6 |
| C18:1 n-7 *cis* | nd | 132.7±24.7 | 123.6±9.9 | 258.7±49.4 | 32.4 |
| C20:1 n-9 *cis* | nd | 185.7±54.8 | 191.8±30.5 | 458.4±92.3 | 36.5 |
| C22:1 n-9 *cis* | nd | 77.2±19.0 | 81.28±11.6 | 190.3±50.2 | 39.2 |
| ΣMUFA | 13.74±1.1 | 2303.2±70.0 | 1991.0±341.0 | 3318.0±557.0 | 32.4 |
| C18:2 n-6 *cis* | nd | 166.7±16.8 | 140.2±29.0 | 192.3±34.2 | 24.5 |
| C20:4 n-6 *cis* | nd | 22.4±2.5 | 25.47±3.7 | 64.69±11.63 | 41.4 |
| Σomega-6 | - | 189.2±15.6 | 165.6±25.6 | 256.9±45.8 | 33.3 |
| C20:5 n-3 *cis* | nd | 56.62±6.3 | 64.16±7.9 | 180.1±33.8 | 23.1 |
| C22:6 n-3 *cis* | nd | 60.22±11.1 | 83.8±13.5 | 228.3±48.2 | 23.1 |
| Σomega-3 | - | 116.8±9.2 | 147.9±21.4 | 408.4±82.0 | 23.1 |
| ΣPUFA | - | 306.0±23.8 | 313.6±7.2 | 665.3±127.8 | 25.2 |

545 Lipolysis: {(FA in undigested NL - FA in D120 min NL)/ FA in undigested NL} x100. nd: not
546 detected; ΣSFA: total saturated fatty acids; ΣMUFA: total monounsaturated fatty acids;
547 ΣPUFA: total polyunsaturated fatty acids.

548

**Figure 1**

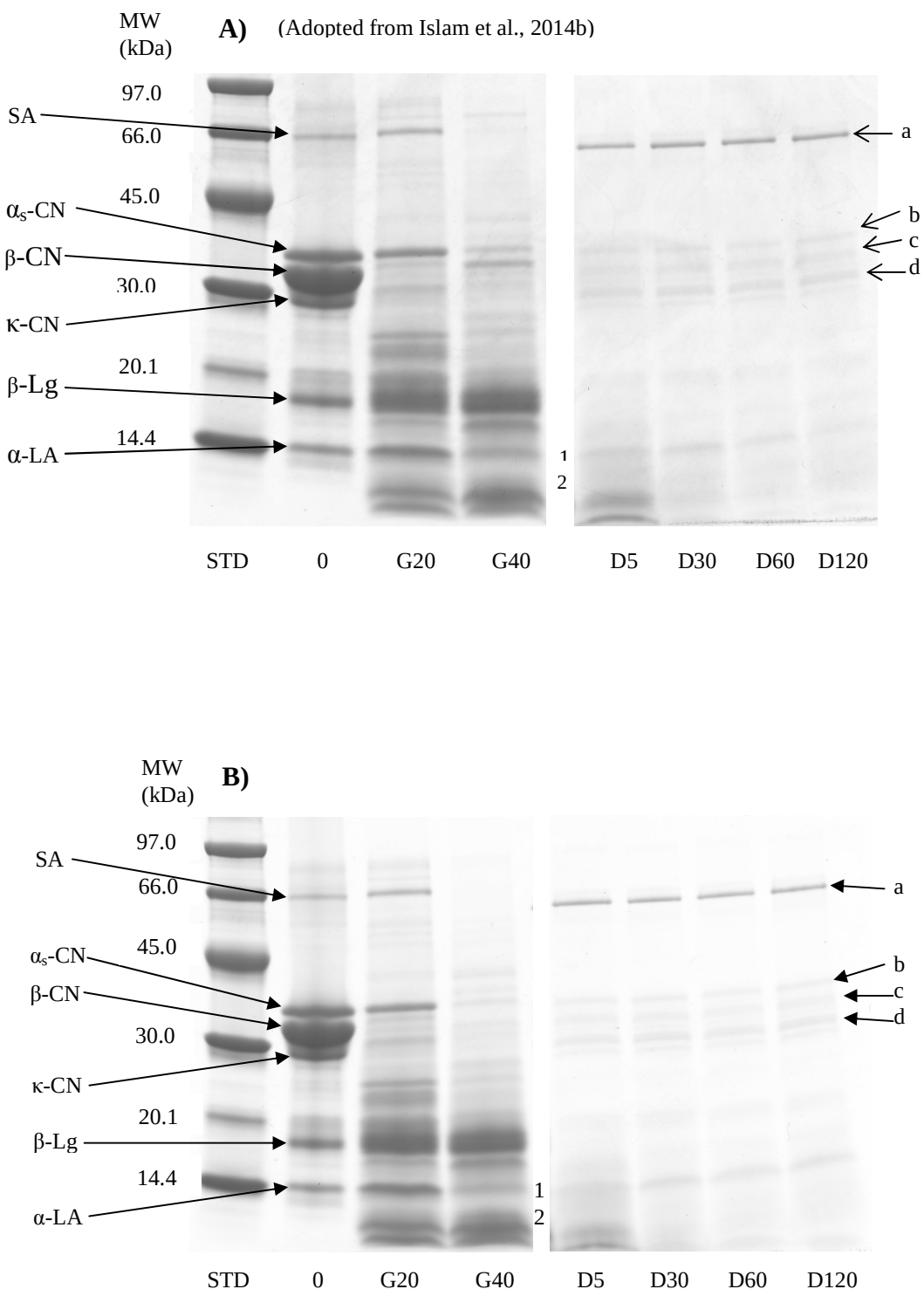

A)
(Adopted from Islam et al., 2014b)
MW (kDa)
97.0
66.0
45.0
30.0
20.1
14.4
SA
αs-CN
β-CN
κ-CN
β-Lg
α-LA
a
b
c
d
1
2
STD
0
G20
G40
D5
D30
D60
D120
B)
MW (kDa)
97.0
66.0
45.0
30.0
20.1
14.4
SA
αs-CN
β-CN
κ-CN
β-Lg
α-LA
a
b
c
d
1
2
STD
0
G20
G40
D5
D30
D60
D120

**Figure 2**

$\alpha_{s1}$-Casein (Accession number: O62823)

```
G20   1   MKLLILTCLVAVALARPKQPIKHQGLPQGVLNENLLRFFVAPFPEVFGKEKVNELSTDIG   60
G40   1   MKLLILTCLVAVALARPKQPIKHQGLPQGVLNENLLRFFVAPFPEVFGKEKVNELSTDIG   60
D5    1   MKLLILTCLVAVALARPKQPIKHQGLPQGVLNENLLRFFVAPFPEVFGKEKVNELSTDIG   60
D120  1   MKLLILTCLVAVALARPKQPIKHQGLPQGVLNENLLRFFVAPFPEVFGKEKVNELSTDIG   60

G20   61  SESTEDQAMEDIKQMEAESISSSEEIVPISVEQKHIQKEDVPSERYLGYLEQLLRLKKYN   120
G40   61  SESTEDQAMEDIKQMEAESISSSEEIVPISVEQKHIQKEDVPSERYLGYLEQLLRLKKYN   120
D5    61  SESTEDQAMEDIKQMEAESISSSEEIVPISVEQKHIQKEDVPSERYLGYLEQLLRLKKYN   120
D120  61  SESTEDQAMEDIKQMEAESISSSEEIVPISVEQKHIQKEDVPSERYLGYLEQLLRLKKYN   120

G20   121  VPQLEIVPNLAEEQLHSMKEGIHAQQKEPMIGVNQELAYFYPQLFRQFYQLDAYPSGAWY  180
G40   121  VPQLEIVPNLAEEQLHSMKEGIHAQQKEPMIGVNQELAYFYPQLFRQFYQLDAYPSGAWY  180
D5    121  VPQLEIVPNLAEEQLHSMKEGIHAQQKEPMIGVNQELAYFYPQLFRQFYQLDAYPSGAWY  180
D120  121  VPQLEIVPNLAEEQLHSMKEGIHAQQKEPMIGVNQELAYFYPQLFRQFYQLDAYPSGAWY  180

G20   181  YVPLGTQYPDAPSFSDIPNPIGSENSGKTTMPLW 214
G40   181  YVPLGTQYPDAPSFSDIPNPIGSENSGKTTMPLW 214
D5    181  YVPLGTQYPDAPSFSDIPNPIGSENSGKTTMPLW 214
D120  181  YVPLGTQYPDAPSFSDIPNPIGSENSGKTTMPLW 214
```

$\alpha_{s2}$-Casein (Accession number**: Q3Y443)**

```
G20    1  MKFFIFTCLLAVALAKHTMEHVSSSEESIISQETYKQEKNMAIHPSKENLCSTFCKEVIR 60
G40    1  MKFFIFTCLLAVALAKHTMEHVSSSEESIISQETYKQEKNMAIHPSKENLCSTFCKEVIR 60
D5     1  MKFFIFTCLLAVALAKHTMEHVSSSEESIISQETYKQEKNMAIHPSKENLCSTFCKEVIR 60
D120   1  MKFFIFTCLLAVALAKHTMEHVSSSEESIISQETYKQEKNMAIHPSKENLCSTFCKEVIR 60

G20    61  NANEEEYSIGSSSEESAEVATEEVKITVDDKHYQKALNEINQFYQKFPQYLQYLYQGPIV 120
G40    61  NANEEEYSIGSSSEESAEVATEEVKITVDDKHYQKALNEINQFYQKFPQYLQYLYQGPIV 120
D5     61  NANEEEYSIGSSSEESAEVATEEVKITVDDKHYQKALNEINQFYQKFPQYLQYLYQGPIV 120
D120   61  NANEEEYSIGSSSEESAEVATEEVKITVDDKHYQKALNEINQFYQKFPQYLQYLYQGPIV 120

G20    121  LNPWDQVKRNAVPITPTLNREQLSTSEENSKKTVDMESTEVITKKTKLTEEDKNRLNFLK 180
G40    121  LNPWDQVKRNAVPITPTLNREQLSTSEENSKKTVDMESTEVITKKTKLTEEDKNRLNFLK 180
D5     121  LNPWDQVKRNAVPITPTLNREQLSTSEENSKKTVDMESTEVITKKTKLTEEDKNRLNFLK 180
D120   121  LNPWDQVKRNAVPITPTLNREQLSTSEENSKKTVDMESTEVITKKTKLTEEDKNRLNFLK 180

G20    181  KISQHYQKFTWPQYLKTVYQYQKAMKPWTQPKTKVIPYVRYL 222
G40    181  KISQHYQKFTWPQYLKTVYQYQKAMKPWTQPKTKVIPYVRYL 222
G5     181  KISQHYQKFTWPQYLKTVYQYQKAMKPWTQPKTKVIPYVRYL 222
D120   181  KISQHYQKFTWPQYLKTVYQYQKAMKPWTQPKTKVIPYVRYL 222
```

β-Casein (Accession number**: Q9TSI0**)

```
G20  1   MKVLILACLVALALARELEELNVPGEIVESLSSSEESITHINKKIEKFQSEEQQQMEDEL   60
G40  1   MKVLILACLVALALARELEELNVPGEIVESLSSSEESITHINKKIEKFQSEEQQQMEDEL   60
D5   1   MKVLILACLVALALARELEELNVPGEIVESLSSSEESITHINKKIEKFQSEEQQQMEDEL   60
D120 1   MKVLILACLVALALARELEELNVPGEIVESLSSSEESITHINKKIEKFQSEEQQQMEDEL   60

G20  61    QDKIHPFAQTQSLVYPFPGPIPKSLPQNIPPLTQTPVVVPPFLQPEIMGVSKVKEAMAPK  120
G40  61    QDKIHPFAQTQSLVYPFPGPIPKSLPQNIPPLTQTPVVVPPFLQPEIMGVSKVKEAMAPK  120
D5   61    QDKIHPFAQTQSLVYPFPGPIPKSLPQNIPPLTQTPVVVPPFLQPEIMGVSKVKEAMAPK  120
D120 61    QDKIHPFAQTQSLVYPFPGPIPKSLPQNIPPLTQTPVVVPPFLQPEIMGVSKVKEAMAPK  120

G20  121   HKEMPFPKYPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQPPQPLPPTVMFPPQSVLSL  180
G40  121   HKEMPFPKYPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQPPQPLPPTVMFPPQSVLSL  180
D5   121   HKEMPFPKYPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQPPQPLPPTVMFPPQSVLSL  180
D120 121   HKEMPFPKYPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQPPQPLPPTVMFPPQSVLSL  180

G20  181   SQSKVLPVPQKAVPYPQRDMPIQAFLLYQEPVLGPVRGPFPIIV 224
G40  181   SQSKVLPVPQKAVPYPQRDMPIQAFLLYQEPVLGPVRGPFPIIV 224
D5   181   SQSKVLPVPQKAVPYPQRDMPIQAFLLYQEPVLGPVRGPFPIIV 224
D120 181   SQSKVLPVPQKAVPYPQRDMPIQAFLLYQEPVLGPVRGPFPIIV 224
```

κ-Casein (Accession number**: A8KRP5**)

```
G20    1   MMKSFFLVVTILALTLPFLGAQEQNQEQPIRCEKEERFFNDKIAKYIPIQYVLSRYPSYG 60
G40    1   MMKSFFLVVTILALTLPFLGAQEQNQEQPIRCEKEERFFNDKIAKYIPIQYVLSRYPSYG 60
D5     1   MMKSFFLVVTILALTLPFLGAQEQNQEQPIRCEKEERFFNDKIAKYIPIQYVLSRYPSYG 60
D120   1   MMKSFFLVVTILALTLPFLGAQEQNQEQPIRCEKEERFFNDKIAKYIPIQYVLSRYPSYG 60

G20    61  LNYYQQKPVALINNQFLPYPYYAKPAAVRSPAQILQWQVLPNTVPAKSCQAQPTTMTRHP 120
G40    61  LNYYQQKPVALINNQFLPYPYYAKPAAVRSPAQILQWQVLPNTVPAKSCQAQPTTMTRHP 120
D5     61  LNYYQQKPVALINNQFLPYPYYAKPAAVRSPAQILQWQVLPNTVPAKSCQAQPTTMTRHP 120
D120   61  LNYYQQKPVALINNQFLPYPYYAKPAAVRSPAQILQWQVLPNTVPAKSCQAQPTTMTRHP 120

G20    121  HPHLSFMAIPPKKNQDKTEIPTINTIVSVEPTSTPITEAIENTVATLEASSEVIESVPET 180
G40    121  HPHLSFMAIPPKKNQDKTEIPTINTIVSVEPTSTPITEAIENTVATLEASSEVIESVPET 180
D5     121  HPHLSFMAIPPKKNQDKTEIPTINTIVSVEPTSTPITEAIENTVATLEASSEVIESVPET 180
D120   121  HPHLSFMAIPPKKNQDKTEIPTINTIVSVEPTSTPITEAIENTVATLEASSEVIESVPET 180

G20    181  NTAQVTSTVV 190
G40    181  NTAQVTSTVV 190
D5     181  NTAQVTSTVV 190
D120   181  NTAQVTSTVV 190
```

β-Lactoglobulin (Accession number: **C3W955**)

```
G20    1  MKCLLLALGLALACGAQAIIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVY 60
G40    1  MKCLLLALGLALACGAQAIIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVY 60
D5     1  MKCLLLALGLALACGAQAIIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVY 60
D120   1  MKCLLLALGLALACGAQAIIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVY 60

G20    61  VEELKPTPEGDLEILLQKWENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKY 120
G40    61  VEELKPTPEGDLEILLQKWENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKY 120
D5     61  VEELKPTPEGDLEILLQKWENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKY 120
D120   61  VEELKPTPEGDLEILLQKWENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKY 120

G20    121  LLFCMENSAEPEQSLACQCLVRTPEVDDEALEKFDKALKALPMHIRLSFNPTQLEEQCHV 180
G40    121  LLFCMENSAEPEQSLACQCLVRTPEVDDEALEKFDKALKALPMHIRLSFNPTQLEEQCHV 180
D5     121  LLFCMENSAEPEQSLACQCLVRTPEVDDEALEKFDKALKALPMHIRLSFNPTQLEEQCHV 180
D120   121  LLFCMENSAEPEQSLACQCLVRTPEVDDEALEKFDKALKALPMHIRLSFNPTQLEEQCHV 180
```

**Figure 3**

A) Peptides from $\alpha_{s1}$-casein (gastric phase)

```
BFG-03      ---------------------SDIPNPIGSENSGKTTM   17
BSG-07      ---------------------SDIPNPIGSENSGKTTM   17
BFG-07      -----HI------------QKEDVPSERY---------   12
BSG-08      ------I------------QKEDVPSERY---------   11
BSG-09      ---PLGT-----QYPD-APLFSDIPN------------   17
BFG-05      YYVPLGT-----QYPD-APSF-----------------   15
BSG-10      YYVPLGT-----QYPD-APSF-----------------   15
BFG-06      YYVPLGT-----QYPD-APLF-----------------   15
BSG-11      YYVPLGT-----QYPD-APLF-----------------   15
BFG-02      ----------------GIHAQQKEPMIGVNQEL-----   17
BSG-05      ------AEEQLHSMKEGIHAQQKEPMIGVNQEL-----   27
BFG-08      EIVPNLAEEQLHSM-----------------------   14
BFG-09      EIVPNLAEEQLHSMKE---------------------   16
BSG-06      EIVPNLAEEQLHSMKE---------------------   16
BFG-04      ---R--------P-KQ-PIKHQGLPQGVLNENL-----   20
BSG-01      ---R--------P-KQ-PIKHQGLPQGVLNENL-----   20
BFG-01      -FVA--------PFPE-VFGKEKVNEL-----------   17
BSG-02      -FVA--------PFPE-VFGKEKVNELS----------   18
BFG-10      -------------LEQ-LLRLKKYNVPQL---------   15
BSG-03      -------------LEQ-LLRLKKYNVPQL---------   15
BFG-11      -------------FRQ-FYQL-----------------   7
BSG-04      -------------FRQ-FYQL-----------------   7
BFG-12      ------------------YQLDAYPSGAW---------   11
BSG-12      ---------------------DAYPSGAW---------   8
Consensus:  YYVPLGTEEQLHQYPEGAPQFQKVPNPGLNENLGKTTM
```

B) Peptides from $\alpha_{s1}$-casein (duodenal phase)

```
BSD-01      ----SDIPNPIGSENSGK------  14
BFD-05      ----SDIPNPIGSENSGK------  14
BSD-05      EGIHAQQKEPMIGVNQEL------  18
BFD-03      EGIHAQQKEPMIGV----------  14
BSD-04      ------------EIVPNLAEEQLH  12
BFD-07      ------------EIVPNLAEEQLH  12
BFD-02      QPI-KHQGLPQGV-----------  12
BSD-03      QPI-KHQGLPQGVLNENL------  17
BSD-06      QPI-KHQGLPQGVLNENL------  17
BFD-01      -----HQGLPQGVLNENL------  13
BFD-06      -----------HIQKEDVPSE---  10
BFD-04      ------YYVPLGTQYPDAPS----  14
BSD-02      ------YFYPQ-------------  5
Consensus:  QPIHKHQGLPQGVLNENLPEEQLH
```

C) Peptides from $\alpha_{s2}$-casein (gastric phase)

```
BSG-01      VYQYQKAMKPWTQPKTNVIPYVRYL 25
BSG-02      LYQGPIVLNPWDQVKRNAVPITPTL 25
BFG-01      LYQGPIVLNPWDQVKRNAVPITPTL 25
            :**   .::** * * *.:* .  *
Consensus:  LYQGPIVLNPWDQVKRNAVPITPTL
```

D) Peptides from $\alpha_{s2}$-casein (duodenal phase)

```
BFD-03      ------NQFYQKFPQ-   9
BFD-01      --LYQGPIVLNPWDQV   14
BFD-02      ----------FAWPQ-   5
BSD-01      --ITVDDKHYQ-----   9
BFD-05      --ITVDDKHYQ-----   9
BSD-02      TKLTEEDKNRLNFL--   14
BFD-04      --LTEEDKNRLN----   10
Consensus:  --LTEDDKHYQNFPQ-
```

E) Peptides from β-casein (gastric phase)

```
BSG-06      -----QSWM-----------HQPPQPLPPTV----M-------------------------   16
BFG-04      -----QSWM-----------HQPPQPLPPTV----M-------------------------   16
BSG-05      --------M-----------HQPPQPLPPTV----MFPPQSVL-------------------   20
BFG-05      --------M-----------HQPPQPLPPTV----MFPPQSVL-------------------   20
BSG-01      -------GVSKVK--EAMAPKHKEMPFPK--YPVEPFTESQSLTL-----------------   34
BFG-06      -------GVSKVK--EAMAPKHKEMPFPK--YPVEPFTESQS-------------------   31
BSG-02      ---------------AMAPKHKEMPFPK--YPVEPFTESQSLTLTDVENLHLPLPLL---   40
BFG-03      --------------------------------PVEPFTESQSLTLTDVENLHLPLPLL---   26
BFG-01      ------------------------------------------TDVENLHLPLPLLQSW   15
BSG-03      ----------------------------------------LTLTDVENLHLPLPLLQSW   18
BFG-02      ---------------------------------------TLTDVENLHLPLPLLQS-   17
BSG-04      --LYQEPVLGPVRGPFPIIV-----------------------------------------   18
BFG-07      FLLYQEPVLGPVRGPFPIIV-----------------------------------------   20
BSG-08      ------------------LQDKIHPFAQTQS----------------------------   13
BSG-09      --SLSQSKVLPVP--------QKAVPYPQRDMPIQAFL----------------------   28
BFG-08      --SLSQSKVLPVP--------QKAVPYPQRDMPIQAFL----------------------   28
BSG-07      ----------------------------PVVVPPFLQPEIMGVSKVKE-----------   20
BFG-11      ----------------------------PVVVPPFLQPEIMGVSKVKE-----------   20
BSG-10      -----QSLVYPFPGPIPK-------------------------------------------   13
BFG-09      -----QSLVYPFPGPIPK-------------------------------------------   13
BSG-11      -------LVYPFPGPIPNSLPQNIPPLTQT-------------------------------   23
BFG-10      -------LVYPFPGPIPKSLPQNIPPLTQT-------------------------------   23
BFG-12      -------LVYPFPGPIPKSLPQNIPPLTQTPVVVPPFLQPEIM------------------   36
Consensus:  FLLLQQSLVYPVPGPIPKALHQKIQPLPQTVVPVEPFLESQILTLTDVENLHLPLPLLQSW
```

F) Peptides from β-casein (duodenal phase)

```
BFD-08      --------------SWMHQPPQPLPPTV-----------------   14
BSD-04      --------------WMHQPPQPLPPTV----MFPPQ---SV----   20
BFD-09      --------------WMHQPPQPLPPTV----MFPPQ---SV----   20
BSD-05      ---------------MHQPPQPLPPTV----MFPPQ---SVLS--   21
BFD-10      ---------------MHQPPQPLPPTV----MFPPQ---SVL---   20
BFD-14      --------AVPYPQ-------------------------------   6
BSD-06      --AQTQSLVYPFPGPIPK---------------------------   16
BFD-04      --AQTQSLVYPFPGPIPK---------------------------   16
BSD-07      ------------------SLPQNIPPLTQTPVVVPPFLQPEIMGVS   28
BFD-06      ------------------SLPQNIPPLTQTPVVVPPFLQPEIMGVS   28
BFD-07      --------VYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEIM---   35
BSD-08      -------LVYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEIM---   36
BFD-05      -------LVYPFPGPIPNSLPQNIPPLTQT----------------   23
BSD-01      SLTLTDVENLHLPLPL-----------------------------   16
BFD-11      SLTLTDVENLHLPLPL-----------------------------   16
BSD-02      -LTLTDVENLHLPLPLL----------------------------   16
BFD-12      -LTLTDVENLHLPLPLL----------------------------   16
BFD-13      --------VLPVPQ-------------------------------   6
BSD-09      --LYQEPVLGPVRGPFPIIV-------------------------   18
BFD-02      --LYQEPVLGPVRGPFPIIV-------------------------   18
BSD-03      ---QMEDELQDKIHPF-----------------------------   13
BFD-01      ---QMEDELQDKIHPF-----------------------------   13
BSD-10      ----------HKEMPFPK---------------------------   8
BFD-03      ----------HKEMPFPK---------------------------   8
Consensus:  SLTLTEVEVLPFPGPFPHQPPQPLPPTVQTPVMFPPFLQPEIMGVS
```

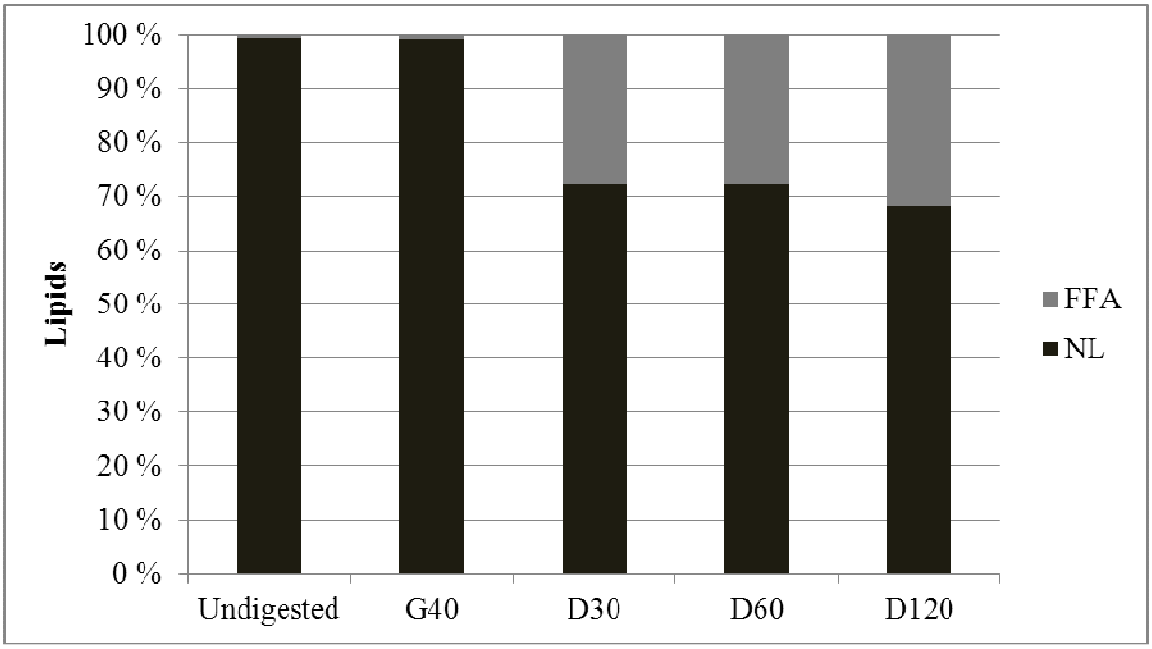
G) Peptides from κ-casein (gastric phase)

```
BSG-02      --------------------MAIPPKKNQDKTEIPTINT  19
BFG-01      --------------------MAIPPKKNQDKTEIPTINT  19
BSG-07      -------------------YYQQKPVAL-----------  9
BFG-03      ----------------GLNYYQQKPVAL-----------  12
BSG-06      ---------LINNQFLPYPYYA-KPAAVRSPAQILQ---  26
BFG-04      ---------LINNQFLPYPYYA-KPAAVRSPAQILQ---  26
BSG-03      ----------TRHPHPHLSF-------------------  10
BFG-05      ----------TRHPHPHLSF-------------------  10
BSG-01      FNDKIAKYIPIQYVL------------------------  15
BFG-02      FNDKIAKYIPIQYVL------------------------  15
BSG-05      -------YVLSRYPSY-----------------------  9
BFG-06      -------YVLSRYPSY-----------------------  9
BSG-04      --------VLSRYPSYGLNY-------------------  12
Consensus:  FNDKIAKYVLIRYPSYGLNYYAIKPAALQDKAEILQINT
```

H) Peptides from β-lactoglobulin (duodenal phase)

```
BSD-01      ----KIDALNENK--VLV-----   12
BFD-01      ----KIDALNENK--VLV-----   12
BSD-03      VYVEELKPTPEGDLEILLQ----   19
BSD-04      VYVEELKPTPEGDLE--------   15
BFD-03      VYVEELKPTPEGDLEILLQ----   19
BSD-02      --------TPEVDDEALEKFDKA   15
BFD-02      --------TPEVDDEALEKFDK-   14
Consensus:  VYVEEIKPTPEGDLEVLEKFDK-
```

803 **Figure 4**




804

805

## Figure Legend

**Figure 1.** Protein degradation profile of A) skimmed buffalo milk and B) skimmed buffalo milk fortified with cod liver oil after human gastric (G) and duodenal (D) digestion. Lane 1, Low molecular weight marker. Lane 2, undigested milk. Lane 3-6, follow up gastric 20 min (G20) and 40 min (G40) and duodenal 5 min (D5), 30 min (D30), 60 min (D60) and 120 min (D120). MW, molecular weight; kDa, kilo dalton; SA, serum albumin; CN, casein; β-Lg, β-lactoglobulin; α-LA, α-lactalbumin; D5: 1 and 2, α-lactalbumin with β-lactoglobulin; a, amylase; b, carboxypeptidase, chymotrypsin , elastase, lipase, gastricsin and amylase; c, carboxypeptidase, elastase, lipase, trypsin and amylase; d, elastase, carboxypeptidase, chymotrypsin, amylase, lipase and trypsin.

**Figure 2.** Comparative peptide regions derived from the different milk proteins of buffalo skimmed (grey) and omega-3 enriched buffalo skimmed milk (underlined) after *ex vivo* gastric (G) and duodenal (D) digestion. Single letter amino acid code used. bold and Italic residues, signal peptide. The pH at G20 and G40 was 5.0 and 2.5, respectively and at D5 and D120 was 7.0. G20 and G40, gastric digestion for 20 and 40 min, respectively; D5 and D120, duodenal digestion for 05and 120 min, respectively.

**Figure 3 (A-H).** Multiple sequence alignment (software: CLUSTAL O (1.2.0)) of peptides generated from different proteins of omega-3 enriched buffalo skimmed milk (BF) and skimmed (BS) buffalo milk by gastric (G) and duodenal (D) digestion. Numbers on the left is the serial number of the minimal overlapped peptides. Numbers on the right indicates the number of amino acid residues in that peptide. Consensus obtained from Jalview 2.8. ., residues

827 with weakly similar properties and conserved;  :, residues with strongly similar properties and
828 conserved; *, residue which is fully conserved.

829 **Figure 4.** Proportion (%) of lipolysis of neutral lipids (NL) of omega-3 enriched buffalo
830 skimmed milk and subsequent formation of free fatty acids (FFA) during digestion with human
831 gastric (G) and duodenal (D) juices. G40, gastric digestion at pH 2.5 for 20 min after 20 min
832 gastric digestion at pH 5.0; D30, D60 and D120, duodenal digestion for 30, 60 and 120 min,
833 respectively at pH 7.0.

PAPER IV

# *Ex Vivo* Digestion of Milk from Red Chittagong Cattle Focusing Proteolysis and Lipolysis

**Mohammad Ashiqul Islam*, Dag Ekeberg, Elling-Olav Rukke and Gerd Elisabeth Vegarud**

Department of Chemistry, Biotechnology and Food Science,

Norwegian University of Life Sciences, P. O. Box 5003, NO-1432 Aas, Norway

*Email addresses*:

mohammad.islam0@nmbu.no

dag.ekeberg@nmbu.no

elling-olav.rukke@nmbu.no

gerd.vegarud@nmbu.no

**Running head: *Ex Vivo* Proteolysis and Lipolysis of Red Cattle Milk**

*Corresponding author: Tel.: +47-64965900, Fax: +47-64965901

*Email address*: mohammad.islam0@nmbu.no or m.a.islam@bau.edu.bd

Department of Chemistry, Biotechnology and Food Science,

Norwegian University of Life Sciences, P. O. Box 5003, NO-1432 Aas, Norway

**ABSTRACT:** *Ex vivo* digestion of proteins and fat in Red Chittagong Cattle milk from Bangladesh was carried out using human gastrointestinal enzymes. This was done to investigate the milk protein digestion in this bovine breed with a special focus on the degradation of the allergenic milk proteins; $\alpha_{s1}$-casein and β-lactoglobulin and also to record the generation of peptides. Lipolysis of the milk fat and release of fatty acids were also under consideration. After 40 min gastric digestion all the $\alpha_s$-caseins were digested completely while β-lactoglobulin remained intact. During 120 min duodenal digestion β-lactoglobulin was reduced, however, still some intact β-lactoglobulin was observed. The highest number of peptide was identified from β-casein and all most all the peptides from κ-casein and β-lactoglobulin were identified from the gastric and duodenal samples, respectively. No lipolysis was observed in the gastric phase of digestion. After 120 min duodenal digestion, milk fat showed 48% lipolysis. Medium and long chain fatty acid showed 6-19% less lipolysis than the short chain fatty acids. Among the unsaturated fatty acids C18:1Σothers showed highest lipolysis (81%) which was more than three times of C18:2Σall and all other unsaturated fatty acids showed lipolysis ranging from 32% to 38%. The overall digestion of Bangladeshi Red Cattle milk was more or less similar to the digestion of Nordic bovine milk (Norwegian Red Cattle). (**Key Words:** Gastric digestion, Duodenal digestion, $\alpha_{s1}$-casein, β-lactoglobulin, Peptide, Fatty acid).

## INTRODUCTION

The use of *ex vivo* digestion model is important for understanding the mechanisms involved in food digestion and to mimic the human digestion. There are some other purposes as well for using such models are - e.g. investigation of bioactive components, study the survivability of drugs through the gastrointestinal (GI) tract, investigation of the digestibility of food allergens, to design food-based delivery system in the GI tract and to study the structural changes of ingested components.

According to Ekmekcioglu (2002) and Kalantzi et al. (2006), the most challenging physiological parameters are the individual variation in enzymes, acid and bile salt secretion, substrate availability and retention time in the gastric and intestine. The commercial enzyme (s) preparations are purified from different animal species. Generally, the same enzymes purified from different animal species are likely to vary in specificity, functional enzymatic parameters and stability (Furlund et al., 2013). But, to date, most of the *in vitro* studies regarding protein degradation and peptide generation have been done by using the commercial proteases, mainly of porcine or bovine origin. Whereas, human GI juices is a complex mixture of enzymes, with their isoforms and inhibitors, and bile salts. The sample characteristics, enzyme activity, ionic composition, used mechanical stresses and the digestion duration profoundly affect the results of *in vitro* digestion (Hur et al., 2011) and simulation of *in vivo* condition will never be complete (Boisen and Eggum, 1991; Coles et al., 2005). However, making the compromise between accuracy and ease of utilization, *in vitro* model digestion could be used as a rapid screening tool for foods with different composition and structures (Hur et al., 2011). Coles et al. (2005) suggest the use of single enzymes in *in vitro* model digestion but according to Boisen and Eggum (1991), use of a mixture of enzymes is more realistic and Eriksen et al. (2010) concluded that human digestive juices are preferred.

59 The milk protein digestion and peptides from *in vitro* digested milk has been done by several
60 authors (Almaas et al., 2006; Inglingstad et al., 2010; Almaas et al., 2011; Furlund et al., 2013;
61 Devle et al., 2014; Tidona et al., 2014; Islam et al., 2014b). But still more knowledge is needed
62 because of high variability in milk composition between breeds (Miranda et al., 2004; Abd El-
63 Salam and El-Shibiny 2011; Medhammar et al., 2012; Islam et al., 2014a) and in *in vitro*
64 digestion protocol used (Hur et al., 2011; Kopf-Bolanz et al., 2012; Furlund et al., 2013; Islam
65 et al., 2014b). The structure of milk and milk protein composition may have an influence on its
66 digestibility (Almaas et al., 2006). Tidona et al. (2014) found more rapid degradation of β-
67 lactoglobulin (β-Lg)-I when β-Lg-II is absent in donkey's milk. The heterogeneity in the amino
68 acid composition of milk may results in variation in peptides formation and content after
69 proteolysis (Ulleberg, 2011). The rapid degradation pattern of allergenic milk proteins, β-Lg
70 and $\alpha_{s1}$-casein, in Bangladeshi buffalo milk (Islam et al., 2014b) also put some more interest to
71 check other bovine milk from Bangladesh. In a previous study, Islam et al. (2014a) concluded
72 that buffalo and Red Chittagong Cattle (RCC) milk showed the highest compositional
73 characteristics for nutritional and technological properties.
74 Very few studies have been conducted regarding the milk lipid digestion and according to
75 Miled et al. (2000), in general, few studies have been reported on lipid digestion. Lipid
76 digestion is more complex than the protein digestion regarding the enzymes and physiological
77 conditions in the gut. Factors like food matrix and buffering capacity, emulsion type (oil
78 based/water based), individual secretion of both lipolytic enzymes and bile salts affect the
79 hydrolysis of dietary lipids. According to Boisen and Eggum (1991), digestion of one nutrient
80 may affect the digestion of others. Devle et al. (2014) and Islam et al. (2014b) showed different
81 effect of milk lipids on the protein digestion in cow (Norwegian Red Cattle) and buffalo milk.
82 The objective of the present study was to investigate the digestion of milk from Red Chittagong

Cattle with the special focus on lipolysis and proteolysis of the allergenic proteins, $\alpha_{s1}$-casein and β-lactoglobulin.

## MATERIALS AND METHODS

### Milk sample

Mixed whole milk from nineteen Red Chittagong Cattle (RCC) was collected from Bangladesh Livestock Research Institute (BLRI) dairy farm. Sampling was done from the morning milk. The animals were at different number and stage of lactation and the milk production during the sampling time varied between 1.3 to 5.0 L. The samples were preserved by bronopol (1 tablet/40 mL milk; D & F control systems, Inc. USA) with minimum delay after they were milked. All milk samples were kept at -20 ºC and transferred to the Norwegian University of Life Sciences and stored at -20 ºC until used. Detailed composition of RCC milk was reported by Islam et al. (2014a) and according to them the true protein and fat content of the RCC milk used herein were 38 and 42 g/kg milk, respectively.

### Gastrointestinal (GI) enzymes

Human GI enzymes as human gastric juices (HGJ) and human duodenal juices (HDJ) was collected and prepared according to the method of Ulleberg et al. (2011). In brief, aspiration of juices was done on six healthy, fasted (for at least 8 hrs), adult (20-37 years old) volunteers at Lovisenberg Diakonale Hospital, Oslo, Norway. A triple lumen tube (Maxters catheters, Marceille, France) was used for this purpose. The protocol used was approved by the Norwegian Ethical Committee.

### Ex vivo digestion

A two phase digestion, gastric and duodenal was carried out according to the method described by Devle et al. (2014) and Islam et al. (2014b). The details of the *ex vivo* digestion model are given in Table 1. The digestion was carried out at 37 ºC in a water bath for a total of 120 min

then the reaction was stopped by placing them into -20 ºC (protein samples) or adding 20 mL choloroform:methanol (2:1) and then placing into -20 ºC (lipid samples). The digestion was done in triplicate.

**Milk protein degradation profile**

The proteins were separated by SDS-PAGE using a modified method described by Islam et al. (2014b). The digested samples were mixed with sample buffer and applied on a precast gel (6.5 – 200 kDa; mini PROTEAN® TGX™ precast gels, Tris Glycine extended, Bio-Rad laboratories, Inc. Made in United States) and ran for 35 min at 200 V. Then it was fixed, stained with Commassie Brilliant Blue, destained and kept in preservation solution.

**Protein identification**

A method described by Devle et al. (2014) and Islam et al. (2014b) were used to identify the protein bands in the SDS-PAGE. In brief, the identification of each band was done by nano-UPLC/Q-Exactive MS both from Thermo Fisher Scientific, Bremen, Germany. The UPLC was equipped with a trap column (Acclaim PepMap100, C18, 5 µm, 100 Å, 300 µm i.d. x 5 mm, Thermo Fisher Scientific, Bremen, Germany) and a 50 cm x 75 µm analytical column (Acclaim PepMap RSLC C18, 2 µm, 100 Å, 75 µm i.d. x 50 cm, nanoViper, Bremen, Germany). The Q-Exactive MS (Thermo Fisher Scientific, Bremen, Germany) was in full scan (300-1600 m/z) followed by (up to) 5 MS2 scans at resolution (R) 70.000 and 35000, respectively and the used neutral collision energy (NCE) was 28. For MS/MS, 1=z>5 (‘z’ is the charge) precursors were excluded. An in-house Mascot (v.2.4) server was used for the database search. The data base was National Center for Biotechnology Information (NCBI), number: 20130131 (22749596 sequences; 7819872540 residues).

**Identification of peptides**

Peptides were identified by using the method described by Islam et al. (2014b) according to the methods of Furlund et al. (2013) and Qureshi et al. (2012). A nanoACQUITY$^{TM}$ UPLC$^{®}$ (Waters, Milford, USA) and Q-TOF Ultima MS (Micromass Ltd., Manchester, UK) was used for this purpose. The columns in the UPLC were 5-µm symmetry C18 trap column (180 µm × 20 mm; Waters, Milford, USA) and 1.7-µm BEH C18 analytical column (75 µm × 100 mm; Waters, Milford, USA).

**Multiple sequence alignment (MSA) of the peptides**

The MSA was done to identify the minimal overlapped peptides and their position in the protein sequence. A method described by Islam et al. (2014b) was used with modifications. The software used was Clustal omega (version: CLUSTAL O (1.2.1); http://www.ebi.ac.uk/Tools/msa/clustalo/) and Jalview 2.8.0b1.

**Lipid analysis**

The total lipid was first extracted and then separated by solid phase extraction (SPE) into neutral lipid (NL) and free fatty acid (FFA). The fatty acid methyl esters (FAMEs) were identified by Gas Chromatography-Mass Spectrometry (GC-MS). A modified method according to Devle et al. (2014) as described by Islam et al. (2014b) was followed in lipid analysis. In brief, total lipid was extracted by 20 mL of chloroform and methanol mixture (2:1) added immediately after the digestion. The SPE was carried-out on a liquid handling robot (Gilson, GX-274 ASPEC, Middleton, USA). Elution of NL and FFA were done with 5 mL chloroform and diethyl ether:acetic acid (98:2), respectively. The FAMEs of NL and FFA were prepared by using sodium-methanolate and boron trifluride-methanol complex, respectively. In GC (Agilent 6890 series, Agilent Technology, Wilmington, DE, USA), 50 m CP-Sil 88 capillary column with ID 0.25 and 0.20 µm thickness (Varian, Middelburgh, The Netherlands)

was used. The coupling Autospec Ultima MS was from Micromass Ltd. Manchester, England using electron ionization ion source (mass ranze *m/z* 40-600).

## RESULTS AND DISCUSSION

### Protein degradation

The protein degradation pattern during the *ex vivo* digestion is shown in Figure 1. The majority of the caseins were digested after initial 20 min gastric digestion and appeared completely digested after 40 min gastric phase.

The whey protein serum albumin was degraded during the gastric digestion for 40 min while β-lactoglobulin and some of the α-lactalbumin were resistant. After duodenal digestion still some β-lactoglobulin was intact after 120 min and identified in band 1 (lane D5) in Figure 1 together with with fragments of serum albumin. The other bands, 2, 3 and 4 (lane D5) in Figure 1 were the blend of β-lactoglobulin, α-lactalbumin and serum albumin. In the duodenal digested samples, some bands that appear at approximately 30-60 kDa (Figure 1; a, b, c and d) are the digestive enzymes present in the duodenal juices (Devle et al., 2014).

In all model digestion studies, the type, amount and activity of the enzymes, as well as pH used appear to influence the caseins digestion during the gastric phase (Almass et al., 2006). Kopf-Bolanz et al. (2012) reported a complete digestion of all the caseins after 30 min *in vitro* gastric digestion, while Gallier et al. (2012) showed a total degradation of caseins after 45 min using commercial enzymes of animal origin. When human gastrointestinal enzymes were used, Devle et al. (2014) reported a complete casein digestion after 40 min. The results of the present study on bovine milk are in agreement with these results. However, Islam et al. (2014b) showed traces of $\alpha_s$-caseins in buffalo milk after 40 min gastric digestion and not total casein degradation. Tidona et al. (2014) reported very low degradation of the caseins in donkey's milk after 30 min gastric digestion. These two reports indicate the importance of species variation

and are in agreement with Inglingstad et al. (2010) in a study on human, equine, goat and bovine milk digestion.

The present study showed some intact β-lactoglobulin after 120 min duodenal digestion and this is in line with the report on bovine milk by Devle et al. (2014), Gallier et al. (2012) and Inglingstad (2010). However, Kopf-Bolanz et al. (2012) obtained almost complete digestion of bovine β-lactoglobulin by commercial gastric (120 min) and pancreatic enzymes (30, 60, 90 and 120 min). However, another important result obtained by, Islam et al. (2014b) showed all most complete hydrolysis of β-lactoglobulin in full fat buffalo milk after 5 min duodenal digestion. So, genetic factors for the degradation of β-lactoglobulin may be of importance as also being reported by Tidona et al. (2014) in donkey. Another factor important for the digestion of β-lactoglobulin seems to be the bile salts where a higher concentration than ca. 2-3 mM is required (Gass et al., 2007). The presence of α-lactalbumin in band 2, 3 and 4 (D5, Figure 1) confirmed by UPLC-MS, indicate the hydrolysis of α-lactalbumin as Kopf-Bolanz et al. (2012) reported on the complete digestion of α-lactalbumin after 30 min duodenal digestion using commercial enzymes.

**Peptides**

The total number of identified peptides from the different milk proteins during the different phases of *ex vivo* digestion is shown in Table 2. The minimal overlapped peptides from the different milk proteins after gastric and duodenal phase digestion with their corresponding position in the protein sequence are given in Table 3. Maximum number of peptides was identified from the β-casein followed by $\alpha_{s1}$-casein, κ-casein, $\alpha_{s2}$-casein and β-lactoglobulin. Most of the peptides from κ-casein and β-lactoglobulin were identified from gastric and duodenal phase of digestion, respectively (Table 2). This is in agreement with the results obtained on buffalo milk by Islam et al. (2014b). The sequence coverage of the identified

minimal overlapped peptides (Table 3) corresponded well with the number of total identified peptides (Table 2); β-casein showed more extensive hydrolysis, next was $\alpha_{s1}$-casein followed by κ-casein, $\alpha_{s2}$-casein and β-lactoglobulin. The presence of proline in all most all the peptides and the hydrophobicity of all the peptides are notable. These results are also in agreement with the results showed by Islam et al. (2014b) and Islam et al. (2014c). Proline is known as a helix breaker in the protein structure and may be, the proteolytic enzymes have less access to the hydrophobic sequence for further proteolysis. The extent of hydrolysis of the proteins, especially the caseins (Figure 1) was not evident neither by the number of identified peptides (Table 2) nor by the protein sequence coverage by the minimal overlapped peptides (Table 3). This may be explained by the detection limit of the UPLC/Q-TOF MS that can identify peptides with the lowest molecular weight of 0.80 kDa. Peptides with lower molecular size as di-, tri- and tetra-peptides and free amino acids were not detected in this study. According to Kopf-Bolanz et al. (2012) 50% of the total milk proteins were degraded into di- and tri-peptides and 10% of the proteins were degraded to the free amino acids. They also mentioned that absence of bile salts may reduce the degradability and conclude that the size distribution of the proteins and peptides in the range of 5 kDa and tripeptides is unclear. However, the digestion condition used in the present study and in the study of Kopf-Bolanz et al. (2012) is different.

**Lipolysis and generation of free fatty acids during ex vivo digestion**

Milk fat consists of 95% triacylglycerol (Haug et al., 2007) and more than 95% of the milk fat can be absorbed (Mu and Hoy, 2004). But before absorption, the fat needs to be digested. The pre-duodenal (lingual and gastric) lipase and duodenal (pancreatic) lipase hydrolyze the triacylglycerol to free fatty acids and monoacylglycerol. These lipases attack ester bonds at *sn*-1 and *sn*-3 position of the triacylglycerol (Tiruppathi and Balasubramania, 1982; Rogalska et al., 1990; Carriere et al., 1994; Miled et al. 2000; Armand, 2007). Different reports are prevailed

225 regarding the exact contribution of the pre-duodenal lipases (Carriere et al. 1993; Armand et al.,
226 1999; Pafumi et al., 2002; Mu and Hoy, 2004; Gallier et al., 2013) to the total lipolysis of
227 triacylglycerol and according to Jensen (2002) it could be 25 to 40% of the triacylglycerol.
228 The lipolysis of neutral lipid of Red Chittagong Cattle milk fat during the gastric and duodenal
229 *ex vivo* digestion and subsequent release of free fatty acids are given in Figure 2. There was no
230 lipolysis observed after 40 min gastric digestion. This is in agreement with the reported gastric
231 lipolysis of full fat bovine milk, full fat buffalo milk, and for 2% cod liver oil enriched buffalo
232 skimmed milk (Devele et al., 2014; Islam et al., 2014b and Islam et al., 2014c). The possible
233 reasons are as mentioned earlier by different authors (Devele et al., 2014; Islam et al., 2014b
234 and Islam et al., 2014c) – *firstly*, the optimum pH for the gastric lipase activity is 5 to 6
235 (Carriere et al., 1993) and *secondly*, insufficient secretion of gastric lipases because the
236 volunteers were in a semi-fasting condition and not stimulated for lipid digestion. However, it
237 has been reported that gastric digestion of milk fat is important for further duodenal lipolysis
238 (Jensen 2002; Ye et al., 2011; Gallier et al., 2012). A sharp rise of free fatty acids (33%) were
239 shown after 30 min duodenal digestion. The proportion of free fatty acid after 60 min duodenal
240 digestion was 15.6% higher than at 30 min and the proportion after 60 and 120 min were more
241 or less similar. The sharp increase in free fatty acids after 30 min duodenal digestion is in line
242 with the results reported by Devle et al. (2014) in cows milk and Islam et al. (2014b) in buffalo
243 milk. The bile salts concentration was 2.4 mM in the aspirates in the present study whereas, the
244 aspirates used by Devle et al. (2014) has only 1.0 mM bile salts. Moreover, the milk fat globule
245 size of the Red Chittagong Cattle milk (3.4 μm) was smaller than in the buffalo milk (12.3 μm)
246 as reported by Islam et al. (2014a). Bile salts are important for accelarated lipolysis by creating
247 small lipid micelles. The present study ends up with 48% lipolysis of the neutral lipid after 120
248 min duodenal digestion. Final lipolysis after 120 min duodenal digestion observed in cow and

249 buffalo milk were 40 and 35%, respectively (Devle et al., 2014; Islam et al., 2014b). In the
250 present study few inconsistent fatty acids (C13:0, C20:0, C17:1 n-7, C18:3 n-3, C20:4 n-6)
251 were also observed that are included in the results presented in Figure 2 but not shown in Table
252 4. Part of this inconsistency may arise from the phospholipids. However, we did not take
253 phospholipids in consideration as Devle et al. (2014) reported a non-significant digestion of the
254 phospholipids.
255 The changes in the concentration of free fatty acids during the different steps of *ex vivo*
256 digestion are given in Table 4 which also includes the lipolysis (%) of individual fatty acids.
257 The changes in the free fatty acid concentration stay close to the lipolysis reported in Figure 2
258 when the standard deviations were taken into consideration. The standard deviation of
259 undigested, G40, D30, D60 and D120 were 0.10, 0.11, 5.50, 2.10 and 6.77, respectively (for
260 Figure 2). The average lipolysis (%) of short chain fatty acids (C6:0-C8:0), medium chain fatty
261 acids (C10:0-C16:0) and long chain fatty acid (>C17:0) was 52, 33.3 and 46.5, respectively.
262 This is in agreement with the lipase preferred positions; *sn*-1 and *sn*-3, where short chain fatty
263 acids are more abundant at that position followed by long chain and medium chain fatty acids
264 (Angers et al., 1998; Blasi et al., 2008 and Maansson, 2008). The unsaturated fatty acids, C14:1
265 n-5, C16:1 n-7 and C18:1 n-9 showed almost similar lipolysis, ranging from 31.9 to 33.7%
266 though their presence at lipase preferred position of the triacylglycerol is different (Blasi et al.,
267 2008) and mainly depends on the size of the triacylglycerol (Angers et al., 1998). The lipolysis
268 of total saturated fatty acids and total unsaturated fatty acids showed little variation, 2.3% more
269 in total saturated fatty acids. This is in contrary to the results of cow's milk (Devle et al., 2014),
270 however, Islam et al. (2014b) reported more lipolysis in total saturated fatty acids than the total
271 unsaturated fatty acids in buffalo milk. According to Blasi et al. (2008) "saturated fatty acids
272 were prevalently esterified in *sn*-3 position, while monounsaturated fatty acids in *sn*-2 position,

with some exceptions". The stereospecific distribution of the fatty acids in the triacylglycerol comes out with considerable variability (Parodi, 1979; Angers et al., 1998; Blasi et al., 2008; Maansson et al., 2008).

## CONCLUSIONS

In full fat milk from Red Chittagong Cattle, all the $\alpha_s$-caseins were digested after 40 min gastric digestion, while some β-lactoglobulin was still intact after 120 min duodenal digestion. The β-casein was degraded more extensively and gave the highest number of peptides. All the peptides identified from the different proteins were rich in proline along with other hydrophobic amino acids like Alanine, leucine, isoleucine, valine, phenylalanie, methionine and tryptophan. The milk fat showed 48% lipolysis. Short chain fatty acid showed higher lipolysis than the medium and long chain fatty acids and so was the total saturated fatty acids compared to total unsaturated fatty acids. The digestion of the Red Chittagong Cattle milk from Bangladesh showed similar digestion pattern like the Nordic cows milk (Norwegian Red Cattle).

289 **REFERENCES**


290 Abd El-Salam, M. H. and S. El-Shibiny. 2011. A comprehensive review on the composition
291 and properties of buffalo milk. Dairy Sci. Technol. 91:663-699.

292 Almaas, H., A.-L. Cases, T. G. Devold, H. Holm, T. Langsrud, L. Aabakken, T. Aadnoey and
293 G. E. Vegarud. 2006. In vitro digestion of bovine and caprine milk by human gastric
294 and duodenal enzymes. Int. Dairy J. 16:961-968.

295 Almaas, H., E. Eriksen, C. Sekse, I. Comi, R. Flengsrud, H. Holm, E. Jensen, M. Jacobsen, T.
296 Langsrud and G. E. Vegarud. 2011. Antibacterial peptides derived from caprine whey
297 proteins, by digestion with human gastrointestinal juice. Br. J. Nutr. 106:896-905.

298 Angers, P., E. Tousignant, A. Boudreau and J. Arul. 1998. Regiospecific analysis of fractions of
299 bovine milk fat triacylglycerols with the same partition number. Lipids. 33:1195-1201.

300 Armand, M. 2007. Lipases and lipolysis in the human digestive tract: where do we stand? Curr.
301 Op. Clin. Nutr. Metab. Care. 10:156-164.

302 Armand, M., B. Pasquier, M. Andre, P. Borel, M. Senft, J. Peyrot, J. Salducci, H. Portugal, V.
303 Jaussan and D. Lairon. 1999. Digestion and absorption of 2 fat emulsions with different
304 droplet sizes in the human digestive tract. Am. J. Clin. Nutr. 70:1096-1106.

305 Blasi, F., D. Montesano, M. De Angelis, A. Maurizi, F. Ventura, L. Cossignani, M. S.
306 Simonetti and P. Damiani. 2008. Results of stereospecific analysis of triacylglycerol
307 fraction from donkey, cow, ewe, goat and buffalo milk. J. Food Comp. Anal. 21:1-7.

308 Boisen, S. and B. Eggum. 1991. Critical evaluation of in vitro methods for estimating
309 digestibility in simple-stomach animals. Nutr. Res. Rev. 4:141-162.

310 Carriere, F., J. Barrowman, R. Verger and R. Laugier. 1993. Secretion and contribution to
311 lipolysis of gastric and pancreatic lipases during a test meal in humans.
312 Gastroenterology. 105:876-888.

313 Carriere, F., Y. Gargouri, H. Moreau, S. Ransac, E. Rogalska and R. Verger. 1994. Gastric
314 lipases: cellular, biochemical and kinetic aspects. In: Lipases: Their Structure,
315 Biochemistry and Application (Ed. P. Woolley and S. B. Petersen). Cambridge
316 University Press. pp. 181-205.
317 Coles, L., P. Moughan and A. Darragh 2005. In vitro digestion and fermentation methods,
318 including gas production techniques, as applied to nutritive evaluation of foods in the
319 hindgut of humans and other simple-stomached animals. Anim. Feed Sci. Technol.
320 123:421-444.
321 Devle, H., E. K. Ulleberg, C. F. Naess-Andresen, E.-O. Rukke, G. E. Vegarud and D. Ekeberg.
322 2014. Reciprocal interacting effects of proteins and lipids during ex vivo digestion of
323 bovine milk. Int. Dairy J. 36:6-13.
324 Ekmekcioglu, C. 2002. A physiological approach for preparing and conducting intestinal
325 bioavailability studies using experimental systems. Food Chem. 76:225-230.
326 Eriksen, E. K., H. Holm, E. Jensen, R. Aaboe, T. G. Devold, M. Jacobsen and G. E. Vegarud.
327 2010. Different digestion of caprine whey proteins by human and porcine
328 gastrointestinal enzymes. Br. J. Nutr. 104:374-381.
329 Furlund, C. B., E. K. Ulleberg, T. G. Devold, R. Flengsrud, M. Jacobsen, C. Sekse, H. Holm
330 and G. E. Vegarud 2013. Identification of lactoferrin peptides generated by digestion
331 with human gastrointestinal enzymes. J. Dairy Sci. 96:75-88.
332 Gallier, S., A. Ye and H. Singh. 2012. Structural changes of bovine milk fat globules during in
333 vitro digestion. J. Dairy Sci. 95:3579-3592.
334 Gallier, S., J. Cui, T. D. Olson, S. M. Rutherfurd, A. Ye, P. J. Moughan and H. Singh. 2013. In
335 vivo digestion of bovine milk fat globules: Effect of processing and interfacial structural
336 changes. I. Gastric digestion. Food Chem. 141:3273-3281.

337 Gass, J., H. Vora, A. F. Hofmann, G. M. Gray and C. Khosla. 2007. Enhancement of dietary
338 protein digestion by conjugated bile acids. Gastroenterology. 133:16-23.
339 Haug, A., A. T. Hostmark and O. M. Harstad. 2007. Bovine milk in human nutrition–a review.
340 Lipids Heal. Dis. 10.1186/1476-511X-6-25.
341 Hur, S. J., B. O. Lim, E. A. Decker and D. J. McClements. 2011. *In vitro* human digestion
342 models for food applications. Food Chem. 125:1-12.
343 Inglingstad, R. A., T. G. Devold, E. K. Eriksen, H. Holm, M. Jacobsen, K. H. Liland, E. O.
344 Rukke and G. E. Vegarud. 2010. Comparison of the digestion of caseins and whey
345 proteins in equine, bovine, caprine and human milks by human gastrointestinal
346 enzymes. Dairy Sci. Technol. 90:549-563.
347 Islam, M. A., M. K. Alam, M. N. Islam, M. A. S. Khan, D. Ekeberg, E. O. Rukke and G. E.
348 Vegarud. 2014a. Principal milk components in buffalo, holstein cross, indigenous cattle
349 and Red Chittagong Cattle from Bangladesh. Asian-Aust. J. Anim. Sci.
350 http://dx.doi.org/10.5713/ajas.2013.13586.
351 Islam, M. A., D. Ekeberg, E. O. Rukke and G. E. Vegarud. 2014b. *Ex vivo* digestion of proteins
352 and fat in buffalo milk. Int. Dairy J. (Sub. Man.).
353 Islam, M. A., D. Ekeberg, E. O. Rukke and G. E. Vegarud. 2014c. *Ex vivo* digestion of omega-3
354 enriched buffalo skimmed milk. J. Func. Foods. (Sub. Man.).
355 Jensen, R. G. (2002). The composition of bovine milk lipids: January 1995 to December 2000.
356 J. Dairy Sci. 85:295-350.
357 Kalantzi, L., K. Goumas, V. Kalioras, B. Abrahamsson, J. B. Dressman and C. Reppas. 2006.
358 Characterization of the human upper gastrointestinal contents under conditions
359 simulating bioavailability/bioequivalence studies. Pharma. Res. 23:165-176.

Kopf-Bolanz, K. A., F. Schwander, M. Gijs, G. Vergeres, R. Portmann and L. Egger. 2012. Validation of an in vitro digestive system for studying macronutrient decomposition in humans. J. Nutr. 142:245-250.

Maansson, H. L. 2008. Fatty acids in bovine milk fat. Food Nutr. Res. 10.3402/fnr.v52i0.1821.

Medhammar, E., R. Wijesinha-Bettoni, B. Stadlmayr, E. Nilsson, U. R. Charrondiere and B. Burlingame. 2012. Composition of milk from minor dairy animals and buffalo breeds: a biodiversity perspective. J. Sci. Food Agri. 92:445-474.

Miled, N., S. Canaan, L. Dupuis, A. Roussel, M. Riviere, F. Carriere, A. de Caro, C. Cambillau and R. Verger. 2000. Digestive lipases: From three-dimensional structure to physiology. Biochimie. 82:973-986.

Miranda, G., M.-F. Mahe, C. Leroux and P. Martin. 2004. Proteomic tools to characterize the protein fraction of Equidae milk. Proteomics. 4:2496-2509.

Mu, H. and C.-E. Hoy. 2004. The digestion of dietary triacylglycerols. Prog. Lipid Res. 43:105-133.

Pafumi, Y., D. Lairon, P. L. de la Porte, C. Juhel, J. Storch, M. Hamosh and M. Armand. 2002. Mechanisms of Inhibition of Triacylglycerol Hydrolysis by Human Gastric Lipase. J. Biol. Chem. 277:28070-28079.

Parodi, P. W. 1979. Stereospecific distribution of fatty acids in bovine milk fat triglycerides. J. Dairy Res. 46:75-81.

Qureshi, T. M., G. E. Vegarud, R. K. Abrahamsen, and S. Skeie. 2012. Characterization of the Norwegian autochthonous cheese Gamalost and its angiotensin I-converting enzyme (ACE) inhibitory activity during ripening. Dairy Sci. Technol. 92:613-625.

382 Rogalska, E., S. Ransac and R. Verger. 1990. Stereoselectivity of lipases. II. Stereoselective
383 hydrolysis of triglycerides by gastric and pancreatic lipases. J. Biol. Chem. 265:20271-
384 20276.
385 Tidona, F., A. Criscione, T. G. Devold, S. Bordonaro, D. Marletta and G. E.Vegarud. 2014.
386 Protein composition and micelle size of donkey milk with different protein patterns:
387 Effects on digestibility. Int. Dairy J. 35:57-62.
388 Tiruppathi, C. and K. A. Balasubramania. 1982. Purification and properties of an acid lipase
389 from human gastric juice. Biochim. Biophy. Acta (BBA) - Lipids and Lipid Metab.
390 712:692-697.
391 Ulleberg, E. K. (2011). In vitro digestion of caprine whey proteins by human gastrointestinal
392 juices: Effect of whey hydrolysates and peptides on in vitro cell responses. PhD thesis,
393 Norwegian University of Life Sciences, Aas, Norway.
394 Ulleberg, E. K., I. Comi, H. Holm, E. B. Herud, M. Jacobsen. and G. E. Vegarud. 2011. Human
395 gastrointestinal juices intended for use in in vitro digestion models. Food Dig. 2:52-61.
396 Ye, A., J. Cui and H. Singh. 2011. Proteolysis of milk fat globule membrane proteins during in
397 vitro gastric digestion of milk. J. Dairy Sci. 94:2762-2770.
398

399 **Table 1.** Details on the *ex vivo* digestion model

| Sample (1 mL milk) | Steps | pH | Duration (min) | pH adjusted by | Added enzymes and others |
|---|---|---|---|---|---|
| Gastric phase | G20 | 5.0 | 20 | 2M HCL | 711 unit pepsin activity/g milk protein. |
| | G40 | 2.5 | 20 | | |
| Duodenal phase | D5 | 7.0 | 5 | 2M NaOH | 558 unit proteolytic activity/g milk protein. The added duodenal juice also contained 889 unit lipase activity/mL and 2.4 mM bile salts. |
| | D30 | | 30 | | |
| | D60 | | 60 | | |
| | D120 | | 120 | | |

400

401

402 **Table 2.** Number of peptides identified from Red Chittagong Cattle milk at different stage of

403 *ex vivo* digestion from different milk protein.

| Protein | G20 | G40 | D5 | D120 |
|---|---|---|---|---|
| $\alpha_{s1}$-CN | 15 | 12 | 13 | 02 |
| $\alpha_{s2}$-CN | 04 | 04 | 07 | 01 |
| β-CN | 36 | 35 | 40 | 31 |
| κ-CN | 10 | 08 | 03 | - |
| β-Lg | 01 | 01 | 03 | 05 |

404 All the identified peptides counted. CN, Casein; Lg, lactoglobulin; G20, Gastric
405 digestion for 20 min at pH 5; G40, Gastric digestion for 20 min at pH 2.5; D5 and D120,
406 Duodenal digestion for 5 and 120 min, respectively at pH 7.

407 **Table 3.** Minimal overlapped gastric and duodenal peptides from different milk protein of *ex vivo* digested Red Chittagong Cattle milk

| EMW (Da) | Position | Peptide | Rich in ……residues |
|---|---|---|---|
| Gastric peptides from $\alpha_{s1}$-Casein (accession number: B5B3R8) | | | |
| 1000.452 | 165-171 | FRQFYQL | Hydrophobic and side chain containing |
| 1384.794 | 114-124 | LRLKKYKVPQL | Hydrophobic and basic |
| 1499.726 | 95-106 | HIQKEDVPSERY | Side chain containing, hydrophobic and acidic |
| 1890.858 | 141-157 | GIHAQQKEPMIGVNQEL | Hydrophobic and side chain containing |
| 1948.838 | 39-55 | FVAPFPEVFGKEKVNEL | Hydrophobic |
| 2214.998 | 195-214 | SDIPNPIGSENSEKTTMPLW | Hydrophobic and side chain containing |
| 2346.169 | 16-35 | RPKHPIKHQGLPQEVLNENL | Hydrophobic and side chain containing |
| Consensus | | RPPPIGKEKVPQLNL | Hydrophobic, side chain containing and basic |
| Duodenal peptides from $\alpha_{s1}$-Casein (accession number: B5B3R8) | | | |
| 1236.623 | 40-50 | FVAPFPEVFGK | Hydrophobic |
| 1298.677 | 119-129 | YKVPQLEIVPN | Hydrophobic and side chain containing |
| 1336.628 | 95-105 | HIQKEDVPSER | Side chain containing, hydrophobic and acidic |
| 1956.862 | 195-213 | SDIPNPIGSENSGKTTMPL | Side chain containing and hydrophobic |

| | | | |
|---|---|---|---|
| 1965.025 | 19-35 | HPIKHQGLPQEVLNENL | Hydrophobic and side chain containing |
| 2019.977 | 140-157 | EGIHAQQKEPMIGVNQEL | Hydrophobic and side chain containing |
| Consensus | | IPQPEGVPNEGVEL | Side chain containing, hydrophobic and acidic |
| Gastric peptides from $\alpha_{s2}$-Casein (accession number: P02663) | | | |
| 1196.68 | 181-189 | KISQRYQKF | Hydrophobic and side chain containing |
| 1221.59 | 58-67 | VVRNANEEEY | Hydrophobic, side chain containing and acidic |
| 2831.49 | 114-138 | LYQGPIVLNPWDQVKRNAVPITPTL | Hydrophobic and side chain containing |
| 2924.65 | 111-135 | LQYLYQGPIVLNPWDQVKRNAVPIT | Hydrophobic and side chain containing |
| Consensus | | LYQGPIVLNPWDQVKRNAVPIT | Hydrophobic and side chain containing |
| Duodenal peptides from $\alpha_{s2}$-Casein (accession number: P02663) | | | |
| 1245.61 | 85-94 | KITVDDKHYQ | Side chain containing, hydrophobic and acidic |
| 1733.88 | 166-179 | TKLTEEEKNRLNFL | |
| 1738.81 | 96-109 | ALNEINQFYQKFPQ | Hydrophobic and side chain containing |
| 2039.03 | 114-130 | LYQGPIVLNPWDQVKRN | Hydrophobic and side chain containing |
| 2044.98 | 111-127 | LQYLYQGPIVLNPWDQV | Hydrophobic and side chain containing |
| Consensus | | LYQGPIKLNEWDQVYQNF | Hydrophobic and side chain containing |

| | | | |
|---|---|---|---|
| Gastric peptides from β-Casein (accession number: P02666) | | | |
| 1243.66 | 109-120 | GVSKVKEAMAPK | Hydrophobic and basic |
| 1511.716 | 60-72 | LQDKIHPFAQTQS | Hydrophobic and side chain containing |
| 1624.78 | 16-29 | RELEELNVPGEIVE | Hydrophobic and acidic |
| 1873.978 | 143-158 | TDVENLHLPLPLLQSW | Hydrophobic and side chain containing |
| 2015.03 | 140-157 | LTLTDVENLHLPLPLLQS | Hydrophobic and side chain containing |
| 2178.17 | 96-115 | PVVVPPFLQPEVMGVSKVKE | Hydrophobic |
| 2253.22 | 205-224 | FLLYEQPVLGPVRGPFPIIV | Hydrophobic |
| 2876.36 | 179-204 | SLSQSKVLPVPQKAVPYPQRDMPIQA | Hydrophobic and side chain containing |
| 2881.476 | 156-180 | QSWMHQPHQPLPPTVMFPPQSVLSL | Hydrophobic and side chain containing |
| 2901.51 | 129-154 | PVEPFTESQSLTLTDVENLHLPLPLL | Hydrophobic and side chain containing |
| 3935.19 | 73-108 | LVYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEVM | Hydrophobic and side chain containing |
| Consensus | | LLYEQPVPGPIVPLPQKIPQTPVPVPPFLQPEVLGLT DVENLHLPLPLLQS | Hydrophobic and side chain containing |
| Duodenal peptides from β-Casein (accession number: P02666) | | | |
| 1470.67 | 56-67 | TEDELQDKIHPF | Hydrophobic, acidic and side chain containing |

| | | | |
|---|---|---|---|
| 1624.78 | 16-29 | RELEELNVPGEIVE | Hydrophobic and acidic |
| 1887.01 | 139-155 | SLTLTDVENLHLPLPLL | Hydrophobic and side chain containing |
| 1893.94 | 206-222 | LLYQEPVLGPVRGPFPI | Hydrophobic and side chain containing |
| 1993.08 | 207-224 | LYQEPVLGPVRGPFPIIV | Hydrophobic and side chain containing |
| 2004.98 | 143-159 | TDVENLHLPLPLLQSWM | Hydrophobic and side chain containing |
| 2277.06 | 121-139 | HKEMPFPKYPVEPFTESQS | Hydrophobic and side chain containing |
| 2681.28 | 156-178 | QSWMHQPHQPLPPTVMFPPQSVL | Hydrophobic and side chain containing |
| 3935.10 | 73-108 | LVYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEVM | Hydrophobic and side chain containing |
| Consensus | | LLDQENLHGPVRGPFPILIEMPQPPVEPFLQEQS | Hydrophobic and side chain containing |
| Gastric peptides from κ-Casein (accession number: P02668) | | | |
| 1796.97 | 39-53 | FSDKIAKYIPIQYVL | Hydrophobic and side chain containing |
| 1267.59 | 52-60 | VLSRYPSYGLN | Side chain containing, hydrophobic |
| 1197.58 | 117-126 | ARHPHPHLSF | Hydrophobic and side chain containing |
| 1108.51 | 63-71 | YYQQKPVAL | Hydrophobic and side chain containing |
| 1536.84 | 88-100 | VRSPAQILQWQVL | Hydrophobic and side chain containing |
| 2861.30 | 72-96 | INNQFLPYPYYAKPAAVRSPAQILQ | Hydrophobic and side chain containing |

| | | | |
|---|---|---|---|
| Consensus | | PYKPVAVRSPAQILQ | Hydrophobic and side chain containing |
| Duodenal peptides from β-lactoglobulin (accession number: P02754) | | | |
| 1942.94 | 57-73 | VYVEELKPTPEGDLEIL | Hydrophobic and acidic |
| 1634.71 | 141-154 | TPEVDDEALEKFDK | Acidic and hydrophobic |
| 1064.53 | 108-116 | VLVLDTDYK | Hydrophobic |
| Consensus | | VKTPEDEL | Acidic and hydrophobic |

408 EMW, Experimental molecular weight; Position, in the whole protein sequence; Consensus was generated from Jalview 2.8.0b1.

409 Residues properties were obtained from CLUSTAL O (1.2.1).

410

411 **Table 4.** Fatty acid (FA) concentrations (mean±STD μg/mL milk) in the free fatty acid fraction
412 of Red Chittagong Cattle milk during digestion with human gastric (G) and duodenal (D) juices.
413 Lipolysis (%) of individual FA in neutral lipid (NL) fraction is also presented herein

| Fatty acids | G40 | D30 | D60 | D120 | Lipolysis (%) |
|---|---|---|---|---|---|
| C6:0 | 2.4±0.7 | 70.0±8.3 | 82.9±3.8 | 120.8±3.8 | 54.8 |
| C8:0 | 2.2±0.8 | 135.2±27.1 | 191.2±14.3 | 227.9±7.3 | 49.1 |
| C10:0 | 8.8±0.7 | 374.8±24.7 | 449.1±26.1 | 503.2±16.9 | 31.2 |
| C12:0 | 14.8±1.4 | 499.1±36.8 | 578.9±38.7 | 650.7±24.7 | 30.7 |
| C14:0 | 40.5±5.3 | 1627.0±107.0 | 1900.2±74.1 | 2143.7±55.4 | 33.0 |
| C15:0 | nd | 278.9±28.5 | 349.6±13.3 | 385.6±11.3 | 35.6 |
| C16:0 | 150.6±20.6 | 5161.0±472.0 | 6164.0±304.0 | 7114.0±278.0 | 36.1 |
| C17:0 | nd | 151.0±20.1 | 304.2±176.9 | 223.4±10.4 | 49.2 |
| C18:0 | 84.9±7.7 | 1786.0±218 | 2302.1±107.3 | 2687.8±135.2 | 43.88 |
| ΣSFA | 304.1±36.7 | 10083.1±927.3 | 12321.9±396.2 | 14057.3±506.2 | 36.6 |
| C10:1 n-6 | 2.2±0.2 | 60.5±7.4 | 80.4±7.8 | 82.5±4.2 | 37.6 |
| C14:1 n-5 | 4.9±2.6 | 249.5±20.7 | 308.3±15.1 | 332.0±10.1 | 31.9 |
| C16:1 n-7 | nd | 348.4±30.7 | 443.0±21.9 | 475.6±12.0 | 33.7 |
| C18:1 n-9 | 45.4±8.0 | 3990.0±480.0 | 5072.0±244.0 | 5744.0±216.0 | 32.7 |
| C18:1 Σothers | nd | 103.8±4.1 | 137.0±12.4 | 157.9±5.8 | 80.8 |
| C18:2 Σall | nd | 244.1±8.9 | 317.0±12.4 | 323.7±9.5 | 24.4 |
| ΣUSFA | 52.5±10.8 | 4996.3±546.6 | 6357.3±307.7 | 7115.6±251.0 | 34.3 |

414 nd, not detected; ΣSFA, total saturated fatty acids; ΣUSFA, total unsaturated fatty acids

415

**Figure 1.**

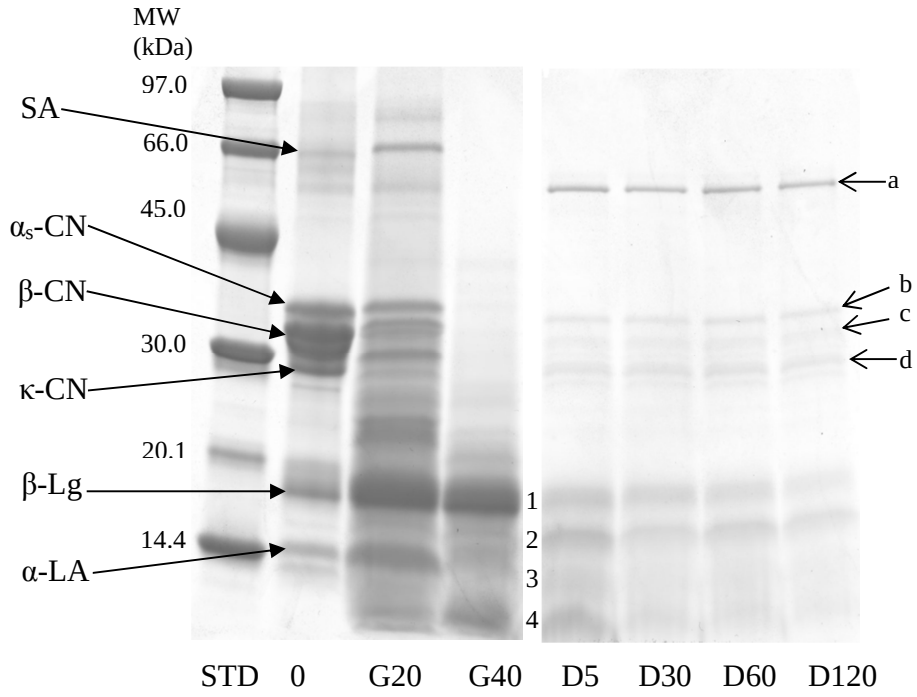
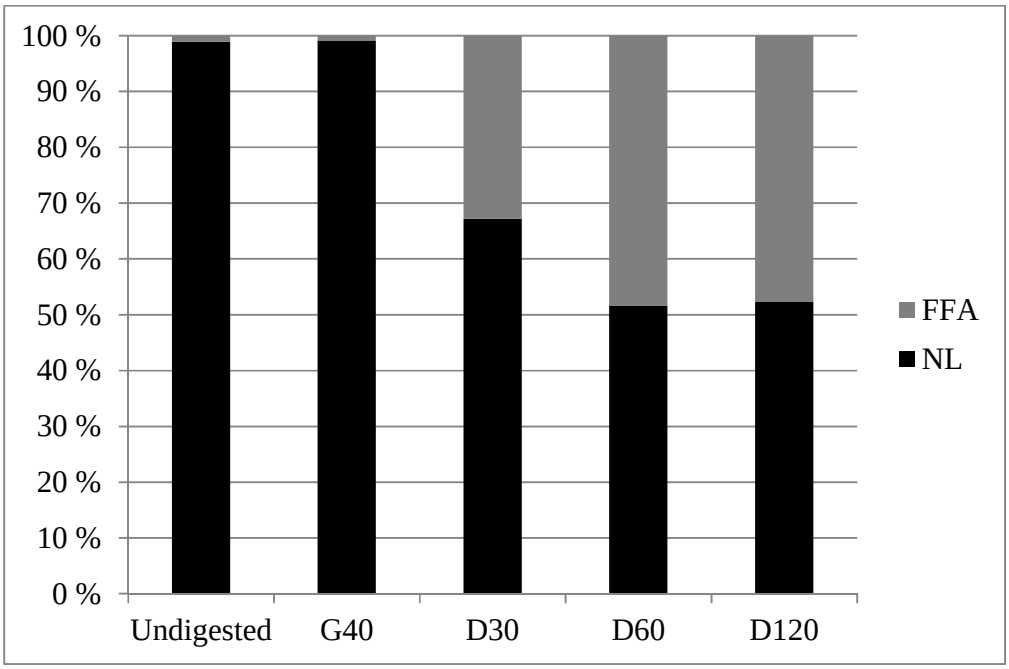



**Figure 2**



## Figure Legend

**Figure 1.** Protein degradation profile of Red Chittagong Cattle milk after *ex vivo* gastric (G) and duodenal (D) digestion. MW, molecular weight; kDa, kilo dalton; SA, serum albumin; CN, casein; β-Lg, β-lactoglobulin; α-LA, α-lactalbumin; STD, low molecular weight marker; 0, undigested sample; G20, Gastric digestion for 20 min at pH 5.0; G40, gastric digestion for 20 min at pH 2.5; D5, D30, D60 and D120, duodenal digestion for 5, 30, 60 and 120 min, respectively at pH 7.0; D5:1, β-lactoglobulin and serum albumin; D05:2, 3 and 4, β-lactoglobulin, α-lactalbumin and serum albumin; a, amylase; b, carboxypeptidase, chymotrypsin, elastase, lipase, gastricsin and amylase; c, carboxypeptidase, elastase, lipase, trypsin and amylase; d, elastase, carboxypeptidase, chymotrypsin, amylase, lipase and trypsin.

**Figure 2.** Proportion (%) of neutral lipid and free fatty acid during the *ex vivo* digestion of Red Chittagong Cattle milk. G40, gastric digestion at pH 2.5 for 20 min after 20 min gastric digestion at pH 5.0; D30, D60 and D120, duodenal digestion for 30, 60 and 120 min, respectively at pH 7.0. Undigested and D120 are from duplicate data where others are from triplicate data.

**Curriculum Vitae of Mohammad Ashiqul Islam**

| | |
|---|---|
| Born on: | 08th September, 1981 |
| Country of origin: | Bangladesh |
| Married to: | Rawnak Jahan (in 2008) |
| Children: | Mohammad Aaban Islam (in 2010), Mohammad Ahnaf Islam (in 2012) and expecting one more In Sha Allah in 2014. |
| School: | Sadardi Govt. Primary School, Bhanga, Faridpur, Bangladesh (Class 1-4) |
| | Hitaishi High School, Faridpur Sadar, Faridpur, Bangladesh (Class 4-5) |
| | Faridpur Zilla School, Faridpur Sadar, Faridpur, Bangladesh (Class 5-10) and obtained Secondary School Certificate (SSC) in the year 1997 |
| | Govt. Yasin College Faridpur, Faridpur Sadar, Faridpur, Bangladesh (Class 11-12) and obtained Higher Secondary Certificate (HSC) in the year 1999 |
| | Bangladesh Agricultural University, Mymensingh-2202, Bangladesh in the session 1999-2000 and obtained Bachelor of Science in Animal Husbandry in the year 2003 (held in 2005) |
| | Bangladesh Agricultural University, Mymensingh-2202, Bangladesh and obtained Master of Science in Dairy Science in the year 2006 |
| | Norwegian University of Life Sciences, Aas-1432, Norway (PhD study) and doctoral degree defense will take place on 13th June, 2014 |
| First job: | Lecturer, Department of Dairy Science, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh (2006-2008) |
| Present profession: | Assistant Professor, Department of Dairy Science, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh (2008 to date) |
| Looking for: | Post-Doctoral or equivalent research position |