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## Ex vivo Digestion of Milk from Red Chittagong Cattle Focusing Proteolysis and Lipolysis

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**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 protein digestion in this bovine breed's milk with an especial focus on the degradation of the allergenic milk proteins;  $\alpha_{s1}$ -casein and  $\beta$ -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 of gastric digestion, all the  $\alpha_s$ -caseins were digested completely while  $\beta$ -lactoglobulin remained intact. During 120 min of duodenal digestion  $\beta$ -lactoglobulin was reduced, however, still some intact  $\beta$ -lactoglobulin was observed. The highest number of peptides was identified from  $\beta$ -casein and almost all the peptides from  $\kappa$ -casein and  $\beta$ -lactoglobulin were identified from the gastric and duodenal samples, respectively. No lipolysis was observed in the gastric phase of digestion. After 120 min of duodenal digestion, milk fat showed 48% lipolysis. Medium (C10:0 to C16:0) and long ( $\geq$ C17:0) chain fatty acids showed 6% to 19% less lipolysis than the short (C6:0 to C8:0) chain fatty acids. Among the unsaturated fatty acids C18:1 $\Sigma$ others showed highest lipolysis (81%) which was more than three times of C18:2 $\Sigma$ all 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,  $\beta$ -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.

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 are 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. 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). Few suggest the use of single enzymes in *in vitro* model digestion but the use of a mixture of enzymes is more realistic and some workers

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prefer to use human digestive juices.

Milk protein digestion and peptides from *in vitro* digested milk has been studied by several authors (Almaas et al., 2006; Inglingstad et al., 2010; Almaas et al., 2011; Furlund et al., 2013; Devle et al., 2014; Tidona et al., 2014; Islam et al., 2014b). But still more knowledge is needed because of high variability in milk composition between breeds (Miranda et al., 2004; Abd El-Salam and El-Shibiny 2011; Medhammar et al., 2012; Islam et al., 2014a) and in *in vitro* digestion protocol used (Hur et al., 2011; Kopf-Bolan et al., 2012; Furlund et al., 2013; Islam et al., 2014b). The structure of milk and milk protein composition may have an influence on its digestibility (Almaas et al., 2006). Tidona et al. (2014) found more rapid degradation of  $\beta$ -lactoglobulin ( $\beta$ -Lg)-I when  $\beta$ -Lg-II is absent in donkey's milk. The heterogeneity in the amino acid composition of milk may result in variation in peptide formation and content after proteolysis (Ulleberg, 2011). The rapid degradation pattern of allergenic milk proteins,  $\beta$ -Lg and  $\alpha_{s1}$ -casein, in Bangladeshi buffalo milk (Islam et al., 2014; unpublished data) also increased interest in checking other bovine milk from Bangladesh. In a previous study, Islam et al. (2014a) concluded that buffalo and Red Chittagong Cattle (RCC) milk showed the highest compositional characteristics for nutritional and technological properties. The dairy potentialities, disease resistance, tolerance to harsh environmental conditions along with low input supply makes the RCC a potential interesting dairy genetic resource. Accordingly, several studies have been conducted regarding the management of the RCC, its productive and reproductive performances, phenotypes and genotypes and recently on principal milk components but studies have yet to test the nutritional quality of the milk.

Very few studies have been conducted regarding the milk lipid digestion and according to Miled et al. (2000), in general, few studies have been reported on lipid digestion. Lipid digestion is more complex than protein digestion regarding the enzymes and physiological conditions in the gut. Factors like food matrix and buffering capacity, emulsion type (oil based/water based), individual secretion of both lipolytic enzymes and bile salts affect the hydrolysis of dietary lipids. The digestion of one nutrient may affect the digestion of others. Devle et al. (2014) and Islam et al. (2014; unpublished data) showed different effects of milk lipids on the protein digestion in cow (Norwegian Red

Cattle) and in buffalo milk, respectively. The objective of the present study was to investigate the digestion of milk from RCC using human gastrointestinal enzymes with a special focus on lipolysis, and proteolysis of the allergenic proteins,  $\alpha_{s1}$ -casein and  $\beta$ -lactoglobulin.

## MATERIALS AND METHODS

### Milk sample

Mixed whole milk from nineteen RCC cows was collected from Bangladesh Livestock Research Institute dairy farm. Sampling was done from the morning milk. The animals were at different parity number and stage of lactation and the individual milk production during the sampling time varied between 1.3 to 5.0 L. The management (especially feeding) of the animals under sampling was described by Islam et al. (2014a). The samples were preserved by bronopol (1 tablet/40 mL milk; D & F control systems, Inc. Boston, MA, USA) with minimum delay after the cows were milked. All the milk samples were kept at  $-20^{\circ}\text{C}$  and transferred to the Norwegian University of Life Sciences and stored at  $-20^{\circ}\text{C}$  until used. Detailed composition of RCC milk including milk protein and fatty acid composition 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 enzymes

Human GI enzymes as human gastric juices and human duodenal juices were 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 to 37 years old) volunteers at Lovisenberg Diakonale Hospital, Oslo, Norway. A triple lumen tube (Masters 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 digestion 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  $37^{\circ}\text{C}$  in a water bath for different period

**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 (G) phase	G20	5.0	20	2 M HCL	711 unit pepsin activity/g milk protein.
	G40	2.5	20		
Duodenal (D) phase	D5	7.0	5	2 M 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	7.0	30		
	D60	7.0	60		
	D120	7.0	120		

All the digestion steps from G40 to D120 were followed by the preceding digestion step(s).

of time corresponding to the digestion steps (Table 1); then the reaction was stopped by placing them into  $-20^{\circ}\text{C}$  (protein samples) or by adding 20 mL of chloroform and methanol (2:1) mixture and then placing into  $-20^{\circ}\text{C}$  (lipid samples). The digestion experiments were performed in triplicate.

### Milk protein degradation profile

The proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a modified method described by Islam et al. (2014b). The digested samples were mixed with the sample buffer (1:2) and applied on a precast gel (6.5 to 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 Coomassie Brilliant Blue, destained and kept in preservation solution. Six gels were run.

### Protein identification

A method described by Devle et al. (2014) and Islam et al. (2014b) was used to identify the protein bands in the SDS-PAGE. In brief, the identification of bands of interest was done by nano-ultra performance liquid chromatography (UPLC)/Q-Exactive Mass Spectrometry (MS), both from Thermo Fisher Scientific, Bremen, Germany. The UPLC was equipped with a trap column (Acclaim PepMap100, C18, 5  $\mu\text{m}$ , 100  $\text{\AA}$ , 300  $\mu\text{m}$  i.d.  $\times$  5 mm, Thermo Fisher Scientific, Bremen, Germany) and a 50  $\text{cm}\times$  75  $\mu\text{m}$  analytical column (Acclaim PepMap RSLC C18, 2  $\mu\text{m}$ , 100  $\text{\AA}$ , 75  $\mu\text{m}$  i.d.  $\times$  50 cm, nanoViper, Bremen, Germany). The Q-Exactive MS was in full scan mode (300 to 1,600  $m/z$ ) followed by (up to) 5 MS2 scans at resolution 70,000 and 35,000, respectively and the used neutral collision energy 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). A nanoACQUITY UPLC (Waters, Milford, MA, USA) and quadrupole-time-of-flight (Q-TOF) Ultima MS (Micromass Ltd., Manchester, UK) was used for this purpose. The columns in the UPLC were 5- $\mu\text{m}$  symmetry C18 trap column (180  $\mu\text{m}\times$  20 mm; Waters, USA) and 1.7- $\mu\text{m}$  BEH C18 analytical column (75  $\mu\text{m}\times$  100 mm; Waters, USA). Identification of peptides was done in duplicate.

### Multiple sequence alignment of the peptides

The multiple sequence alignment (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 content 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-magnetic sector 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) that was added immediately after the digestion. The SPE was carried-out on a liquid handling robot (Gilson, GX-274 ASPEC, Middleton, WI, 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 trifluoride-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  $\mu\text{m}$  thickness (Varian, Middelburgh, The Netherlands) was used. The coupling Autospec Ultima MS was from Micromass Ltd. Manchester, England using electron ionization ion source (mass range  $m/z$  40-600). It was done in triplicate.

### Statistical analysis

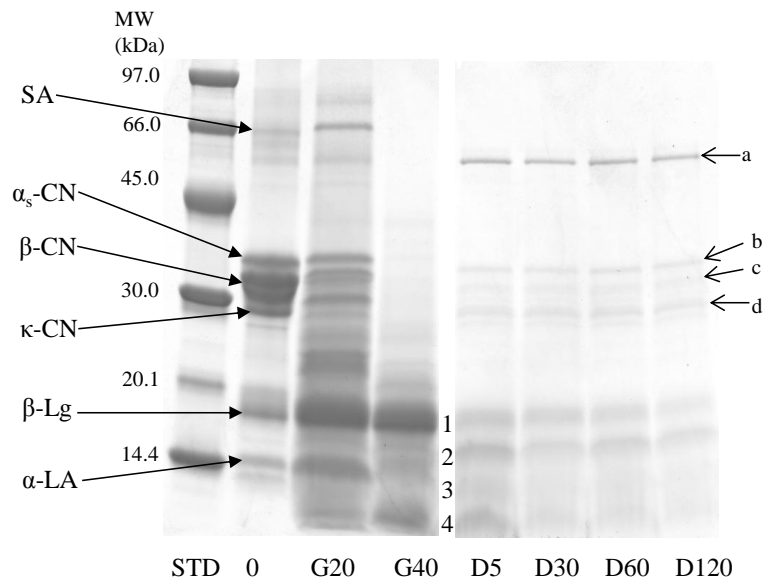
One way Analysis of Variance was done by using Minitab 17 (Minitab Ltd., Brandon Court, Coventry, UK). In case of significant difference, Tukey's test was employed for mean separation.

## 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 of gastric digestion and appeared completely digested after 40 min of gastric phase.

The whey protein serum albumin was degraded during the gastric digestion for 40 min while  $\beta$ -lactoglobulin and some of the  $\alpha$ -lactalbumin were resistant. After 120 min of duodenal digestion, some  $\beta$ -lactoglobulin was found intact (Figure 1: lane D5, band 1) together with the fragments of serum albumin. The other bands, 2, 3 and 4 (lane D5) in Figure 1 were the blend of  $\beta$ -lactoglobulin,  $\alpha$ -lactalbumin and serum albumin. In the duodenal digested samples, some bands that appear at approximately 30 to 60 kDa (a, b, c, and d in Figure 1) are the digestive enzymes present in the



**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;  $\beta$ -Lg,  $\beta$ -lactoglobulin;  $\alpha$ -LA,  $\alpha$ -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,  $\beta$ -lactoglobulin and serum albumin; D05:2, 3, and 4,  $\beta$ -lactoglobulin,  $\alpha$ -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.

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-Bolan 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. (2014; unpublished data) found traces of  $\alpha_s$ -caseins in buffalo milk after 40 min of gastric digestion. Tidona et al. (2014) reported a very low degradation of the caseins in donkey's milk after 30 min of gastric digestion. These two findings 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  $\beta$ -lactoglobulin after 120 min of duodenal digestion and this is in line with the report on bovine milk by Devle et al. (2014), Gallier et al. (2012) and Inglingstad et al. (2010). However, Kopf-Bolan et al. (2012) obtained almost complete digestion of bovine  $\beta$ -lactoglobulin by commercial gastric (120 min) and pancreatic enzymes (30, 60, 90, and 120 min). However, another important result obtained by Islam et al. (2014;

unpublished data) showed almost complete hydrolysis of  $\beta$ -lactoglobulin in full fat buffalo milk after 5 min of duodenal digestion. So, genetic factors for the degradation of  $\beta$ -lactoglobulin may be of importance as was also reported by Tidona et al. (2014) in donkey milk digestion. Another factor important for the digestion of  $\beta$ -lactoglobulin seems to be the bile salts;  $\geq 2$  mM concentration may accelerate the digestion of the  $\beta$ -lactoglobulin (Gass et al., 2007). The presence of  $\alpha$ -lactalbumin in band 2, 3, and 4 (D5, Figure 1) confirmed by UPLC-MS, indicate the hydrolysis of  $\alpha$ -lactalbumin and Kopf-Bolan et al. (2012) reported the complete digestion of  $\alpha$ -lactalbumin after 30 min of duodenal digestion using commercial enzymes.

### Peptides

The total number of identified peptides from the different milk proteins during the different phases of *ex vivo*

**Table 2.** Number of peptides identified from Red Chittagong Cattle milk proteins at different stages of *ex vivo* digestion

Protein	G20	G40	D5	D120
$\alpha_{s1}$ -CN	15	12	13	02
$\alpha_{s2}$ -CN	04	04	07	01
$\beta$ -CN	36	35	40	31
$\kappa$ -CN	10	08	03	-
$\beta$ -Lg	01	01	03	05

G20, Gastric digestion for 20 min at pH 5; G40, Gastric digestion for 20 min at pH 2.5; D5 and D120, Duodenal digestion for 5 and 120 min, respectively at pH 7; CN, Casein; Lg, lactoglobulin.

**Table 3. i)** Minimal overlapped gastric and duodenal peptides from different milk proteins of *ex vivo* digested Red Chittagong Cattle milk

EMW (Da)	Position <sup>1</sup>	Peptide	Rich in .....residues <sup>2</sup>
Gastric peptides from $\alpha_{s1}$ -Casein (accession number <sup>3</sup> : 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 <sup>4</sup>		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	VVRNANEEY	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	TKLTEEEKNRLNLF	
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		LYQGPIKLNEWQVYQNF	Hydrophobic and side chain containing
Gastric peptides from $\beta$ -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	FLLYEQPVLGVPVRGPFPIIV	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	LVYFPFGPIPNSLPQNIPPLTQTPVVVPPFL QPEVM	Hydrophobic and side chain containing
Consensus		LLYEQPVPGPIVPLPQKIPQTPVPVPPFLQP EVLGLTDVENLHLPLPLLQS	Hydrophobic and side chain containing

EMW, experimental molecular weight.

<sup>1</sup> Position in the whole protein sequence. <sup>2</sup> Residues properties were obtained from CLUSTAL O (1.2.1).<sup>3</sup> For the whole protein sequence. <sup>4</sup> Consensus was generated from Jalview 2.8.0b1.

digestion is shown in Table 2. The minimal overlapped peptides from the different milk proteins after gastric and duodenal phase of digestion with their corresponding position in the protein sequence are given in Table 3 (i and

ii). Maximum numbers of peptides were identified from the  $\beta$ -casein followed by  $\alpha_{s1}$ -casein,  $\kappa$ -casein,  $\alpha_{s2}$ -casein and  $\beta$ -lactoglobulin. Most of the peptides from  $\kappa$ -casein and  $\beta$ -lactoglobulin were identified from gastric and duodenal

**Table 3. ii)** Minimal overlapped gastric and duodenal peptides from different milk proteins of *ex vivo* digested Red Chittagong Cattle milk (Continued)

EMW (Da)	Position <sup>1</sup>	Peptide	Rich in .....residues <sup>2</sup>
Duodenal peptides from $\beta$ -Casein (accession number <sup>3</sup> : 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	LLYQEPVLGPVGRPFPI	Hydrophobic and side chain containing
1993.08	207-224	LYQEPVLGPVGRPFPIIV	Hydrophobic and side chain containing
2004.98	143-159	TDVENLHLPLLLQSWM	Hydrophobic and side chain containing
2277.06	121-139	HKEMPFKYPVEPFTESQS	Hydrophobic and side chain containing
2681.28	156-178	QSWMHQPHQLPPTVMFPPQSVL	Hydrophobic and side chain containing
3935.10	73-108	LVYFPFGPIPNLQNIPLTQTPVVVPPFLQ PEVM	Hydrophobic and side chain containing
Consensus <sup>4</sup>		LLDQENLHGPVGRPFPIIEMPQPPVEPFLQ EQS	Hydrophobic and side chain containing
Gastric peptides from $\kappa$ -Casein (accession number: P02668)			
1796.97	39-53	FSDKIAKYIPIQYVL	Hydrophobic and side chain containing
1267.59	52-60	VLSRYPYGLN	Side chain containing, hydrophobic
1197.58	117-126	ARHPHPLSF	Hydrophobic and side chain containing
1108.51	63-71	YYQKPVVAL	Hydrophobic and side chain containing
1536.84	88-100	VRSPAQLQWQVL	Hydrophobic and side chain containing
2861.30	72-96	INNQLFPYYPYAKPAVRSPAQLQ	Hydrophobic and side chain containing
Consensus		PYKPVAVRSPAQLQ	Hydrophobic and side chain containing
Duodenal peptides from $\beta$ -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

EMW, experimental molecular weight.

<sup>1</sup> Position in the whole protein sequence. <sup>2</sup> Residues properties were obtained from CLUSTAL O (1.2.1).

<sup>3</sup> For the whole protein sequence. <sup>4</sup> Consensus was generated from Jalview 2.8.0b1.

phase of digestion, respectively (Table 2). This is in agreement with the results obtained on buffalo milk by Islam et al. (2014; unpublished data) and Islam et al. (2014b). The sequence coverage of the identified minimal overlapped peptides (Table 3, i and ii) corresponded well with the number of total identified peptides (Table 2);  $\beta$ -casein showed more extensive hydrolysis, next was  $\alpha_{s1}$ -casein followed by  $\kappa$ -casein,  $\alpha_{s2}$ -casein and  $\beta$ -lactoglobulin. The presence of proline in almost 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. (2014; unpublished data) and Islam et al. (2014b). 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, i and ii). 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 an absence of bile salts may reduce the degradability and concluded that the size distribution of the proteins and peptides in the range of 5 kDa and tripeptides was unclear. However, the digestion conditions used in the present study and those in the study of Kopf-Bolanz et al. (2012) are 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) lipases and duodenal (pancreatic) lipase hydrolyze the triacylglycerol to FFAs and monoacylglycerol. These lipases attack ester bonds at *sn*-1 and *sn*-3 position of the

triacylglycerol (Rogalska et al., 1990; Carriere et al., 1994; Miled et al., 2000; Armand, 2007). Different reports exist regarding the exact contribution of the pre-duodenal lipases (Carriere et al., 1993; Pafumi et al., 2002; Mu and Hoy, 2004; Gallier et al., 2013) to the total lipolysis of triacylglycerol and according to Jensen (2002) it could be 25% to 40% of the triacylglycerol.

The lipolysis of NL of RCC milk fat during the gastric and duodenal *ex vivo* digestion and subsequent release of FFAs are given in Figure 2. No lipolysis was observed after 40 min of gastric digestion. This is in agreement with the reported gastric lipolysis of full fat bovine milk, full fat buffalo milk, and 2% cod liver oil enriched buffalo skimmed milk (Devle et al., 2014; Islam et al., 2014; unpublished data; Islam et al., 2014b). The possible reasons are as mentioned earlier by different authors (Devle et al., 2014; Islam et al., 2014b) – *firstly*, the optimum pH for the gastric lipase activity is 5 to 6 (Carriere et al., 1993) and *secondly*, insufficient secretion of gastric lipases because the volunteers were in a semi-fasting condition and not stimulated for lipid digestion. However, it has been reported that gastric digestion of milk fat is important for further duodenal lipolysis (Jensen 2002; Ye et al., 2011; Gallier et al., 2012). A sharp rise of FFAs (33%) was shown after 30 min of duodenal digestion. The proportion of FFA after 60 min of duodenal digestion was 15.6% higher than at 30 min and the proportion after 60 and 120 min were more or less similar. The sharp increase in FFAs after 30 min duodenal digestion is in line with the results reported by Devle et al. (2014) in cows milk, Islam et al. (2014; unpublished data) in buffalo milk and Islam et al. (2014b) in 2% cod liver oil

fortified buffalo skimmed milk. The bile salts concentration was 2.4 mM in the aspirates used in the present study, whereas, the aspirates used by Devle et al. (2014) had only 1.0 mM bile salts. Moreover, the milk fat globule size of the RCC milk (3.4  $\mu\text{m}$ ) was smaller than in the buffalo milk (12.3  $\mu\text{m}$ ) as reported by Islam et al. (2014a). Bile salts are important for accelerating lipolysis by creating small lipid micelles. The present study ended with 48% lipolysis of the NL after 120 min of duodenal digestion. Final lipolysis after 120 min of duodenal digestion observed in cow and buffalo milk was 40% and 35%, respectively (Devle et al., 2014; Islam et al., 2014; unpublished data). In the present study few inconsistent fatty acids (C13:0, C20:0, C17:1 n-7, C18:3 n-3, C20:4 n-6) were also observed and are included in the results presented in Figure 2 but not shown in Table 4. Part of this inconsistency may arise from the phospholipids. However, we did not take phospholipids in consideration as Devle et al. (2014) reported a non-significant digestion of the phospholipids.

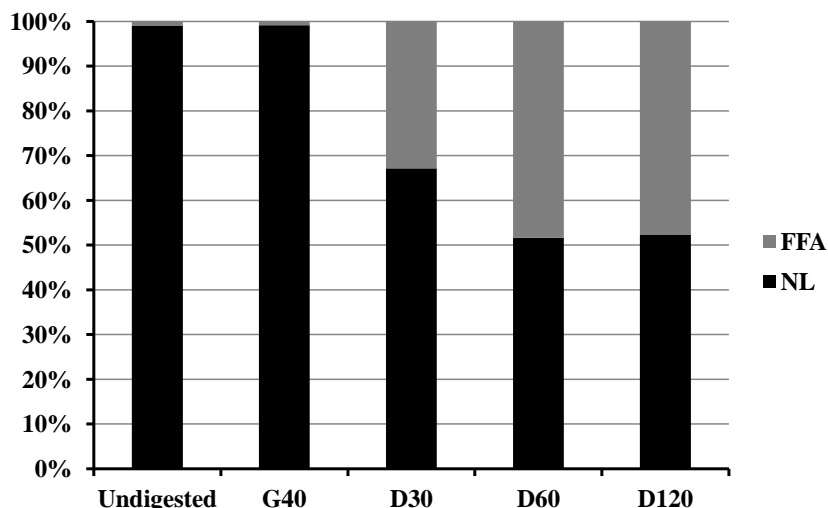
The changes in the concentration of FFAs during the different steps of *ex vivo* digestion are given in Table 4 which also includes the lipolysis (%) of individual fatty acids. Statistical analysis revealed that the concentration of the fatty acids were significantly ( $p \leq 0.001$ ) different in different digestion steps except C17:0. Few of them were highest in D120 step, some were found similar between D30 and D60 steps and few were found similar among D30, D60, and D120 steps. The changes in the FFA concentration stays close to the lipolysis reported in Figure 2 when the standard deviations were taken into consideration. The standard deviation of undigested, G40, D30, D60, and D120

**Table 4.** Individual fatty acid (FA) concentrations (mean $\pm$ STD  $\mu\text{g/mL}$  milk) in the free fatty acid fraction of Red Chittagong Cattle milk during digestion with human gastric (G) and duodenal (D) juices, and their lipolysis

Fatty acids	G40	D30	D60	D120	p-value	Lipolysis (%)
C6:0	2.4 <sup>D</sup> $\pm$ 0.7	70.0 <sup>C</sup> $\pm$ 8.3	82.9 <sup>B</sup> $\pm$ 3.8	120.8 <sup>A</sup> $\pm$ 3.8	0.000	54.8
C8:0	2.2 <sup>C</sup> $\pm$ 0.8	135.2 <sup>B</sup> $\pm$ 27.1	191.2 <sup>A</sup> $\pm$ 14.3	227.9 <sup>A</sup> $\pm$ 7.3	0.000	49.1
C10:0	8.8 <sup>D</sup> $\pm$ 0.7	374.8 <sup>C</sup> $\pm$ 24.7	449.1 <sup>B</sup> $\pm$ 26.1	503.2 <sup>A</sup> $\pm$ 16.9	0.000	31.2
C12:0	14.8 <sup>C</sup> $\pm$ 1.4	499.1 <sup>B</sup> $\pm$ 36.8	578.9 <sup>A</sup> $\pm$ 38.7	650.7 <sup>A</sup> $\pm$ 24.7	0.000	30.7
C14:0	40.5 <sup>D</sup> $\pm$ 5.3	1,627.0 <sup>C</sup> $\pm$ 107.0	1,900.2 <sup>B</sup> $\pm$ 74.1	2,143.7 <sup>A</sup> $\pm$ 55.4	0.000	33.0
C15:0	nd	278.9 <sup>B</sup> $\pm$ 28.5	349.6 <sup>A</sup> $\pm$ 13.3	385.6 <sup>A</sup> $\pm$ 11.3	0.001	35.6
C16:0	150.6 <sup>D</sup> $\pm$ 20.6	5,161.0 <sup>C</sup> $\pm$ 472.0	6,164.0 <sup>B</sup> $\pm$ 304.0	7,114.0 <sup>A</sup> $\pm$ 278.0	0.000	36.1
C17:0	nd	151.0 $\pm$ 20.1	304.2 $\pm$ 176.9	223.4 $\pm$ 10.4	0.267	49.2
C18:0	84.9 <sup>D</sup> $\pm$ 7.7	1,786.0 <sup>C</sup> $\pm$ 218	2,302.1 <sup>B</sup> $\pm$ 107.3	2,687.8 <sup>A</sup> $\pm$ 135.2	0.000	43.9
$\Sigma$ SFA	304.1 <sup>D</sup> $\pm$ 36.7	10,083.1 <sup>C</sup> $\pm$ 927.3	12,321.9 <sup>B</sup> $\pm$ 396.2	14,057.3 <sup>A</sup> $\pm$ 506.2	0.000	36.6
C10:1 n-6	2.2 <sup>C</sup> $\pm$ 0.2	60.5 <sup>B</sup> $\pm$ 7.4	80.4 <sup>A</sup> $\pm$ 7.8	82.5 <sup>A</sup> $\pm$ 4.2	0.000	37.6
C14:1 n-5	4.9 <sup>C</sup> $\pm$ 2.6	249.5 <sup>B</sup> $\pm$ 20.7	308.3 <sup>A</sup> $\pm$ 15.1	332.0 <sup>A</sup> $\pm$ 10.1	0.000	31.9
C16:1 n-7	nd	348.4 <sup>B</sup> $\pm$ 30.7	443.0 <sup>A</sup> $\pm$ 21.9	475.6 <sup>A</sup> $\pm$ 12.0	0.001	33.7
C18:1 n-9	45.4 <sup>C</sup> $\pm$ 8.0	3,990.0 <sup>B</sup> $\pm$ 480.0	5,072.0 <sup>A</sup> $\pm$ 244.0	5,744.0 <sup>A</sup> $\pm$ 216.0	0.000	32.7
C18:1 $\Sigma$ others	nd	103.8 <sup>C</sup> $\pm$ 4.1	137.0 <sup>B</sup> $\pm$ 12.4	157.9 <sup>A</sup> $\pm$ 5.8	0.001	80.8
C18:2 $\Sigma$ all	nd	244.1 <sup>B</sup> $\pm$ 8.9	317.0 <sup>A</sup> $\pm$ 12.4	323.7 <sup>A</sup> $\pm$ 9.5	0.000	24.4
$\Sigma$ USFA	52.5 <sup>C</sup> $\pm$ 10.8	4,996.3 <sup>B</sup> $\pm$ 546.6	6,357.3 <sup>A</sup> $\pm$ 307.7	7,115.6 <sup>A</sup> $\pm$ 251.0	0.000	34.3

STD, standard deviation; nd, not detected; SFA, saturated fatty acid; USFA, Unsaturated fatty acid.

Lipolysis (%) of individual FA in neutral lipid (NL) fraction is also presented herein.



**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; 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.

samples were 0.10, 0.11, 5.50, 2.10, and 6.77, respectively (for Figure 2). The average lipolysis (%) of short chain fatty acids (C6:0-C8:0), medium chain fatty acids (C10:0-C16:0) and long chain fatty acids ( $\geq$ C17:0) was 52.0, 33.3, and 46.5, respectively. This is in agreement with the lipase preferred positions; *sn*-1 and *sn*-3, where short chain fatty acids are more abundant followed by long chain and medium chain fatty acids (Angers et al., 1998; Blasi et al., 2008; Maansson, 2008). The unsaturated fatty acids, C14:1 n-5, C16:1 n-7, and C18:1 n-9 showed almost similar lipolysis, ranging from 31.9% to 33.7% though their presence at lipase preferred positions of the triacylglycerol is different (Blasi et al., 2008) and mainly depends on the size of the triacylglycerol (Angers et al., 1998). The lipolysis of total saturated fatty acids and total unsaturated fatty acids showed little variation, 2.3% more in total saturated fatty acids. This is in contrary to the results of cow's milk (Devele et al., 2014), however, Islam et al. (2014; unpublished data) reported more lipolysis in total saturated fatty acids than the total unsaturated fatty acids in buffalo milk. According to Blasi et al. (2008) "saturated fatty acids 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 milk fat triacylglycerol comes out with considerable variability (Parodi, 1979; Angers et al., 1998; Blasi et al., 2008; Maansson et al., 2008).

## CONCLUSION

In full fat milk from Red Chittagong Cattle, all the  $\alpha_s$ -caseins were digested after 40 min of gastric digestion. However, some  $\beta$ -lactoglobulin was still intact after 120

min of duodenal digestion. The  $\beta$ -casein was degraded more extensively and contributed to 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, phenylalanine, methionine and tryptophan.

The milk fat showed 48% lipolysis. Short chain fatty acids showed higher lipolysis than the medium and long chain fatty acids and so were the total saturated fatty acids compared to the total unsaturated fatty acids. The Red Chittagong Cattle milk from Bangladesh showed a similar digestion pattern to Nordic cow's milk (Norwegian Red Cattle).

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