Review of Literature
2. Review of Literature

Colostrum is the complex physiological fluid secreted by the mammary glands of mammals that fulfill the nutritional needs of neonates. It comprises of vitamins, minerals and amino acids, putative permeability fraction and fraction containing enzymes, proteins, peptides and bioactive components such as glycoconjugates, growth and tissue repair factors, phagocytes antimicrobial compounds (Hooijdonk et al, 2000; Figure 2). In addition, it also endows the newborn with components that protect against infection from a wide range of pathogens (Jackson and Nazar, 2006). These immune-enhancing components are significantly unique from those associated with mature milk and impart passive immunity to the newborn thus preventing infections (Gopal and Gill, 2000).

Figure 2. Colostrum composition
(Source: www.foodandnutritionjournal.org)

Colostrum first fascinated the Western medicinal community for its potential health benefits in the late eighteenth century. Some of the protective properties of colostrum can be attributed to immunoglobulins. However, the effectiveness of
colostrum is not only limited to the protection against infection by immunoglobulins. It has high concentrations of lactoperoxidase, lactoferrin growth hormones and growth factors such as transforming growth factor β (TGF-β1 and β2) and insulin-like growth factor (IGF-1), which are beneficial in anti-aging (Korhonen et al., 2000; Shah, 2000; Elfstand et al., 2002; Tripathi and Vashishtha, 2006; Korhonen, 2012). In addition, the macromolecules in colostrum are resistant to enzymatic digestion in the gastrointestinal tract and have the ability to inhibit the adherence of enteropathogens to the gastrointestinal tract of the neonates (Uruakpa, 2002). Glycoconjugates of bovine milk exist in two different forms as glycolipids and glycoproteins. The significant role of the glycoconjugates as bioactive molecules is to provide immunity against pathogens in neonatal health which led to vast commercial/pharmaceutical exploitation of colostrum. As a result, bovine colostrum was prescribed for many conditions, including immune system boosting. Hence, bovine colostrum is considered as a universal donor of colostrum to human (Godhia and Patel, 2013). Infact, buffalo milk is renowned for its significant nutritive value owing to the presence of nearly twice as much quantity of fat, lactose, casein, whey proteins and mineral components compared to cow milk (Table 2). India contributes to about 53% of the total buffalo milk production in the world (FAO, 2012, 2016). Since ages, buffalo milk has been valued for its essential chemical composition which influences the nutritive properties (Ahmad et al., 2013). The triglyceride content present in buffalo milk (9-12%) makes it thicker than cow milk. It is also a good source of conjugated linoleic acid, a group of polyunsaturated fatty acids. Potential physiological effects include anti-adipogenic, anti-diabetogenic, anti-carcinogenic, and anti-atherosclerotic properties (Khedkar et al., 2016).
Table 2. Comparison of Cow, Buffalo and Human Milk

<table>
<thead>
<tr>
<th>Nutrients</th>
<th>Cow</th>
<th>Buffalo</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water, g</td>
<td>88.0</td>
<td>84.0</td>
<td>87.5</td>
</tr>
<tr>
<td>Energy, kcal</td>
<td>61.0</td>
<td>97.0</td>
<td>70.0</td>
</tr>
<tr>
<td>Protein, g</td>
<td>3.2</td>
<td>3.7</td>
<td>1.0</td>
</tr>
<tr>
<td>Fat, g</td>
<td>3.4</td>
<td>6.9</td>
<td>4.4</td>
</tr>
<tr>
<td>Lactose, g</td>
<td>4.7</td>
<td>5.2</td>
<td>6.9</td>
</tr>
<tr>
<td>Minerals, g</td>
<td>0.72</td>
<td>0.79</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Source: Tomer et al, (2011)

Colostrum in particular contains significant number of leukocytes (5x10^6 cells/ml), which decreases tenfold in mature milk. Colostral leukocytes are primarily composed of lymphocytes (23%), neutrophils (38%) and macrophages (40%), which phagocytose microbes (Quigley, 2001). Lymphocytes, including T cells, natural killer cells, and antibody-producing B cells, make up 10% of the leukocytes in human breast milk. Each of these cells contributes to the overall cellular immune system in animals. This evidence suggests that these cells survive passage through the infant’s gastrointestinal system where they are absorbed and exert influence on infant’s immune response (Oddy, 2002).

**Whey proteins**

Colostrum or milk contains heterogeneous mixture of proteins (caseins and whey) and peptides. Whey is the liquid by-product produced during cheese manufacturing process after the precipitation of caseins. In cheese processing, there are two types of whey; acidic whey obtained from a process of curd formation (cottage cheese) and sweet, derived from the manufacture of rennet-produced cheeses (Smith et al, 2016). **Whey proteins constitute about 60-80% of total colostrum**
whereas, caseins predominate in mature milk accounting for approximately 80% of the total protein content. However, the whey protein composition is not the same in all mammals. Human milk is characterized by high concentration of whey proteins, constituting about 70% of the total protein content (Liao et al, 2011). It is made up of multitude of proteins that remain soluble providing heterogeneous mixture of proteins having biological value (Smithers, 2008). The worldwide production of liquid whey is estimated to about 180 million tonnes in 2013 (Dairy processing handbook, Chapter 15). Whey can be converted into lactose-free whey powder, condensed whey, whey protein concentrates (WPC) and whey protein isolates (WPI) all of which are currently commercially available (Whey Protein Institute, 2003). The major whey proteins are β-lactoglobulin(β-lg), α-lactalbumin(α-la), immunoglobulins(Ig’s), serum albumin(SA) besides, whey also contains numerous minor proteins, called low abundance proteins, such as lactoferrin (LF), lactoperoxidase (LP), proteose peptone (PP), osteopontin (OPN), lysozyme (LZ), among others; LF and LP are the most abundant minor proteins (Jovanovic et al, 2007; Santos et al, 2012).

Whey is a significant resource for proteins associated with immune-stimulating, antibacterial, antithrombotic, antihypertensive and opioid-like properties (Sharma et al, 2011). The Ig’s, LZ, and LF present in whey can resist the low pH and the digestive enzymes of upper gastrointestinal (GI) tract owing to the presence of trypsin inhibitor in colostrum, thus allowing these proteins to remain biologically active (Dallus et al, 2012). Thus whey protein is a complete, high quality globular protein with a rich amino acid profile plays an important role in tissue growth and repair (Anandharamakrishnan et al, 2007). The potential health benefits attributed to whey proteins has increased greatly in recent years (Marshall, 2004). Gauthier et al. (2006) assessed the immunomodulatory activity of whey protein hydrolysates by
focusing on their effects on promotion of phagocytic activity, cytokine secretion, antibody production, lymphocyte activation and proliferation. Whey proteins are also reportedly involved in the *in vitro* suppression of lymphocyte mitogenesis and alloantigen-induced proliferation when included in murine lymphocytes culture (Oliver et al, 1989; Barta et al, 1991). Whey is also used in the manufacture of iron protinate, an anti-anaemic preparation (Dalev, 1994). Whole whey proteins have been reported to prevent breast and intestinal cancer (Hakkak et al, 2001). It is also known to aid in the treatment of HIV patients (Clare et al, 2003). Many bioactive components derived from whey have been explored for their ability to confer specific health benefits to modulate adiposity, enhance immune function, for antioxidant activity and as a popular supplement among athletes and bodybuilders (Hayes & Cribb, 2008). The multifunctional property associated with whey includes anti-hypertensive, anti-oxidant, hypocholestrolaemic, opioid, anti-microbial, immunomodulatory and cytomodulatory activities (Korhonen and Pihlanto, 2006). Many peptides of β-lg derived after various proteases have been identified to possess moderate to high angiotensin I- converting enzyme activity (Hernandez-Ledesma et al, 2008).

The glycosylation of immune protective proteins in milk also renders them partially resistant to digestion and supports their biological functions in the distal gut. In addition, these milk glycoproteins (Kiyohara et al, 2011; Oda et al, 2013) and glycopeptides (Liepke et al, 2002) possess glycans, which structurally resemble HMOs and can serve as growth substrates for infant-borne bifidobacteria. Interestingly, some bifidobacteria even possess specific endoglycosidases that enable these strains to cleave the glycan portion away from the cognate glycoproteins, thus freeing the glycan substrate for consumption. Colostrum IgG provides a multitude of
functions—fixation of complement, opsonization, agglutination of bacteria and neutralizing toxins and viruses (Korhonen and Marnila, 2002). The diverse proteins and peptides that comprise whey have essential biofunctional characteristics to modulate some regulatory processes. Owing to the unique nutritional and physiological functions, whey has earned the status of a “functional food” in the preparations, fractions or extracts containing bioactive compounds of varying purity, that are used as ingredients by manufacturers in the food designed to reduce or control chronic diseases and promote health (Lopez-Rubio et al, 2012). Hence, a detailed study on whey based glycoprotein like IgG would be worthwhile to unravel the structural and functional significance of it in early life of new born.

Immunoglobulin G (IgG)

Immunoglobulins (Igs) are the family of globular whey proteins with antimicrobial and other protective biological activities. They are the principal glycoproteins that protect the gut mucosa against pathogenic microorganisms, and in colostrum they confer passive immunity from mother to neonate (Korhonen et al, 2000). Four classes of Igs have been identified in lacteal secretions- IgG, IgA1, IgA2 and IgM (Kulkarni et al, 1981; Table 3). In bovine colostrum and milk, IgG is the major immune component, with low levels of IgA and IgM (Lilius & Marnila, 2001). IgG is the major whey glycosylated protein that makes up 80-90% of the total Igs in colostrum. Earlier investigations indicated the presence of only two subclasses of IgG in bovine colostrum (IgG1 and IgG2) (El-Loly, 2007). IgG1 (~75%) is many fold greater in concentration than IgG2 of colostral whey (Larson et al, 1980). The estimated levels of total immunoglobulins in buffalo colostrum IgG, IgM and IgA were found to occur in the ratio 85-90%, 7% and 5% respectively (Singh et al, 1993; Dang et al, 2009). The immunoglobulin fraction in buffalo colostrum responsible for
increased phagocytic activity was found to be enriched with IgG (Dang et al, 2007a; Dang et al, 2009). In fact, IgG from ovine colostrum was widely utilized for the immunological supplementation of foods, specifically in infant formulae (Gapper et al, 2007).

Table 3. Immunoglobulins in ruminant colostrum and milk

<table>
<thead>
<tr>
<th>Ig mg/ml</th>
<th>Colostrum</th>
<th>Milk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total IgG</td>
<td>32-212</td>
<td>0.72</td>
</tr>
<tr>
<td>IgG1</td>
<td>20-200</td>
<td>0.6</td>
</tr>
<tr>
<td>IgG2</td>
<td>12.0</td>
<td>0.12</td>
</tr>
<tr>
<td>IgA</td>
<td>3.5</td>
<td>0.13</td>
</tr>
<tr>
<td>IgM</td>
<td>8.7</td>
<td>0.04</td>
</tr>
</tbody>
</table>

(Source: Adar et al, 2009)

IgG is the heterodimeric multifunctional protein with a molecular weight of about 150,000 Daltons. It is composed of two polypeptide chains, heavy (H, 50 kDa) and light (L, 25 kDa) chains (Kobata, 2008). The light chains characteristically fold into two domains and heavy chains into four domains (Wright et al, 1991) that are connected to one another by disulfide bridges and appear as Y-shape molecule. The stem of the Y is the Fc region and it consists mainly of two identical halves of the H chains. Each of the arms of the Y contains one complete L chain and half of one of the H chains. The two arm is referred to as the Fab region, which has two antigen binding fragments (Fab)2 and is said to be divalent. It is the antigen binding fragment of the antibody molecule (Janeway et al, 2001). The upper hinge-towards the amino-terminal segment allows variability of the angle between the Fab arms (Fab-Fab flexibility) as well as rotational flexibility of each individual Fab. The flexibility of
the lower hinge region (towards the carboxy-terminal) determines the position of the Fab-arms relative to the Fc region (Fab-Fc flexibility). Hinge-dependent Fab-Fab and Fab-Fc flexibility may be important in triggering further effector functions such as complement activation and Fc receptor binding. IgG has a single N-linked biantennary structure at Asn297 that is buried between the CH2 domains (Nimmerjahn and Ravetch, 2005).

**Isolation and purification of IgG**

Scientific and commercial interest in the biological properties and nutritional value of colostrum IgG has prompted the development of various methods for isolating and purifying IgG. Kinghorn et al. (1995) used capillary zone electrophoresis to quantify the four major bovine whey proteins (BSA, β-LG, α-LA and IgG) in both liquid whey samples and reconstituted WPC powder. Separation and quantification of the major bovine milk proteins was carried out by reverse-phase-HPLC (Bobe et al, 1998). Later, simultaneous Precipitation and salting out were widely used to selectively precipitate and concentrate bovine whey proteins (Xu et al, 2000). Kumar et al. (2013) applied various membrane separation techniques like ultra-filtration, microfiltration, reverse osmosis and cation exchange resin as molecular sieve for the isolation of colostral whey.

Various analytical approaches based on either separation techniques or immuno assays are employed for the purification and quantitation of IgG from colostrum and milk samples (McKinney and Parkinson, 1987; Konecny et al, 1994; Levieux and Ollier, 1999; Korhonen et al, 2000; Gapper et al, 2007; Bergmann-Leitner et al, 2008; Menegatti et al, 2012). Using immobilized egg yolk, bovine IgG subclasses were isolated from milk (Akita and Li-Chan, 1988). Subsequently,
microfiltration based affinity purification with membrane filtration was initially used to isolate LF and IgG from cheddar cheese whey (Chen and Wang, 1991). Subsequently, bovine IgG subclasses were isolated from immobilized egg yolk antibodies from bovine milk using immunoaffinity chromatography technique (Akita & Li-Chan, 1998). Later, preparative scale HPLC was found very effective for the purification of polyclonal antibodies from bovine serum (Jan and Leokadia, 2004) while, monoclonal antibodies from transgenic goat milk were purified using ultrafiltration (Baruah et al, 2006). In addition, Copestake et al. (2006) used protein G to quantitate IgG in colostrum based powder. Small scale production of IgG was achieved by ammonium sulfate or ethanol precipitation followed by chromatographic separation (El-Loly and Farrag, 2007). The most suitable protocol for isolation and purification of IgA and IgG from bovine colostrum was reported by Liu (2007) which involves salting out, ultra-filtration and gel chromatography; which provided theoretical and experimental foundation for industrial production of IgA and IgG. Mian-bin et al. (2009) purified IgG from bovine colostrum using serial cation-anion exchange chromatography. Bereli (2006) developed cost effective procedure for purification of human plasma IgG using Poly hydroxyethyl methacrylate-N-methacyrloyl-(I)-histidine methyl ester monoliths with purity of 95.3%. Prasanna et al. (2010) successfully showed that Cu$^{2+}$ and Ni$^{2+}$ loaded convective interaction media- iminodiacetic acid monolithic for the purification of both polyclonal and monoclonal IgG. Although microfiltration based affinity purification with membrane filtration was used to isolate LF and IgG from cheddar cheese whey (Tong et al, 2012) while, Kavaz et al (2012) developed another effective separation parameter for IgG purification by using magnetic chitosan nanoparticles. The method developed by Menegatti et al. (2013) involving cyclo hexamer appears to be an attractive candidate for developing a
cost-effective and robust chromatographic resin to purify monoclonal antibodies. More recently, a protein A-mimetic peptide ligand was used as an alternative affinity ligand for selective isolation of bovine IgG with a purity of >85% from skim milk, acid whey and colostrum (Billakanti et al, 2014).

**Proteomic characterization of IgG**

Generally characterization of glycoprotein IgG involves various analytical approaches based on either separation techniques or immune assays employed for the purification and quantitation of IgG from serum and milk samples (McKinney et al, 1987; Konecny et al, 1994; Levieux and Ollier, 1999; Korhonen et al, 2000; Gapper et al, 2007; Bergmann-Leitner et al, 2008; Menegatti et al, 2012; Tong et al, 2012). Among them, liquid chromatography-mass spectrometry (LC-MS) is currently the most powerful analytical tool for structural characterization of antibodies due to its superior resolution, sensitivity and accuracy. LC coupled to tandem mass spectrometry (LC-MS/MS) has become a promising platform for quantifying therapeutic proteins including antibody molecules in biological samples (Ezan and Bitsch, 2009; Goetze et al, 2011; Wu et al, 2011; Li et al, 2012; Mesmin et al, 2012).

Chougule and Aparna, (2011) used Liquid chromatography-Electro-spray ionization-mass spectrometry (LC-ESI/MS) to characterize β-lactoglobulin, a major lipocalin present in whey from buffalo colostrum. Proteomic analysis of bovine colostrum whey was studied by Golinelli et al. (2011) using two-dimensional electrophoresis in order to analyze minor proteins by MALDI-TOF/TOF MS. Reliable quantification of human IgG Fc region by LC-MS/MS has emerged as a promising assay platform for the bioanalysis of therapeutic protein candidates (Furlong et al, 2012). Boutz et al. (2014) demonstrated a strategy to improve the accuracy of antibody identification by
shotgun proteomics through the use of high mass accuracy LC-MS/MS and high stringency filters applied to groups of peptide-spectral matches.

Characterization of IgG using *in vitro* digestion with proteolytic enzymes is well-studied. The primary digestive enzymes in humans for proteins are pepsin, in the stomach, followed by trypsin and chymotrypsin, in the small intestine (Hur et al, 2011), which digest IgG to Fab dimers (~100 kDa) and monomer Fab fragments (~50 kDa) (Porter, 1959). The digestion of IgG at the hinge region with papain produces two active domains, F(ab)_2 and Fc (Jasion and Burnett, 2015). Furthermore, modulating the reaction time, pH and temperature during enzymatic digestion will result in a variety of active domain fragments. Pepsin is known to be highly effective in digesting all redundant low molecular weight serum components leaving F(ab)V2 portion intact. Ig fragmentation and subsequent sequence determination by means of MS is challenging. The structural analysis of intact and S–S bond reduced IgG was studied by employing Orbitrap Electron Transfer Dissociation- tandem mass spectrometry (ETD MS/MS) (Fornelli et al, 2012). Jones and Landon (2002) digested ovine antiserum and its purified IgG for therapeutic use. By selectively cleaving an antibody with proteases by using reducing agents, it is possible to engineer fragments with discrete characteristics. It is also possible to express small fragments with genetic engineering techniques directly (Harmsen and De Haard, 2007). In some cases it may be desirable to remove the Fc domain, which may mediate unwanted binding events, leaving a bivalent Fab’2 fragment, where small size is important for better tissue penetration a Fab’ (derived from Fab’2) or Fab may be preferred (Holliger and Hudson, 2005). Such antibody fragments are commonly used as the foundation for drug molecules as they have lower immunogenicity than intact antibodies. Analysis of IgG using a unique enzyme combination IgG-degrading enzyme of *Streptococcus*
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*pyogenes* (IdeS) and endoglycosidase S (EndoS), utilizing RPC18 LC-ESI/MS studied by Frediksson et al. (2010). Recently endopeptidase, IdeS was used to cleave heavy chains below the hinge region, producing F(ab’)2 and Fc fragments, following reduction of disulfide bonds, three antibody domains which could be released and further characterized by LC-MS, capillary isoelectric focusing (An et al, 2014).

**Glycome characterization of IgG**

Glycoconjugates are the biomolecules either-proteins or lipids that are conjugated to glycan/ saccharide moiety. Glycosylation is the process that involves the enzyme catalyzed attachment of glycan moiety to proteins, the important post-translational modification that is vital for the function of proteins. Two types of protein glycosylation exist: N-linked and O-linked. N-glycans are found on proteins with a consensus sequence NXT/S where N is asparagine, X is any amino acid except proline, and the third amino acid can be either threonine (T) or serine (S) and, in rare cases, cysteine. O-glycosylation may occur at any serine or threonine residue with no single common core structure or consensus protein sequence. Because glycosylation is dictated by a set of competing glycosyltransferases, the glycosylation patterns of glycoproteins are very complicated. The population of glycans occurring at a given glycosylation site is often heterogeneous such that a specific N-glycosylation and O-glycosylation site may be occupied by a number of structurally distinct glycans and described as microheterogeneity (An et al, 2009). Moreover, glycosylation changes significantly during inflammation, sepsis and cancers (McCarthy et al, 2014). It also limits access to protease and peptidase cleavage sites and determines the ability of the remaining glycopeptide to persist and transit to lower parts of the intestine.
IgGs form immune complexes with antigen pathogen subsequently activating effector mechanism such as antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) ultimately resulting in the clearance and destruction (Arnold et al, 2007). Non-fucosylated IgG is known to exhibit an increased ADCC (Zeitlin et al, 2011) and antiviral activities (Shade and Anthony, 2013) in a mouse model system (Nimmerjahn et al, 2005; Junttila et al, 2010). However, sialylation of the Fc N-glycans is majorly responsible for the immunosuppressive property of IgG (Kaneko et al, 2006). Hence glycosylation of IgG is vital for its various roles to neutralize antigens, cell recognition, cell-cell interaction, signaling events, influencing the gut microbiota and prevention of adherence of pathogens to the neonatal intestine (Alfaleh et al, 2011). The glycosylation of immune protective proteins in milk also renders them partially resistant to digestion and supports their biological functions in the distal gut (Newburg and Walker, 2007). In addition, milk glycoproteins (Kiyohara et al, 2012; Oda et al, 2013) and glycopeptides (Liepke et al, 2002) possess glycans, which structurally resemble human milk oligosaccharides and can serve as growth substrates for infant-borne bifidobacteria. Buffalo colostrum whey proteins were found to enhance the growth of *Bifidobacterium bifidus* (Aparna and salimath, 1999). Interestingly, some bifidobacteria even possess specific endoglycosidases that enable these strains to cleave the glycan portion away from the cognate glycoproteins, thus freeing the glycan substrate for consumption. More recently structural and functional characteristics of bovine milk protein glycosylation was reviewed by O'Riordan et al. (2014) indicating various glycoforms of whey and their implications in neonatal health.
Quantitative study of human and bovine polyclonal IgG and monoclonal IgG1 were characterized by fluorophore assisted carbohydrate electrophoresis (FACE) and high pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The analysis revealed (Saba et al, 2002) presence of predominant N-linked structures having core-fucosyl asialyl biantennary chains with varying galactosylation including minor amounts of afucosyl, bisected, and monosialyl oligosaccharides. Later, gel-separated nonsialylated N-glycoproteins was introduced as a fast and sensitive method of characterization by Larsen et al. (2005). The method takes advantage of enzymatic treatment followed by selective purification and characterization of the glycopeptides using graphite powder micro columns in combination with mass spectrometry. The human milk analysis revealed a 6% high mannose, 57% sialylation, and 75% fucosylation distribution, while, 10% high mannose, 68% sialylation, and 31% fucosylation distribution was observed in the bovine milk analysis. Although certain compositional similarities were identified in both milk oligosaccharides, such studies also identified remarkable difference in the structures and relative abundances of the different oligosaccharides types in both milk sources (Totten et al, 2012). The presence of terminal fucose residues on the milk glycoproteins was thus a defining feature of human rather than bovine milk (Newburg, 1999). Glycomic approaches toward profiling N-glycans from a wide range of biological samples typically employ the release of the glycans via PNGase F treatment prior to MS analysis (Kita et al, 2007). N-glycolylneuraminic acid (NeuGc) prevents determining composition based strictly on accurate mass because combinations of fucose (Fuc) and NeuGc can yield masses equivalent to oligosaccharides containing N-acetylneuraminic acid (NeuAc) and hexose (Hex). Garrido et al. (2012) analyzed N-glycans using nano-flow liquid chromatography
coupled with quadrupole time-of-flight mass spectrometry following chromatographic separation on a porous graphitized carbon chip. In all, 38 N-glycan compositions were observed in the human milk sample while the bovine milk sample revealed 51 N-glycan compositions. Although NeuAc sialylation was observed in both milk samples, the NeuGc residue was only observed in bovine milk and marks major difference between human and bovine milks. This study is the first MS based confirmation of NeuGc in milk protein bound glycans as well as the first comprehensive N-glycan profile of bovine milk proteins. Hence, MS-based analysis appears as an efficient method for identification and quantification of oligosaccharides in glycomic studies and endorses the power of MS for glycopeptide characterization with high sensitivity in glycoproteomic research. Thus, MS has emerged as the leading tool for oligosaccharide analysis with high sensitivity for more than a decade (Park and Lebrilla, 2004).

Similarly, Yang and Hancock (2004) developed analytical approach to study glycosylation patterns by multi-lectin affinity column containing concanavalin A (Con A) from human serum, the captured glycopeptides were specific, efficient and reproducible. Similarly, glycan analysis of oligosaccharides released from IgG-Fc was developed by employing HPLC, MALDI-TOF MS and MS/MS techniques (Wada et al, 2007). A disease-related glycosylation change of non sialylated N-glycoproteins from a 2-D gel spot was analyzed by MALDI-TOF/TOF which enables the analysis of large glycopeptides over 4000 Dalton and low-abundance glycopeptides (Hao et al, 2010). Both the sialylated and non-sialylated glycopeptides of human plasma IgG were analyzed by intermediate pressure matrix-assisted laser desorption ionization fourier transform ion cyclotron resonance mass spectrometry (MALDI-FTICR-MS) which analyses at much shorter time and allow high resolution profiles compared to
LC-MS (O’Connor et al, 2002). However, MALDI-FTICR-MS with dihydroxybenzoic acid (DHB) and α-cyano-4-hydroxycinnamic acid (CHCA) matrices provided robust and reproducible IgG glycopeptides profiles (Selman et al, 2010). The oligosaccharides profile in bovine milk and dairy streams can also now be accomplished using novel methodologies such as microchip liquid chromatography separation and high performance MS techniques, including TOF and quadrupole time-of-flight analyzers (qTOF) (Wu et al, 2010; Wu et al, 2011). Takimori et al (2011) analyzed early bovine milk N-glycome during the early lactation stage, using chemoselective glycoblotting technique and MALDI-TOF/TOF MS analysis. In this method the glycans derived are selectively captured onto novel high-density hydrazide beads for highly efficient purification of oligosaccharides from complex biological samples. In addition, a total of 39 N-glycans were enzymatically released from bovine milk whey protein concentrate by 2-D HPLC mapping technique and MALDI-TOF (Suzuki, 2012). Hong et al (2013) developed a method for the absolute quantitation of human IgG and its glycoforms directly from serum using multiple reaction monitoring (MRM) on a triple quadrupole mass spectrometer (QqQ-MS).

A complete structural assignment of neutral and sialylated oligosaccharide of plasma-derived human Ig’s was separated by RP chromatography and analyzed by ESI-MS, and the released oligosaccharides were separated on porous graphitic carbon (PGC) (Stadlmann et al, 2008). Human serum IgG subclasses (IgG1, IgG2, IgG3, IgG4) glycosylation profiles vary during pregnancy, rheumatoid arthritis and several diseases. The distinct difference between IgG subclasses was obtained by a robust method of nano LC-MS (Wuhrer et al, 2007). Dallas et al (2011) employed nano flow liquid chromatography (nanoLC) with a graphitized carbon chip and orthogonal time-of-flight (TOF) mass spectrometry for N-glycan profiling of human milk. 52 N-
glycans were identified; this ability to separate and detect heterogeneous mixtures with high resolution peaks and mass accuracy makes this technique excellent. Another strategy to analyze IgG Fc glycosylation on biopharmaceuticals involves ESI-high-resolution-MS/MS (Reusch et al, 2012). In addition, glycan sequencing by LC-ESI-CID-MS/MS improved speed and sensitivity (Jensen et al, 2012). Fong et al. (2011) demonstrated a hydrophilic interaction chromatography high-performance liquid chromatography-high resolution selected reaction monitoring-mass spectrometry (HILIC HPLC-HRSRM-MS) method for measuring different oligosaccharides in bovine milk, bovine colostrum and infant formulas. N-linked glycans from human and bovine polyclonal immunoglobulin G characterized by fluorophore-assisted carbohydrate electrophoresis (FACE) and high pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) both MALDI-MS and HPLC/MS methods generated results in good agreement with HPAEC-PAD and FACE data (Saba et al, 2002). IgG N-glycans from human serum was fluorescently labeled and profiled using Ultra Performance Liquid Chromatography Capillary Electrophoresis with Laser Induced Fluorescence Detection (UPLC-CE-LIF; Mittermayr et al, 2011). High mass accuracy obtained with high resolution mass spectrometers allows determination of the glycoform composition on intact monoclonal antibodies based on accurate mass with a typical 15-2 ppm mass accuracy error. High resolution mass accuracy of Fourier transform ion cyclotron resonance (FT-ICR) is an ideal tool for oligosaccharide analysis (Park and Lebrilla, 2005). Quantitative glycan profiling was established using high resolution HPLC with 2-aminobenzoic acid labeling which allows an accurate determination of sialylation levels. Glycomic content in Fc and Fab was determined using an internal standard for accurate glycan recoveries (Jefferis, 2005; Arnold et al., 2007; Kobata, 2008; Anumula, 2012).
Functional attributes of IgG

Milk provides the ultimate model for functional food development being endowed with nutritional, immunological and biologically active components (Hedley et al, 2006). Shrinivas et al, (2010) reported 90 useful components of bovine colostrum with therapeutic potential to the human being due to its versatile composition and the property to stimulate immune system.

Anti-adhesive property colostrum/milk oligosaccharides

Mammalian milk or colostrum usually contains a variety of saccharides. Human milk or colostrum has been reported to contain more than 100 oligosaccharides (Ballard and Morrow, 2013). Milk oligosaccharides are known to be one of the selective pressures enforced on the gut microbiota and exert prebiotic effect (Coppa et al, 2006). The committee of the European Society of Pediatric Gastroenterology, Hepatology and Nutrition in 2004 emphasized the role of intestinal microbiota especially bifidobacteria in breast-fed infants in the inhibition of colonization of pathogen (Knol et al, 2005) and also modulation of the systemic immunologic and inflammatory responses (Figure 3).

At present, there is limited data on oligosaccharides of buffalo colostrum or milk (Aparna and Salimath, 1996) compared to human milk or colostrum, whereas fewer number of oligosaccharides have been identified in cow, goat and sheep (Urashima et al, 1997; Martinez-Ferez et al, 2006). A glycan isolated from human milk was shown to stimulate the growth of Lactobacillus bifidus and was termed the ‘Bifidus Factor’ (Gyorgy et al, 1974). Saksena et al. (1999) found that processed oligosaccharide mixture of buffalo milk stimulated of delayed-type hypersensitivity and non-specific immune responses in teams of macrophage migration index while,
buffalo colostrum acidic glycoproteins were found to act as bifidogenic substances enumerating the growth of *Bifidobacterium bifidus* (Aparna and Salimath, 1999).

Milk oligosaccharides were shown to strongly inhibit hemagglutination mediated by enterotoxigenic *E. coli* (ETEC) and uropathogenic *E. coli* (UPEC) strains, however, the inhibitory activity decreased when the oligosaccharides were desialylated (Martin-Sosa et al, 2002). Fucosylated oligosaccharides were found to bind to members of the *calici* virus family of enteric pathogens which cause diarrhoea in humans, especially in infants (Marionneau and Ruvoen, 2002).

![Figure 3. Glycan epitopes as soluble receptors preventing bacterial adherence](image)

A) A pathogen has multiple glycan-recognizing receptors on its surface (such as adhesins, fimbriae or pili) which can bind to epithelial cells of the gastrointestinal or respiratory tract. Once bound, the pathogens can cause infection. B) Ingestion of glycan-containing molecules (glycoproteins, glycolipids or soluble oligosaccharides) with identical glycan epitopes as recognized by the pathogen results in limited adhesion and a reduced risk of infection (Peterson et al, 2013).

It has long been recognized that breast-fed infants exerts a competitive inhibition on enteric pathogenic bacteria and undoubtedly ‘fine-tunes’ the immune response against infections. Several *in vitro* studies show that fucosylated
glycoconjugates of human milk inhibit binding of Campylobacter to intestinal cells in vivo, a bacterium known to cause diarrhea world wide (Ruiz-Palacios, 2003).

Some of the biological functions attributed to human milk oligosaccharides include prebiotic activity, anti-adhesive activity, anti-inflammatory properties, modification of the entire complement of cell surface sugars, development of the brain, influencing growth-related characteristics of intestinal cells and other uncharacterized effects (Newburg et al, 2005; Bode, 2006; Kunz and Rudloff, 2006; Hickey, 2012; Gilda et al, 2013). Few clinical studies have been reported in the literature, which help strengthen the link between the in vitro anti-adhesive properties of defined oligosaccharides and in vivo prevention (Espinosa et al, 2007). Sialic acid is the most important structural element in the acidic fraction of bovine milk oligosaccharides. Earlier studies demonstrate that sialylated oligosaccharides, such as 3'-sialyllactose and 6'-sialyllactose are potent inhibitors of pathogen adhesion and these structures are present in both human and bovine milk (Gopal and Gill, 2000; Boehm and Stahl, 2007). Sialic acids also comprise the terminal functional residue of brain gangliosides and glycoproteins, such as neural cell adhesion molecule and have important roles in the development of the infant brain (McJarrow et al, 2009). In addition, bovine milk oligosaccharides being much less complex than those found in human milk also posses anti-adhesive effects against certain pathogens (Wang et al, 2001; Hakkarainen et al, 2005; Tao et al, 2009; Zivkovic and Barile, 2011). So far, the antiadhesive effect of human milk neutral oligosaccharides has been documented for enteropathogenic E.coli, Vibrio cholerae, and Salmonella fyris pathogenic bacteria causing diarrhea in infants (Coppa et al, 2006). The overall process of binding involves the assembly of a solvated polyhydroxylated glycan on the surface of cells, with a solvated protein-combining site (adhesin) present on the pathogenic agent; the
surface on the glycan is complementary to the protein combining site. Coppa et al. (1990) observed that a mixture of neutral oligosaccharides, mainly tri & tetrasaccharides, inhibited the adhesion of uropathogenic *E. coli* to human uro-epithelial cells, while, as far as enteropathogenic *E. coli* is concerned, Cravioto et al. (1991) showed that fucosyl-tetra-pentasaccharides were able to inhibit its adhesion to HEp-2 cells. This study confirms the capacity of fucosyl-oligosaccharides containing fractions to inhibit the adhesion of enteropathogenic *E. coli*. Colostrum or milk derived Ig concentrates from of hyperimmununized cows was found to be effective in prevention of some infections with *Shigella flexneri* and virulent strains of *E. coli* (Tacket et al, 1992). Further, human milk oligosaccharides were shown to be effective anti-adhesive agents using different epithelial cell lines; mainly by sialyl-oligosaccharides as confirmed by animal models (rhesus monkeys) (Mysore et al, 1999). Studies on hyperimmune powdered colostrum extract formulated as tablets evaluated its efficacy 30 participants, found that active tablet formulations (400mg) were more effective than placebo in protecting volunteers against the development of diarrhoea caused by enterotoxigenic *E. coli* (Otto et al, 2011). In addition, Gal/GalNAc HMOs significantly reduce *Entamoeba histolytica* attachment and cytotoxicity in co-cultures with human intestinal epithelial cell lines (Jantscher-Krenn et al, 2012), which facilitates parasite attachment as well as phagocytosis of intestinal epithelial cells.

In addition to glycans, milk is also the rich source of antimicrobial proteins and peptides capable of exerting antimicrobial activities comparable to antibiotics (Joerger, 2003; Severin and Wenshui, 2005; Lopez-Exposito and Recio, 2008). This effect was due to the synergistic activity of naturally occurring peptides and defence proteins besides Ig’s, LF, LP and LZ and is greater than the individual contribution
(Severin and Wenshui, 2005; Benkerroum, 2008). Milk peptides are known to exhibit antimicrobial activity against a broad range of Gram positive and Gram negative bacteria including the food pathogens *Listeria monocytogenes* (Clare 2003; Floris et al, 2003). Several immunomodulatory peptides have been found in bovine and human milk (Gill et al, 2000; Politis and Chronopoulou, 2008). In fact, some immune peptides derived from casein have been used by human and murine macrophages to protect against *Klebsiella pneumoniae* infection in mice (Smacchi and Gobetti, 2000). Incidentally, bovine colostrum IgG indicated its pivotal role in reducing enterohemorrhagic *E. coli* infection, thereby offers protection to non breast-fed children (Vilte et al, 2008).

**Colostrum/ Milk Prophylaxis**

About 75 preclinical studies have documented the nutritive benefits of the bovine Ig-containing protein preparations in terms of improving feed intake, growth, and sometimes feed conversion (Petschow et al, 2014). Oral prophylaxis with hyper immune bovine milk Ig provided 90% protection against clinical diarrhoea caused by oral challenge with ETEC (Tacket et al, 1988). Orally administered bovine Ig concentrates reduce the severity of enteropathy in animals (Bosi et al, 2004; Corl et al, 2007) modulate gut barrier function and permeability (Moreto and Perez-Bosque, 2009). The hyperimmune milk products were also tested for prophylaxis and treatment of bacterial and virus infections. A colostrum product, containing neutralizing antibody to different serotypes of human rotavirus (Ebina, 1996) was used for infants, with advantageous results. Bovine hyperimmune anti-*Cryptosporidium* colostrum Ig preparation was also effective in prophylaxis of volunteers challenged with the parasite *Cryptosporidium parvum* (Okhuysen et al, 1998). Recent trials in humans demonstrate that Serum-derived bovine Ig /protein
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(SBI) isolate contained >90% protein, over 50% was IgG. SBI was found safe and improved the nutritional status and gastrointestinal symptoms like chronic loose and frequent stools, abdominal discomfort, bloating, urgency in patients with enteropathy associated with diarrhea-predominant IBS (IBS-D) or HIV infection (Asmuth et al, 2013; Wilson et al, 2013). Thus in a potential approach to control AIDS-associated cryptosporidiosis, the passive administration of hyperimmune bovine colostrum, either prior to the onset of advanced immunosuppression or in high-risk situations was found ideal. In addition, colostrum-derived anti-HIV antibodies offered a cost-effective option for preparing the significant quantities of broadly neutralizing antibodies that would be needed in a low-cost topical combination HIV-1 microbicide (Kramski et al, 2012). Thus based on the earlier research findings and observations made so far on the Ig in colostrum/serum, its involvement in host defense response against pathogenic microbes including physiological functions carried out by them can be summarized below.

- **Opsonization**: Antibodies enhance phagocytic engulfment of microbial antigens (Bacterial and viral particles. IgG and IgM Abs has a combining site for the Ag and a site for cytophilic association with phagocytes are ingested with increased efficiency.

- **Toxin Neutralization**: Toxin-neutralizing antibodies (antitoxins) react with a soluble bacterial toxin and block the interaction of the toxin with its specific target cell or substrate in the host.

- **Agglutination and Precipitation**: Antibodies combine with the surfaces of microorganisms or soluble antigens and cause them to agglutinate or precipitate. This reduces the number of separate infectious units and makes them more readily phagocytosed because the clump of particles is larger in
size. Also, floccules or aggregates of neutralized toxin may be removed by phagocytes.

- **Activation of Complement:** Antibodies combined with the surface antigens of microbes activate the complement cascade which has four principal effects related to host defense: 1. Induction of the inflammatory response 2. Attraction of phagocytes to the site of immunological encounters 3. Opsonization of cells which increases efficiency of phagocytosis 4. Lysis of bacteria or viruses.

- **Antibody-dependent cell cytotoxicity (ADCC):** IgG enable certain cells (Natural Killer or NK cells) through cell-mediated immunity to recognize and kill opsonized target cells. NK cells attach to opsonized target cells by means of an IgG Fc receptor and kill by an extracellular mechanism after attachment. Similarly, certain other types of cells including monocytes and neutrophils also act in way. (Source: Todar, 2011).

**Klebsiella pneumoniae**

**Scientific Classification**

- **Domain:** Bacteria
- **Phylum:** Proteobacteria
- **Class:** Gammaproteobacteria
- **Order:** Enterobacteriales
- **Family:** Enterobacteriaceae
- **Genus:** Klebsiella
- **Species:** *K. pneumoniae*

*Klebsiella pneumoniae* is one of the leading causes of hospital acquired infections globally and is recognized as an important opportunistic pathogen (Stahlhut
et al, 2009). Other than respiratory tract infections, *K. pneumoniae* causes urinary tract and wound infections, pneumonia, septicemia, soft tissue infections, suppurative lesions and bacteremia are frequently severe, affecting incapacitated patients with a suppressed immune status and neonates in intensive care units with mortality rates as high as 70% (Guggenbichler et al, 2011). A few strains of *K. pneumoniae* have also been implicated as the cause of bloody diarrhoea (Brisse and Verhoef, 2001). *Klebsiella* are non-motile, aerobic, Gram negative rod and measures 0.3~1.0 μm in diameter and 0.6~6.0 μm long, that posses a prominent polysaccharide capsule (Figure 4). However, five main classes of virulence factors were identified namely capsule, lipopolysaccharide (LPS), siderophores, adhesins (pili, fimbriae, outer membrane proteins) and exotoxins.

*Klebsiella pneumoniae* has earned notoriety largely due to its resistance to the “last resort” antimicrobials such as third generation-cephalosporins and carbapenems, which significantly narrows, or in some scenarios completely removes the therapeutic options for the treatment of multidrug- resistant (MDR) *K. pneumoniae* infections (Munoz-Price et al, 2013). Clearance of *K. pneumoniae* from the host system requires effective augmentation of host defense mechanisms, wherein bacterial surface plays a major role. Three components of bacterial surface can logically be counted upon in the development of immunity: polysaccharide capsule (CPS), lipopolysaccharide (LPS) and outer membrane proteins (Omp). So far, common vaccines used against *Klebsiella* infections are based on CPS and also LPS to a certain extent (Yadav et al, 2005). However, CPS and LPS provide only type specific protection against these infections as at least 77 K antigen types and 8 LPS (O-antigens) serotypes exist. Omps exist as trimers and act as water filled channels that allow the hydrophilic molecules across the membrane. Majority of Omps are conserved among the members
of Gram negative bacilli. These are involved in antibiotic resistance/susceptibility mechanisms and contribute to the virulence of the organisms. OmpK36 is produced by majority of extended-spectrum beta-lactamases (ESBL) producing *K. pneumoniae* members and it is also reported to contribute to resistance or reduced susceptibility to carbapenems in ESBL producing *K. pneumoniae* strains (Mena et al, 2006; Skurnik et al, 2010). OmpA is one of one of the major proteins of outer membranes of Gram negative bacteria and is highly conserved among the members of *Enterobacteriaceae* (Pichavant et al, 2003). OmpA is a potent carrier protein that binds to and activates macrophages and dendritic cells (Jeannin et al., 2000). OmpA has been shown to possess potent carrier properties and also induces specific humoral and cytotoxic responses (Jeannin et al, 2002). Therefore, OmpA and OmpK36 could be considered as ideal targets while designing anti- *Klebsiella* drugs since these targets are carried by all the strains unlike O and K antigens.

*Enterococcus faecalis* and *K. pneumoniae* frequently infect the human urinary tract and therefore cause chronic inflammation in prostate, with periodic aggravations (Motrich, 2007; Pavone-Macaluso, 2007). *K. pneumoniae* was identified in another major hospital in the Kathmandu valley (Tada et al, 2013). Eighteen *K. pneumoniae* strains were identified from an outbreak of extended-spectrum beta-lactamases (ESBL) producing *K. pneumoniae* (Narciso et al, 2011). Snitkin et al. (2012) performed whole-genome sequencing on *K. pneumoniae* isolates to gain insight into why the outbreak progresses despite early implementation of infection control procedures. Both integrated genomic and epidemiological analysis was used to trace the outbreak form patients. *K. pneumoniae*, multidrug- resistant (MDR) recently, being singled out as an “urgent threat to human health” by the U.S. centers for disease control and Prevention (CDC), the World Health Organization (WHO), the
Government of the United Kingdom and other international healthcare organizations (Centers for disease control & Prevention, 2013; UK Department for Environment Food & Rural Affairs, 2013). Hence, a targeted therapy or a food formulation possessing anti-microbial property would be a rational basis to combat *K. pneumonia* infections.