Discussion
5. Discussion

Buffalo colostrum an ultimate alternative model next to bovine milk as functional food is endowed with nutritive immune active components. Milk proteins, especially whey protein components, α-lactalbumin, β-lactoglobulin, glycomacropeptide and immunoglobulins are known to be the key resource for bioactive components. Buffalo milk is superior to cow milk in terms higher protein content (11.42%) (Mane and Chatli, 2015) and important minerals, namely calcium (92%), iron (37.7%) and phosphorus (118%), it is nearly twice as rich as cow milk (Varrichio et al, 2007), hence long been valued for its important nutritive properties and for its application in dairy industry.

Milk can be fractionated by precipitation into its four components: fat, casein, whey and lactose (Brans et al, 2004). The two major families of proteins in milk are caseins (insoluble, 80%) and whey (soluble, 20 %) proteins. Whey represents a rich and heterogeneous mixture of globular proteins with varied functional attributes for nutritional, biological, and food purposes (Krissansen, 2007). Whey protein and casein are termed as high quality proteins based on human protein requirements, digestibility and their amino acid availability (Boye et al, 2012). Caseins are classified as α-, β- and k-caseins, whey is composed of β-lactoglobulin, α-lactalbumin, serum albumin, immunoglobulins, lactoperoxidase, lactoferrin, glycomacropeptide, lysozyme and several minor proteins with different pharmacological activities (Mohanty et al, 2016). Whey comprises of about 60 indigenous enzymes and has found potential applications in therapeutics, food industry (El-Loly, 2007) and as supplements for cell culture media (Jenkins & McGuire, 2006).

β-lactoglobulin plays an important role in developing passive immunity along with immunoglobulin G and in its transference to the newborn, and in regulation of
phosphorus metabolism at the mammary gland. α-lactalbumin and serum albumins contributes to reduce the risk of breast and colon cancer. Lactoferrin possesses antimicrobial activity and lactoperoxidase play important role cell-mediated pathogen killing (Fadæi, 2012). Peptides showing opioid (Clare et al, 2000) and angiotensin I-converting enzyme (ACE) inhibitor activity (Poussa & Korpela, 2003) were found in α-lactalbumin and β-lactoglobulin and have shown to inhibit wide array of tumors via apoptosis (Parodi, 2007). Hence, whey proteins are considered as superior dietary proteins which have richer taste due to its contents of milk fat, protein, lactose, total dry matter, vitamins and minerals. Thus, there has been considerable commercial interest in large scale production of purified whey proteins. A commercially available product Prolibra™, a specialized whey fraction (high in leucine, bioactive peptides and milk calcium) has been proved to promote loss of body mass and preservation of lean muscle (Frestedt, 2008. However, the present study was carried out to isolate and purify a major whey protein from buffalo colostrum and to determine its biological activity.

5.1 Isolation, purification and characterization of Immunoglobulin G (IgG)

Isolation of individual whey proteins have gained much attention now days to adapt to dietetic requirement. The defatted and dialyzed colostrum adjusted to pH 4.6 facilitated precipitation of casein leaving acidic proteins, these acidic proteins is commonly referred to as whey proteins. The yield of whey proteins was found to be 14 - 17%. This is in analogy to the existing report on the enrichment of whey proteins in early milk Rohit. (2012). In addition, the concentration of whey proteins in water buffaloes is much higher (11.42%) compared to cow and humans (Lonnerdal, 2004; Pandya and Haenlin, 2009). During our earlier study, we had observed co-
fractionation of a major protein along with β-lactoglobulin (Rohit & Aparna, 2011). Hence, we subjected the whey sample for 60% ammonium sulphate precipitation. A fractionation scheme for the economically interesting proteins, such as IgG, lactoferrin and lactoperoxidase several alternative procedures have been developed for industrial-scale to fulfill the growing demand by food manufactures for cost-effective multifunctional ingredients. Whey purification using ultrafiltration and demineralization by diafiltration or electrodialysis, forms the basis for the earliest commercially feasible method (Baldasso et al, 2011). However, whey protein is prone to denaturation which makes the fractionation of whey very expensive (Marshall, 2004; Liang et al, 2006). Additionally, ultrafiltration is neither sufficient for the complete removal of lactose, nor for the isolation of single pure protein (Rossano et al, 2001). Within the last decade there has been increasing interest in liquid chromatographic processes because of the growing biotechnology industry and the special needs of the pharmaceutical industries (Smithers, 2008).

Immunoglobulin G (IgG) belongs to the family of glycoproteins found in abundance in colostrum and milk, which plays a major role in helping defend the host against infection. They are composed of 82-96% protein and 4-18% carbohydrate (Yap, 1993). Their general structure has been conserved through evolution. It is composed of two heavy and two light chains with multiple homologous protein domains possessing a characteristic fold of a series of anti-parallel β-strands rolled up into a globular β-barrel structure stabilized by a conserved disulphide bond between two of the strands (Montano & Morrison, 2000). In the present investigation, we have aimed at purification and characterization of IgG from buffalo colostrum to unravel the structure-function relationship of the major whey protein. The Igs, the major component of whey has increased demand for highly purified forms with marked
antimicrobial activities (Hernandez-Ledesma et al, 2011). The IgG was purified from buffalo colostrum following fractionation using ammonium salt precipitation and purification on Sephadex-G100. Adopting similar strategy we have earlier purified β-lactoglobulin (Rohit and Aparna, 2011). The microfiltration based affinity purification with membrane filtration was initially used to isolate LF and IgG from cheddar cheese whey (Jyh-Ping and Wang, 1991) and later using immobilized egg yolk antibodies bovine milk IgG subclasses were isolated (Akita et al, 1998). Bovine and caprine colostrum IgG were isolated by microfiltration combined with ultrafiltration has led to 90% purity (Piot et al, 2004). Copestake et al. (2006) used protein G to quantitate IgG in colostrum based powder. Similarly, many Ig’s available commercially have been purified by affinity chromatography using either protein A / G ligands (Farid, 2006; Hober et al, 2007) which is more expensive. Several chromatographic techniques like immobilized metal chelate chromatography, immunoaffinity and cation-exchange chromatography have been applied to improve the yield and purity of major fraction of whey IgG (Korhonen and Pihlanto, 2007; Roque et al, 2007). Wu and Xu, (2009) purified IgG from bovine colostrum using serial cation-anion exchange chromatography while, the isolation and purification of Ig’s from colostral or cheese whey based on ultrafiltration (UF) or a combination of UF and chromatography (Korhonen et al, 2000), reverse phase HPLC (El-Loyl, 2007) seemed to be the most cost-effective approach. More recently Menegatti et al. (2014) developed a method using cyclo hexamer, appear to be an attractive candidate for developing a cost-effective and robust chromatographic resin to purify monoclonal antibodies. Although the available methods can be exploited for the purification of IgG from either serum or milk, the method developed in the present investigation is simple for the rapid purification of colostrum or milk IgG with highest purity.
SDS-PAGE is commonly used to analyze the molecular weight determination and distribution of peptides of the whey samples (Jiménez et al, 2012; Bonnaillie et al, 2014). The NATIVE and SDS-PAGE patterns of purified protein led to the determination of the molecular weight as 150 kDa supported by peptide mass fingerprint data revealing the subunits of IgG corresponding to light chain (25kDa) and heavy chain (50kDa) respectively. The peptide mapping was used to identify primary sequence of proteins. Earlier studies indicated that IgGs can be digested into peptides using in-gel proteolytic digestion followed by their separation by LC-MS/MS analysis (Tun-Li Shen et al, 2005; Gundry et al, 2010). Trypsin is the most used enzyme to hydrolyze protein into peptides prior to LC-MS detection as it offers adequate charge states and better b/y ion signals under collision-induced dissociation (CID) fragmentation (Baldwin, 2004). CID is the most common fragmentation method in peptide mapping, CID breaks amide bonds on the peptide backbone and generates b and y product ions, which are further analyzed by the mass analyzer and produce MS/MS spectrum (Beardsley et al, 2004). Similarly, LC-MS/MS approach was established to quantify therapeutic monoclonal antibodies using whole antibody (Li et al, 2012) as internal standard to overcome the limitations of current methods. More recently, a universal surrogate peptide sequence unique to the human Fc region of the therapeutic protein in plasma samples was developed by Furlong et al. (2012) based on LC-MS/MS analysis. Usually both MS and MS/MS are acquired for peptide identification. Thus, in the present investigation also protein identification was based on both peptide mass fingerprinting data complemented by LC-MS/MS analysis.
5.2 Fragmentation and Peptide profiling of Immunoglobulin G

The LC-MS/MS is an important tool in characterizing the amino acid sequence and post-translational modifications of therapeutic antibodies. Identifying changes in the antibody is important in monitoring possible changes in activity and/or immunogenicity. In order to get a better insight to the proteomic sequence of colostrum based IgG, we digested IgG using pepsin and pancreatin mimicking in vivo gastrointestinal simulated digestion and analyzed the generated peptides by HR-LC/MS technique. Pepsin digestion of IgG yields an F(ab’2) fragment that includes the two antigen-binding (Fab) sites while, pancreatin further digests the protein. Intact Ig, F(ab’2) and other antibody formats are being exploited in the development of antibody therapeutics (Carter, 2006). Holt et al. (2003) reported that IgG heavy-chain antibodies interfere with various biological processes and may make it a good candidate for human therapy. Remarkable progress was observed in high-resolution MS analyze proteins without digestion (Reid and McLuckey, 2002, Kelleher, 2004). The use of a cysteine proteinase from Streptococcus pyogenes (IdeS) to characterize the sequence and post-translational modifications (PTMs) of monoclonal antibodies has also been reported by Chevreux et al. (2011). Recent studies show that an endopeptidase-IdeS, cleaves heavy chains below the hinge region, producing F(ab’2) and Fc fragments. Following reduction of disulfide bonds, three antibody domains (LC, Fd, and Fc/2) could be released and further characterized by liquid chromatography/mass spectrometry and capillary isoelectric focusing (Yan An et al, 2014). However, short peptide motifs are beneficial than whole antibody itself. The proteomic data generated in the present study adds information to the buffalo IgG to the already existing information in the data base for human and bovine source. Although the proteomic coverage was not extensive in spite of using combination of
enzymes supports the fact that macromolecules in colostrum resist gastrointestinal digestion (Hurley and Theil, 2011) and thus they contribute to growth and development of the newborn. However, high glycosylation protecting the cleavage sites cannot be ruled out. Further, LC-MS/MS approach was developed to quantify monoclonal antibodies and human Fc region of the therapeutic proteins in plasma samples (Li et al, 2012; Furlong et al, 2012). Thus LC MS/MS based analysis has become an important tool in characterizing the amino acid sequence and post-translational modifications of therapeutic antibodies which identifies changes in the antibody relying to possible changes in activity and/or immunogenicity.

5.3. Glycomic characterization of Immunoglobulin G

MALDI-TOF/MS is a widely used approach for profiling released glycans directly or in a derivatized form (Wuhrer et al, 2007). Due to the structural complexity and heterogeneity of glycans, N-glycans were derived enzymatically by PNGase from purified IgG coupled with chemoselective blotting from buffalo colostrum prior to MALDI-TOF-MS analysis. A sum total of 54 N-glycans were identified in the present study where in the core structures of IgG glycans were found similar to human serum and bovine milk oligosaccharides. Like buffalo colostrum IgG, presence of highly sialylated oligosaccharides was also a characteristic feature of day one bovine colostrum (Takimori et al, 2011). Interestingly, more than 25% of the N-glycans of buffalo colostrum were found more similar to the usual structural elements of human serum IgG (Johannes et al, 2008). Further, the serum IgG from mouse, cow, goat, sheep, horse and rhesus monkey were found to contain only NeuGc while, those of dog, guinea pig, rat, cat possessed mixture of both NeuGc and NeuAc like buffalo.
colostrum IgG albeit, in different proportions (Raju et al, 2000) but human serum IgG is reported to have only NeuAc (Johannes et al, 2008).

Although mass spectrometric analysis coupled to HPLC has been used extensively for the elucidation and characterization of human milk oligosaccharides from various species (Zaia 2004; Ninonuevo et al, 2006; Broberg, 2007; Pfenninger et al, 2008; Tao et al, 2009) Capillary electrophoresis (CE) with UV or laser induced fluorescence detection has been used successfully for the quantification of human milk oligosaccharides (Newburg et al, 2000; Shen et al, 2000). The combined use of two novel techniques, chemoselective glycoblotting and MALDI-TOF/TOF mass spectrometry (Shen et al, 2000) allows both purification and precise analysis of common oligosaccharides and glycopeptides from native glycoproteins. Similarly, glycan analysis of oligosaccharides released from IgG-Fc has been developed by employing HPLC, MALDI-TOF MS and MS/MS techniques (Nishimura et al, 2004). Owing to glycan separation, PGC particularly affords a suitable means for separation of glycan isomers and also detailed MS/MS information providing compositions and connectivity of separated glycan isomers (Wada et al, 2007). In addition, the HILIC HPLC-HRSRM-MS method reported allows the absolute quantification of five different bovine milk oligosaccharides simultaneously (Chu et al, 2009). The N-glycosylation analysis of secretory IgA from human colostrum has been performed at the glycopeptide level using in-gel trypsin digestion and subsequent LC/MS and LC-FTICR-MS (Renfrow et al, 2007) and LC-MS/MS (Deshpande et al, 2010). High resolution HPLC with 2-aminobenzoic acid (2AA) labeling for determination of sialylation levels was established recently for human plasma derived IgG fragments (Anumula, 2012). Recently, ion mobility spectrometry (IMS) technology is used for glycan analysis (Pagel et al, 2013), which discriminates structural isomers of glycans.
which are not confirmed by conventional MS methods. In addition, tandem MS methods such as TOF/TOF, QIT-TOF, LTQ FT-ICR, and LTQ-Orbitrap are used to analyze the sequence of glycans (Liu et al, 2014). But, the glycoblotting technique adopted in the present study yielded comprehensive profile of N-glycans with the higher order of glycan identification.

It is well known that Fc glycans with no Fuc in core GlcNAc, substantially increase the ADCC activity as they bind to FcγRIIIa receptor with significantly increased affinity. The improved binding of these non-fucosylated N-glycans is said to be independent of IgG sub classes (Niwa et al, 2005). In the light of this information, buffalo colostrum IgG containing higher order of non-fucosyl N-glycans along with high mannose oligosaccharides can be successfully exploited for the development of novel antibodies to elicit effective ADCC activity (Robert et al, 2012). 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 FCγRIIIa binding and ADCC (Hodoniczky et al, 2005). Owing to structural characterization of sialyl oligosaccharides from colostrum IgG, they are implicated as potential therapeutic agents in glycan interaction associated with some of the diseases like cancer, rheumatoid arthritis and influenza (Kai-Ting et al, 2013). Furthermore, 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 (Menon et al, 2009). Since colostral IgG is known for its potential function as an anti-inflammatory agent or immune modulator, in the initial phase of neonatal growth and development may contribute towards antibody effector functions and its stability. In the light of this information, we evaluated the
antibacterial action of IgG on *K. pneumoniae* as it is emerging as an important multi-resistant pathogen in humans.

**Functional attributes of Immunoglobulin G (IgG)**

*K. pneumoniae*, multidrug resistant organism is being studied extensively during recent times for its incidence often leading to high mortality. Our results show the dose dependent inhibition of *K. pneumoniae* by IgG. The antibacterial property of buffalo colostum IgG was confirmed by MIC and turbidometric assay. The most commonly used methods to determine the minimal inhibitory concentration (MIC) of antimicrobial agents are the agar dilution and broth dilution methods. The MIC is defined as the lowest concentration of substance that inhibits the growth of the microbes under controlled conditions (Lambert and Pearson, 2000). Madureira et al, (2007) studies showed the biological activity of whey proteins. The antimicrobial activity of whey peptides were reported against different types of bacteria, yeast and filamentous fungi. This potential might be due to low pH and presence of lactic acid, LF (iron 64 binding protein to sequester iron from bacteria inhibiting its growth and metabolism), LP (catalyse the oxidation of thiocyanate in hypothiocyanate ion which cause damage to bacterial cells) and Ig’s. The antimicrobial capacities of the whey protein products evaluated in disk susceptibility tests using bacterial strains Gram-positive *S. aureus*; Gram-negative- *K. pneumoniae, P.aeruginosa, Salmonella sp.* and *E. coli* (Jiménez et al, 2012). The study was further supported by adopting substantially more sensitive detection method to quantify the antimicrobial effects of IgG as compared to the less sensitive standard agar disc diffusion assay. The microtiter assay applied allowed the detection of antimicrobial effects of whey samples at low concentration levels (Dubber and Harder, 2008). However, similar studies on the bacteriolytic property of HMBA against *H. pylori* (Belagihalli et al,
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2011) and protein degradative activity of leaf extract on \textit{S. aureus} (Anas et al, 2008) have been reported.

The SEM analysis revealed the anti-adhesive properties of IgG containing oligosaccharide residues recognized by lectins expressed by the pathogens are most likely to modulate adhesion. Oligosaccharides are important components of milk or colostrum with bioefficacy as prebiotics, anti-infectives, and immune system modulators and as a possible source of sialic acid for neural function (Fong, 2011). Earlier investigations on protective role of milk glycogonjugates that act as soluble receptor analogs of epithelial cell surface and tend to compete with microbes for binding sits and these oligosaccharides have therapeutic and prophylactic potential in the gastrointestinal tract when they are consumed, because they can act as ‘decoys’ which can mimic host carbohydrate receptors for microbial lectins (Lane et al, 2010). In fact, Vilte et al. (2008) have shown that bovine colostrum IgG can reduce enterohemorrhagic \textit{E. coli} infection in neonates implicating its probable role in offering antibacterial protection to non breast fed infants in analogy to bactericidal effect of human milk oligosaccharides. Both \textit{in vivo} and \textit{in vitro} clinical studies proved anti-adhesive property of oligosaccharides (Prieto et al, 2007), these human milk oligosaccharides are more effective when present as mixture rather than an individual components. Sialylated oligosaccharides prevent the binding of certain septic \textit{E. coli} strains (Parkkinen et al, 1983) and inhibition of \textit{H. influenzae} (Andersson et al, 1986). Since cell morphological changes under adverse environmental conditions are the most visible parameters of bacterial adaptation (Shi and Xia, 2003), the changes observed in the present study with and without IgG treatment to \textit{K. pneumoniae} supports the same concept and the deformation observed
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in the present study is in conformity with the bacterial suicide response hypothesis
proposed by Aldsworth et al. (1999).

Adaptive responses in bacteria range from rapid transient changes in motility
to long-term global reorganizations of gene expression and cell morphology. As
evidenced in the present study, the expression of a new protein, CheY may be one
such response. CheY constitute a regulatory system involved in the transmission of
sensory signals from the chemoreceptors to the flagellar motors. It has been reported
that the flagella and chemotaxis proteins are important for cell adhesion and invasion
(Khoramian-Falsafi et al, 1990). CheY in bacteria is known to induce tumbling by
interacting with the flagellar switch protein FliM, inducing a change from
counterclockwise to clockwise rotation of the flagellum. Change in the rotation state
of a single flagellum can disrupt the entire flagellar bundle and cause a tumble as
evidenced by ultra-sensitive response of the motor to the CheY-P signal and
clustering of chemoreceptors (Cluzel et al, 2000; David and Sourjik, 2004).
Subsequently, the signaling protein CheY, was shown to bind the rotor protein FliN to
control the direction of flagellar rotation in *E. coli* (Sarkar et al, 2010). However, the
only report on bovine colostrum IgG indicated its pivotal role in reducing
enterohemorrhagic *E. coli* infection, thereby offering protection to non breast-fed
children (Vilte et al, 2008). Although not many reports are available from natural food
source to combat *K. pneumoniae* infection, reports from Chinese traditional medicine
and herbal extracts from medicinal plants are shown to have bactericidal property on
*K. pneumoniae* (Singh et al, 2012; Lin et al, 2013). However, human milk peptides
with immunomodulatory and antibacterial properties are of clinical relevance to the
proximal intestinal tract (Dallas et al, 2014). LF derived peptides have been shown to
suppress the intestinal overgrowth and bacterial translocation of enterobacteria in
mice (Teraguchi et al, 1995). It was also known to effectively eradicate *H. pylori* (Di Mario et al, 2003). Other *in vivo* studies on milk protein and peptides investigated its antiviral and immunomodulatory effects, and other host-protective activities such as cancer prevention, as well as clinical applications (Tomita et al, 2002; Marshall, 2004). Hence it would be rational approach to utilize colostrum IgG as anti-adhesive drug candidate to treat various infectious diseases including *K. pneumoniae*. But, expression of signaling protein in the present study in response to IgG needs a detailed investigation.

Earlier studies indicated in the role of porins in exhibiting higher antibiotic resistance as accomplished in *E. coli* (strain K-12) with special reference to two major porins, OmpC and OmpF (Domenech-Sanchez et al, 2000). OmpF, functional pore is somewhat larger than that of OmpC; indeed, it is easier for molecules to pass through the OmpF pore (Llobet et al, 2009). In *K. pneumoniae*, two major porins, OmpK35 and OmpK36, are analogous to OmpF and OmpC, respectively. Clinically, most of the Extended-spectrum beta-lactamases ESBL-producing *K. pneumoniae* strains express only OmpK36, whereas the majority of *K. pneumoniae* that do not produce ESBLs synthesize both OmpK35 and OmpK36 (Hernandez-Alles et al, 1999). Reports suggest that OmpK36 may play an important role in the resistance or reduced susceptibility to carbapenems in *K. pneumoniae* that produce ESBL or AmpC-type β-lactamases (Wang et al, 2009). In the light of this information, we planned to verify the interaction of small peptides to OmpK using molecular docking studies.

Molecular modelling has proven increasingly important in helping to design novel ligand receptor structure-based interaction and pharmacophore-based drug designing. Hence, the bioactivity of the IgG peptides unravelled in the present investigation was verified with osmoporin. The small peptide sequences
Osmoporins are a transmembrane channel which allows diffusion of antibiotics and many other substances and is considered as an ideal target for developing a drug against several bacterial infections. A reasonable concordance was observed between in vitro and in silico results with antibiotic combinations. In a similar study on clinical strains of E. coli identified sequence alterations of charged and polar residues within the pore of the related OmpC porin that altered antibiotic susceptibility without affecting pore size (Lou et al., 2011). The preliminary study carried out using ovine β-casein hydrolyzate after pepsin, trypsin and chymotrypsin digestion was used as a biosensor for quorum sensing molecules and it indicated that the antimicrobial peptides from ovine β-casein may interfere with cell-signaling in bacterial populations (Gómez-Ruiz et al, 2005). This report is in analogy to our observations with IgG peptides interacting with porin as evidenced by the expression of a signaling protein. Hence, the findings of the present study with reference to biologics can be exploited further to treat/cure various microbial infections as food derived components induce no side effects.