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# Comprehensive characterization of bioactive peptides from Buffalo (*Bubalus bubalis*) colostrum and milk fat globule membrane proteins

N. Brijesha, H.S. Aparna *

*DOS in Biotechnology, University of Mysore, Manasagangotri, Mysore 570 006, India*

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ABSTRACT

Milk fat is dispersed in milk as small spherical globules stabilized in the form of emulsion by its surrounding membrane, often referred to as fat globule membrane (FGM). Buffalo, a major milking mammal of Asia and second most milking mammal across the globe presents physicochemical features different from that of other ruminant species containing higher content of lipids and proteins. The present study describes characterization of FGM proteins isolated from both buffalo milk and colostrum. A detailed proteomic analysis of peptides generated by *in vitro* gastrointestinal simulation digestion of buffalo milk and colostrum FGM fractions was performed by nLC-ESI MS/MS. The peptide based clustering of FGM proteins unravelled association of membrane proteins in fat transport, enzymatic activity, general transport, defence, cell signalling, membrane/protein trafficking protein synthesis/binding/folding including unknown functions. Gene annotation, STRING and YLoc analyses provided putative insights into major secretory pathways in milk and colostrum FGM peptides, interactive protein networks including their sub cellular localization. The peptides of milk and colostrum FGM offered cellular protection as powerful antioxidants indicated their promising perspectives in commercial formulations and nutraceuticals.

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## 1. Introduction

Milk is a mammalian specific biological fluid while, colostrum is the early lacteal secretion produced after parturition to nourish and promote growth and development of the newborn in its new environment. Apart from various components present in milk, the milk fat synthesized in mammary epithelial cells are enveloped by cell membrane referred as milk fat globule membrane (MFGM) whose composition and properties are completely different from those of either milk or plasma (Nguyen et al., 2015; Robenek et al., 2006; Zou et al., 2015). The trilayered structure consisting of a complex mixture of proteins, glycoproteins, enzymes, phospholipids and glycosphingolipids accounts for 2–6% of the total mass of the fat globules (Singh, 2006). In milk, the MFGM enables the fat to remain dispersed and ensures structural integrity, protection and stability in the aqueous phase (Ye, Singh, Taylor, & Anema, 2002).

Extensive research has been carried out on human and bovine MFGM on the identification of proteins using proteomic techniques (El-Loly, 2011; Smoczyński, Staniewski, & Kiełczewska, 2012; Sui et al., 2014). The major MFGM proteins reported to date includes mucin 1 (MUC1), xanthine dehydrogenase/oxidase (XDH/XO), periodic acid Schiff (PAS) III, cluster of differentiation 36 (CD36), butyrophilin (BTN), PAS 6/7 or lactadherin, adipophilin (ADPH), proteose peptone 3 (PP3) and fatty acid - binding protein (FAB). Mather (2000) reported glycosylated proteins while more recent studies unravelled glycoproteomics of both human and bovine MFGM (Dallas et al., 2014; Murgiano et al., 2009; O'Riordan, Kane, Joshi, & Hickey, 2014).

More than 97 million tons of Buffalo milk produced each year represents the second largest volume of milk produced globally after cow's milk (FAOSTAT, 2012). The major constituent of the buffalo milk is fat fraction, which represents almost twice the fat content of bovine milk (Solah, Staines, Honda, & Limley, 2007). Ménard et al. (2010) reported that buffalo milk is also one of the richest milks from a compositional point of view having fat globules that are significantly larger in size compared to bovine milk fat globules. Previous authors have isolated buffalo MFGM (Abou-Dawood, Moussaa, El-Demerdash, & Ahmed, 1988; D'Ambrosio et al., 2008) and the proteomic analysis identified 50 proteins from buffalo MFGM similar to bovine MFGM proteins (D'Ambrosio et al., 2008; Fong, Norris, & MacGibbon, 2007). Recently, an insight into inter-species complexity of MFGM from Holstein, Jersey, yak, buffalo, goat, camel, horse, and human was established by an iTRAQ proteomic approach (Yang, Zheng, Zhao, Zhang, & Han, 2015). More recent studies by Nguyen et al. (2015, 2016) not only revealed the microstructure and also showed the dynamics of the biological membrane surrounding the buffalo milk fat globule as a function of temperature.

* Corresponding author at: Department of Studies in Biotechnology, University of Mysore, Mysore 570 006, Karnataka, India.
*E-mail address:* aparna@appbot.uni-mysore.ac.in (H.S. Aparna).

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MFGM appears to be an important source of nutraceutical components including polar lipids and membrane proteins (Spitsberg, 2005). MFGM can also be used as "natural" emulsifiers, in preventing flocculation and coalescence of fat globules in milk and protecting the fat against enzyme action (Evers et al., 2008). Human MFGM proteins such as mucins and PAS 6/7 including immunoglobulin were reported to play important role in various cell processes and defence mechanisms against bacteria and viruses in the newborn (Silvestre et al., 2005Spitsberg, 2005). Similarly, inhibitory properties of mucin 1, PAS 6/7, and PP3 during rotavirus were reported (Bojsen et al., 2007; Inagaki et al., 2010; Kvistgaard et al., 2004). In addition, XDH/XO, a major protein of MFGM plays an antimicrobial defensive role in the neonatal gut complementing endogenous enzyme of the intestinal epithelium (Harrison, 2006). Its activity is relatively high in bovine milk compared to milk from other species (*e.g.* goat, sheep, camel, donkey, mare and human milk (Uniacke-Lowe, 2011 & Gantner, Mijić, Baban, Škrtić, & Turalija, 2015). MFGM glycoproteins were also known to prevent adhesion of the colonic microbiota and resulted in increased bacterial butyrate production (Struijs et al., 2013).

The research focus on MFGM has enhanced our understanding on different proteomes of human and bovine MFGM and provided insights for their inclusion in commercial food formulations with potential health benefits. Therefore in the present study, FGM proteins isolated from buffalo milk and colostrum were subjected for *in vitro* gastrointestinal simulation digestion using hydrolytic enzymes, pepsin and pancreatin in order to generate bioactive peptides. The small peptides (<3 Da) were characterized by nLC-ESI MS/MS and their functional clustering, interaction and localization studies were carried out by gene annotation, STRING and YLoc analyses. The cellular response to oxidative stress was evaluated in the presence of FGM peptides by chemical and cell based assays.

## 2. Materials and methods

### 2.1. Chemicals

The colostrum (first day) and milk samples were collected from healthy buffaloes. 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), Pepsin and pancreatin, dichlorodihydro fluorescein diacetate (DCF), 4-(2-hydroxyethyl)-1-piperazineethanesulfonicacid (HEPES), 2, 4-Dinitrophenol (DNP), homovanillic acid (HVA), o-phthaldehyde, di-thio-bis-nitrobenzoic acid (DTNB), enhanced Chemiluminescence detection reagents and hyperfilm ECL were from Sigma Chemical Co. USA. All other reagents were of HPLC or analytical grade.

### 2.2. Isolation of MFGM

The colostrum and milk fat globule membranes (CFGM & MFGM) were extracted from cream as described by Basch, Greenberg, and Farrell (1985) with minor modifications. Briefly, the cream was washed sequentially (4500 ×*g*, 10 min, 4 °C) in phosphate buffer saline (PBS, 10 mM, pH 7.2) and distilled water. FGM thus collected was suspended in distilled water and allowed to crystallize at 4 °C for 20 h. The separated fat and serum fractions were warmed (45 °C, 30 min) to melt the fat and washed again with distilled water to recover the residual serum. The total serum was centrifuged (5000 ×*g*, 15 min, 4 °C), washed twice in acetone (1:4, v/v) to remove fat (8000 ×*g*, 20 min, 4 °C), allowed to dry and stored at −20 °C until further analysis.

### 2.3. In vitro digestion

*In vitro* digestion of FGM was performed as described by Wu and Ding (2002). CFGM & MFGM in 3.5% KCl-HCl buffer (100 mM, pH 2) were mixed with pepsin (4%, w/w) and incubated for 4 h at 37 °C. Reaction was terminated by boiling for 10 min and neutralized using NaOH (2N). The suspension was centrifuged (10,000 ×*g*, 30 min) and digestion was continued with pancreatin (4%, w/w) at 37 °C for 4 h. The reaction was arrested by keeping in boiling water bath (10 min) followed by centrifugation (10,000 ×*g*, 30 min). Supernatants passed through Amicon filter (<3 kDa) were sequentially extracted using extraction buffer 1 (300 μl, 60% acetonitrile [ACN] containing 0.1% formic acid) and buffer 2 (150 μl, 100% ACN) respectively. Supernatant collected from both the extraction steps were pooled and dried in Speed Vac. The peptides reconstituted in 0.1% formic acid (100 μl) were desalted on C-18 solid phase extraction disks (3M Empore) (Rappsilber, Ishihama, & Mann, 2003) and quantified as described by Church, Swaisgood, Porter, and Catignani (1983).

### 2.4. nLC-ESI MS analysis of peptides

nLC-ESI MS separation of peptides (<3 kDa) was carried out on Agilent 6550 iFunnel QTOF mass spectrometer (Agilent Technologies) coupled to an Agilent 1260 Infinity Capillary Pump and 1260 Infinity Nano flow Pump LC system. The samples were loaded on to a Polaris - HR Chip-3 C18 reverse-phase separation column (150 mm × 75 μm, 3 μm, Agilent Technologies). The solvent system consisted of water containing 0.1% formic acid (A) and ACN: $H_2O$ (90:10) containing 0.1% formic acid (B). The peptides were separated using a linear gradient of 3% to 97% solvent B in 90 min at a flow rate of 0.3 ul/min. Mass spectra were acquired in positive ion mode by scanning *m/z*-range from 100 to 3200. The data was analyzed using MassHunter Software LC/MS Data Acquisition; Version: B.05.01. LC was interfaced directly with a Q-TOF Mass Spectrometer and MS/MS data of the peptides was obtained in CID using Nitrogen as collision gas. The data files were processed using Spectrum Mill software (Agilent Technologies) in Swiss-Prot and peptides were identified.

### 2.5. Bioinformatics and statistical analysis of proteins

Protein-protein interaction for protein dataset was obtained from STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database v9.1 (www.stringdb-org).Accumulated peptide sequences of FGM proteins identified by MS/MS were uploaded into YLoc software to predict their subcellular localization. Analysis of the identified FGM-enriched proteins of colostrum and milk associated with annotated functions was performed by gene ontology (GO) annotation software (http://david.abcc.ncifcrf.gov/home.jsp). All assay methods were carried out in triplicates. Data was analyzed separately for each experiment and subjected to Arcsine transformation and analysis of variance (ANOVA). Further, the experimental results were subjected to Tukey's HSD at ($P < 0.05$) [SPSS tool (version 8)].

### 2.6. ABTS assay

ABTS radicals (ABTS*) were generated by reacting ABTS with potassium persulphate (2.45 mM) as described by Re et al. (1999). The absorbance of the solution was adjusted to 0.7 at 734 nm using PBS (150 mM, pH 7.5). The reaction was initiated by adding test samples (50 μl) at different concentrations to ABTS (950 μl) working solution. The reaction mixture was incubated for 5 min at room temperature and the absorbance was measured at 734 nm. Percentage inhibition was calculated and the dose response curve was plotted.

### 2.7. Experimental design

Venous blood was collected from healthy, non-smoking human volunteers as per the guidelines of Institutional human Ethical Committee (IHEC-UOM No. 59/PhD/2011-12) University of Mysore, Mysore. Blood samples were collected in the presence of EDTA were categorized into Group I-Control blood, Group II - blood treated with 2, 4 DNP (100 μM/ml), Group III - blood treated with 2, 4 DNP pre incubated with MFGM peptides (200 μg) for 10 min at 37 °C, Group IV - blood treated

with 2, 4 DNP pre incubated with CFGM peptides (200 μg) for 10 min at 37 °C, Group V - blood treated with MFGM peptides (200 μg) alone, Group VI-blood treated with CFGM peptides (200 μg) alone. 2 ml of blood was used in each group. The tubes were incubated (37 °C, 2 h), centrifuged (2000 ×*g*, 15 min) and respective serum samples were used for biological assays.

*2.7.1. Reactive oxygen species (ROS)*

Reactive oxygen species (ROS) was quantified by adopting the method of Driver, Kodavanti, and Mundy (2000) using DCF-DA. Briefly, serum samples (10 μl) from each group were incubated with Locke's solution (150 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 5 mM HEPES, 2 mM $CaCl_2$, 10 mM glucose, pH 7.4) for 30 min at room temperature followed by the addition of DCF (10 μl). The reaction mixture was incubated at 37 °C for 30 min and fluorescence was measured with excitation and emission wavelengths set at 480 nm and 530 nm respectively using Variokan multimode plate reader (Thermo scientific, USA). ROS level was quantified using DCF standard and expressed as pmole DCF formed/min/mg protein.

*2.7.2. Hydrogen peroxide ($H_2O_2$)*

Endogenously generated $H_2O_2$ was determined as described by Barja (2002). Serum sample (0.2 mg) was added to the reaction mixture containing HEPES (200 μl) buffer saline and HVA (10 μl). After incubating for 30 min at 37 °C, fluorescence was measured at excitation and emission wavelengths of 312 nm and 420 nm respectively using Variokan multimode plate reader (Thermo scientific, USA).

*2.7.3. The reduced glutathione (GSH)*

The reduced glutathione (GSH) level was measured by flurometric method (Chandrashekar, 2010) with minor modifications. Briefly, serum samples (0.1 mg protein) were added to 1 ml of formic acid (0.1 M) and centrifuged (10,000 ×*g*, 10 min). An aliquot of the supernatant was added to sodium phosphate buffer (0.1 M, pH 8, 5 mM EDTA) containing buffered formaldehyde (1:4 v/v, 0.1 M formaldehyde:0.1 M $Na_2HPO_4$) and o-phthaldehyde (100 μl). The reaction mixture was incubated at 37 °C for 45 min, samples were excited at 345 nm and the resulted fluorescence was measured at 425 nm. The concentration of GSH was expressed as μg GSH/mg protein.

*2.7.4. Catalase*

The catalase activity was determined by measuring the hydrolysis of $H_2O_2$ at 240 nm (Aebi, 1984). Aliquots of serum samples (0.05 mg) were added to the reaction mixture (1 ml) containing $H_2O_2$ (8.8 mM) in sodium phosphate buffer (0.1 M, pH 7). The decrease in absorbance was monitored for 3 min and the activity was expressed as μmol $H_2O_2$ decomposed/min/mg protein (€-43.6/mM/cm).

*2.7.5. Acid phosphatase (ACP) & alkaline phosphatase (ALP)*

The assays were performed as described by Tenniswood, Bird, and Clark (1976). ACP and ALP were measured by the amount of enzyme that converts *p*-nitrophenyl phosphate to *p*-nitrophenol/mg protein. The amount of PNPP released was calculated using standard curve.

*2.7.6. LDH*

The serum LDH level was measured as per the manufacturer's instructions using commercial kit (Siemens autopak LDH kit, India) at 340 nm and expressed as activity/min.

## 3. Results and discussion

*3.1. Isolation of FGM from milk and colostrum*

The yield of FGM was 2 g and 8.4 $gL^{-1}$ while, the protein content was estimated to be 500 μg and 700 μg per mg dry weight of FGM from milk and colostrum respectively. A recent review by Holzmüller and Kulozik (2016) reported that factors such as number of washing steps, type and volume of washing solution, temperature and g-force differ greatly across different studies and thus clear conclusions cannot be drawn as a best method of FGM isolation. But the method adopted in the present study resulted in good yield with minimal co contaminants and all the major FGM proteins were identified (Supplementary file 1).

*3.2. In vitro digestion and nLC - ESI MS/MS of MFGM and CFGM peptides*

*In vitro* gastric digestion provides a practical and easy approach to imitate the oral administration of bioactive peptides. In this context, *in vitro* gastrointestinal digestion of FGM samples carried out yielded 40 ± 0.14% to 60 ± 0.17% peptides in permeates. Small peptides were found enriched in MFGM over CFGM. The resultant peptides analyzed by nLC - ESI MS/MS elicited unambiguous identification of 75 and 40 proteins in milk & colostrum FGM respectively (Tables 1 and 2). The number of residues in the identified peptides varied from 5 to 22. Using these peptide tags MFGM and CFGM proteins were catalogued into nine functional classes based on their biological activity. Accordingly, membrane and vesicular trafficking proteins accounted for 27.5 & 17.8% in MFGM and CFGM respectively. Similarly, proteins belonging to other classes included protein synthesis, binding and folding (7 & 13.33%), enzymatic activity (17 & 18%), cell signalling (15 & 6.9%), immune function (2.3 & 4.44%), fat transport/metabolism (6 & 9%), milk proteins (3.5 & 15%) general transport function (8 & 6.7%) and unknown function/s (14 & 9%) in MFGM and CFGM proteins. Few studies have focussed on FGM *in vitro* simulation digestion (Gallier et al., 2013; Singh & Gallier, 2014; Trung et al., 2013; Ye, Cui, & Singh, 2011) indicating variations in the degree of FGM protein digestion. But the present study aided in characterizing putative peptide fragments of various FGM proteins which gives ample scope to explore their bioactivities. In addition, peptide based MFGM and CFGM functional clusters were found in close relation to other mammalian FGM samples (Yang et al., 2015.

*3.3. Identification and functional clustering of MFGM and CFGM proteins*

The most abundant membrane proteins identified in MFGM included BTN, lactadherin and XDH/XO and among them, majority of the peptide sequences were from XDH/XO followed by BTN1A1 and lactadherin. But in CFGM sample, only BT1A1 was identified. BTN is known to be expressed during lactation and appears to be essential for milk fat globule production (Heid & Keenan, 2005; Robenek et al., 2006). XDH/XO a major protein of MFGM is a well-known and extensively characterized redox/purine catabolizing enzyme. Purified XDH/XO was shown to possess antibacterial effect by inhibiting the growth of *Staphylococcus aureus*, *Escherichia coli* and *Salmonella enteritidis* (Harrison, 2006). Similarly, lactadherin found in MFGM play a possible role in membrane vesicle secretion such as budding or shedding of plasma membrane and exocytosis of endocytic multivesicular bodies (Oshima, Aoki, Kato, Kitajima, & Matsuda, 2002). Although a greater number of proteins have been identified based on 2D-electrophoresis (D'Ambrosio et al., 2008), the strategy adopted in the present study was to create a peptide library of FGM proteins following *in vitro* gastrointestinal simulation digestion. Further, absence of peptide sequences in a closer match to FGM proteins in CFGM may be due to limited proteolysis or extensive glycosylation affecting proteolytic digestion as already reported (O'Riordan et al., 2014).

Besides major membrane proteins, we also identified several other proteins associated to membrane and vesicular trafficking in early milk. Their proper recruitment and assembly was known to be effected by Ras super family members which are regulatory GTP-binding proteins (Cai, Reinisch, & Ferro-Novick, 2007; Kahn et al., 2006) involved in microlipid droplet budding through ADP-ribosylated factor-1. Other membrane and vesicular trafficking proteins identified were Rab proteins, low molecular-weight GTP-binding proteins that coordinate stages of transport in the secretory pathway and annexins, which

**Table 1**
The identified proteins of MFGM with their name, accession number, sequence, mass, species, score and SPI %.

| Protein name | Accession no | Sequence | $MH^+$ (Da) | Species | Score | SPI % |
|---|---|---|---|---|---|---|
| Milk protein | | | | | | |
| Alpha-S1-casein | P02662.2 | (F)FVAPFPEV(F) | 905.477 | Bovine | 18.03 | 98.2 |
| Alpha-S1-casein | P02662.2 | (R)FFVAPFPEV(F) | 1052.545 | Bovine | 10.29 | 62 |
| Alpha-S2-casein | P02663.2 | (Y)LYQGPIV(L) | 789.451 | Bovine | 3.7 | 48.3 |
| Fat transport/metabolism | | | | | | |
| Acid ceramidase | Q17QB3.3 | (I)ITEDKEGHLLHGRN(L) | 1618.83 | Bovine | 3.2 | 52.2 |
| Fatty acid desaturase 2 | A4FV48.1 | (A)LQDIIGS(L) | 745.409 | Bovine | 3.3 | 49.5 |
| Lipoyltransferase 1 | O46419.1 | (H)WLPLE(I) | 657.361 | Bovine | 6.15 | 63.9 |
| Perilipin-2 | Q9TUM6.1 | (D)YLVNNTPLN(W) | 1047.547 | Bovine | 3.73 | 50.3 |
| 15-Hyroxyprostaglandin dehydrogenase | Q3T0C2.1 | (N)LVSVISGTY(L) | 938.519 | Bovine | 3.25 | 46 |
| Enzymatic activity | | | | | | |
| Carboxypeptidase | Q17QK3.1 | (K)FTGSDKARV(I) | 980.516 | Bovine | 3.04 | 59.5 |
| D-glucuronyl C5-epimerase | O18756.2 | (G)YFYPIQ(I) | 830.408 | Bovine | 3.73 | 44.7 |
| Dopamine beta-hydroxylase | P15101.2 | (C)YVTELPDG(F) | 893.425 | Bovine | 4.59 | 53.5 |
| Galactose-3-O-sulfotransferase 4 | Q5E9W5.1 | (G)LPFPPE(L) | 699.371 | Bovine | 5.64 | 57.9 |
| Inositol hexakisphosphate | A7Z050.1 | (A)LEGELTPI(L) | 871.477 | Bovine | 4.62 | 54.8 |
| Lon protease homolog 2 | Q3SX23.1 | (T)LRGLVLPVGG(I) | 980.625 | Bovine | 3.73 | 43.3 |
| Methionine synthase | Q4JIJ3.1 | (A)IKIPL(L) | 583.418 | Bovine | 6.5 | 63.3 |
| Monoacylglycerol lipase ABHD6 | Q1LZ86.1 | (T)LALPI(L) | 526.36 | Bovine | 4.14 | 66.5 |
| Polypeptide *N*-acetylgalactosaminyltransferase 1 | Q07537.1 | (G)WLEPL(L) | 657.361 | Bovine | 8.87 | 59.5 |
| Probable inactive glycosyltransferase 25 family member 3 | A7MB73.1 | (W)LAAVGDNYAAVV(W) | 1162.61 | Bovine | 5.82 | 50.3 |
| Prostaglandin G | O62664.2 | (G)LLPGLMV(Y) | 742.453 | Bovine | 7.29 | 66.9 |
| Retinoid isomerohydrolase | Q28175.3 | (I)WLEPEV(L) | 772.388 | Bovine | 3.47 | 55 |
| Tryptophan 2,3-dioxygenase | Q2KIQ5.1 | (V)ITRMHRVVV(I) | 1110.656 | Bovine | 3.19 | 47.1 |
| 5,6-dihydroxyindole-2-carboxylic acid oxidase | Q8WN57.2 | (H)LSPNDPI(F) | 755.393 | Bovine | 5.37 | 46.2 |
| Xylulose kinase | Q3SYZ6.1 | (H)FDRDLVE(F) | 893.436 | Bovine | 9.52 | 67.6 |
| General transport | | | | | | |
| Calcium-binding mitochondrial carrier protein SCaMC-2 | Q0V7M4.1 | (C)LYVPL(I) | 604.37 | Bovine | 8.04 | 80.3 |
| Coatomer subunit beta | A0JN39.1 | (L)LEPLmPA(I) | 770.412 | Bovine | 4.62 | 42.5 |
| Electron transfer flavoprotein-ubiquinone oxidoreductase | Q2KIG0.1 | (A)LGVEVYPGYAAAEV(L) | 1437.726 | Bovine | 3.09 | 65.8 |
| Ferritin | Q2YDI9.1 | (F)LPKHISTSLV(F) | 1094.657 | Bovine | 5.12 | 56.7 |
| Mitochondrial import receptor subunit TOM40B | A6QR22.1 | (F)LTWQFDGE(Y) | 995.447 | Bovine | 5.54 | 49.7 |
| Stromal interaction molecule 1 | Q58CP9.1 | (L)FGPPL(L) | 530.297 | Bovine | 4.34 | 56.3 |
| Transportin-1 | Q3SYU7.2 | (L)LPHILPL(L) | 802.519 | Bovine | 5.71 | 62 |
| Immune function | | | | | | |
| Platelet glycoprotein 4 | P26201.5 | (N)YIVPIL(W) | 717.455 | Bovine | 4.08 | 48.6 |
| Platelet glycoprotein 4 | P26201.5 | (L)WGYTDPF(L) | 885.378 | Bovine | 6.68 | 51.1 |
| Cell signalling | | | | | | |
| Calcineurin subunit B type 2 | Q2TBI5.3 | (A)LSVKEFTSMPE(L) | 1267.624 | Bovine | 6.71 | 58 |
| Calpain-2 catalytic subunit | Q27971.2 | (M)IQLDL(I) | 601.356 | Bovine | 5.98 | 60.7 |
| COP9 signalosome complex subunit 6 | A6QQ21.1 | (E)IIESPL(F) | 671.397 | Bovine | 5.63 | 65.1 |
| Cyclic nucleotide-gated olfactory channel | Q03041.1 | (Q)LAEMDAPQQRRGG(F) | 1428.701 | Bovine | 5.43 | 58.9 |
| Diacylglycerol kinase alpha | A0JN54.1 | (R)FPRPV(Y) | 615.361 | Bovine | 4.36 | 55.7 |
| Extracellular matrix protein | Q3MHH9.1 | (C)LANGMIM(Y) | 749.368 | Bovine | 4.3 | 55.7 |
| F-box only protein 28 | Q2NL16.1 | (Y)FDEKIVPI(L) | 960.54 | Bovine | 4.01 | 51.3 |
| Mothers against decapentaplegic homolog 2 | Q1W668.1 | (S)FVKGWGAE(Y) | 893.452 | Bovine | 6.25 | 44.9 |
| Pleiotrophin | P21782.2 | (K)IPCNWKKQ(F) | 1073.556 | Bovine | 3.97 | 42.5 |
| Programmed cell death protein 2 | Q2YDC9.1 | (G)LPGPPE(L) | 609.324 | Bovine | 3.93 | 52 |
| Rho-associated protein kinase 2 | Q28021.1 | (D)IEQLRSQ(L) | 873.479 | Bovine | 3.85 | 51.1 |
| Rho-related GTP-binding protein RhoU | A5D7J5.1 | (E)IRCHCPKAPI(I) | 1251.645 | Bovine | 3.01 | 48.4 |
| Tensin-1 | Q9GLM4.1 | (S)LSALVYQHS(I) | 1017.536 | Bovine | 7.19 | 60.5 |
| Membrane/protein trafficking function | | | | | | |
| Butyrophilin subfamily 1 member A1 | P18892.2 | (K)LIPLQPSQGVP(−) | 1148.667 | Bovine | 11.73 | 87.2 |
| Butyrophilin subfamily 1 member A1 | P18892.2 | (L)IPLQPSQGVP(−) | 1035.583 | Bovine | 11.17 | 91.7 |
| Butyrophilin subfamily 1 member A1 | P18892.2 | (F)WAVEL(Y) | 617.329 | Bovine | 5.74 | 66.3 |
| Chloride channel CLIC-like protein 1 | Q1LZF8.1 | (L)LLVNPI(W) | 668.434 | Bovine | 5.07 | 55.8 |
| Disintegrin and metalloproteinase domain-containing protein 10 | Q10741.1 | (T)LPFHSVI(Y) | 812.467 | Bovine | 9.66 | 86.2 |
| Excitatory amino acid transporter 1 | P46411.1 | (V)IVLPL(L) | 554.391 | Bovine | 5.15 | 69.9 |
| Interferon alpha | Q95141.1 | (N)LYPmVHIS(L) | 959.502 | Bovine | 5.8 | 78.8 |
| Lactadherin | Q95114.2 | (W)LQIDLGSQKRVTG(I) | 1414.801 | Bovine | 12.72 | 68.8 |
| Lactadherin | Q95114.2 | (F)IQVAGRSGDKIF(I) | 1290.717 | Bovine | 6.88 | 63.6 |
| Neuromedin-U receptor 2 | Q58CW4.1 | (N)IQRPSRKSVTKm(L | 1430.826 | Bovine | 5.5 | 59.1 |
| Probable dolichyl pyrophosphate Glc1Man9GlcNAc2 alpha-1, 3-glucosyltransferase | Q0P5D9.1 | (T)LDYPPF(F) | 751.366 | Bovine | 6.21 | 51.2 |
| Putative phospholipase B-like 2 | Q2KIY5.1 | (R)LLAVSGPTWDQ(L) | 1186.61 | Bovine | 3.71 | 46 |
| Transcription factor HES-1 | Q3ZBG4.1 | (K)LGSPAGEAAKV(F) | 999.547 | Bovine | 3.59 | 43.3 |
| Trafficking protein particle complex subunit 9 | Q32PH0.1 | (E)LSVGARSDEEL(I) | 1175.59 | Bovine | 3.5 | 48.9 |
| 72 kDa type IV collagenase | Q9GLE5.1 | (N)LDAVVDLQGGGHS(Y) | 1267.628 | Bovine | 3.39 | 53.1 |
| Xanthine dehydrogenase/oxidase | P80457.4 | (E)LSLSPDAPGGM(I) | 1044.503 | Bovine | 13.92 | 87.6 |
| Xanthine dehydrogenase/oxidase | P80457.4 | (E)LSLSPDAPGGMIE(F) | 1286.63 | Bovine | 7.06 | 61.9 |
| Xanthine dehydrogenase/oxidase | P80457.4 | (S)FTVPF(L) | 610.323 | Bovine | 6.29 | 71.6 |
| Xanthine dehydrogenase/oxidase | P80457.4 | (A)LKIPISKI(Y) | 911.629 | Bovine | 4.25 | 51.1 |

**Table 1** (*continued*)

| Protein name | Accession no | Sequence | MH$^+$ (Da) | Species | Score | SPI % |
|---|---|---|---|---|---|---|
| Xanthine dehydrogenase/oxidase | P80457.4 | (K)FKNQLFPM(I) | 1024.528 | Bovine | 4.04 | 41.5 |
| Xanthine dehydrogenase/oxidase | P80457.4 | (F)YRTPNLGYS(F) | 1070.526 | Bovine | 4.03 | 44.7 |
| Xanthine dehydrogenase/oxidase | P80457.4 | (A)IDIGQVEGA(F) | 901.462 | Bovine | 3.34 | 43.5 |
| Xanthine dehydrogenase/oxidase | P80457.4 | (F)LSADDIPGSNETG(L) | 1275.57 | Bovine | 7.7 | 60.3 |
| 15-Hyroxyprostaglandin dehydrogenase | Q3T0C2.1 | (N)LVSVISGTY(L) | 938.519 | Bovine | 3.25 | 46 |
| Protein synthesis/binding/folding | | | | | | |
| Inter-alpha-trypsin inhibitor heavy chain H1 | Q0VCM5.1 | (F)LRQNEV(L) | 758.415 | Bovine | 5.36 | 42.5 |
| Leucine-rich repeat-containing protein 72 | A6H759.1 | (Y)LEEKDTGPAQM(L) | 1218.567 | Bovine | 4.61 | 48.3 |
| 39S ribosomal protein L1, mitochondrial | A6QPQ5.1 | (S)LPYPF(I) | 636.339 | Bovine | 3.65 | 46.2 |
| Plasminogen | P06868.2 | (D)FVCRAFQ(Y) | 927.451 | Bovine | 3.77 | 44.9 |
| T-complex protein 11-like protein 2 | A7Z033.1 | (A)LTEGLPE(L) | 758.393 | Bovine | 4.83 | 60.3 |
| Zinc finger protein ZFP69 | A7MBI1.1 | (T)FKDISVDFTQEE(W) | 1457.68 | Bovine | 3.48 | 40.5 |
| Unknown function | | | | | | |
| DNA mismatch repair protein Msh2 | Q3MHE4.1 | (Q)IPTVNN(L) | 657.357 | Bovine | 3.53 | 47.1 |
| Amyotrophic lateral sclerosis 2 chromosomal region candidate gene 8 protein homolog | Q58CW6.1 | (K)IQELVSQG(I) | 873.468 | Bovine | 3.64 | 47.8 |
| Eyes absent homolog 2 | Q58DB6.1 | (G)LIGAPKRETW(L) | 1170.663 | Bovine | 5 | 53.2 |
| PAX-interacting protein 1 | A0JNA8.1 | (N)LNWTPAEVPQ(L) | 1154.584 | Bovine | 5.01 | 52.3 |
| Probable cytosolic iron-sulfur protein assembly protein ClAO1 | Q32PJ6.1 | (T)LSGFHSRTI(Y) | 1017.548 | Bovine | 6.34 | 59.8 |
| Protein BANP | Q0VCW3.1 | (A)LEATCKS(L) | 808.387 | Bovine | 3.4 | 42.9 |
| Protein FAM110B | Q2KJ38.1 | (N)FARANSDI(I) | 893.448 | Bovine | 5.22 | 40.1 |
| Protein FAM13A | Q8HYW0.1 | (S)FVSEVPE(L) | 806.393 | Bovine | 6.55 | 53.5 |
| Protein RRNAD1 | Q5E9V4.1 | (G)FHAELLPI(F) | 939.53 | Bovine | 3.77 | 62.4 |
| Protein inturned | F1MDL2.2 | (E)WLDSVQKNGE(L) | 1175.569 | Bovine | 3.42 | 43.7 |
| Transcriptional adapter 2-alpha | Q3SZP8.1 | (A)IPFHSADDPPRPT(F) | 1449.712 | Bovine | 3.33 | 47.6 |
| Uncharacterized protein C12orf31 homolog | Q2TBR9.1 | (V)LmKDVQE(I) | 862.434 | Bovine | 3.99 | 44.7 |

include a group of calcium-dependent membrane aggregating proteins which can initiate contacts between secretory vesicle membranes, which subsequently fuse (Wu, Yates, Neville, & Howell, 2000). Proteins like monocyte differentiation antigen CD 36 and Toll like receptor (CD 282) were found in both MFGM and CFGM, which are involved in defence mechanism. Additional components associated to defence/immunity, inter-α-trypsin inhibitor heavy chain H1were found in MFGM and early innate immune molecules, interferon regulatory factor 3 and Toll-like receptor 2 proteins in CFGM.

Lipid homeostasis is important for milk fat globules and it should be noted that number of proteins are specifically involved in lipid metabolism as identified in MFGM fraction. These proteins span a variety of processes in lipid metabolism, including lipid *de novo* synthesis (Lipoyltransferase 1, Xylulose kinase; Xylulokinase, Adipophilin/Perilipin-2, and Fatty acid desaturase 2). In CFGM, 1-acyl-*sn*-glycerol-3-phosphate acyltransferase, δ1-AGPAT 4 and Long-chain-fatty-acid–CoA ligase ACSBG1 were present for the same function. We also identified proteins involved in milk fat globule lipid droplet formation and secretion (BTN and XDH/XO), lipid storage (perilipin-2) in MFGM.

Toll like receptors-2 (CD 282) are mainly present on antigen presenting cells and signal the host during pathogen occurrence (D'Ambrosio et al., 2008). This protein present in CFGM is directly involved in activation of innate immunity. Further, the number of minor proteins identified in this study emphasizes multiple functions of MFGM and CFGM proteins, which besides affording nutrition to the newborn through its major components, also promotes neonatal development and protection of the digestive tract from pathogens, as well as helps maintaining optimal mammary gland function.

### 3.4. YLoc, STRING and gene annotation of MFGM and CFGM proteins

All of the identified FGM-enriched proteins were categorized into biological processes, cellular components, and molecular functions according to their annotation (Fig. 1). The biological processes were cellular process, localization, transport, signal transduction, and response to stimulus. The most prevalent cellular components were located in the membrane and extracellular region; other proteins were assigned to the endoplasmic reticulum, vesicle, golgi apparatus and ribosome. The most predominant molecular function was binding activity; other major functional categories were catalytic and hydrolase activity. Thus based on their subcellular localization prediction, the proteins were classified in YLoc (- an interpretable web server for predicting subcellular localization) and were divided into extra cellular (11.4%), nucleus (14.8%), cytoplasm (22.7%), membrane associated (29.5%), endoplasmic reticulum (4.5%), peroxisome (4.5%), mitochondria (4.5%), lysosome (4.5%) and golgi apparatus (4.5%) in milk while, colostrum proteins compartmentalized as extra cellular (20%), nucleus (13%), cytoplasm (30%), membrane associated (15.5%), peroxisome (4.5%), mitochondria (15.5%) and lysosome (2.2%). However, it needs to be pointed out that the higher amount of membrane-associated proteins were found in MFGs while, higher order of cytoplasmic proteins was obvious in CFGs. Therefore the subtle boundaries among classes, the manifold and intertwined protein functions and different roles that the same protein play in different cellular or tissue contexts create difficulties in generation of GO classifications and consequently hinder their comparability among studies (Yang et al., 2015). This applies especially since the results derived by GO classification algorithms and based on scientific knowledge researchers have corrected or left unchanged by "purists" of GO classifications. For example, xanthine dehydrogenase/oxidase (XDH/XO) is a cytoplasmic enzyme, but it has been correctly classified as membrane-associated of MFGM proteins (Bianchi et al., 2009). Similarly, platelet glycoprotein 4, interferon regulatory factor 3 and toll-like receptor 2 proteins comes under defence/immune functions but these are membrane associated proteins.

The protein-protein interaction of MFGM and CFGM proteins was performed using STRING analysis software. The milk proteins were found to interact with various protein assemblies (Fig. 2). The largest network involved interaction of 72 kDa type IV collagenase precursor protein between disintegrin and metalloproteinase domain-containing protein 10, plasminogen precursor, mothers against decapentaplegic homolog 2 and 15-hydroxyprostaglandin dehydrogenase protein. In addition, the chain also linked other proteins like prostaglandin G/H synthase 1 and tryptophan 2, 3-dioxygenase. The interaction between 72 kDa type IV collagenase precursor protein and plasminogen precursor involved specific actions like activation, binding, catalysis, expression and inhibition. Similarly, the same protein (72 kDa type IV collagenase) had interaction with mothers against decapentaplegic homolog 2 protein, showing specific actions of activation expression and expression with inhibition. Protein FAM13A & Rho-related GTP-binding protein RhoU-tensin-1, uncharacterized protein C12 or f31

**Table 2**
The identified proteins of CFGM with their name, accession number, sequence, mass, species, score and SPI %.

| Proteins name | Accession no | Sequence | $MH^{+}$ (Da) | Species | Score | SPI % |
|---|---|---|---|---|---|---|
| Milk proteins | | | | | | |
| Alpha-S1-casein | P02662.2 | (F)FVAPFPEV(F) | 905.477 (9) | Bovine | 20.7 | 100 |
| Alpha-S1-casein | P02662.2 | (F)FVAPFPEVFGKEKVNE(L) | 1836.953 (2) | Bovine | 7.93 | 47.9 |
| Alpha-S1-casein | P02662.2 | (R)FFVAPFPEV(F) | 1052.545 (5) | Bovine | 17.02 | 90.5 |
| Beta-casein | P02662.2 | (V)LGPVRGPFPI(I) | 1052.625 (3) | Bovine | 12.83 | 62.1 |
| Beta-casein | P02662.2 | (L)YQEPVLGPVRGPFPI(I) | 1668.911(4) | Bovine | 7.55 | 48.7 |
| Beta-casein | P02662.2 | (T)LTDVENLHLPLPL(L) | 1473.831 (2) | Bovine | 4.94 | 49.3 |
| Beta-casein | P02662.2 | (L)YQEPVLGPVRGPFPIIV(−) | 1881.063 (2) | Bovine | 4.21 | 50.5 |
| Fat transport/metabolism function | | | | | | |
| Abhydrolase domain | Q5EA42.1 | (L)LAIPKSLSEKRENVm(F) | 1714.952 | Bovine | 3.24 | 40.1 |
| Sphingomyelin phosphodiesterase | Q0VD19.1 | (F)LGPVPV(Y) | 581.366 | Bovine | 3.89 | 66.1 |
| Long-chain-fatty-acid–CoA ligase ACSBG1 | Q2KHW5.1 | (V)LEKYKDV(I) | 894.493 | Bovine | 4.88 | 40.3 |
| 1-acyl-sn-glycerol-3-phosphate acyltransferase delta | Q5E9R2.1 | (A)YVPI(I) | 491.286 | Bovine | 4.75 | 79.3 |
| Enzyme activity | | | | | | |
| Aldehyde dehydrogenase family 8 member A1 | Q0P5F9.1 | (K)LNLPPRNQAG(Y) | 1079.596 | Bovine | 3.69 | 41.8 |
| Cyclin-dependent kinase 2 | Q5E9Y0.1 | (G)IPLPL(I) | 552.376 | Bovine | 7.5 | 76 |
| DIS3-like exonuclease 1 | A0JN80.2 | (T)LNVNKHRAQ(I) | 1079.607 | Bovine | 3.6 | 50.8 |
| Galactokinase | A6H768.2 | (S)LVPLSDPKLAV(L) | 1151.703 | Bovine | 3.78 | 52 |
| Glycerol kinase | Q0IID9.1 | (I)LPNVRSSSEI(Y) | 1101.59 | Bovine | 4.01 | 46.1 |
| Methyltransferase-like protein 13 | A5PK19.1 | (G)LKAVFPL(L) | 787.508 | Bovine | 5.91 | 61 |
| Vitamin K-dependent protein C | P00745.1 | (S)FIKVPVVPYNACVHAmENK(I) | 2216.135 | Bovine | 3.95 | 49.6 |
| 5-aminolevulinate synthase | Q3ZC31.1 | (E)LAELHQKDSA(L) | 1111.574 | Bovine | 5.23 | 61.7 |
| Immune functions | | | | | | |
| Interferon regulatory factor 3 | Q4JF28.1 | (D)LSPGGPSNLTMASEKPPQ(F) | 1810.9 | Bovine | 5.87 | 52.9 |
| Toll-like receptor 2 | Q95LA9.1 | (N)YVSGILEVE(F) | 1008.525 | Bovine | 4.82 | 56.8 |
| General transport | | | | | | |
| Hemoglobin subunit beta | P02070.1 | (R)LLVVYPWTQR(F) | 1274.726 | Bovine | 12.99 | 82.5 |
| Transportin-1 | Q3SYU7.2 | (L)LPHILPL(L) | 802.519 | Bovine | 6.18 | 54.7 |
| 39S ribosomal protein L18 | Q3ZBR7.1 | (M)IEGGVVLQEPRRI(Y) | 1465.849 | Bovine | 4.74 | 49.4 |
| Membrane/protein trafficking | | | | | | |
| Butyrophilin subfamily 1 member A1 | P18892.2 | (L)IPLQPSQGVP(−) | 1035.583 | Bovine | 7.48 | 79.1 |
| Cleft lip and palate transmembrane protein 1-like protein | A2VE61.1 | (S)LPVLVPAG(I) | 765.487 | Bovine | 5.28 | 55.6 |
| GTP-binding protein 10 | Q3MHG6.1 | (L)LVAEGGLGGKL(L) | 1013.599 | Bovine | 3.52 | 54.2 |
| Prolactin-releasing peptide receptor | Q4EW11.1 | (L)LRDLDPHA(I) | 936.49 | Bovine | 3.05 | 49.1 |
| Ras-like protein family member 12 | Q08E00.1 | (V)FGKPRAGGGQQSAPLEVNLA(I) | 1997.056 | Bovine | 3.89 | 41.1 |
| TM2 domain-containing protein 2 | Q2TA35.1 | (L)LLHCVSRSHSHNATAEPE(L) | 2044.962 | Bovine | 3.64 | 43.1 |
| Trafficking protein particle complex subunit 9 | Q32PH0.1 | (S)LRPQKmKSSLGPSVSAKSPF(I) | 2145.185 | Bovine | 3.63 | 41.9 |
| UPF0577 protein KIAA1324-like homolog | A7E2Z9.1 | (N)LSSVGSLMNGPSFTSKGTK(Y) | 1897.969 | Bovine | 3.29 | 45.7 |
| Protein synthesis/binding/folding | | | | | | |
| Eukaryotic translation initiation factor 2D | Q58CR3.1 | (G)LVVPPAG(L) | 652.403 | Bovine | 8.51 | 74.6 |
| Flotillin-1 | Q08DN8.1 | (G)ISVVSYTLKD(I) | 1124.62 | Bovine | 3.65 | 40.7 |
| Phosphatase and actin regulator 4 | F1MCY2.1 | (K)LSQRPTVAE(L) | 1000.542 | Bovine | 3.55 | 44.2 |
| Ribosome-releasing factor 2 | A6QNM2.1 | (G)YVPLAEImG(Y) | 992.512 | Bovine | 4.09 | 50.5 |
| RNA-binding protein 44 | E1BC15.1 | (G)IEIKGKSVNVR(L) | 1242.753 | Bovine | 3.55 | 41.3 |
| 28S ribosomal protein S10 | P82670.2 | (H)IFKKHRVQYEmRT(L) | 1735.943 | Bovine | 5.28 | 46.8 |
| Cell signalling | | | | | | |
| Alpha-internexin | Q08DH7.1 | (N)IGQLENDLRNTKSEmARH(L) | 2112.061 | Bovine | 3.87 | 42.9 |
| Cell death activator CIDE-3 | F1MN90.1 | (C)LNVKATLYGT(Y) | 1079.61 | Bovine | 3.79 | 61.5 |
| NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 13 | Q95KV7.3 | (E)LYGLRASEEV(L) | 1136.595 | Bovine | 4.66 | 42 |
| Unknown function | | | | | | |
| POC1 centriolar protein homolog A | Q2TBP4.1 | (T)LHGHQGPATTVAFSRTGEY(F) | 2028.989 | Bovine | 4.17 | 40.8 |
| Protein FAM13A | Q8HYW0.1 | (S)FVSEVPE(L) | 806.393 | Bovine | 3.45 | 41.5 |
| Protein strawberry notch homolog 2 | A0JND4.1 | (M)LAVGPAmDGELPPHEAPPAGSV(L) | 2112.043 | Bovine | 3.16 | 52.7 |
| Zinc finger SWIM domain-containing protein 8 | A7E305.1 | (E)LSAKQVAFH(I) | 1000.557 | Bovine | 3.03 | 41.4 |

homolog-calpain-2 catalytic subunit proteins formed another network. The galactose-3-O-sulfotransferase 4 and Polypeptide *N*-acetyl galactosaminyltransferase 1, ferritin-thyroglobulin precursor, α-S1-casein antioxidant peptide and α-S2-casein Casocidin-1, transportin-1 and pleiotrophin, xanthine dehydrogenase/oxidase and perilipin-2 were the other protein networks identified in the present study. In colostrum, we observed small networks involving ribosome-releasing factor 2 with GTP-binding protein 10, 28S ribosomal protein S10, phosphatase and actin regulator 4. Other interactions were interferon regulatory factor and toll-like receptor 2 precursor; trafficking protein particle complex subunit 9 and methyltransferase-like protein 13 and hemoglobin subunit β and 5-aminolevulinate synthase. Thus interaction of various FGM proteins derived from both buffalo milk and colostrum were assigned. The study imparts knowledge on the prevailing communications among protein assemblies in milk and colostrum FGM.

The functional clustering of all the identified proteins led to their clustering into 8 and 5 different gene functional groups in MFGM and CFGM proteins (Supplementary files 2A & 2B). As more number of peptides was characterized in MFGM, gene annotation studies revealed more clusters involving in various cellular activities. Similarly in CFGM a major cluster involving in glycoprotein, signal, glycosylation, disulfide bond, secretion and also integral component of membrane signal peptide was identified suggesting the functional assemblies of these gene

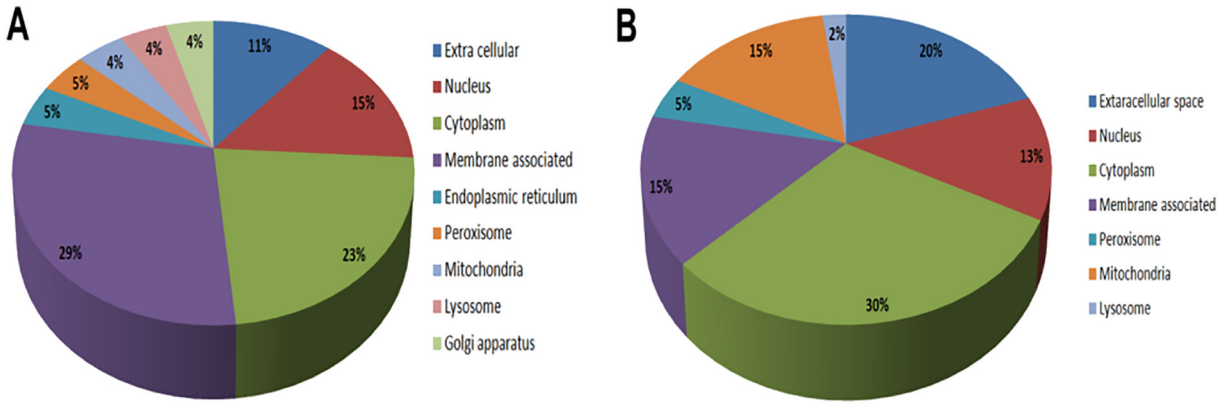


**Fig. 1.** Pie chart representation of identified proteins in sub cellular organelles in MFGM (A) & CFGM (B).

clusters. In addition, the data also provided clustering related gene report as 2D heat map where green area showed highly common annotations and black-green area indicated difference in annotations (Supplementary files 3A & 3B).

### 3.5. Radical scavenging activity of MFGM and CFGM peptides

Owing to potential health benefits of MFGM proteins, the peptides generated from both milk and colostrum FGM were analyzed for antioxidant activity. The antioxidant property of the peptide fractions shown by ABTS assay was confirmed by performing cell based antioxidant assays like GSH, $H_2O_2$, ROS, catalase, LDH, ACP and ALP using DNP, a known oxidant on blood components. In ABTS free radical scavenging assay, MFGM and CFGM samples showed 54 & 58%, 66 & 70%, 73 & 78, 81 & 88% scavenging at 250, 500, 750 and 1000 μg of peptides respectively (Fig. 3A). The results indicated increased radical scavenging activity with increasing concentration of the peptides. However, CFGM peptides were more effective antioxidants than MFGM peptides.

The level of glutathione (GSH) declined in serum to 28% after DNP treatment compared to control. But, decreased level of GSH elevated to 63 and 69% when DNP was pre incubated with MFGM and CFGM peptides while, peptides alone had marginal effect (Fig. 3B). On the endogenous antioxidant markers, more elevation in $H_2O_2$ level was observed (Fig. 3C) in DNP treated sample (135%) but was reduced to 56 and 61% when peptides were pre incubated with DNP. Similarly, significant enhancement in the level of ROS (153%) was observed in DNP treated group which up on pre incubation with peptide fractions of MFGM and CFGM decreased to 40 and 48% respectively. However peptides alone had very little effect (Fig. 3D). In addition, the catalase activity was also found high when serum was treated with DNP group (19 fold) which in presence of MFGM and CFGM peptides decreased to 11 and 10 folds while, there were no observable changes in peptides alone treated group (Fig. 3E).

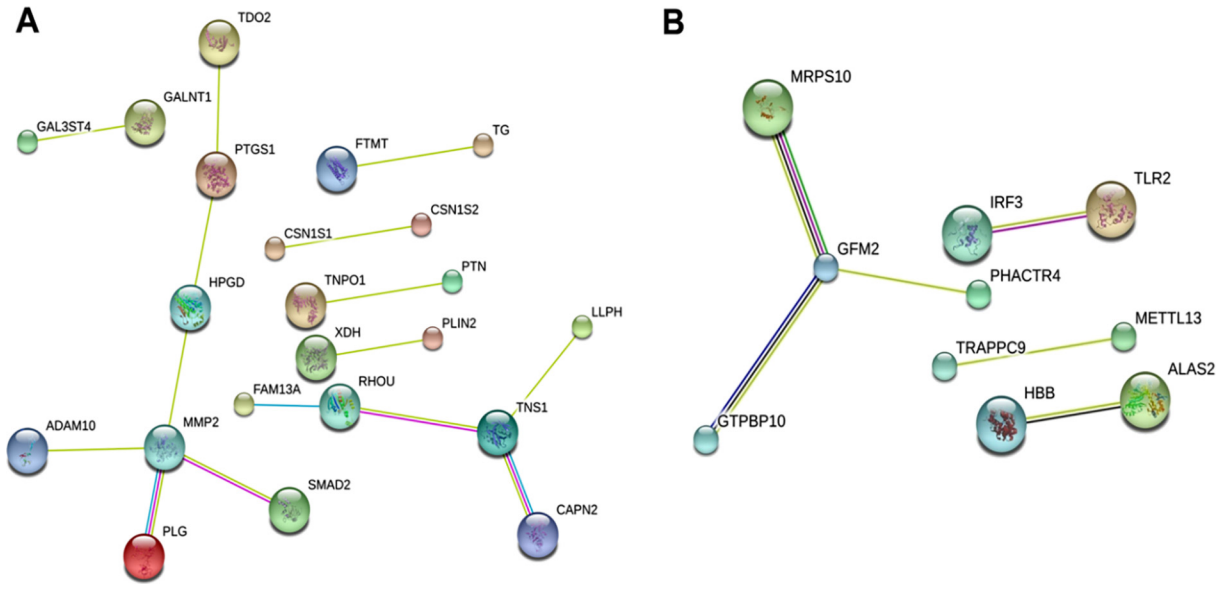


**Fig. 2.** STRING prediction of associated network among MFGM (A) & CFGM (B) proteins. The node represents the differentially accumulated protein while the different colored edges represents different evidences for the predicted functional relationship between proteins: green line - neighbourhood genome evidence; dark blue line - co-occurrence evidence; pink line - experimental evidence; light blue line - database evidence; black line - co expression evidence; yellow line - text-mining evidence. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

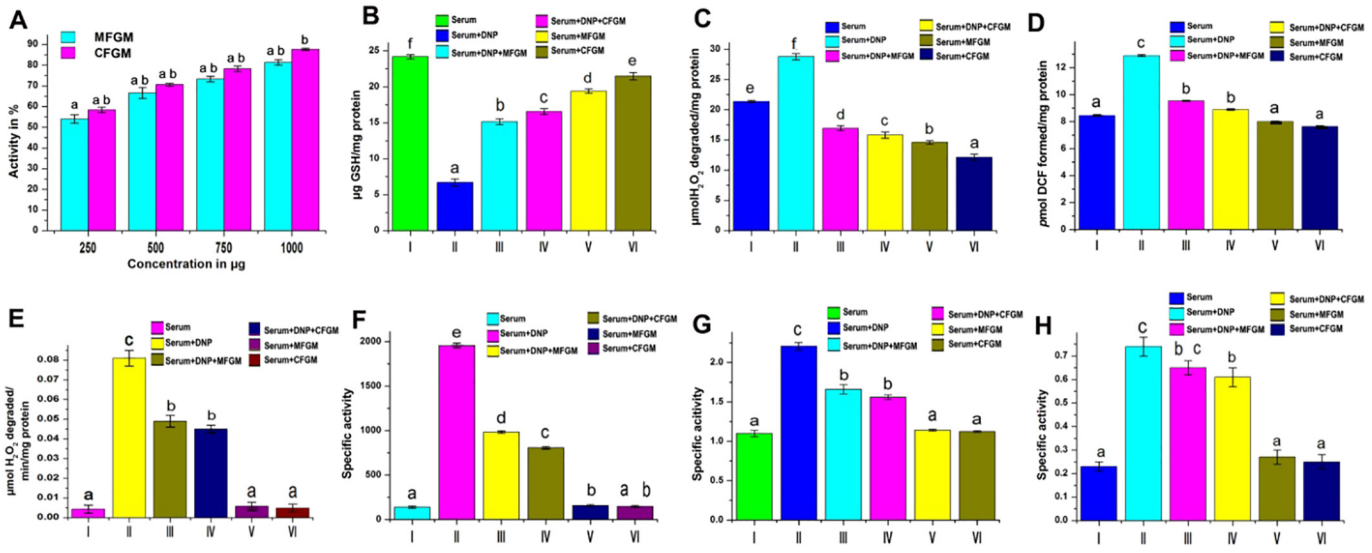


**Fig. 3.** Antioxidant activity of FGM peptides from Buffalo milk and Colostrum. (A) ABTS (B) GSH (C) $H_2O_2$ (D) ROS (E) Catalase (F) LDH (G) ACP (H) ALP I-control, II-blood treated with DNP, III-blood treated with DNP pre incubated with MFGM peptides, IV-blood treated with DNP pre incubated with CFGM peptides, V-blood treated with MFGM peptides alone, VI-blood treated with CFGM peptides alone. Bars indicate ± SE. Means designated with the same letter are not significantly different according to Tukey's HSD at $P < 0.05$.

In the lysosomal enzyme assay, there was 14 fold elevations in LDH in DNP treated group which reverted to 6 and 4 folds respectively when peptides of MFGM and CFGM were pre incubated with DNP (Fig. 3F). Peptide alone treated group was found unaltered. In the ACP assay, MFGM and CFGM peptides offered 50% and 57% protection respectively compared to DNP treated sample (200%). The peptides alone treated group remained same in CFGM and very little change was noticed in MFGM peptides (Fig. 3G). Similarly, there was 56% decrease in ALP level when DNP was pre incubated with CFGM group and 39% in MFGM peptides treated group when compared with DNP alone treated group (320%). But, neither of the peptides altered ALP level when treated alone (Fig. 3H).

The antioxidant efficacy of FGM peptides corroborated with various cell based assays strongly suggest newer prospective functional role offered by FGM proteins. Since majority of the earlier studies emphasize antibacterial role of MFGM proteins (Clare, Zheng, Hassan, Swaisgood, & Catignani, 2008), the peptides characterized in the present study unfolds their potential role in protecting the newborn against various environmental insults. Further, the higher degree of cytoprotection offered by CFGM peptides against oxidative stress was noteworthy.

## 4. Conclusions

The FGM proteins isolated from both buffalo milk and early milk were subjected for *in vitro* simulation digestion mimicking digestive system of human beings. The peptides derived from both the samples were analyzed by nLC-ESI MS/MS to unravel their sequences and to match the component proteins of FGM. The peptide libraries created facilitated clustering of milk and colostrum FGM proteins in to various functional groups involved in lipid metabolism and energy production, protein synthesis and secretion, transport, cell signalling, catalytic and also in immune function. The STRING and DAVID analyses revealed interactive protein networks and subcellular localization in both the samples. Although several studies implicate antibacterial activity of FGM proteins, the powerful antioxidant effect offered by peptides gives newer insights for their functional utility in neonatal health care. In addition, the peptide libraries created for FGM proteins from buffalo milk and colostrum in the present study provide ample scope to explore newer functionalities for these peptides.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.foodres.2017.03.037.

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