Summary and Conclusion
6. Summary and Conclusions

Colostrum constitutes a fascinating group of biomolecules that are able to contribute, high value added markets for the dairy industry. Bovine colostrum has been known to provide essential food for newborns as it is a rich source of immune and growth factors, conveying passive immunity to the offspring. The milk-derived preparations have found broad application in the food industry, production of infant formulas; the products have found application as preventive or therapeutic measures for a broad array of pathological states in neonates, infants, adults and the elderly with no adverse effects. Many of these milk-derived proteins and peptides may represent a supplementary treatment to conventional therapy and have been commercialized as ingredients of nutraceuticals (FitzGerald et al, 2004). In addition to Igs, colostrum is rich in other components that have documented beneficial effects in promoting the differentiation, growth, and health of a variety of tissues and organs (Playford et al, 2000). Colostral IgG has its potential function as an anti-inflammatory agent or immune modulator. The presence of various glycans on IgG not only increases microheterogeneity, but also affects antibody effector functions and its stability. Terminal Gal, GlcNAc and Man residues affect C1q binding and CDC activity whereas, terminal NeuAc, Man, core Fuc and bisecting GlcNAc residues are known to affect receptor binding and ADCC (Hodoniczky et al, 2005). Owing to structural characterization of sialyl oligosaccharides from IgG, they are implicated as potential therapeutic agents in glycan interaction associated with diseases like cancer, rheumatoid arthritis and influenza (Dube et al, 2005). Further, the presence of Man₉GlcNAc₂, a high mannose type of N-glycan often recognized by dendritic cell surface receptor during viral infection suggests its functional utility in developing antiviral inhibitors (Shade and Anthony, 2013).
6.1 Isolation, purification and characterization of Whey protein

In the present study, we have focused on purification and characterization of immunoglobulin G (IgG) from buffalo colostrum. We isolated whey proteins from first day sample of buffalo colostrum with an average yield of 14 - 17%. The heterogeneous whey proteins were purified on Sephadex G-100 and the major fraction was subjected for further analysis. The major protein was found homogenous with Native-PAGE and SDS-PAGE analysis indicated its dimeric nature (Fragment I and II) with approximate molecular masses of 25 and 49 k Da. The exact molecular mass was found to be 147.848 k Da as determined by MALDI-TOF MS. The peptide mass fingerprint revealed the identity of protein as immunoglobulin G with fragment I (25 kDa) showing 82 % sequence homology to Homo sapiens immunoglobulin light chain variable region while, fragment II (49 kDa) having 90 % sequence homology to Orytologous cruniculus immunoglobulin heavy chain V-DJ region. The LC- ESI-MS/MS analysis was performed with CID to further confirm the identity of purified protein. The identification of double charged ion pairs with m/z - 497²⁺ (LLIYGATSR) and 664²⁺ (VYNEYLPAPIVR) corresponded to light and heavy chains, facilitated the detection of peptide sequences of IgG, which was in compliance to IgG Kappa chain V–III region NG9 from Homo sapiens while, fragment II yielded peptide sequence VYNEYLPAPIVR was in homology to immunoglobulin γ heavy chain constant region from Cervus lelaphus hispanicus.

6.2 Fragmentation and Peptide profiling of Immunoglobulin G

The purified buffalo colostrum IgG as confirmed by LC-MS/MS analysis was digested by a combination of protease enzymes pepsin and pancreatin to generate peptide sequences. The peptide mixture analyzed by HR-LC-MS/MS was searched against non redundant NCBI database. Search results revealed 25 identifiable peptide
sequences corresponding to Ig G heavy and light chains of mouse, human and bovine species. Most of the sequence identity was from human immunoglobulin and a very few match was found from mouse and bovine species.

6.3. Glycomic characterization of Immunoglobulin G

Quantization of total glycome was performed by adopting chemoselective glycoblotting technique coupled to MALDI-TOF MS analysis. IgG after trypsinization followed by PNGase treatment released N-linked glycans from the protein. A sum of 54 N-glycans was identified in the colostrum IgG where in, complex sialyl oligosaccharides were found in abundance compared to hybrid and high mannose types. However, non fucosylated glycans were found present in higher proportions than fucosylated oligosaccharides, as promising molecules to elicit ADCC. In order to validate the structural assignments of abundant IgG N-glycans, MALDI-TOF/TOF analysis was carried out for m/z - 1768, 1914, and 2073. The spectra clearly explicit the structural confirmation of \((\text{Hex})_2 (\text{HexNAc})_2 + (\text{Man})_3(\text{GlcNAc})_2, (\text{Hex})_2 (\text{HexNAc})_2 (\text{Deoxyhexose})_1 + (\text{Man})_3(\text{GlcNAc})_2\) and \((\text{HexNAc})_2 (\text{NeuAc})_1 + (\text{Man})_3 (\text{GlcNAc})_2\) oligosaccharides accounting for 51% of the total N-glycans structures.

6.4 Functional Attributes of Immunoglobulin G

6.4.1 Antibacterial effect of Immunoglobulin G on Klebsiella pneumoniae

*Klebsiella* strain was identified as *Klebsiella pneumoniae* using 16s rRNA amplification followed by sequence analysis and Blastn search. Antibacterial activity was determined by MIC (31.3 µg) and growth inhibition assay by turbidometry and viable cell count methods. Anti-adhesive role established by SEM studies revealed the morphological changes in the cell wall of *K. pneumoniae* cells after IgG treatment.
compared to control. Glycan enriched fraction was found to be more potent than intact IgG. The effect of IgG on the proteome of *K. pneumoniae* was analysed by resolving the protein mixture on SDS-PAGE after treating the cells with IgG. Majority of the low molecular weight proteins (<35 kDa) were found missing probably either due to IgG effect or lowering in the protein content because of bactericidal effect of IgG. Interestingly, expression of a new protein (~14 kDa) was obvious in the treated cells which up on in-gel trypsin digestion followed by MALDI-TOF analysis showed 77% homology to chemotaxis protein (CheY).

**6.4.5 In silico studies of Immunoglobulin G peptides**

Based on the *in vitro* results the probable interaction of IgG peptides with *K. pneumoniae* was studied *in silico* which involved molecular docking using Schrodinger software by targeting outer membrane porin (OSM) of *K. pneumoniae*. The short peptides obtained from IgG fragmentation by pepsin and pancreatin were used to examine their interaction on *K. pneumoniae* outer membrane protein target. The overall dock scores (GScore) for 5 ligands including ampicillin - 4GCP, FDVWGTGT, IVKPGASV, IKSRFI and FIFPP were -4.9, -4.8, -4.7, -4.6 and -4.3 respectively. The Gscore of IgG peptides were found closer to the score of standard antibiotic ampicillin suggesting their probable interaction to the drug target.

**Conclusions**

The advanced proteomic and glycomic tools facilitated identification and molecular characterization of novel protein, Immunoglobulin G (IgG) from buffalo colostrum from first day colostrum. The IgG with bound N-glycans to Asn297 is known to play key roles in mediating IgG stability and undergo characteristic glycosylation changes, in addition to its biological efficacy as prebiotics, anti-
inflammatory and immune modulators and as a possible source of sialic acid for the growth and development of nervous system being present in colostrum. The present study unravelled the structural complexity and functional ability of colostrum IgG. The purified IgG was characterized by employing MALDI-TOF/MS, LC-MS/MS and HR-LC/MS analyses. The glycomic analysis revealed the presence of 54 N-glycans. Like bovine milk, buffalo colostrum IgG also contained both NeuAc and NeuGc unlike human milk which possesses only NeuAc. The unique functional ability of buffalo colostrum IgG was its potent antibacterial effect on *K. pneumoniae*, a multidrug resistant pathogen. The above findings suggest that the glycoproteins in buffalo colostrum may confer different levels of protection against human pathogens like it was observed with *K. pneumoniae* and the bound glycans could serve as mimetics of pathogen adhesion sites. Hence, buffalo colostrum IgG with higher order of non-fucosylated and sialylated oligosaccharides can effectively enhance ADCC and anti-inflammatory responses, which can have profound impact on its functional utility in therapeutic interventions or as nutraceuticals in commercial food formulations.