Studies on the Adherence Properties of Plant Lectins and Bacterial Adhesins and their Inhibition by Prebiotic Oligosaccharides and Bovine Colostrum Fractions

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STUDIES ON THE ADHERENCE PROPERTIES OF PLANT LECTINS AND BACTERIAL
ADHESINS AND THEIR INHIBITION BY PREBIOTIC OLIGOSACCHARIDES AND
BOVINE COLOSTRUM FRACTIONS

by

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STUDIES ON THE ADHERENCE PROPERTIES OF PLANT LECTINS AND BACTERIAL ADHESINS AND THEIR INHIBITION BY PREBIOTIC OLIGOSACCHARIDES AND BOVINE COLOSTRUM FRACTIONS

María Ximena Maldonado-Gómez, M.S.

University of Nebraska, 2011

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To initiate colonization and infection, most microbial pathogens must first recognize and adhere to cells in the host tissues. Adherence inhibition, therefore, can be used as a strategy for preventing infectious disease. Prebiotic carbohydrates, in addition to their ability to influence the colonic microbiota, are also able to inhibit attachment of pathogenic bacteria to the epithelial cells that line the intestinal tract. However, the mechanism for this inhibition is not well understood, in part, because the receptor sites on the target cells have not been identified. In this research, we developed a simplified model using plant lectins and HEp-2 tissue culture cells to simulate bacterial adhesins-receptors interactions and to identify putative ligands located on the surface of target cells. The effect of lectin cognate ligands and two prebiotic carbohydrates, galactooligosaccharide (GOS) and polydextrose (PDX) was measured with this approach. We hypothesized that GOS would inhibit adherence of those lectins whose cognate ligands were structurally similar to GOS. All of the lectins that were able to bind to the target cells were inhibited by nearly 100% in the presence of 1 mg/ml of the cognate ligands. When prebiotics were added, inhibition of lectin binding was also observed. The inhibition was generally
proportional to the structural similarity between the prebiotic and the cognate ligands. In particular, GOS significantly inhibited attachment of most of the lectins, consistent with the established role of GOS as an anti-adherence agent. In contrast, PDX did not significantly inhibit attachment of lectins, suggesting that it is structurally dissimilar to the HEP-2 receptor sites. The results support the hypothesis that cell binding inhibition is caused by competition of the GOS for the lectin. Subsequent anti-adherence experiments were completed to assess the anti-adherence properties of several bovine colostrum fractions against four different bacterial strains, enteropathogenic *Escherichia coli*, enterotoxigenic *Escherichia coli*, *Cronobacter sakazakii* and *Salmonella enterica* serovar Typhimurium. An ultrafiltration permeate and an oligosaccharide fraction significantly inhibited binding of all four pathogens to HEP-2 tissue culture cells. In contrast, a nanofiltration retentate inhibited *Salmonella* adherence, but not other pathogens, while a colostrum whey fraction had no effect for any of the test organisms. The effectiveness of the inhibitory fractions was mainly attributed to the presence of oligosaccharides and/or peptides, although lactose may have also contributed to adherence inhibition. Several methods for extracting oligosaccharides and removing lactose from bovine colostrum were evaluated. Enzymatic hydrolysis and microbial fermentation were effective methods for degrading lactose. In contrast, the physical-chemical separation methods (i.e. crystallization, affinity chromatography and nano-filtration) were limited in their ability to separate and enrich oligosaccharides from bovine colostrum. The results obtained from this study provide evidence to support the concept that bovine milk components can promote consumer health by preventing or reducing enteric infections. These constituents could be used as antimicrobial agents in foods and/or in pharmacological applications. In particular, bovine colostrum whey appears to be a suitable source for these bioactive compounds.
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When I decided to start my master in science my family shared these words with me: “travels a thousand journeys and let each one take you further than the last, speak lightly and always smile when you say you're Colombian”. After two years of intense work I can say that graduate school was not an easy journey, but it was completely worth it. I learn invaluable lessons and I sincerely thank for the encouragement received.

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Preface

This thesis is comprised of five chapters. Chapter 1 provides a review of the current literature on general principles of bacterial adherence, strategies for preventing bacterial adherence, and the application of milk oligosaccharides as inhibitory agents. Chapter 2 describes a study on the effect of prebiotic carbohydrates on the binding kinetics of lectins to epithelial tissue culture cells. In Chapter 3, our efforts to separate oligosaccharides and remove or degrade lactose from bovine colostrum are described. Chapter 4 describes results obtained when assessing the anti-adherence effect of colostrum oligosaccharides and several commercial colostrum whey fractions against enteropathogenic Escherichia coli, enterotoxigenic Escherichia coli, Enterobacter sakazakii and Salmonella enterica serovar Typhymurium. Chapter 5 provides a conclusion section that summarizes the major research findings presented in this thesis.
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Chapter 1

General principles of bacterial adherence, strategies for its prevention and the use of milk oligosaccharides as inhibitor agents.
1. Introduction

For most bacterial pathogens, recognition and adhesion to the host cells are essential to initiate colonization and infection. The importance of adherence in microbial virulence and pathogenesis has led to considerable research on understanding the specific structures and mechanisms involved in these processes. In this review, the structural features and general principles involved in bacterial adherence to host cells will be reviewed. Strategies for preventing bacterial adhesion and preventing or reducing bacterial infections will also be described. In particular, the use of receptor analogs to block or interfere with pathogen-host cell interactions will be discussed. This discussion will include descriptions of natural and synthetic glycans that have shown to be effective against bacterial pathogens, such as oligosaccharides derived from milk and colostrum. Potential techniques to obtain these oligosaccharides will be described.

2. Bacterial Adhesion

Virtually all microbial pathogens must adhere to the surface of host tissue cells in order to initiate infections. The adherence step serves several important functions. First it provides a basis for tissue recognition such that the bacterium is able to adhere to specific target cells. In addition, adherence provides resistance against environmental shear forces, such as those that occur in the intestinal tract and that would otherwise cause the bacteria to become displaced. While bacteria are bound to the host surfaces their ability to acquire nutrients increases, which further enhances their ability to survive and invade the host. Attachment also gives the bacteria substantially greater resistance to the clearance and killing by immune factors, bacteriolytic enzymes and antibiotics.

To facilitate adherence, Van der Waal’s and Coulombic forces bring the bacteria to close proximity to the host mucosal surface (Busscher & Weerkamp 1987). Then hydrophobic interactions reversibly bind the microorganism to the surface of the epithelial cell (Ofek, Hasty et al. 2003). These early non-specific events are followed by more permanent and more specific
adherence events. The latter are mediated by structures on the bacterial surface, called adhesins, and their complementary ligands located on the mucosal surface of the host. This highly stereospecific interaction leads to a firm adhesion (Ofek & Sharon 1990; Hasty et al. 1992; Ofek & Doyle 1994; McCoy et al. 1994; Ofek, et al. 2003). Detachment would be expected only when all the adhesin-receptor interactions are coordinately reversible, the probability of which is low (Ofek et al. 2003).

It is also worth noting that adherence and molecular target motifs can be found not only in naturally occurring cells and tissues, but also in coated abiotic materials such as glass and plastic. The latter case is especially important in industrial environments, since bacterial biofilms contaminate work surfaces and are the cause of clogged tubes and machinery (Kumar and Anand, 1998; Mattila-Shadholm and Wirtranen, 1992)

2.1. Adhesins

Adhesins are specialized components or appendages common to many bacteria. Typically protein in nature, adhesins recognize and bind very specific receptors on the surface of the target host cells. Adhesins, therefore, are the primary means for determining tissue tropism of the pathogen. For many bacterial pathogens, adhesins are considered as essential virulence factors that are required for the bacteria to infect mucosal tissues in the respiratory, urinary and gastrointestinal tracts (Connell et al. 1997; Klemm et al. 2006, 2007; Roos et al. 2006). Although, many bacterial pathogens have the ability to express different adhesins, each adhesin is often expressed at different stages during infection. It is the concerted action of the different adhesins that allows infections to occur (Klemm et al., 2010).

There are three types of adhesin-receptor interactions, based on differences in the chemical nature of the adhesin-ligand reaction (Figure 1). The first type is similar to the classic lectin-carbohydrate interaction that is common in the plant kingdom (section 3). Glycan recognition is mediated either by a lectin on the bacterial surface or a lectin on the host surface.
Among bacterial pathogens, the former predominates and contains the largest group of adhesins so far described. The second category involves recognition between a protein on the surface of the bacteria and a complementary protein on the mucosal surface (e.g. Streptococcus pyogenes protein F binds to fibronectin). The third type, involves the binding between hydrophobins. A hydrophobin is a molecule or location on the bacterial surface that is involved in adhesion by interacting via hydrophobic structures. Commonly, these interactions are mediated via lipids and hydrophobic moieties of proteins. Hydrophobic adhesion has not received much attention because it is usually responsible for weak and reversible interactions that are difficult to measure (Ofek et al. 2003).

**Molecular structures of bacterial adhesins.** For adhesins to bind to the target moiety it is necessary that they be positioned at the exterior of the cell. Many adhesins, including the auto transporter adhesins TibA and AIDA of E. coli, are located directly on the bacterial surface (Benz and Schmidt, 1992; Sherlock et al., 2004, 2005). However, proximity of the adhesin to the “body” of the cell can cause steric hinderance and reduce the interaction between the ligand and the receptor. To solve this problem, bacteria have evolved numerous ways to position adhesins and maximize successful interactions. Most bacterial adhesins are organized as thin thread-like organelles called fimbriae or pili. Indeed, a majority of gram-negative and gram-positive pathogens produce these structures (Foster 2004, Odenbreit 2005; Pizarro-Cerda and Cossart 2006). Fimbriae are heteropolymers with lengths of about 1.0 µm. They are composed of a structural protein, which serves as a platform for display of the actual adhesin which is located in the tip of the organelle (Hahn et al. 2002; Klemm et al. 2010, Klemm and Schembri 2000). Fimbriae can be evenly distributed over the surface of the bacterium; however, in some cases they are located preferentially on one part of the bacterial surface (Ofek et al., 2003). Based on their morphological appearance, fimbriae have been classified according to four general categories: (1) Fimbriae with rigid structures measuring approximately 7 nm in diameter with various lengths (e.g., type 1 and P fimbriae); (2) Thin, flexible fimbriae of 2 or 3 nm in diameter (e.g., 987P
fimbriae); (3) Thinner and much more flexible structures such as curli; and (4) Type IV fimbriae, usually flexible pili of 4 to 6 nm in diameter which often form in bundles (Orskov and Orskov, 1990).

Finally, there are several non-fimbriae surface materials that have also been implicated in bacterial attachment, but that lack a specific target molecule. Included are capsules, lipopolysaccharides, flagella and cellulose. Not all the species of bacteria or even all the strains produce them.

2.2. Enteric pathogens adhesion

2.2.1. Enteropathogenic E. coli (EPEC)

Enteropathogenic *Escherichia coli* (EPEC) is one of several pathovars of *E. coli* that cause disease in humans. Worldwide, EPEC is a leading cause of infant diarrhea. A characteristic mechanism of EPEC pathogenesis is the formation of attaching and effacing (A/E) lesions. A/E lesions are characterized by a series of events that include the destruction of the intestinal microvilli, intimate adherence of bacteria to the epithelium, formation of a pedestal, and aggregation of actin and other constituents of the cytoskeleton. The genes that encode for the production of A/E factors are located on the locus of enterocyte effacement (LEE) (McDaniel et al., 1995). The expression of this island of pathogenicity results in the synthesis of intimin, a type III secretion system, a number of secreted (Esp) proteins, and Tir, the translocated intimin receptor (Nataro and Kaper 1998). The intimate adherence of the EPEC is mediated by the outer membrane intimin, which is encoded by the eae gene. Intimin binds the protein Tir, which is inserted into the host cells membrane to serve as receptor (Kenny et al., 1997).

Adherence of EPEC to host cells follows a pattern called localized adherence (LA). In LA, bacteria bind to the tissue culture cells as microcolonies or bacterial clusters (Scaletsky et al., 1984). This pattern is associated with the presence of the EAF plasmid which encode for a bundle-forming pili (BFP). BFP is responsible for interconnecting bacteria within microcolonies
and therefore promote colony stabilization. EAF enhances the efficiency of A/E lesions, but is not essential for their formation. Furthermore, different EPEC strains show diverse adherence patterns besides LA; these include localized-like adherence (LAL), diffuse adherence pattern (DA) or aggregative adherence pattern. Each is mediated by different adhesins; LAL is mediated mainly by intimin, DA by the Afa adhesin and AA by an aggregative adhesin (Pelayo et al., 1999, Trabulsi et al., 2002).

It has been suggested that flagella and flagellins of EPEC also contribute to adherence. EPEC flagella bind to mucins present in the gastrointestinal tract at the mucosal surface. In addition, EPEC flagella also have affinity for Extracellular Matrix (ECM) proteins, collagen, laminin, and fibronectin. The ability to bind ECM proteins could contribute to host colonization when the intestinal barrier is disrupted (Erdem et al., 2007).

2.2.2. Enterotoxigenic E. coli (ETEC)

ETEC is the most common cause of food and water-borne E. coli-mediated human diarrhea worldwide. It is also responsible for traveler’s diarrhea. ETEC infections occur in animals as well, and the organism is a major problem for animal producers since it attacks pigs and calves, resulting in significant financial losses. ETEC adhere to the microvilli of the small intestinal epithelial cells, and although morphological lesions are not produced, enterotoxins are secreted that act locally on the enterocytes. (Nagy and Fekete, 1999. Among the major determinants of ETEC virulence are the plasmid-encoded colonization factors (CF) (Yamamoto & Yokota, 1983; Echeverria et al., 1986), surface structures that allow bacteria to attach to the intestinal mucosa. In addition, there are several non-CF chromosomally-encoded virulence factors that are implicated with ETEC virulence (Turner et al., 2006). It has been suggested that CFs may be responsible for bringing the bacterium closer to the cell, and once the bacterium is closer, non-CF virulence factors will enhance the strength and intimacy of the interaction (Turner et al., 2006).
**Colonization factors.** More than 20 CFs have been identified and characterized. Usually, a four-gene operon encodes for these CFs. Expression of these genes results in the biosynthesis of a periplasic chaperone, an outer membrane usher protein, a major fimbrial subunit, and a minor subunit. The minor subunit, which is located in the tip of the fimbriae, is responsible for the binding of the bacteria to the host cell receptor. Only a few cognate receptors of the CFs are fully characterized. That there is a large number of different CFs suggests that there is a vast diversity of receptors, which makes the identification and characterization of the adhesin ligands more complicated. It is known that CFs receptors are glycoconjugates in the eukaryotic cells’ membrane. The type of oligosaccharide structure present in the glycoconjugates confer ETEC specificity towards tissues and cells (Lindahl and Wadstrom, 1984; Blomberg et al., 1993; Erickson et al., 1994).

### 2.2.3. *Salmonella enterica* serotype Typhimurium

*Salmonella* Typhimurium is responsible for a systemic infection referred to as murine typhoid fever. Fimbriae are important virulence factors in *Salmonella* Typhimurium and are involved in invasion and the stimulation of an inflammatory response. Four distinct fimbrial operons have been characterized, referred to as fim, lpf, pef and agf (Baumler et al.,1995; Clegg et al., 1987; Collinson et al., 1996; Friedrich et al., 1993). Mutations in individual fimbrial biosynthesis genes result in only a slight decrease of *S.* Typhimurium virulence. This suggests that the absence of any adhesin can be compensated by the use or synthesis of alternate adhesins. When all of the fimbrial operons are inactivated, virulence in mice is reduced significantly, however, *Salmonella* is still able to adhere to the host cells and cause lethal infection (Velden et al., 1998).
2.2.4. *Enterobacter sakazakii*

*E. sakazakii* is an opportunistic pathogen responsible for causing meningitis, necrotizing enterocolitis, and sepsis, especially in neonates. These are very serious infections, with mortality rates ranging from 40 to 80%. Dried infant formula is the principal product associated with *E. sakazakii* infections. Information on the mechanisms of pathogenicity is limited. Two types of adhesion patterns, mixed pattern and localized adherence, have been observed, *in vitro*, using Hep-2 and Caco-2 cells. Mannose seems to be a cognate ligand for at least one of the adhesins expressed by this microorganism. However, mannose-independent adherence also has been reported (Mange et al., 2006). Although operons coding for type 1 and curli fimbriae have been predicted (Lehnet et al., 2006; Zogaj et al., 2003), it has also been suggested that frimbrial structures are not involved in adherence under experimental conditions (Mange et al., 2006). Rather, fibronectin-mediated binding was suggested as a nonspecific adherence mechanism of *E. sakazakii*. Interaction with fibronectin, a major glycoprotein component of the extracellular matrix of eukaryotic tissue, would bring the bacteria closer to the epithelial cells facilitating a specific interaction between bacterial ligands and host receptors (Mohan-Nair and Venkitanarayanan, 2007).

2.3. Ways to prevent bacterial adherence

Since adherence is a prerequisite to initiate colonization and infection, preventing bacterial adhesion would appear to be an ideal strategy for reducing infectious disease. Several approaches have been evaluated, with each showing different levels of success and feasibility. These methods have involved both industrial and biomedical applications and are based on the following interactions: (i) changing surface interactions by coating inert surfaces with inhibitory macromolecules; (ii) metal chelators that interfere with bacterial growth, attachment and biofilm formation; (iii) prevention of adhesin biosynthesis; (iv) anti-adhesive vaccines; and (v) adhesion inhibition with receptor analogs. The latter three will be covered in more detail.
2.3.1. Interference of adhesins biosynthesis

This strategy is based on the inhibition of fimbriae biosynthesis by specific chemical agents. The biosynthesis of fimbriae involves a helper protein that assists the assembly of large heterolymeric organelles. The best characterized fimbriae group, assembled via the chaperone-usher-dependent pathway, is synthesized by the action of an usher placed in the outer membrane. A periplasmic chaperone assists the usher by positioning structural components to the outer membrane (Klemm et al. 2010; Waksman and Hultgren 2009). This chaperone-usher dependent pathway has been successfully disrupted by chemical interference at the assembly level of the fimbriae biogenesis (Pinker et al., 2006). In this study, a class of bi-cyclic 2-pyridones, called pilicides, were used to inhibit fimbriae synthesis. Adherence of either type I or P type fimbriae to bladder cells and subsequent biofilm formation were reduced by approximately 90% in different E. coli strains. The authors suggested that pilicides could be designed to target conserved regions on other chaperones and ushers, which would make it possible to interrupt the assembly of a range of different fimbriae (Åberg and Almqvist 2007; Larsson et al. 2005; Pinkner et al. 2006; Svensson et al. 2001).

2.3.2. Anti-adhesive vaccines

Inhibition of bacterial adhesion can also be achieved by immunization with the adhesin. The immunization would block the adhesion-receptor interaction by inducing the production of adhesin-specific antibodies in the host. For most of the attempts done to make anti-fimbriae vaccines, the major structural proteins of the fimbriae were the targets of the immune response. However this approach has failed due to the constant sequence variation of these proteins. In contrast, in cases where the immune response is directed against the adhesin itself (i.e., the component that interacts with the host receptor), the results have been more successful. For example, the vaccine for piglets based on K88 fimbriae from ETEC strains decreased the incidence of diarrhea (Rutter and Jones 1973). Nevertheless, some vaccines targeting specific
adhesins were less successful. For instance, although vaccination against FimH reduced UPEC infection of the bladder of tested animals (Langermann et al. 1997, 2000, 2003), it did not prevent UPEC infection in humans. FimH trigger a severe auto-immune disease, pauciimmune focal necrotizing glomerulonephritis. It is caused by the molecular mimicry between FimH and the human LAMP-2 protein that is expressed by many human cell types (Kain et al. 2008).

2.3.3. Inhibition with receptor analogs

One of the major types of adhesin-receptor interactions is the binding of bacterial adhesins to the sugar moieties of glycoproteins or glycolipids. The molecular structures of many of the target receptors, however, are still unknown and the techniques to elucidate the interactions between the active site of the adhesin and the sugar are complicated. For those cases where the interactions have been studied, the information about the adhesins and their cognate ligands have been used to design and develop receptor mimetics or decoys. For example, it is known that uroplakin IA and Ib (mannose-rich glycoproteins) are the primary physiological receptors in the urinary tract for FimH (Min et al. 2002; Zhou et al. 2001). Furthermore, several proteins that contain one or more N-linked mannose structures can also been recognized by FimH. This type of fimbrial adhesion has been successfully blocked by a variety of natural and synthetic saccharides which also are able to inhibit biofilm formation (Bouckaert et al. 2005; Nagahori et al. 2002 Schembri and Klemm 2001). However, the low affinity of the receptor analogs has been a significant obstacle in developing carbohydrate based therapies. A solution that may overcome this limitation is to make suitable chemical transformations of the saccharide analogs. For instance alkyl-substituted mannosides have affinities for FimH in the nanomolar range (Bouckaert et al. 2005).

Another strategy to develop an effective blocking adhesion therapy is to use a mixture of active receptor analogs. A heterogenic blend of carbohydrates could block a greater variety of pathogens in a more efficient way since most bacteria use more than one adhesin or lectin to
adhere to host cells. Furthermore, individual lectins of pathogens are often able to bind more than one carbohydrate moiety. Therefore, the more diverse the decoys, the more effective will be the defense mechanism. Milk oligosaccharides are good examples of a natural mixture that contains effective binding inhibitors. They have shown to be efficient agents blocking bacterial, as well as viral adherence (Kunz, et al., 2000; Shah, 2000; Ruiz-Palacios et al., 2003; Morrow et al., 2005; Newburg et al., 2005; Bao et al., 2007). It is suggested that the composition of human milk OS changes during lactation in response to needs of the neonate. These OS cannot be metabolized; therefore, OS are able to reach the colon where, among other functions, they act as receptor decoys and protect the infant from infections. Milk OS as pathogen binding inhibitors will be discussed in more detail in section 4.1.2.

3. Lectins

Lectins are a heterogeneous group of oligomeric proteins that have a common activity, but different sizes, structures, molecular organization, and active sites (Lis and Sharon, 1998). They are defined as “proteins of non-immunoglobulin nature capable of specific recognition and reversible binding to carbohydrate moieties of complex carbohydrates without altering covalent structure of any of the recognized glycosyl ligands” (Kocourek & Horejsi, 1983). Apart from complex carbohydrates, oligosaccharides, glycoproteins, and glycolipids, lectins can also bind to free monosaccharaides. In fact, lectins specificity is often determined by hemagglutinating experiments using simple carbohydrates as inhibition agents.

Typically, lectins are di- or polyvalent, with each molecule containing two or more carbohydrate linking sites. This characteristic gives them the ability to agglutinate cells, as each reaction site is capable of binding two or more carbohydrates on the surface of different cells, bringing them together and causing their precipitation. Lectins can also induce precipitation of polysaccharide or glycoprotein molecules in solution by forming cross-links between independent molecules. The sugar ligands for which the lectins are specific are able to inhibit both the
agglutination and precipitation reactions. Usually, hemagglutinating or erythrocyte agglutination are used for lectin detection and characterization.

Lectins are found in most organisms, ranging from simple organisms such as viruses and bacteria, to complex organisms such as plants and animals. Their binding specificity confer them the capability to serve as recognition molecules within a cell, between cells, or between organisms (Chrispeels and Raikhelb, 1991).

3.1. Bacterial Lectins

Lectins are the primary type of adhesin identified in bacteria (Ofek and Doyle, 1994; Sharon et al., 2000), and they therefore mediate the adhesion of the organisms to the host cell. Even though several different adhesins of pathogenic bacteria have been identified (Table 1), the interactions between the lectins and their carbohydrate receptors have not yet been studied in depth. The molecular basis of these interactions requires the use of complex techniques. For example FimH and PapG adhesins of uropathogenic E. coli and adhesin F17-G of enterotoxigenic E.coli were crystallized in order to elucidate their three dimensional structures and to understand their mode of interaction (Choudhury et al., 1999; Donson et al., 2001; Sung et al., 2001)

In gram positive bacteria, lectins may be located on the peptide-glycan matrix or are extended beyond the cell wall from the cytoplasmic membrane (Ofek et al., 2003). In gram negative bacteria, the lectins can be presented as amorphous or seemingly amorphous structures, as part of the fimbriae, or as other types of outers membrane components (Ofek and Doyle, 1994).

The classification of bacterial lectins is usually based on their sugar specificity. Specificity can be determined by inhibiting the adhesion to host cells or glycan arrays, using free carbohydrates, in competition experiments. To establish sugar specificity it is necessary to account for the primary sugar specificity as well as the fine sugar specificity. The primary sugar
specificity refers to the simplest saccharide structure that inhibits the lectin adhesion. The fine sugar specificity defines the structure of the most potent carbohydrate inhibitor (Ofek et al., 2003). Not only is the type of carbohydrate important in defining lectin specificity, it is also essential to consider the position within the saccharide at which the lectin recognizes the sugar. Most bacterial lectins recognize terminal sugar structures; however, some also are able to recognize internal sequences. For instance, the tip of adhesion PapG of P fimbriae recognizes internal Gal(a1\(\rightarrow\)4)Gal sequences in glycolipids on host cell surfaces (Striker et al., 1995; Stromberg et al., 1990). In the case of glycolipids, the lectin binding appears to be dependent on the linkage of fatty acids to the sugar moieties. Detailed analysis of the fine sugar specificities of lectin-bearing bacteria is an extremely useful tool to establish the suitable carbohydrates for the development of anti-adhesion therapy of microbial infections.

### 3.2. Plant lectins

Plant lectins were the first to be identified and therefore have been more extensively studied than microbial lectins. Many theories about their function have been proposed, however, their biological role is still not well understood. One proposed function is to enhance the symbiosis between the plant and nitrogen fixing bacteria (mainly rhizobia and leguminous plants). Another proposed function is to defend the plant against phytopathogenic fungi and animal predators (Lis and Sharon, 1998). For example, some lectins have strong insecticidal properties. Plant lectins have also received attention for causing a type of food poisoning. Specifically, raw or insufficiently cooked beans cause nausea followed by vomiting and diarrhea due to *Phaseolus vulgaris* agglutinin (PHA). This lectin binds to intestinal cells and becomes endocytosed, leading to hyperplasia and hyperthrophy of the small intestine (Peumans and Van Damme 1995).

Lectin specificity has been well studied and many of the ligands have been identified (Table 2). Although plant lectins certainly interact with monosaccharides, their affinity for simple
sugars is usually low when compared to more complex carbohydrate structures (Garcia-Pino et al., 2007; Mandal et al., 1994). Techniques that have been used to elucidate the lectin specificity include dot blot assays with multiple oligosaccharides (Baldwin et al., 2000; Matsui et al., 2001) and microarrays with either immobilized glycans or immobilized lectins (Blixt et al. 2004; Kuno et al., 2005). Plant lectins have been classified according to their ability to agglutinate cells, their carbohydrate specificity, their carbohydrate-binding domains, and their evolutionary relationships with other proteins. Based on recent genome/transcriptome analyses, plant lectins were classified into twelve distinct families of evolutionary and structurally related lectin domains. The carbohydrate-binding domains are named in alphabetical order: *Agaricus bisporus* agglutinin homologs, amaranthins, class V chitinase homologs, cyanovirin family, *Euonymus europaeus* agglutinin family, *Galanthus nivalis* agglutinin family, proteins with hevein domains, jacaclins, proteins with a legume lectin domain, LysM domains and *Nicotiana tabacum*. Each lectin domain has its own characteristic overall fold with one or more carbohydrate-binding sites (Van Damme et al., 2008).

Plant lectins have many direct and indirect applications in biomedicine and biotechnology. They are widely used as reagents for the study of glycoconjugates in solution and cells and also for cell characterization and separation. For example, an important application of SBA is to purge human bone marrow cells from haploidentical donors for transplantation into children born with severe combined immune deficiency. This procedure has been effective in over 75% of the children treated (Reisner et al., 1983). Research on the lectins field is critical since the characterization of additional native lectins and the development of modified derivatives will lead to the discovery of novel applications for these proteins.
4. Milk as source of bioactive carbohydrates

4.1. Colostrum and mature milk

The composition of milk from bovine and other mammals progressively changes post parturition to meet the changing requirements of the neonate. The lacteal secretion during these first days of milking is called colostrum or “early milk”, the composition of which is quite different from “mature milk”. In the specific case of bovine milk, colostrum is secreted during the first four days of lactation (Gopal et al., 2000). After this period, milk enters a transitional stage in which the composition will continuously change until it reaches a constant composition corresponding to the mature milk profile. The function of milk in these first days is not only to satisfy the nutritional requirements of the newborn, but also to provide protection against infection while the infant’s immune system is still developing. In addition to normal nutrients such as proteins, carbohydrates, fats, vitamins and minerals, colostrum contains many other biologically active constituents. These include growth factors, antimicrobial compounds, and immune-enhancing components.

Colostrum differs from normal milk in several important ways. First, it is significantly higher in fat, protein, vitamins, and immunoglobulins, and is lower in lactose (Table 3). These components (especially immunoglobulins) decline rapidly after the first day of milking. Therefore, contrary to milk, it is difficult to assume a “typical” composition profile for bovine colostrum. The composition and physical characteristics also vary greatly with individual breeds, feeding regimen, length of dry periods of cows, and time post parturition.

4.2. Bioactive colostrum components.

Human milk contains several constituents that have biological activity, including oligosaccharides, glycolipids and glycoproteins, mucins and gangliosides. Some of these components are reported to protect the infant against pathogenic bacteria and other infectious agents. For example, colostrum glycoproteins and glycolipids were protective against
enterotoxigenic *E. coli* (Newburg et al. 1990), and an inhibitory effect of components derived from the milk-fat globule membrane against rotavirus has also been demonstrated. The bioactivity of these fractions was attributed to lactoferrin (Superti et al., 1997, 2001) and/or mucinous proteins (Yolken et al., 1992; Kanamaru et al., 1999), including but not exclusively MUC1 (Kvistgaard et al., 2004).

4.2.1. Milk oligosaccharides

Most of the studies on the bioactive properties of milk have focused on the oligosaccharide fraction, in particular, those found in human milk. Although oligosaccharides are also found in bovine milk, they are present at significantly lower concentrations. Human milk contains 12-14 g/L, and human colostrum contains as much as 23g/L (Coppa et al., 1999; Boehm and Stahl 2007). In contrast, the concentration of oligosaccharides in the milk of domestic mammals is 10 to 100 time less (Boehm and Stahl, 2003). In the specific case of bovine milk, oligosaccharides are present at a concentration between 0.7 and 1.2 g/L during the early lactation period (Veh et al., 1981; Tao et al. 2008), decreasing to 0.1- 0.6 g/L for mature milk. In addition, the structural heterogeneity of human milk oligosaccharides (HMO) is much greater than oligosaccharides found in milk from bovines and other animals, a property that appears to have important implications (see below).

4.2.1.1. Composition of milk oligosaccharides. Milk oligosaccharides consist of oligomers of 3 to 15 monosaccharides covalently linked through glycosidic bonds (Figure 2). Among the major monomers found in human milk oligosaccharides are D-glucose (Glc), D-galactose (Gal), L-fucose (Fuc), N-acetylglucosamine (GlcNAc), and N-acetyl neuraminic acid (NeuAc) (Rivero-Urgell and Santamaria-Orleans,2001; Boehm and Stahl, 2007). In contrast, bovine milk contains N-glycolylneuraminic acid (NeuGc), but fucose, present in human milk, is generally absent in bovines. The largest single OS species in bovine milk is a heptamer containing Glc, Gal, NeuGc
and GlcNA. However, tri and tetrasaccharides account for the majority of the BMO, with sialyl-lactose the most abundant. Bovine milk OS are built with a disaccharide or trisaccharide core. The disaccharides lactose and lactosamine are the most common cores (Ninonuevo et al., 2006); whereas, the trisaccharide core, consisting of lactose linked to Galβ-(1-3) or β-(1-6), is either not present or at very low abundance.

Overall, about 200 different OS species have been identified in human milk, compared with only 40 OS species in bovine milk. Furthermore, the diversity of linear and branched oligosaccharide structures is more complex in human milk compared to those found in bovine milk. In addition, the number of isomers in human milk exceeds the number of isomers in bovine milk (Tao et al., 2008). In bovine colostrum, acidic anionic OS account for up to 70% of the total OS content, with sialyl-lactose as the most abundant. The latter OS accounts for as much as 30% of total OS in bovine colostrum (Tao et al., 2009). In contrast, acidic OS are minor components in human milk, representing less than 20% of the total OS in colostrum and mature milk. As noted above, a unique characteristic of bovine milk compared with human milk is the presence of NeuGc-containing OS. Nevertheless, this OS is only present in bovine colostrum; its concentration decreases to undetectable levels in mature milk. Bovine milk, however, does not contain fucose-containing OS, whereas 70% of the human milk OS are fucosylated.

In the same way that OS concentrations change over time, the composition of bovine milk OS also varies within the different lactation stages. Although the total amount of OS decreases continuously during the first days of lactation, (Figure 3) the concentration of total neutral OS increases with time. For example, the concentration of the neutral oligosaccharide lacto-N-neotetraose [LNnT; Gal (β1-4) GlcNAc (β1-3) Gal(β1-4) Glc], increased slightly after 6 days of lactation and decreased again once milk reaches the mature stage. The previous behavior of the neutral OS and the dramatic decrease of NeuAc linked OS resulted in a large change in the acidic/neutral OS proportion. Anionic OS decreased from 73% to 53.7% after 6 days, while the
neutral OS concentration increased from 22% to 43% in the transition stage and remained relatively constant until milk reached the mature stage (Tao et al., 2009) (Figure 3b).

4.2.1.2. Biological functions. There is now considerable interest in understanding the biological functions of milk OS. Several possible biological activities have been suggested, based mostly on research using human milk OS. In addition, non-milk OS that have structural similarities to the core molecules of HMO have also been studied. The latter include the galactooligosaccharides (GOS), OS derived from lactose via transgalactosylation reactions. Both the milk-derived and lactose-synthesized OS are known to have prebiotic activity, in that they specifically stimulate the growth of beneficial bacteria in the intestinal tract (LoCasio et al., 2007; Ward et al., 2007; Marcobal et al., 2010; Sela et al., 2011). They also provide postnatal stimulation of the immune system (Eiwegger et al., 2004; Braunstein et al., 1997; Recigno et al., 2009), and enhance defense against bacterial and viral infections (Kunz et al., 2000, Morrow et al., 2005; Newburg et al., 2005; Bao et al., 2007).

4.2.1.2.1. Prebiotic activity of milk OS. Recent studies have shown that mixtures of HMO promote the growth of several species of bifidobacteria. In particular, *Bifidobacterium longum* bv. *infantis* and *Bifidobacterium bifidum* have been shown to have higher ability to ferment HMO than other species (György et al. 1954; LoCasio et al., 2007; Marcobal et al., 2010). Most of the HMO that promote growth of bifidobacteria are neutral glycans, however, it was recently reported that *B. infantis* can also metabolize acidic oligosaccharides (Sela et al., 2011).

Based on *in vitro* assays, the protein and non-protein components in human milk and bovine milk have also been reported to promote growth of several bifidobacteria, including *Bifidobacterium bifidum var pennsylvanicus, Bifidobacterium infantis,* and *Bifidobacterium breve* (Petschow et al., 1990). This phenomenon is supported by *in vivo* studies in which breast-fed infants have a much higher percentage of *Bifidobacterium bifidum* than formula-fed infants. In
contrast, formula fed infants have relatively low numbers of bifidobacteria, and a more diverse microflora with and enterococci, bacteroides and clostridia predominating (Lankaputhra, 1997; Lankaputhra & Shah, 1998; Goldman, 2000). Nevertheless, it has been found that HMO consumption is not exclusive to bifidobacteria species, as *Bacteroides fragilis* and *Bacteroides vulgatus* are evidently able to metabolize free glycans and reach moderate levels. In contrast, *Enterococcus, Streptococcus, Veillonella, Eubacterium, Clostridium,* and *Escherichia coli* had no or very low growth showing that HMO promote the growth of selective beneficial bacteria (Ward et al., 2007).

### 4.2.1.2.2. Milk oligosaccharides as pathogen adherence inhibitors.

The virulence of most pathogenic microorganisms depends on their ability to adhere to the host’s epithelial surface. Adhesion related virulence molecules are often lectins, glycan-binding proteins which bind to oligosaccharides on the epithelial cell surface (Sharon N. 1996). Binding sites for microorganisms are abundant in the gut since the intestinal mucosa is one of the most heavily glycosylated tissues (Nagler-Anderson 2001). The principal targets for viruses and bacteria are the sialylated and fucosylated OS (Bohem & Moro, 2008.), which are the two types of OS present in mammalian milks. This suggests that milk oligosaccharides serve as decoys, blocking pathogen adhesion, and protecting the breastfed infant against infections (Newburg et al., 2005). In fact, HMO and the glycans present on different cell types are synthesized by common glycosyl- and fucosyltransferases. (Newburg, 2000; Erney et al., 2000)

Milk OS are responsible for inhibiting the adherence of pathogens to epithelial cells (Table 5) (Kunz, et al., 2000, Morrow et al., 2005; Newburg et al., 2005; Bao et al., 2007) and blocking the toxic effects of bacterial toxins such *E. coli* heat-stable enterotoxin (Cravioto et al., 1991; Schwertmann et al., 1999). Fucosylated OS fraction of human milk, specifically α1,2-linked fucosylated glycans, protect against *C. jejune*, enterotoxigenic *E. coli*, its stable toxin and major strains of cacilviruses by inhibiting binding to host cell receptors (Cravioto et al., 1991;
Furthermore, it has been demonstrated that the concentration of α1,2-linked fucosylated glycans in the mother’s milk is inversely related to the incidence of diarrhea caused by *Campylobacter*, the stable toxin of enterotoxigenic *E. coli* and cacilviruses (Ruiz-Palacios et al., 2003; Morrow et al. 2004; Newburg et al. 2004; Morrow et al, 2005).

Human milk OS showed a strong inhibitory capacity against ETEC and UPEC adhesion, processes in which sialic acid seems to play an important role. After native OS are desialylated the levels of inhibition decrease. Since pure sialic acid does not inhibit adherence, it is thought that the acid residue is involved in recognition and the neutral parts of the OS are responsible for the binding. Bovine milk OS are also shown to be quite good inhibitors of UPEC strains, and less effective against ETEC strains for which human milk OS are shown to be better binding inhibitors. (Martín-Sosa et al. 2002). *Helicobacter pylori* have been shown to be inhibited by 3’-sialyllactose, 6’-sialyllactose and 6’-sialyllactosamine isolated from bovine milk to different extents, with 3’sialyllactose being the most active OS (Simon et al., 1997). Non enteric pathogens are also inhibited by human milk, for example *Streptococcus pneumoniae* is inhibited by sialylated oligosaccharides ending in NeuAca2-3(or 6)Galb1, and to a less extent by OS that terminate with lactosamine (Galb1-4GlcNAcb1) (Anderson et al., 1986; Barthelson et al. 1998).

Native and enzyme-modified whey products are effective inhibitors of association and invasion of *Salmonella* Typhimurium, *E. coli* O157:H7 and *Enterobacter sakazakii* to human CaCo-2 cells. In particular whey products which were treated with porcine pancreatic lipase (PPL) were better inhibitors of association and invasion. Such increase in the inhibitory effect is thought to be the consequence of the presence of active peptides or free fatty acids that were latent within the untreated material (Halpin et al. 2010) Neither the invasion nor the adherence inhibitions is attributed to the milk OS, which could be due to the very low concentration of these compounds in this fraction.
It has been suggested that the main role of anionic OS is to block the adhesion of pathogenic bacteria to the epithelial surface, whereas the neutral OS are responsible of the development of beneficial intestinal microbiota. The premise that supports this hypothesis is based on the newborn immune system development state and concentration of acidic OS in the different lactation stages. In the first days of life the newborn’s immune system is not well developed, therefore, the defense against pathogens has to be based on external factors. Consequently, sialylated milk OS which are in high concentration in colostrum would be responsible for blocking pathogens infections during this stage. After some time has elapsed, the immune system is well developed and it’s able to control microbial infections; by this time acidic OS concentration has decreased significantly, while some of the neutral OS concentration increase, suggesting that neutral OS feed selective colonic bacteria promoting the infant health. Although this hypothesis is consistent, it cannot be insured that acidic OS exclusively protect from pathogens and neutral OS feed selective bacteria. There is enough evidence that shows that several fucosylated OS inhibit microbial adhesion to epithelial cells and acidic OS can also be metabolized by a Bifidobacterium specie. Therefore, neither the anionic nor the neutral OS have an exclusive function.

4.2.1.2.3. Other functions of milk OS. In the specific case of sialylated OS, it has been suggested that increased production of gangliosides (important components in the membrane receptors and cell surfaces of the nervous system), serve as a source of sialic acid for newborn infants. Although all mammals have the capacity to synthesize sialic acids, newborn infants typically incorporate exogenous sialic acids because of their immaturity and rapid growth. The development of the brain exceeds their capacity to produce it and, therefore diet is an important source for sialic acids (Tao et al. 2008). It has been shown that sialic acid-containing oligosaccharides also reduce the adhesion of leukocytes to endothelial cells, an indicator of an immune regulatory effect of certain HMO (Kunz and Rudloff, 2008). Other immunodulatory
effects that have been proposed include the decrease of allergic reactions in the skin (Gruber et al., 2010) and stimulation of the production of cytokins in blood-borne immune cells (Eiwegger et al., 2004). Milk OS can also have a direct effect on the immune system; OS can be taken up by the dendritic cells (Braunstein et al., 1997) which will have many potential downstream effects derived from the many known immune-regulating functions of dendritic cells (Recigno et al., 2009).

4.2.1.3. Extracting oligosaccharides from milk.

Most of the functions attributed to milk oligosaccharides have been established using HMO. However, for obvious reasons, human milk components are not food grade and their use in food systems would not be legal. Despite this restriction, there is still much interest in obtaining large amounts of human OS or human-like OS for food or pharmaceutical applications. Accordingly, several approaches have been considered, based on both in-vivo and in-vitro technologies.

The first strategy relies on exploiting the the natural biosynthesis of milk OS in the mammary gland. Although not all of the specific reactions are known, it is possible to synthesize HMO either in tissue culture models (Ackland et al, 2001), transgenic animals (Prieto et al., 1995), or genetically engineered bacteria (Priem et al., 2002). However, these methods are biochemically rather complicated and would also presumably require legal approval before they could be implemented as accepted processes for the production of human consumption products.

A second approach for obtaining oligosaccharides from milk would be to identify animal species or breeds that produce milk with high levels of OS. The most important variables to be taken into account would include composition, availability, production size, and price. Some of the animals that have been considered for this purpose include elephants, goats and cows. Elephant milk OS, in particular, closely resemble human milk OS, with respect to composition. However, to our knowledge there are no commercial applications for milk from elephants. Goat
milk is also a rich source of OS and also has structural diversity that is greater than that of sheep and cow. Bovine milk has been considered as a suitable source of OS because it is produced in very high quantities, is easily accessible, and the oligosaccharide-containing byproducts (i.e., whey) have a very low price. Until a few years ago, most of the whey produced during cheese manufacture was simply discarded. Recently, industry interest in whey utilization has increased significantly, as it now used to enhance cheese yield or produce high value alternative products. During 2010, total United States cheese manufacture resulted in the production of 4.2 million tons of whey (USDA, 2011). Therefore, the harvesting of the bioactive oligosaccharide fraction could increase the value of whey.

Finally, another potential source of OS is bovine colostrum, and the protein-free or colostrum whey fraction, in particular. Although the colostrum processing industry is not nearly as developed as for bovine mature milk, there is still a large amount of colostrum sub-products available for use as a source of OS and other milk bioactive compounds. Most of these colostrum products are marketed in Japan and the Far East. Bovine colostrum is relatively cheap since dairy producers must keep milk free of colostrum to fulfill legal regulations. In addition, since the US currently prohibits colostrum products in human foods, the existing product demand (at least domestically) is relatively low, which keeps the price low as well. Furthermore, the OS concentration is significantly higher than in mature milk, representing an additional advantage of colostrum whey.

As mentioned before, a unique characteristic of bovine colostrum, in contrast with human milk, is the presence of NeuGc-linked OS. Although it is not clear, it has been postulated that the consumption of NeuGc is associated with risk or incidence of disease. In effect, an excess of NeuGc intake provokes an immune response in adults (Varki, 2001). Since NeuGc is present to a wide extent in most mammalian cells, its absence in human cells has been explained as an evolutionary phenomenon. The lack of NeuGc might be a strategy to decrease susceptibility or create resistance against certain pathogens. Microbes that cause important diarrheal diseases in
farm animals have a strong preference for NeuGc and humans are thus immune to these infections (Lanne et al., 1995; Varki, 2001). It will be necessary to study the health implications of NeuGc-linked OS consumption and its interaction with human pathogens in order to determine whether the presence of NeuGc in bovine milk would be a problem for the use of colostrum as a source of OS. It is possible that the use of NeuGc in moderated doses is safe enough to inhibit pathogen binding in any significant level.

4.2.1.4. Fractionation, separation, and analysis of milk OS

Several physical and chemical processes have been used to separate biologically valuable OS compounds from milk. These include chromatography, gel and capillary electrophoresis, elective precipitation and membrane technology. The only technique that has been efficiently implemented at a large scale is membrane separation using either nano or conventional platforms. The effectiveness of nanofiltration and reverse osmosis has been evaluated using human, goat and bovine milk or whey, showing satisfactory results. The permeate from cheese whey ultrafiltration was shown to be a rich source of milk OS with composition similar to those in human milk. In the particular case of the whey obtained in the production of Gorgonzola cheese, 115 different OS were identified, with 7 having the same composition as human milk OS. (Barile et al., 2009). To facilitate the separation of lactose and the OS, nanofiltration has also been combined with enzymatic treatment which enhances the separation by increasing the size difference between the contained sugars. (Sarney et al., 2000; Roth et al., 2001; De-Frees, 2002; Martinez-Ferez et al., 2006; Barile et al., 2009). The other technologies have not been implemented in the industry due to high capital cost, low productivity and the high difficulty of operation.
5. Oligosaccharides potential separation technologies

5.1. Nanofiltration

There are four major membrane separation technologies used in bioprocessing. These include nanofiltration (NF), microfiltration (MF), ultrafiltration (UF) and reverse osmosis (RO). All of these separate particles in a fluid based on their size, shape, and charge, using pressure as the driving force. The different separation methods differ from each other in the pore size, the membrane material, and the pressure exerted over the membrane. MF uses the biggest pore size followed by UF, NF and RO. Membranes for NF have pore sizes less than 0.002 µm, which approximates a molecular weight cut-off between 100 and 1200 Daltons (Wisconsin center of dairy research, 2004). While small components will pass through a NF membrane, components larger than the membrane cut-off, will be retained despite the driving force. The charge of the molecules is also a determinant of the separation. Ions with the same charge as the membrane will be retained due to an electrostatic repulsion, whereas ions with opposite charge will pass through (Zhang, 1998). The feed material that is retained and eventually gets concentrated is called retentate and the portion that crosses the membrane is the permeate or the filtrate.

Nanofiltration has a wide range of applications in different industrial settings, including the pharmaceutical, textile, chemical, agricultural, water production, and electronic and optical industries. The dairy industry was among the first users of nanofiltration, as it can be used to concentrate and fractionate liquid whey. The various product streams include lactose, whey permeates, and bioactive oligosaccharides (Bird, 1996; Koyuncu et al., 1999; Sarney et al., 2000; Rektor and Vatai, 2004; Martinez-Ferez et al., 2006). Other applications include the clarification and concentration of fruit juices (Warczok et al., 2004), the treatment of waste water from beverage production (Pietraszek, 2001) and the separation of oligosaccharides from carbohydrate mixtures where mono and disaccharides are present. The latter application is important because OS separation is complicated and many difficulties have been encountered when using other technologies. In addition, NF is attractive since it allows the processing of large amounts of
product. Successful separation of oligosaccharides includes the recovery of soy bean oligosaccharides from soybean whey wastewater (Rucka et al., 1996), human milk oligosaccharides from lactose (Sarney et al., 2000), concentration of OS from chicory roots (Kamada et al., 2002), and purification of enzymatically synthesized GOS from lactose feedstocks (Goulas 2002, 2003).

5.2. Lactose crystallization

Crystallization from solution is an important separation and purification process in a variety of industries. Compounds obtained by this process include proteins, sugars such as sucrose, fructose and lactose, catalysts, sodium chloride and other pharmaceutical components. Crystallization is a solid-liquid separation that relies on the formation of solid crystals that will precipitate out of solution. Since crystallization occurs only while the solution is supersaturated, it is necessary to control the relation between solubility and supersaturation to achieve an effective separation. Supersaturation can be achieved by cooling, evaporation or modification of the solvent. The beginning of the crystallization i.e. the start of crystals formation can be spontaneous or induced. In practice, crystallization is induced by adding crystals of the substance of interest to the reactor; this process is known as seeding. It promotes crystal formation of the solute over the surface area of the preformed solids.

Due to the mutarotational property of lactose, the behavior of this disaccharide is quite different from other common sugars. In aqueous solution lactose, $\alpha$ and $\beta$ isomers are in a reversible equilibrium known as mutarotation. In general, the $\alpha$ form of lactose is less soluble. Therefore, $\alpha$ lactose will be the first to crystallize out of a supersaturated solution. After the crystallization of $\alpha$ lactose has occurred, the molecules in suspension will mutarotate to restore the equilibrium. Mutarotation proceeds relatively fast at high temperatures, whereas it is very slow at temperatures closer to the freezing point. Since the ideal conditions to promote mutarotation and crystallization are conflicting, crystallization needs to be performed as the
temperature decreases slowly. In this way, the solution will be supersaturated during the entire process and the molecules will have enough time to restore the equilibrium.

5.3. Affinity chromatography

Chromatography is a widely used separation method applied in industry and laboratory processes. It is a very versatile approach in terms of the wide variety of materials, equipment and techniques that can be used. The separation is based on the distribution of the sample (solute) between the mobile phase and a fixed or stationary phase (Nielsen, 2003). Affinity chromatography is the only chromatography method that is based on the specific interaction between a molecule in solution and the ligand immobilized in the stationary phase. This reversible interaction usually involves biological materials as the stationary phase, including antibodies, enzyme inhibitors and lectins. When the sample passes through the column, the solutes that are complementary to the immobilized ligand will bind to the stationary phase while the rest of the components will be eluted or pass through the column. The bound analyte is then eluted by changing the mobile phase to induce dissociation (Figure 4).

The immobilized ligands can be general or specific. Specific ligands such as antibodies will bind only to their cognate ligand. In contrast, general ligands such as lectins will bind all carbohydrate moieties for which they have affinity (e.g. *Ulex europaeus* EUA-1 lectin will bind molecules containing fucose). The target molecule of the ligands in the stationary phase will determine the type of compounds that can be separated from the sample. Industrial applications of affinity chromatography include preparing recombinant proteins, diagnostic enzymes, monoclonal antibodies, and restriction nucleases, removing trace amounts of contaminants from therapeutic substances, and processing of human plasma (Clonis, 1987). A summary of available affinity media or stationary phase ligands is shown in table 6.
<table>
<thead>
<tr>
<th>Organism</th>
<th>Carbohydrate Specificity</th>
<th>Structure</th>
<th>Target tissue</th>
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<td></td>
<td>Fucα2Galβ3(Fucα4)Gal</td>
<td>GP</td>
<td></td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>Man</td>
<td>GP</td>
<td>Respiratory</td>
</tr>
<tr>
<td>N. gonorrhoea</td>
<td>Galβ4Glc(NAc)</td>
<td>GL</td>
<td>Genital</td>
</tr>
<tr>
<td>N. meningitidis</td>
<td>[NeuAc(α2→3)]0,1</td>
<td>GL</td>
<td>Respiratory</td>
</tr>
<tr>
<td></td>
<td>Galβ4GlcNAcβ3Galβ4GlcNAc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>L-Fuc</td>
<td>GP</td>
<td>Respiratory</td>
</tr>
<tr>
<td></td>
<td>Galβ3Glc(NAc)β3Galβ4Glc</td>
<td>GL</td>
<td></td>
</tr>
<tr>
<td>S. typhimurium</td>
<td>Man</td>
<td>GP</td>
<td>Intestinal</td>
</tr>
<tr>
<td>S. pneumoniae</td>
<td>[NeuAc(α2→3)]0,1</td>
<td>GL</td>
<td>Respiratory</td>
</tr>
<tr>
<td></td>
<td>Galβ4GlcNAcβ3Galβ4GlcNAc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. suis</td>
<td>Galα4Galβ4Glc</td>
<td>GL</td>
<td>Respiratory</td>
</tr>
</tbody>
</table>

Table 1. Carbohydrates attachment sites for bacterial pathogens on animal tissues, Predominant carbohydrate form: glycolipid (GL), glycoprotein (GP), glycophingolipids (GSL). Adapted from Ernst and Magnani, 2009; Ofek et al., 2003 and Sharon 2006
<table>
<thead>
<tr>
<th>Lectin</th>
<th>Plant Specie</th>
<th>Carbohydrate specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Monosaccharides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con A</td>
<td>Canavalia ensiformis</td>
<td>Man/Glc containing α linked Man</td>
</tr>
<tr>
<td>Concanavalin A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WGA</td>
<td>Triticum vulgare (Gramineae)</td>
<td>Man/Glc (low affinity)</td>
</tr>
<tr>
<td>Wheat Germ Agglutinin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PNA</td>
<td>Arachis hypogea</td>
<td>Gal Galβ3GlcNAc</td>
</tr>
<tr>
<td>Peanut Agglutinin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RCA</td>
<td>Ricinus communis (Euphorbiaceae)</td>
<td>Gal oligosaccharides ending in Gal and NAcGal</td>
</tr>
<tr>
<td>ECL</td>
<td>Erythrina cristagalli</td>
<td>Gal Galβ4GlcNAc</td>
</tr>
<tr>
<td>Erythrina Cristagalli Lectin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EEL</td>
<td>Euonymus europaeus (Celastraceae)</td>
<td>Galα3GlcNAc</td>
</tr>
<tr>
<td>Euonymus Europaeus Lectin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SBA</td>
<td>Glycine max</td>
<td>GalNAc/ Gal Oligosaccharides with terminal α- or β-linked NAcGal/ Gal residues</td>
</tr>
<tr>
<td>Soybean Agglutinin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UEA I</td>
<td>Ulex europaeus I</td>
<td>Fu*Fucα6GlcNAc</td>
</tr>
<tr>
<td>Ulex Europaeus Agglutinin I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DBA</td>
<td>Dolichos biflorus</td>
<td>GalNAc α linked NAcGal</td>
</tr>
<tr>
<td>Dolichos Biflorus Agglutinin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHA-E</td>
<td>Phaseolus Vulgaris</td>
<td>-</td>
</tr>
<tr>
<td>Phaseolus Vulgaris</td>
<td>Erythroagglutinin</td>
<td>Galβ4GlcNAcβ2Manβ4-R</td>
</tr>
<tr>
<td>Erythroagglutinin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHA-L</td>
<td>Phaseolus Vulgaris</td>
<td>-</td>
</tr>
<tr>
<td>Phaseolus Vulgaris</td>
<td>Leucoagglutinin</td>
<td>Galβ4GlcNAcβ2 Man</td>
</tr>
</tbody>
</table>

**Table 2.** Relevant plant lectins their origin and carbohydrate specificity. Rudiger and Gabius, 2002; Lis and Sharon, 1986; Iskratsch et al., 2009; Chen et al., 2008
<table>
<thead>
<tr>
<th>Item</th>
<th>Day of lactation</th>
<th>Milk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Specific gravity</td>
<td>1.056</td>
<td>1.040</td>
</tr>
<tr>
<td>Solids, %</td>
<td>23.9</td>
<td>17.9</td>
</tr>
<tr>
<td>Protein, %</td>
<td>14.0</td>
<td>8.4</td>
</tr>
<tr>
<td>Casein, %</td>
<td>4.8</td>
<td>4.3</td>
</tr>
<tr>
<td>IgG, mg/ml</td>
<td>48.0</td>
<td>25.0</td>
</tr>
<tr>
<td>Fat, %</td>
<td>6.7</td>
<td>5.4</td>
</tr>
<tr>
<td>Lactose, %</td>
<td>2.7</td>
<td>3.9</td>
</tr>
<tr>
<td>Vitamin A, ug/L</td>
<td>2950</td>
<td>1900</td>
</tr>
<tr>
<td>Vitamin D, IU/g fat</td>
<td>0.9 to 1.8</td>
<td>0.4</td>
</tr>
<tr>
<td>Riboflavin, ug/ml</td>
<td>4.8</td>
<td>2.7</td>
</tr>
<tr>
<td>Choline, mg/ml</td>
<td>0.70</td>
<td>0.34</td>
</tr>
</tbody>
</table>

Table 3. Composition of colostrum and mature milk (Foley and Otterby, 1978)
Figure 2. Structures of human and bovine milk oligosaccharides. Galactose , Glucose , GlcNAc , NeuAc , Fucose . The HMO structures can be elongated by repeating units of lactosamine (GlcNAc and galactose) and further decorated by sialic acid and fucose. The BMO structures can be further elongated with residues of GlcNAc, galactose, and sialic acids. Adapted from Zivkovic and Barile, 2011.
Table 4. Difference in concentration and composition of human milk, bovine colostrum and bovine mature milk oligosaccharides.

<table>
<thead>
<tr>
<th></th>
<th>Human Milk</th>
<th>Bovine Colostrum</th>
<th>Bovine mature milk</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Concentration</strong></td>
<td>7-12 g/L</td>
<td>0.7-1.2 g/L</td>
<td>0.1-0.6 g/L</td>
</tr>
<tr>
<td><strong>Structures</strong></td>
<td>200 different OS</td>
<td>30-40 OS structures</td>
<td>Less complex with fewer isomers</td>
</tr>
<tr>
<td><strong>Type of OS</strong></td>
<td>High fucosylated (70% of the total OS)</td>
<td>No fucosylated OS</td>
<td>No fucosylated OS</td>
</tr>
<tr>
<td></td>
<td>Anionic OS are minor components (&lt;20%)</td>
<td>Neutral OS represent 22% of the total OS</td>
<td>Over 45% of the total OS are neutral</td>
</tr>
<tr>
<td></td>
<td>NeuAc-linked OS</td>
<td>Over 70% of the total OS are acidic</td>
<td>Acidic OS account for up to 50%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NeuAc -linked OS (73%)</td>
<td>NeuAc-linked OS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NeuGc-linked OS (5%)</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3. a. Changes in the total oligosaccharides (OS) and total anionic OS of bovine milk at different stages of lactation [colostrum, transitional (day 6) and mature milk (day 120)]. RT = retention time. b. Changes in neutral and anionic OS proportions at different stages of lactation. Adapted from Tao et al. 2009
<table>
<thead>
<tr>
<th>Oligosaccharide</th>
<th>Pathogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligosaccharides</td>
<td><em>Streptococcus pneumoniae</em></td>
</tr>
<tr>
<td></td>
<td>Enteropathogenic <em>E. coli</em></td>
</tr>
<tr>
<td></td>
<td>Uropathogenic <em>E. coli</em></td>
</tr>
<tr>
<td></td>
<td>Enterotoxigenic <em>E. coli</em></td>
</tr>
<tr>
<td></td>
<td><em>Listeria monocytogenes</em></td>
</tr>
<tr>
<td>Fucosylated oligosaccharides</td>
<td><em>Campylobacter jejuni</em></td>
</tr>
<tr>
<td></td>
<td><em>Vibrio cholerae</em></td>
</tr>
<tr>
<td></td>
<td>Stable toxin</td>
</tr>
<tr>
<td>Sialylated oligosaccharides</td>
<td><em>Streptococcus pneumoniae</em></td>
</tr>
<tr>
<td>Sialyllactose</td>
<td>Cholera toxin</td>
</tr>
<tr>
<td></td>
<td><em>E coli</em></td>
</tr>
<tr>
<td></td>
<td><em>P. aeruginosa</em></td>
</tr>
<tr>
<td></td>
<td><em>Aspergillus fumigatus conidia</em></td>
</tr>
<tr>
<td></td>
<td>Influeza virus</td>
</tr>
<tr>
<td></td>
<td>Polyomavirus</td>
</tr>
<tr>
<td></td>
<td><em>Helycobacter pylory</em></td>
</tr>
</tbody>
</table>

**Table 5.** Pathogens inhibited by human milk oligosaccharides (Newburg et al., 2005; Chicholwski et al., 2011)
**Figure 4.** Principle of bioselective affinity chromatography. 

a. the support presents the immobilized ligand to the analyte to be isolated.  

b. Only the analyte makes contact with the ligand and attaches to it.  

c. The analyte is recovered by the introduction of an eluent, which dissociates the complex holding the analyte to the ligand. (Adapted from Nielsen, 2003)
<table>
<thead>
<tr>
<th>Target molecules</th>
<th>Potential Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activated / Functionalized</td>
<td>Functional spacer; support matrix; eliminates handling of toxic reagents</td>
</tr>
<tr>
<td>Amino Acid</td>
<td>Serum proteins; proteins; peptides; enzymes; rRNA; dsDNA</td>
</tr>
<tr>
<td>Avidin Biotin</td>
<td>Purification of biotin/avidin &amp; derivatives; biotinylated substances. Biotin derivatives dissociate under nondenaturing conditions.</td>
</tr>
<tr>
<td>Carbohydrate Binding</td>
<td>Soluble glycoproteins; other carbohydrate-containing substances</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>Glycoproteins; lectins; other carbohydrate metabolite proteins. Proper selection can ensure one-step purification. Nonspecific interaction. Mimic biological substrates (substrates, cofactors, effectors); proteins. Optimize purification protocol with different dyes</td>
</tr>
<tr>
<td>Dye Ligand</td>
<td>Purification of glutathione enzymes and GST tagged recombinant proteins</td>
</tr>
<tr>
<td>Glutathione</td>
<td>General affinity ligand, useful for plasma coagulation proteins, nucleic acid enzymes, lipases, etc.</td>
</tr>
<tr>
<td>Heparin</td>
<td>Couple ligands containing free carboxyl groups; proteins</td>
</tr>
<tr>
<td>Hydrophobic Interactions</td>
<td>Immobilized Metal Affinity Chromatography. Uses interactions between protein and chelated metal to separate.</td>
</tr>
<tr>
<td>IMAC</td>
<td>Quantitative determination of antigens, high specificity</td>
</tr>
<tr>
<td>Nucleotide / Coenzyme</td>
<td>Dehydrogenases; kinases; transaminases. Reliable adsorbents.</td>
</tr>
<tr>
<td>Nucleic Acid</td>
<td>mRNA; DNA; rRNA; other nucleic acids and oligonucleotides</td>
</tr>
<tr>
<td>Protein A / Protein G</td>
<td>Purification of immunoglobulins</td>
</tr>
</tbody>
</table>

**Table 6.** Potential applications of affinity chromatography based on the stationary phase target molecules. Adapted from Sigma Aldrich, 2011
References


Nagy, B., and P. Z. Fekete. 1999. Review article Enterotoxigenic Escherichia coli (ETEC) in farm animals. Veterinary research.


Pietraszek, M. Nanofiltracja w uzdatnianiu wody i oczyszczaniu ścieków w przemyśle spożywczym. Techniki Membranowe dla Czystych Technologii 31-34.


Simon, P. M., P. L. Goode, A. Mobasseri, and D. Zopf. 1997. Inhibition of Helicobacter pylori binding to gastrointestinal epithelial cells by sialic Inhibition of Helicobacter pylori
Binding to Gastrointestinal Epithelial Cells by Sialic Acid-Containing Oligosaccharides. Infection and Immunity 65:750-757.


Chapter 2

Inhibition of lectin adherence to tissue culture cells by prebiotic carbohydrates

Maldonado-Gómez M.X., Fangman T., Pinto A., Rupnow J., Hutkins R
Abstract

Prebiotic carbohydrates, in addition to their ability to influence the colonic microbiota, are also able to interfere with how pathogenic bacteria attach to the epithelial cells that line the intestinal tract. For Salmonella, Escherichia coli, and other enteric pathogens, adherence is a receptor-mediated event between bacterial adhesins and their complementary ligands located on the mucosal surface. Some prebiotic carbohydrates are structurally similar to these ligands and inhibit lectin binding. However, the mechanism for this inhibition is not well understood, in part because the receptor sites on the target cells have not been identified. The goal of this research was to measure the effect of two prebiotic carbohydrates, galactooligosaccharide (GOS) and polydextrose (PDX), on the binding kinetics of lectins to epithelial tissue culture cells. To measure adherence, fluorescent-labeled lectins (ConA, WGA, PNA, RCA and ECL) were added individually to cover slips containing HEp-2 tissue culture cells. In some treatments, either the cognate ligand or a prebiotic was added; unbound reactants were removed by washing. Photographs were taken by fluorescence microscopy and the images were analyzed to quantify the spectral component. All the lectins that were able to bind to the target cells were inhibited by nearly 100% in the presence of 1 mg/ml of the cognate ligands. When prebiotics were added (up to 100 mg/ml), inhibition of lectin binding also was observed, depending on the structural similarity between the prebiotic and the cognate ligands. In particular, GOS significantly inhibited attachment of all the lectins except for WGA. In contrast, PDX did not significantly inhibit attachment of the lectins (with the exception of WGA), suggesting that it is structurally dissimilar to the HEP-2 receptor sites. The results from this research support the role of GOS as an anti-adherence agent and also suggest that the receptor sites located on the surface of epithelial HEp-2 cells are structurally similar to GOS. In conclusion, the proposed mechanism of bacterial binding inhibition caused by active oligosaccharides was confirmed.
Introduction

Nearly all microbial pathogens need to adhere to the surface of the host tissue cells in order to cause infections. Adherence provides the means by which the pathogen can resist environmental shear forces, initiate biofilm formation, and begin to colonize or invade host tissue. To accomplish adherence, Van der Waal’s forces, Coulombic forces, and hydrophobic interactions bring the bacteria to a closer proximity and mediate a reversible bond. A more permanent adhesion occurs when structures on the bacterial surface interact with complementary ligands on the mucosal surface. (Busscher & Weerkamp 1987; Ofek et al. 2003). These bacterial structures are specialized components or appendages defined as adhesins. The adhesins-receptor interactions are highly stereospecific and determine tissue tropism of the pathogen. Three types of adhesin-receptor interactions of different chemical nature have been recognized: (1) recognition between a protein on the surface of the bacteria and a complementary protein on the mucosal surface; (2) binding between hydrophobins in both bacterial and tissue cell surfaces; and (3) binding of lectins with carbohydrate moieties. The latter is the most common mechanism and accounts for the primary means by which adherence occurs among bacterial pathogens.

Lectins, which are also found in viruses, plants and animals, are proteins capable of specific recognition and reversible binding to carbohydrate moieties without altering the covalent structure of the glycosyl ligands. Typically, each lectin molecule contains two or more carbohydrate linking sites, and this di- or polyvalency enhances adherence activity. In addition, many bacterial pathogens have the ability to express different adhesins and at different stages of infection. Thus, it is the concerted action of the different adhesins that allows infections to occur (Klemm et al., 2010).

Prebiotic carbohydrates are defined as food ingredients that provide beneficial effects for the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon (Gibson et al., 1995). In addition to their ability to influence the colonic microbiota, however, some prebiotics are also able to interfere with how pathogenic bacteria
attach to the epithelial cells that line the intestinal tract. This adherence inhibitor property has also been attributed to several naturally-occurring carbohydrates, including milk oligosaccharides, mannan oligosaccharides, and pectic oligosaccharides (Kisiela et al. 2006; Ganan et al. 2010). It has been proposed that the bioactivity of such carbohydrates is due to their structural similarity to the glycans present on the surface of host cells. Hypothetically, these free carbohydrates would act as receptor analogs and block the lectin-carbohydrate interaction. Bacteria would recognize these molecules and bind to them instead of binding to the host cell receptor. However, the mechanism of adherence inhibition is not well understood, in part because the receptor sites on the target cells have not been identified and because there are other variables that could also affect the adherence event.

To study and understand the mechanism of adherence inhibition of bioactive carbohydrates, we propose a simplified model in which tissue culture cells are exposed to plant lectins to simulate bacterial lectin-receptor interactions. The use of plant lectins eliminates some of the variables associated with the use of live pathogens used in anti-adherence experiments. Such variables include presence of multiple adhesins with different carbohydrate specificities, expression of adhesions depending on the growth stage and environmental conditions, and the bacterial production of interfering metabolites. In addition, lectins are well characterized and their cognate ligands are known, which allow us to test the possible mechanism of adhesion inhibition. Thus, lectin attachment would be inhibited in the presence of the cognate ligands or carbohydrates with similar structure. Therefore, the goal of this research was to measure the effect of the cognate ligands and two prebiotic carbohydrates, galactooligosaccharide (GOS) and polydextrose (PDX), on the binding kinetics of lectins to epithelial tissue culture cells.
Materials and Methods

Monosacharides and prebiotics oligosaccharides. The monosacharides used in the present study include glucose, galactose, N-acetyl galactosamine, N-acetyl glucosamine (Sigma Chemical Co., St. Lous, MO, USA) and mannose (Acros Organics, New Jersey, USA). Polydextrose (PDX) (Danisco) and galactooligosaccharide (GOS) were tested as potential inhibitors of lectin attachment. Two different GOS products were evaluated. The GOS (F-GOS) from Friesland Campina (FrieslandCampina, New York, USA) contains 53% GOS, and the GOS (G-GOS) from GTC Nutrition (GTC Nutrition, Golden, CO) contained 92% GOS.

Lectins. In total, nine fluorescein-labeled lectins were obtained (Vector Laboratories, Inc., CA, USA) and evaluated, including Concanavalin A (Con A), Wheat Germ Agglutinin (WGA), Peanut Agglutinin (PNA), Ricinus Communis Agglutinin I (RCA), Erythrina Cristagalli Lectin (ECL), Euonymus Europaeus Lectin (EEL), Soybean Agglutinin (SBA), Ulex Europaeus Agglutinin I (UEA I), and Dolichos Biflorus Agglutinin (DBA). Sources and carbohydrate specificity are summarized in table 1.

Tissue culture conditions. HEP-2 cells were obtained from the American Type Culture Collection (Manassas, Virginia). Cells were maintained under tissue culture conditions in minimal essential medium (MEM/EBSS NEAA Modified, HyClone, Thermo Fisher Scientific Inc, Utah, USA) supplemented with 10% fetal bovine serum (HyClone). For inhibition assays, subconfluent monolayers of HEP-2 cells were harvested with 0.25% (vol/vol) trypsin-EDTA solution (HyClone) and seeded into 24 well tissue culture plates at approximately 5 x 10^4 HEP-2 cells per well. Plates were incubated under tissue culture conditions for 30 hours before the experiment.
**Lectins adherence assessment.** Subconfluent HEP-2 cells were prepared as described above. Lectin solutions were prepared in MEM media without FBS (wMEM) and added at a concentration of 7 μg/ml into each well. Cells were incubated at room temperature for 1 hour in the dark to preserve fluorescein fluorescence. Preliminary studies revealed that 1 hour incubation time was sufficient to induce binding. Wells were then washed 2 times with wMEM and 3 times with PBS to remove un-bound lectin. Coverslips were fixed with 10% formaldehyde for 15 min and washed with PBS. Coverslips were placed in slides and analyzed microscopically (see below). All experiments were performed in duplicate.

**Competition experiments.** The same procedure described for the lectin adherence experiments was followed. However, for these competition experiments, 1 ml of the lectin-carbohydrate solution was added. Cognate ligands were added at concentrations of 1, 10 and 100 mg/ml and for oligosaccharides, concentrations of 25, 50 and 100 mg/ml were used.

**Fluorescence microscopy.** The samples were observed under an Olympus FV500 Confocal Laser Scanning Microscope with an Inverted Olympus IX81 microscope and a Plan.Apo 100X oil objective. The 488nm Argon laser was used for the fluorescein stained lectins (Vector Laboratories). For each sample, images from 10 different areas of the coverslip were taken. Each picture contained approximately 50 cells.

**Image Analysis.** MATLAB software (Version 7.8, 2007, The MathWorks, Inc., Natick, Massachusetts) was used to quantify the amount of green contained in each picture. The primary spectral component of green was obtained using the RGB color model. The programmed code separates the green channel from the image, and then adds all of the individual values obtained per pixel. Therefore, the total amount of green present in each picture was calculated.
**Statistical analysis.** Significant differences between the treatments were determined using one-way ANOVA. Post hoc pair-wise comparisons were done using Tukey’s test. Differences in lectins attachment were considered significant when P-values were less than 0.05. GraphPad Prism 5 (version 5.03, GraphPad Software, Inc, 2010) was used to perform statistical tests.

**Results**

**Attachment of lectins to HEp-2 cells.** Nine lectins were assessed for their ability to attach to HEp-2 cells. Only five of the tested lectins, Con A, ECL, PNA, RCA, and WGA, were able to attach to Hep-2 cells. In contrast, cells treated with DBA, SBA, UEA I and EEL lectins did not show any fluorescence signal when observed microscopically.

**Cognate ligands block lectins binding to tissue culture cells.** All of the lectins that were able to bind to HEp-2 cells were inhibited by nearly 100% in the presence of 10 mg/l of at least one of the cognate ligands (Figure 2). Con A was the only lectin for which the presence of the cognate ligand did not inhibit binding (in fact, there was a significant increase in adherence; Figure 2c). The latter was observed when 1 mg/ml of mannose was added. However, using the same monosaccharide but at higher concentrations (i.e., 10 and 100 mg/ml), attachment was effectively inhibited by 42 and 98% respectively. When two different monosaccharaides were used as cognate ligands, different levels of inhibition were obtained (Figure 2a,c,e).

**Prebiotic oligosaccharides inhibit lectins attachment to HEp-2.** Prebiotic oligosaccharides were also able to reduce adherence of the lectins to HEp-2 cells (Figure 1 and 2). The concentrations used to test the oligosaccharides were higher than the cognate ligands since the affinity towards these carbohydrates was unknown and less affinity was expected. However, both types of GOS were highly effective at inhibiting attachment. In fact, RCA, ECL and PNA binding
was completely inhibited by GOS even when the lowest concentration (25 mg/ml) was added (Figure 2a,b,e). GOS showed significant inhibition in most cases, and at least for one type of GOS a concentration of 100 mg/ml was enough to inhibit completely the lectin attachment. In general, G-GOS blocked lectin attachment more efficiently than F-GOS and PDX, with the exception of WGA. When HEp-2 cells were exposed to WGA, PDX was the only prebiotic carbohydrate capable of inhibiting attachment; neither G-GOS nor F-GOS had any significant effect. Nevertheless, PDX was not a very efficient inhibitor since 45% was the maximum level of inhibition reached by PDX for this lectin (Figure 2d). The only case in which PDX blocked the lectin attachment in a substantial manner was in the Con A lectin competition assay. Indeed, higher inhibition of this lectin was obtained in presence of PDX than F-GOS (Figure 2c).

**Discussion**

Plant lectins have been shown to be suitable reagents for characterizing carbohydrate moieties in epithelial cells and other biological systems. In fact, micro arrays and flow cytometry using fluorescently-labeled lectins have been employed to analyze cell surface carbohydrates in several cell lines (Chen, et al. 2007; Alam et al. 1990). In this study, plant derived lectins were used to elucidate the mechanism by which bacterial pathogens adhere in vitro to cell lines. The first step of this approach was to measure lectin attachment directly to HEp-2 cells, which provided a basis for predicting the ligand composition of these cells. Subsequent competition experiments enabled us to assess how similar prebiotic carbohydrates were to these ligands.

**Carbohydrate content of the HEp-2 cell surface.** The observation that several plant-derived lectins, including Con A, ECL, PNA, RCA, and WGA, attach to the human larynx carcinoma cell line implies that the HEp-2 cell surface contains one or more carbohydrates moieties for which these lectins are specific. Consequently, it can be inferred that these carbohydrates likely contain glucose, galactose, α linked mannose, N-Acetyl glucosamine,
dimers or oligosaccharides containing N-Acetylglucosamine and/or glycans with the linkages galactose(β-1,3)N-Acetylgalactosamine or galactose (B1-4)N-Acetylglucosamine. However, to our knowledge, the carbohydrate content of the HEp-2 cell surface has not been characterized.

The adhesins of several pathogenic bacteria are well characterized and their carbohydrate specificity is known (Ohlsen et al., 2009). For example, P-fimbriated uropathogenic *E. coli* strains that bind to HEp-2 cells (Guyer et al., 2002) contain an adhesin specific for glycosphingolipid containing Galα4gal (Ohlsen et al., 2009). Other microorganisms that attach to HEp-2 cells include *S. thyphimurium* (McGowan et al. 2010, unpublish data), *K. pneumoniae* (Favre-Bonte et al., 1999) and *E.coli* Type 1 (Boudeau et al., 2001), all of which bind to mannose residues (Ohlsen et al., 2009; Ofek et al., 2003; Stromberg et al., 1990). In contrast, *S. pneumonia* which also attaches to HEp-2 cells, binds carbohydrates containing the linkages Gal(β-1,3)NAcGal and Gal (B1-4)NAcGlu, (Ohlsen et al., 2009; Idanpaan-Heikkila et al., 1997; Pracht et al., 2005). In consequence, if a pathogen is able to bind to Hep-2 cells it implies that the carbohydrate for which it is lectin is specific is present in the epithelial cell surface.

To assess the ability of prebiotics to act as bacterial adhesin inhibitors, competitive binding experiments were performed. However, instead of using the bacterial adhesins, we used plant derived lectins for which the cognate receptors were already established.

Cognate ligands were added to test the hypothesis that lectin attachment to culture cells would decrease in the presence of free carbohydrates for which these proteins have high binding affinity. Indeed, all of the cognate ligands were able to inhibit lectin attachment, confirming that lectins are able to bind to free carbohydrates, reducing the number of unbound lectins able to bind the epithelial cells receptors.

The extent of inhibition is an indicator of the binding affinity towards a particular monosaccharide. In other words, the sugar with the highest affinity towards a particular lectin will bind the protein the most; as a result, the attachment to HEp-2 cells will be inhibited more
efficiently by this sugar than by any other. For example, RCA adherence was completely inhibited by galactose, whereas NAcGal showed no inhibition at the same concentration (1mg/ml). This behavior is consistent with the reported values of the binding affinity constants \(K_A\) for different galactose and NAcGal containing sugars (Itakura et al., 2007; Houston and Dooley, 1982). For example, \(p\)-Nitrophenyl glycosides of \(\beta\)Gal and \(\beta\)GalNAc were shown to have a \(K_A\) of approximately \(1.8 \times 10^5\) and \(0.3 \times 10^5\) M\(^{-1}\), respectively (Itakura et al., 2007). In a similar way, the addition of galactose at the lowest concentration had no effect on ECL attachment, but it was effectively inhibited by NacGlc. According to Wu et al. (2007) galactose is a much better inhibitor than NAcGlc. The inconsistency may be related to the fact that the binding affinity for these monosaccharides was evaluated in terms of binding inhibition. In Wu et al., \(\alpha\)1-acid glycoprotein was used as the target for ECL. Lectins could target different glycan moieties in each case, consequently galactose and NAcGlc exert different levels of inhibition. In the case that NAcGlc is present in low concentrations or not present at all in \(\alpha\)1-acid glycoprotein, but HEp-2 contain plenty of NAcGlc moieties and ECL is targeting for this monosaccharide, NAcGlc will act as an efficient inhibitor for HEp-2 and have little or no effect for \(\alpha\)1-acid glycoprotein. Accordingly, monosaccharides had to be present in solution at different concentrations to achieve equivalent percentages of inhibition. As revealed by ConA, mannose concentration must be increased by a factor of 10 to reach the same inhibition obtained using glucose. Contradictorily, other authors report that mannose in its pyranose form has a higher binding affinity for Con A than glucose has (Goldtein et al., 1965; Schwarz et al., 1993).

Binding affinity of the tested lectins towards prebiotic carbohydrates was unknown. Nevertheless, it was predicted that lectins attachment would be inhibited by prebiotic oligosaccharides, since their structural monosaccharides and their arrangement are similar to the cognate ligands in most of the cases. In fact, prebiotics were also capable of binding the lectins and reducing the adherence. In general, the greater the similarity between the prebiotic and the cognate ligands, the greater was the inhibition observed. For example, GOS was a more highly
efficient inhibitor for ECL, PNA and RCA lectins. The mentioned lectins are selective for galactose containing glycans and GOS molecules are comprised in the majority by galactose molecules. In contrast, PDX was a poor inhibitor or did not block the adherence at all, because it lacks galactose molecules in its structure (Figure 3). The reason why GTC GOS showed to be a better binding inhibitor among the GOS tested is the difference of the galactooligosaccharides concentration in each of the products. G-GOS contains 90%, while F-GOS contains only 57% (Friesland Campina, GTC Nutrition).

Although PDX has the highest glucose content, G-GOS was shown to be a better inhibitor of Con A attachment. This phenomenon could be the result of the difference in the monomers distribution and OS structures of each of the prebiotics. Possibly, the branched structure of PDX causes steric hindrance, and makes difficult the access of the glucose molecules to the active site of the lectin, and therefore, diminishes its binding affinity. On the other hand, GOS are linear oligosaccharides of smaller sizes. GOS molecules would be able to reach the active site of the lectin more easily, inhibiting to a higher extent its adhesion to the target cells. In fact, the carbohydrate molecular size has been suggested as an explanation of the amylopectin precipitation failure only when large molecules are tested (Goldstein et al. 1965).

Interestingly PDX was able to inhibit WGA although it is not specific for either glucose or galactose containing sugars. The inhibition observed could be due to the presence of organic acid in the PDX mixture. Negative charged molecules might interfere in the lectin attachment to the target cell. Commercial PDX is synthesized using organic acids as catalyst; however some of the acids react and end up in the final product. Organic acid can be either attached to the molecules or free in the mixture. (Allingham 1982). Agglutination of WGA has been inhibited by organic acids such NeuNAc and NeuNGc (Bhavanandan and Katlic, 1979). However, the affinities of acids are lower than other neutral cognate ligands and acid is present in PDX in trace amounts, what make this hypothesis unlikely. An alternative explanation could be a possible affinity towards specific linkages in the PDX polymers. Specificity could be more likely
towards the β 1-6 glycosidic linkage which is the PDX predominant bond. However WGA could also target any other of the possible glycosidic bonds. (Allingham 1982). Specificity for the type of linkages was also observed in ECL competition experiments. This is concluded based on the difference between the percentages of inhibition obtained with GOS and galactose. Although galactose significantly inhibited ELC attachment to HEp-2 cells, its action was not as effective as both types of GOS. The high effectiveness of the galactooligosaccharides may be attributed to the ECL affinity for the linkage Gal(b1-4)NAcGlu which is essentially the same conformation that Gal(b1-4)Glu (GOS glycosidic bond) has. This would imply that ECL has preference for glycans containing Gal(b1-4)NAcGlu over free galactose.

The only case in which the presence of a cognate ligand significantly increased the adherence of the lectin instead of inhibiting the attachment was ConA. This situation was observed only when the lowest mannose concentration was added; when higher concentrations were used the attachment was blocked efficiently. This phenomenon has been also observed when anti-adherence experiments using bacterial pathogens have been performed. McGowan (2011, unpublished data) reported an increase of Salmonella Typhimurium adherence to Hep-2 cells when GOS was added to evaluate its properties as an inhibitory agent. A possible explanation could be the formation of bridges between lectins in the culture cells’ surface, the free monosaccharides and the bacteria. However this theory has not been confirmed and seems to be improbable.

In general, PDX blocked lectins attachment very poorly, suggesting that it is not an effective adherence inhibition agent and that it is structurally dissimilar to the HEp-2 receptor sites. In contrast GOS was shown to be an efficient binding inhibitor. This observation elucidate why PDX has shown to be a poor inhibitor of bacterial adherence while GOS has been found to efficiently inhibit pathogens and their toxins to binding. Some examples include the inhibition of E. coli, E. sakazakii and Vibrio cholerae toxin adherence to epithelial cells and cell surface receptors (Shoaf, et al., 2006; Sinclair et al., 2009; Quintero et al., 2011).
References


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<tr>
<th>Lectin</th>
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<th>Monosaccharides</th>
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Table 1. Plant lectins used in the study; source and carbohydrate specificity. Rudiger and Gabius, 2002; Lis and Sharon, 1986; Iskratsch et al., 2009; Chen et al., 2008; Munson et al., 1989
Figure 1. Microscopic images of HEp-2 cells after competition experiments (100X). Con A vs G-GOS. Amount of green is directly proportional to the amount of bonded lectin and inversely proportional to the percentage of inhibition.
Figure 2. Lectins attachment inhibition by the action of cognate ligands and prebiotic carbohydrates. a. Erythrina Cristagalli Lectin (ECL), b. Peanut Agglutinin (PNA), c. Concanavalin A (Con A), d. Wheat Germ Agglutinin (WGA), d. Ricinus Communis Agglutinin I (RCA).
Figure 3. Structures of the prebiotic oligosaccharides used in the study. Glucose ; galactose

a. Galactooligosaccharides molecule, degree of polymerization between 2-6; containing glycosidic bonds: β 1-3, β 1-4, β 1-6. (GTC Nutrition, 2009)

b. Polydextrose molecule. The R-groups may be hydrogen, glucose, sorbitol, citric acid or a continuation of the polydextrose polymer; containing glycosidic bonds: all possible glycosidic bond, predominately α and β 1-6; Average degree of polymerization 12 (Allingham 1982; Craig et al. 1999).
Chapter 3

Technologies for obtaining oligosaccharide-rich fractions from bovine colostrum
Abstract

The oligosaccharide (OS) fraction of human milk has several important biological functions in the neonate. These OS contribute to the development of the nervous system, stimulate the immune system, protect against bacterial and virus infections, and enhance growth of beneficial bacteria in the intestinal tract. However, since human milk cannot be utilized as commercial source for these components, bovine milk and bovine colostrum by-products have been considered as potential sources of OS. They contain some of the human milk OS, are available in high quantities, and are relative cheap. The existing extraction methods, which mainly involve removal of lactose and concentration of the oligosaccharides fraction, are limited and complicated. The goal of this project was to evaluate several different technologies for obtaining OS-rich fractions from bovine colostrum. We tested nano-filtration, lactose crystallization, affinity chromatography, enzymatic hydrolysis, and bacterial fermentation as methods to degrade or remove lactose and/or separate oligosaccharides. Under the evaluated conditions, the physical-chemical separation methods were limited in their ability to enrich for OS from bovine colostrum. However nano-filtration was a promising option to achieve separation of simple sugars and OS, despite not being able to retain the OS within the retentate fraction. Microbial fermentation and enzymatic hydrolysis were effective methods for degrading lactose.

Introduction

Several important biological functions have been attributed to milk oligosaccharides, including improved gut health and protection against pathogens. These reports have increased interest in obtaining large quantities of milk oligosaccharides for use as food additives and/or pharmaceutical constituents. In addition, non-milk OS that have structural similarities to the core molecules of HMO have also been studied (Moro et al. 2002; Bouhnik et al. 2004; Depeint et al. 2008). The latter include the galactooligosaccharides (GOS), OS derived from lactose via transgalactosylation reactions. Both the milk-derived and lactose-synthesized OS are known to
have prebiotic activity, in that they specifically stimulate the growth of beneficial bacteria in the intestinal tract (LoCasio et al., 2007; Ward et al., 2007; Marcobal et al., 2010; Sela et al., 2011). They also provide postnatal stimulation of the immune system (Eiwegger et al., 2004; Braunstein et al., 1997; Recigno et al., 2009), and enhance defense against bacterial and viral infections (Kunz, et al., 2000, Morrow et al., 2005; Newburg et al., 2005; Bao et al., 2007). Although the process for obtaining non-milk bioactive carbohydrates is well established, the specific OS moieties generally differ only in length, which may limit their biological activity. This is because it has been suggested that the activity or effectiveness of milk OS relies on the heterogeneity of the oligosaccharide fraction (Bode, 2006). Thus, the more structurally diverse are the oligosaccharides, the greater will be their biological activity. This may be especially evident in adherence inhibition, as heterogeneous OS would be expected to block pathogen adherence more effectively than a more homogenous mixture of OS. In addition, a heterogeneous OS mixture may also serve as nutrients for beneficial bacteria with greater specificity, and thereby act as a more effective prebiotic. Nonetheless, for obvious reasons, human milk components are not food grade and their use in food systems would not be legal. Despite this restriction, there is still much interest in obtaining large amounts of human-like OS for food or pharmaceutical applications. Accordingly, several approaches have been considered, based on both in vivo and in vitro technologies.

The first strategy relies on exploiting the the natural biosynthesis of milk OS in the mammary gland. Although not all of the specific reactions are known, it is possible that HMO could be synthesized either in tissue culture (Ackland et al, 2001), transgenic animals (Prieto et al., 1995), or in genetically engineered bacteria (Priem et al., 2002). However, these methods are biochemically rather complicated and would also require considerable regulatory review before they could be considered for human consumption products. A second approach would be to identify animal species or breeds that produce milk with high levels of OS. Animals that have been considered as a source of OS include elephants, goats, and cows. Bovine milk has the
advantage of being produced in very high quantities and at low cost, especially whey. However, the OS concentration is low in milk or whey. In contrast, bovine colostrum contains 10 times more OS than milk, and although the colostrum processing industry is not as developed as for bovine mature milk, there is still a large amount of colostrum sub-products available for use as a source of OS and other milk bioactive compounds. Another unique characteristic of bovine colostrum, in contrast with human milk, is the presence of NeuGc-linked OS. The consumption of NeuGc is associated with risk or incidence of disease. In effect, an excess of NeuGc intake provokes an immune response in adults (Varki, 2001). Further studies must be done to establish if the presence of NeuGc in bovine milk limits the use of colostrum as source of OS. It is possible that NeuGc at low doses does not have health implications but is still an efficient pathogen binding inhibitor.

Several physical and chemical processes have been used to separate biologically or economically valuable OS compounds from milk. These include chromatography, gel and capillary electrophoresis, selective precipitation and membrane technology. The only technique that has been efficiently implemented at a large scale, however, is membrane separation using either nano or conventional platforms (Sarney et al., 2000; Roth et al., 2001; De-Frees, 2002; Martinez-Ferez et al., 2006; Barile et al., 2009). In one of these studies, using whey obtained from Gorgonzola cheese production, as many as 115 different OS were identified, with 7 having the same composition as human milk OS (Barile et al., 2009). In another report, separation of lactose from the OS fraction was facilitated via nanofiltration combined with enzymatic treatment of lactose (Sarney et al., 2000).

In this study, we used several methods to extract the OS fraction from bovine colostrum, and also assessed methods for direct degradation of lactose.
Material and Methods

**Carbohydrates.** Sugars and glycans used in this study included glucose, galactose, lactose (all from Sigma Chemical Co., St. Louis, MO, USA) and galactooligosaccharide (GOS; Purimune, CornProducts International, IL, USA). When large quantities of lactose were required, commercial refined lactose from bovine whey was used (Grande Cheese Company, Brownsville, WI, USA).

**Bovine colostrum samples and its fractionation procedure.** Colostrum from one cow in early lactation (< 1 day) was provided by a local milk producer (Prairieland Dairy, Firth, NE). Milk fat was removed by centrifugation at 4°C and 1000 x g for 30 minutes. Skim colostrum was then mixed with 70% ethanol in a ratio of 1:1 to precipitate proteins. The mixture was allowed to stand for 1 hour at 4°C before it was centrifuged for 40 minutes at 4°C and 1000 x g. The resulting solution was held overnight and centrifuged again at the same conditions to get the maximum separation. Carbohydrate extracts were obtained by evaporating the ethanol using a rotovaporator R-205 (Büchi, Delaware, USA), followed by freeze drying the aqueous solution. Precipitated proteins were also dehydrated by lyophilization. The carbohydrate content of the powders was measured by the phenol-sulfuric colorimetric method (Chaplin and Kennedy, 1986); an FP-528 Nitrogen (LECO, St. Joseph, Michigan) nitrogen/protein analyzer was used to determine total protein content.

**Enzymatic Hydrolysis.** β-Galactosidase (*Aspergillus oryzae*, Sigma) was used to hydrolyze lactose present in the carbohydrate fraction. Colostrum carbohydrate powder and 10 U of enzyme per gram of colostrum carbohydrate were dissolved in 50 mM sodium phosphate buffer (pH 6), filtered sterilized and incubated at 37°C with agitation during 120 hours.
**Bacterial Fermentation.** *Lactobacillus* spp. *1984*, *Lactobacillus helveticus* ATCC 15009, and *Lactococcus lactis* subsp. *lactis* 404 were obtained from the University of Nebraska Lincoln stock culture collection. MRS (Difco, MD, USA) broth and agar were used as growth media for *Lactobacillus* species and Elliker broth (Difco, MD, USA) was used for *L. lactis*. Two types of fermentation media were prepared and used during these experiments; both contained basal growth media-BGM (MRS or Elliker, without carbohydrate, depending on the microorganism). Carbohydrates, either GOS or the freeze dried colostrum extract, at a concentration of 2% (m/v) were added. Before each experiment, frozen stock cultures of each organism were thawed, plated onto the corresponding solid media and grown overnight at 37°C. Two more activations were carried out; a single colony was then inoculated into 10 ml of growth media, incubated overnight, and then inoculated at 1% (v/v) concentration into fresh media. To start the experiment, the fermentation media was inoculated (1% (v/v), and incubated at 37°C, in ambient atmosphere conditions without agitation, for 16 hours. Every 2 hours the OD (620 nm) was measured. pH was adjusted between 7.2 and 7.5 adding NaOH 0.2N to avoid over acidification and maintain cell growth. Simultaneously, at 4 hour intervals, fresh BGM was added to quadruple the volume contained in the fermentation flasks.

**Crystallization.** Supersaturated colostrum carbohydrate solutions were prepared (0.24 g/ml). The solution temperature was increased to 90°C to dissolve lactose and then quickly decreased to 30°C. Solutions were seeded with 1% pure lactose and placed in a water bath at 30°C for 8 hours. Subsequently, tubes were held at 4°C for 34 hours, then centrifuged for 20 min at 4°C and 3200 x g. The supernatant was freeze dried until 30% of the water was removed. Solutions were subjected to a second crystallization following the same procedure.
**Affinity Chromatography.** Columns (2 ml) were assembled using two types of commercial (Vector laboratories, Burlingame, CA, USA) agarose bound lectins, *Phaseolus vulgaris* erythroagglutinin (PHA-E) and *Phaseolus vulgaris* leucoagglutinin (PHA-L). Colostrum carbohydrate extract was passed through the column, and then washed with PBS to elute non-bound carbohydrates. Bound oligosaccharides were eluted using acetic acid (100 mM). The washed carbohydrates and eluted oligosaccharides fractions were collected and concentrated by lyophilization.

**Membrane Technology.** A solution of 2% GOS and 20% refined lactose (Grande Cheese Company, Brownsville, WI, USA) was circulated through a GEA Model R filtration system using a dairy processing sanitary nanofiltration membrane (Model DL3840C-30, DESAL Membrane Products, GEA Filtration, Hudson, USA). The velocity of recirculation was set at 36 gal/min. The trans-membrane pressure was 15 psi and the temperature was held at 32°C to keep lactose in solution. The total process time was 40 minutes.

**Analytical Methods.** Samples from the experiments described above were analyzed by thin layer chromatography (TLC), HPLC and MALDI-TOF MS. However, the results for the latter two methods were inconsistent and will not be discussed. For TLC, silica gel plates (Aluminium sheets 20 x 20 cm, pore size 60 Å, EMD Millipore, Billerica, MA, USA) were used. Plates were developed in a solvent containing 1-butanol, acetic acid, water (22:9:9).
Results

**Microbial fermentation.** One strategy for removing lactose from lactose-OS mixtures is to rely on suitable lactic acid bacteria that preferentially ferment the lactose. Two independent sets of experiments were performed, either with 2% of colostrum carbohydrates or a mixture of 0.5% GOS and 2% lactose. Microorganisms were chosen based on previous experiments (Goin, 2011), in which lactic acid bacteria unable to hydrolyze GOS were identified. Accordingly, *Lactobacillus spp. 1984*, *Lactobacillus helveticus* ATCC 15009, and *Lactococcus lactis* subsp. *lactis* 404 were used in these fermentations. Growth curves for both sets of fermentations showed that regardless of pH adjustment and the addition of nutrients (other than carbohydrates) bacteria populations did not increase after 12 hours of fermentation (Figures 1 and 2). *Lactobacillus spp.* reached higher cell counts when lactose and GOS were added to the media instead of the colostrum carbohydrates. The maximum OD reached by *Lactobacillus* when it was grown in MRS-colostrum carbohydrates was 30% lower than the maximum concentration reached in the GOS-lactose supplemented basal media. When fermentation experiments were done using *L. lactis*, similar results were obtained.

Samples were then analyzed by TLC (Figure 3). Results showed that lactose concentrations decreased over time, disappearing after 16 hours. On the other hand OS concentration appeared to remain constant during the first 12 hours. After 16 hours, OS bands became fainter; however, these samples were 8-times more dilute that the earlier samples.

Similarly, TLC of the 2% colostrum carbohydrate fermentation (Figure 4) also showed that lactose concentrations continuously decreased over time. After 12 hours, no lactose was detected. The putative OS band remained present during the entire fermentation. After 16 hours the band appeared to become wider; however, this may have been due to the observed increase in viscosity of this solution.

**Enzymatic hydrolysis.** Another approach for removing lactose from lactose-OS mixtures is via addition of beta-galactosidases that degrade lactose but not OS. Enzyme-treated solutions
were prepared and analyzed by TLC. At zero time, five bands could be differentiated in the untreated colostrum carbohydrate solution. Bands corresponding to glucose and galactose are present on the top of the TLC plate, followed by the lactose band. The remaining bands correspond to OS (Figure 5). The lactose concentration clearly decreased over time, but was still apparent even after 120 hours of hydrolysis. In contrast, the intensity of the glucose and galactose bands increased during the experiment. Two different OS bands were detected. The OS with the highest molecular weight remained constant during the experiment, whereas the apparent concentration of the low molecular weight OS decreased after 72 hours. In addition, based on the retention factor, the low molecular weight OS detected at 72 and 120 hours appeared to be different from the one identified at time zero.

**Affinity chromatography.** Lectin affinity columns, specific to OS, were used to separate and concentrate OS from lactose-OS solutions. Based on the TLC results, the washed carbohydrates fraction (i.e. carbohydrates that did not bind to the column), contained lactose and OS. The PHA-L washed fraction contained less carbohydrate than the washed fraction obtained with PHA-E. Both were consistent with the concentration of the initial solution passed through the column. Only one OS band was observed in the PHA-E eluted fraction, whereas no OS could be detected in PHA-L eluted fraction (Figure 6).

**Nano-filtration.** Lactose-OS solutions were circulated through nano-pore membranes, and permeate and retentate samples were collected. Samples before and during the circulation were obtained and analyzed by TLC (Figure 7). After accounting for the dilution, the concentrations of lactose and OS in the feed were the highest among the nano-filtration samples. In the three permeate samples, lactose was clearly detected. In addition, however, GOS was observed in permeate fractions. It appeared that both lactose and GOS were present at nearly the same concentration at each time point. Retentate samples also contained lactose and GOS; based on band intensities, it appeared that both lactose and the OS were concentrated in the last fraction.
**Crystallization.** All obtained fractions, including the recovered crystals, contained lactose and oligosaccharides. In fact, the OS concentration of the final solution and the recovered crystals seemed to be nearly the same. However, it was possible to remove some of the lactose by the crystallization method. Based on TLC and accounting for the dilution factor, the lactose concentration in the final solutions was approximately 30 times less than the concentration in the initial solution.

**Discussion**

Separating lactose from OS is technologically challenging due to their similar structures and size. In this study, five methods were assessed for their ability to remove or separate the lactose fraction from bovine colostrum. Although these methods were theoretically suitable for this application, none yielded OS-rich fractions from bovine colostrum.

Crystallization has long been used to remove lactose from milk, whey, and other dairy solutions (Leviton and Leighton, 1938; Nickerson and Moore, 1974; Kyle and Henderson, 1970; Singh et al. 1991). However, when this method was used with the colostrum, a substantial amount of OS accumulated in the crystallized lactose fraction, reducing the OS concentration in solution. Evidently, it appeared that the crystallized lactose had physically trapped the OS molecules.

The use of membrane technology for separating lactose and OS in dairy mixtures is well established (Sarney et al., 2000; Roth et al., 2001; De-Frees, 2002; Martinez-Ferez et al., 2006; Barile et al., 2009). In this study, a nano-filtration approach using a membrane with a cut-off of approximately 150-300 Dalton was carried out. The pore size was large enough, however, to allow the OS to pass through at the same rate during the entire process. Thus, the OS concentrations in the permeate samples (based on TLC) at different stages of the process were generally the same. Similarly, the concentration of OS in the retentate sample did not increase either. Although nano-filtration is a proven technology for separation of simple sugars and OS, appropriate membrane selection and optimizing operating parameters is essential.
Affinity chromatography using ligand-specific lectins is a highly effective process for separating carbohydrates from mixtures. In this study, two lectins (PHA-E and PHA-L) were chosen for their ability to bind complex oligosaccharides. However, the cost of commercial lectin beads limited the size of the columns and the volume of colostrum solution that could be used. Dilute colostrum solutions were prepared, to ensure that the binding capacity of the lectins was not exceeded. If lectins were saturated, then excess OS would pass through the column. The significant amount of OS present in the washed fractions indicated that some or most of the OS were not bound to the lectin or had exceeded the lectin binding capacity. Although the absence of TLC bands in the PHA-L eluted solution suggests that this lectin did not bind any of the OS, it is possible that PHA-L separated a minor fraction of OS that was not detected by TLC (OS concentration is less than the detection limit). Even if both lectins had been able to separate particular OS fractions, affinity chromatography using OS specific lectins may still not be a good approach to separate the carbohydrates from bovine colostrum, as several potentially valuable OS could be lost in the separation process. A separation column using lectins targeting lactose instead of OS could be a better approach. However, if the lactose concentration in the colostrum fraction was very high, the lectins would be easily saturated and a significant amount of lactose would appear in the washed fraction. To completely remove lactose it would be necessary to either use large amount of immobilized lectins or subject the carbohydrate solution to multiple serial purifications. In addition, since milk OS contain lactose in their core molecule, lectins targeting lactose may also bind colostrum OS.

In contrast to generally ineffective methods based on physical separation of lactose from OS, lactose degradation methods were more effective. Two methods were considered, lactose hydrolysis and lactose fermentation. A prolonged enzymatic hydrolysis with β-galactosidase did hydrolyze lactose, as expected, but degradation of the low molecular weight OS also occurred (based on TLC). In order to optimize the process, it will be necessary to identify β-galactosidases from different sources that have the highest selectivity towards lactose and
minimal or no activity on OS. However, because OS hydrolysis occurred during extended incubation, an alternative approach would be to stop the treatment after 48 hours to degrade the maximum amount of lactose and keep the OS fraction intact. In fact, complete lactose degradation without appreciable changes in the OS profile was reported by Sarney et al. (2000) when they hydrolyzed lactose present in human, bovine, ovine and caprine milk during 5 hours. Ultimately, the lactose hydrolysis process still generates the monosaccharides, galactose and glucose, which must then be separated from the OS solution. Thus, enzymatic hydrolysis must be combined with another separation technique. Nanofiltration would be the best option since the hydrolysis increases the difference between the molecular weight of the sugars and facilitates a size-based separation. Indeed milk OS have been separated from a milk carbohydrates mixture after lactose was hydrolyzed by β-galactosidase (Sarney et al. 2000; Roth et al., 2001).

The fermentation method has the advantage of degrading lactose without the appearance of large quantities of monosaccharides. The main metabolite produced by LAB, lactic acid, can easily be neutralized. The efficacy of the bacterial fermentation methods was determined by TLC analysis. However there are several other OS that could be degraded by the bacterial activity, especially the OS trimers. In the case that LAB could utilize OS, fermentation could be also stopped before lactose is completely depleted. LAB will ferment preferentially simple sugars; utilization of more complex sugars represents a major energy expense for the microorganism. According to previous studies of bovine milk OS characterization the glycan detected by TLC should be sialyllactose, which is the most abundant bovine acidic OS; it account for approximately the 30% of the total OS in bovine colostrum (Tao et al., 2009).

It is interesting that the oligosaccharide content of yogurt has been observed to increase during manufacture, suggesting that extracellular β-galactosidases from lactic acid bacteria (and different strains of Aspergillus oryzae) might synthesize oligosaccharides by transgalactosylation (Toba et al., 1981, 1983 and 1986). Thus, it is possible that OS were synthesized in either the enzymatic hydrolysis or the bacterial fermentation processes. However,
this seems a rather remote possibility since the conditions required for OS synthesis are very specific. More likely, the lactic acid bacteria as well as the β-galactosidase used in the experiments did not synthesize OS. It would be necessary to analyze the effects of the bacterial fermentation and enzymatic hydrolysis on each particular OS species to establish if OS are been degraded, synthesized or remained constant during each of these processes.
References


Figure 1. Growth curves of: a) *Lactobacillus spp*. 1984; b) *L. helveticus* ATCC 15009; c) *L. lactis* 404. Growth media carbohydrates: 0.5% GOS and 2% Lactose. Drop in cell numbers at 8 and 12 hours is caused by the basal media addition.
Figure 2. Growth curves of: a) *Lactobacillus spp. 1984*; b) *L. lactis* 404. Growth media carbohydrates: 2% of colostrum carbohydrates. Drop in cell numbers at 8 and 12 hours is caused by the basal media addition.
Figure 3. TLC of 0.5% GOS 2% lactose bacterial fermentation samples. 1, 4 and 12) 2% lactose and GOS standars; 3 to 5) L. lactis fermentation after 0, 12 and 16 h; 6 to 8) Lactobacillus spp. fermentation after 0, 12 and 16 h; 9 to 11) Lactobacillus helveticus fermentation after 0, 12 and 16 h. Samples of 0 h, 12 and 16 h are 10,8 and 16 fold dilutions of the original carbohydrates solutions.
Figure 4. TLC of 2% Colostrum CHOs bacterial fermentation samples. 1 and 2) 2% lactose and GOS standards; 3 to 6) replicates of *L. lactis* fermentation after 0 and 8 hours; 7 to 10) replicates of *Lactobacillus spp.* fermentation after 0 and 8 hours; 11 and 12) replicates of *L. lactis* fermentation after 12 hours; 13 and 14) *Lactobacillus spp.* fermentation after 12 hours. The latest samples (12 hours) are 2 fold dilutions of the original carbohydrates solutions.
Figure 5. TLC of the colostrum CHO enzymatic hydrolisis samples. 1 and 2) 2% glucose and galactose standards; 3 to 13) replicates of colostrum carbohydrates solution after 0, 24, 48, 72 and 120 hours of hydrolysis; 14) Lactose standard.
Figure 6. TLC of affinity chromatography samples. 1 to 4) 2% Glc, Gal, Lac and GOS standards. 5 and 6) Carbohydrates able to bind to PHA-E or PHA-L column after being eluted. Concentrated by 20 fold; 7 and 8) Carbohydrates unable to bind to PHA-E or PHA-L column; 9) colostrum carbohydrate fraction.
Figure 7. TLC of nano-filtration samples. 1 to 4) 2% glucose, galactose, lactose and GOS standards; 5) Starting solution diluted by a factor of 100 (I0); 6) Permeate at 252 psi system pressure (P0); 7) Permeate at 400 psi system pressure (P2); 8) Final permeate (PF); 9) Retentate. (R)
Figure 8. TLC of crystallization samples. 1) correspond to lactose standard; 2 and 3) Replicates of supersaturated initial solution (I0). Spotted samples are 10 fold dilution of the original carbohydrates solution; 4 and 5) Replicates of final solution after crystals removal (F); 6 and 7) Replicates of crystals recovered in the first crystallization (C1); 8 and 9) Replicates of crystals recovered in the second crystallization (C2).
Chapter 4

Adherence Inhibition of Enteric Pathogens to Epithelial cells by Bovine Colostrum

Oligosaccharides and other bovine colostrum fractions
Abstract

To initiate colonization and infection, most microbial pathogens must first recognize and adhere to cells in the host tissues. One suggested strategy for preventing infectious disease is based on adherence inhibition. Exogenous carbohydrates that structurally resemble host cell receptor sites may act as decoys for bacterial adhesins and block pathogen adherence. Milk oligosaccharides and glycoconjugates have been shown to have this ability and can inhibit bacterial attachment. However, this bioactive property is based mostly on human milk oligosaccharides. In this study, in vitro competition experiments were performed to assess the anti-adherence properties of several bovine colostrum fractions against four enteric pathogens, enteropathogenic and enterotoxigenic Escherichia coli, Cronobacter sakazakii, and Salmonella enterica serovar Typhimurium. The UF permeate and the oligosaccharides fraction significantly inhibited binding of all four pathogens to HEp-2 tissue culture cells. None of the other fractions decreased bacterial adhesion of either E. coli strains or C. sakazakii. In contrast, Salmonella T. attachment was also blocked by the NF retentate. The colostrum whey did not decrease adhesion. The results obtained from this study provided evidence to support the concept that bovine milk components can promote consumer health by preventing or reducing recurrence of infections and could be used as food additives and/or pharmaceutical constituents.

Introduction

For most bacterial pathogens, recognition and adhesion to the host cells are essential to initiate colonization and infection. In order to adhere, bacteria have developed specialized structures positioned at the exterior of the cell called adhesins. Adhesins mediate highly stereospecific interactions of recognition and adherence with the host receptors. These adhesin-receptor interactions determine tissue tropism of the pathogen. For many bacterial pathogens, adhesins are considered as essential virulence factors. In fact, pathogens that have lost their ability to express adhesins become avirulent (Connell et al. 1997; Klemm et al. 2006, 2007; Roos et al. 2006).

Several different types of adhesin-receptor interactions of different chemical nature have been
recognized, however, the most common mechanism is the binding of bacterial lectins with carbohydrate moieties on the surface of the host cell. This interaction accounts for the primary means by which adherence occurs among bacterial pathogens (Ofek et al. 2003). Accordingly, the prevention of bacterial adhesion is now regarded as a promising strategy for reducing infectious disease and overcoming the problems of bacterial resistance to antibiotics (Klemm et al., 2010). Several approaches have been evaluated, including the prevention of adhesin biosynthesis (Pinker et al., 2006; Åberg and Almqvist 2007; Larsson et al. 2005; Pinkner et al. 2006; Svensson et al. 2001), the production of anti-adhesive vaccines (Rutter and Jones 1973; Langermann et al. 2003), and adhesion inhibition with receptor analogs (Bouckaert et al. 2005; Nagahori et al. 2002; Schembri and Klemm 2001). The latter is of great interest since several food natural components and synthesized additives have been shown to act as efficient inhibitors of pathogenic bacterial adherence. Among the food components that have been reported to have anti-adherence activity are several prebiotics, including GOS and FOS, mannan oligosaccharides, pectic oligosaccharides and milk glycoconjugates (Quintero el al., 2011; Kisiela et al. 2006; Ganan et al. 2010). The mechanism of action is based on the structural similarity between the exogenous glycans and the epithelial cell receptors. In the presence of soluble glycans, bacterial adhesins will bind to the free glycans instead of the host receptors. Ultimately, the pathogens will be flushed out by intestinal flow.

Bovine milk glycans could be used as food additives and/or pharmaceutical constituents to prevent pathogen adherence, they resemble the receptors of host epithelial cells and therefore could act as efficient decoys. In addition, milk glycans constitute a heterogeneous mixture that may be an advantage in comparison with homogenous mixtures of prebiotic OS, as it has been suggested that the effectiveness of milk OS relies on the variety of glycan configurations. (Bode, 2006). Moreover, according to actual consumer trends, products containing natural components, such as milk fractions, could be easily accepted and preferred over products containing synthetic additives.

Milk glycans that have shown pathogen anti-adherence activity include lactoadherin (Yolken et al., 1992; Newburg et al., 1998; Kvistgaard et al., 2004;), lactoferrin (Superti et al., 1997,2001; Quintero 2011; Wang 2001; de Araujo and Giugliano, 2001), mucins (Schroten et al., 1992; Yolken et al., 1992;
Newburg et al., 1998; Kvistgaard et al., 2004;), gangliosides (Williams et al., 2005), and oligosaccharides (Martin-Sosa et al., 2002; Simon et al., 1997; Kunz, et al., 2000, Morrow et al., 2005; Newburg et al., 2005; Bao et al., 2007). Furthermore, β-lactoglobulin and small peptides have also been shown to inhibit pathogen binding (Ouwehand et al., 1995; Ouwehand et al., 1997 and 1998; Bruck et al. 2006). Most of the attention has focused on milk oligosaccharides, since this fraction contains the greatest variety of carbohydrate species. Milk oligosaccharides consist of oligomer of 3 to 7 monosaccharides. Tri- and tetrasaccharides account for the majority of bovine milk OS. About 40 different OS species have been identified in bovine milk. Anionic OS account for about 70% of the total content, with sialyllactose as the most abundant (Tao et al., 2009). In contrast to human milk, bovine colostrum contains N-glycolylneuraminic acid and lacks fucose, which is common in human milk. It has been suggested that the main role of anionic OS is to block the adhesion of pathogenic bacteria to the epithelial surface whereas the neutral OS are responsible of the development of beneficial intestinal microbiota (Tao et al., 2009). Nonetheless, it has been demonstrated that several human milk fucosylated OS are able inhibit microbial adhesion, as well (Ruvoen-clouet et al., 2006; Morrow et al., 2004; Ruiz-Palacios et al., 2003). Therefore, neutral bovine milk OS may also act as efficient inhibitors.

Bovine colostrum and its byproducts appear to be the best sources of bioactive compounds. Bovine colostrum contains 10 times more OS than mature milk (0.7-1.2 g/L), and although the colostrum processing industry is not as developed as for bovine mature milk, there is still a large amount of colostrum-derived products available. Another advantage of colostrum sweet whey is the presence of glycomacropeptide (GMP) that forms as a result of chymosin-mediated hydrolysis of kappa-casein. GMP has been reported to be an effective adherence inhibitor agent (Neeser et al., 1994; Kawasaki et al., 1993; Simon 1996; Nakajima et al. 2005; Bruck et al., 2006).

The goal of this study was to assess the anti-adherence properties of bovine glycans derived from colostrum and colostrum whey fractions using in-vitro competition experiments.
Material and Methods

**Bovine colostrum samples and oligosaccharides extraction.** Colostrum from a single cow in early lactation (< 1 day) was provided by a local milk producer (PrairieLand Dairy, Firth, NE). Milk fat was removed by centrifugation at 4°C and 1000 x g for 30 minutes. Skim colostrum was then mixed with 70% ethanol in a ratio of 1:1 to precipitate proteins. The mixture was allowed to stand for 1 hour at 4°C before it was centrifuged for 40 minutes at 4°C and 1000 x g. The resulting solution was held overnight and centrifuged again at the same conditions to obtain maximum separation. The carbohydrate-containing supernatant fraction was concentrated using a roto-evaporator R-205 (Büchi, Delaware, USA) to evaporate the ethanol and then lyophilized (Flexi-Dry FD-3-85A-MP, FTS Systems, Stone Ridge, NY). The precipitated proteins were also lyophilized. The carbohydrate contents of the dried fractions were measured by the phenol-sulfuric colorimetric method (Chaplin and Kennedy, 1986), and protein content was determined using an FP-528 Nitrogen (LECO, St. Joseph, Michigan) nitrogen/protein analyzer.

**Bovine colostrum whey fractions.** Three bovine colostrum derived fractions were provided by Sterling Technology (Brookings, SD). According to the manufacturer (Sterling Technology, personal communication), bovine colostrum (between 1 and 3 days of lactation) was treated with chymosin and the whey was collected and filter sterilized. This first fraction is referred to as colostrum whey. The whey was then filtered through a 10 KDa ultra-filtration membrane, and then the permeate was nano-filtrated using a 1 KDa membrane. The permeate of the ultra-filtration (UF permeate) and the retentate of the nano-filtration (NF retentate) correspond to the second and third analyzed fractions.

**Bacterial strains and culture conditions.** *Cronobacter sakazakii* 4603 was obtained from K. Venkitanarayan (Department of Animal Science, University of Connecticut). ETEC strain ATCC 35401 (H104:07) was obtained from Dr. Kuehn (Duke University). EPEC strain E2348/69 (O127:H6) was obtained from M. Donnenberg (University of Maryland School of Medicine, Baltimore). *Salmonella*
Enterica serovar Typhimurium was obtained from the University of Nebraska Lincoln bacterial culture collection. Tryptic Soy Agar and Broth (TSA and TSB; Difco) were used as growth media for EPEC, EPEC and C. sakazakii, and Luria Agar and Broth (LB and LA; Difco, MD, USA) were used for Salmonella. Before each experiment, frozen stock cultures of each organism were thawed, plated onto the corresponding solid media and grown at 37°C overnight. Two more activations were carried out; a single colony was inoculated into 10 ml of growth media, incubated overnight, and then inoculated at 1% (v/v) concentration into fresh media. A final transfer was done, and cells were incubated aerobically for either 4 hours for C. sakazakii, 24 hours for Salmonella, or overnight for ETEC and EPEC. Salmonella and C. sakazakii cultures were centrifuged at 3,184 g for 8 minutes, washed with phosphate-buffered saline (PBS) and re-suspended in minimal essential medium (MEM; Hyclone, Logan, Utah), supplemented with 10% fetal bovine serum (FBS; Hyclone). For ETEC and EPEC experiments, overnight cultures were inoculated at 10% (v/v) in MEM supplemented with bovine serum and incubated for 80 minutes prior to the start of each experiment. MEM used for pathogen solutions was pre-equilibrated overnight at tissue culture conditions (5% CO2, 95% relative humidity, 37°C).

Tissue culture conditions. HEP-2 (CCL-23) were obtained from the American Type Culture Collection (Manassas, Virginia) and maintained under tissue culture conditions in minimal essential medium (MEM/EBSS NEAA Modified, HyClone, Thermo Fisher Scientific Inc, Utah, USA) supplemented with 10% fetal bovine serum (HyClone). For inhibition assays, subconfluent monolayers of HEP-2 cells were harvested with 0.25% (vol/vol) trypsin-EDTA solution (HyClone) and seeded in 24 well tissue culture plates at approximately 5 x 10⁴ HEP-2 cells per well. Plates were incubated under tissue culture conditions for 30 hours before the experiment.

Adherence inhibition assays. Sub-confluent HEP-2 cells were prepared as described above. Colostrum and colostrum fractions were mixed with the bacterial suspension previously prepared (see above), to a final concentration of 20 mg/ml of total carbohydrates or proteins. After washing the monolayers with
PBS, 500 µl of the potential pathogen-inhibitor solutions were added into each well. Cells were incubated at tissue culture conditions for 30 minutes for EPEC and 1 hour for ETEC, C. sakasakii and Salmonella T. Wells were then washed 5 times with PBS to remove non-adherent bacteria, followed by the addition of 1 ml of 0.1% Triton 100x (Fisher, NJ, USA). After 30 minutes, cell de-attachment was completed and the contents were transferred to sterile centrifuge tubes for DNA extraction. In some of the experiments, microscopic counts were also obtained following the protocol described by Quintero (2011). Experiments were performed in triplicate.

**DNA Extraction and Real-time PCR assay for quantification.** Bacterial DNA was extracted from material obtained in the adherence inhibition assays. Samples were centrifuged at 10,000 x g for 5 min and the pellet was re-suspended in 180 µl of buffer ATL from the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). Instructions provided in the manufacturer’s manual for DNA extraction of Gram negative bacteria were followed to complete the extraction. Quantitative real time PCR (qRT-PCR) was performed using a Mastercycler Realplex2 (Eppendorf AG, Hamburg, Germany). Each PCR was done in a 25 µl volume. The reaction mixture comprised 11.25 µl of the 20x SYBR solution and 2.5-µl Real-MasterMix (5Prime), 0.5 µM of each primer, and 1 µl of DNA template (Martínez et al. 2009). Specific primers and amplification program were followed for each organism (Table 1).

**Statistical analysis.** Significant differences between the treatments were determined using one-way ANOVA. Post hoc pair-wise comparisons were done using Tukey’s test. Differences between treatments were considered significant when P-values were less than 0.05. GraphPad Prism 5 (version 5.03, GraphPad Software, Inc, 2010) was used to perform statistical tests and to generate graphical images.
**Results**

**Colostrum carbohydrates fraction.** The colostrum carbohydrate fraction showed significant adherence inhibition for all the pathogens evaluated (EPEC, ETEC, *Salmonella Typhimurium* and *Cronobacter sakazakii*) (Figure 1). The minimum required dose to reach a significant reduction was 60 mg/ml, with the exception of *E. sakazakii* for which a dose of 80 mg/ml was necessary. The extent of the inhibition varied with the microorganisms. In general, colostrum carbohydrates were better inhibitors against EPEC and *E. sakazakii*. The highest level of inhibition observed, 99%, was obtained when these two pathogens were exposed to the colostrum carbohydrates. In contrast, the maximum percentages of inhibition for ETEC and *Salmonella* T. were 83 and 89%, respectively (Table 2).

A clear dose response was observed for EPEC adherence in the presence of colostrum carbohydrates (Figure 1a.). Bacterial attachment decreased progressively as the colostrum carbohydrate concentration increased. However, inhibition did not increase significantly beyond a dose of 110 mg/ml. For the other three microorganisms there was a “maximum effective dose”; after which an increase of the CHOs dose did not further significantly increase the inhibition. The maximum effective dose was 60 mg/ml for ETEC, 100 mg/ml for *E. sakazakii* and 80 mg/ml for *Salmonella* T.

*E. sakazakii* behavior was unique among the organisms evaluated (Figure 1c). When the lowest concentration was tested, the bacterial attachment increased. Nevertheless, the adherence in the presence of 60 mg/ml was not significantly different than the control and doses higher than 80 mg/ml significantly reduced adherence. The same behavior was observed in the microscopic counts, where an initial increase of adherence was followed by a significant decrease of attachment (data not shown).

Because the samples contained significant amounts of lactose (approximately 80% of the total carbohydrate) lactose treatments where included in each of the experiments to establish if the inhibitory effect of the colostrum carbohydrates was due to the oligosaccharides, lactose, or both. For those cases in which the lactose had a significant effect, different doses were tested. None of the *E. coli* strains were significantly inhibited by the highest dose of lactose. On the other hand, *E. sakazakii* and *Salmonella* T. were significantly inhibited by lactose for all the concentrations evaluated (Figures 1c and 1d).
Salmonella T. inhibition slightly increased as a result of the increase in concentration. In contrast, lactose inhibited E. sakazakii attachment to the same extent when exposed to 40, 60 and 80 mg/ml and significantly increased in the presence of 100 and 110 mg/ml. Lactose inhibition accounted for less than 78% of the total inhibition of E. sakazakii and 64% of the total inhibition of Salmonella T. Despite these observations, however, lactose would likely not act as a pathogen inhibitor in vivo because it would instead be hydrolyzed and absorbed before it reached the colon.

Adherence inhibition by commercial colostrum whey fractions was tested against EPEC, E. sakazakii and Salmonella T. However, the dried NF retentate could not be dissolved in saline solution, even after pH adjustment (7.1 - 7.3). Therefore, this material was used in one of two ways: (1) using the entire fraction (including the insoluble part, which stayed in suspension during the incubation period); and (2) using only the soluble fraction of the NF retentate suspension (i.e., the solution resulting after pH adjustment and centrifugation). The only fraction that inhibited adherence of all three microorganisms was the UF permeate (Figure 3). Indeed, this fraction was more efficient than the colostrum carbohydrate fraction. Only 10 mg/ml of the UF permeate were necessary to observe significant inhibition of E. sakazakii and Salmonella T. For EPEC, 20 mg/ml was found to be inhibitory (Table 2).

The NF retentate inhibited attachment of Salmonella T. but not EPEC or E. sakazakii (Figure 4). This fraction achieved nearly the same levels of adherence reduction for Salmonella as the UF permeate (Table 2). Significant adherence reduction was reached with a minimum dose of 10 mg/ml. Therefore, the NF retentate was also more effective than the colostrum carbohydrate fraction. Although both the soluble and the complete NF retentate blocked Salmonella T. adherence, the soluble part of the NF retentate was less effective than the suspension (soluble and insoluble portion). A minimum dose of 60 mg/ml of soluble NF retentate was necessary to significantly inhibit Salmonella T. attachment (Figure 5).

In contrast, the colostrum whey did not inhibit pathogen attachment at any of the concentrations tested. None of the three pathogens evaluated was inhibited by this fraction. Similarly, the NF retentate did not inhibit either EPEC or E. sakazakii binding to HEp-2 cells. This behavior was observed for both subsets of experiments carried out with this fraction (i.e. soluble portion and entire suspension).
Discussion

In vitro tissue culture experiments were conducted to assess the anti-adherence properties of bovine colostrum fractions against EPEC, ETEC, C. sakazakii, and S. Typhimurium. A total of four fractions were evaluated, including colostrum whey, UF permeate, NF retentate and a colostrum carbohydrate fraction. The UF permeate and the carbohydrates fraction significantly inhibited binding of all four pathogens to HEp-2 tissue culture cells, whereas the NF retentate was inhibitory only to Salmonella. The colostrum whey did not decrease adherence for any of the test organisms (Figure 2). The activity of the effective fractions was mainly attributed to the presence of OS and/or GMP. However, lactose and peptides may have also contributed to the adherence inhibition we observed.

Since the mechanism of adherence inhibition depends on the structural similarity of the test glycans to the cognate epithelial cell receptors for the bacterial adhesins, the carbohydrate fraction of colostrum oligosaccharides were the first to be evaluated. Our results confirm the findings of previous studies in which bovine milk OS were shown to be capable of inhibiting bacterial adhesion. Helicobacter pylori was inhibited by 3’-sialyllactose, 6’-sialyllactose and 6’-sialyllactosamine isolated from bovine milk, with 3’sialyllactose as the most active OS (Simon et al., 1997). Similarly, sialylated oligosaccharides were also shown to be effective inhibitors of UPEC and ETEC strains. However human milk OS had greater binding inhibition than bovine OS for ETEC. (Martín-Sosa et al., 2002).

It has been suggested that the two main types of OS that are responsible for pathogen inhibition are the sialylated and fucosylated OS. This is because the principal targets for viruses and bacteria are fucose- and sialic acid-containing glycans (Bohem and Moro, 2008). In fact, studies using human milk OS suggest that both neutral and acidic OS are able to inhibit bacterial and virus attachment (Chichlowski et al., 2010; Ruvoen-Clouet et al., 2006). However, since bovine colostrum lacks fucosylated OS and the acidic OS fraction accounts for more than 70% of the total bovine colostrum OS, the pathogen inhibition observed in this study can be attributed mainly to the colostrum anionic OS.

On the other hand, the colostrum whey besides carbohydrates also contained proteins, including lactoalbumin, lactoglobulin, bovine serum albumin, lysozyme, gangliosides, lactoferrin, GMP,
and lactoadherin. Several of these components have been shown to have anti-adherence properties (de Araujo and Giugliano, 2001; Kvistgaard et al., 2004; Newburg et al., 1998; Ouwehand et al., 1997). However, their anti-adherence activity has been mostly established using fractions or pure components derived from human milk. Limited evidence exists of the activity of the bovine counterparts. Bovine lactoferrin anti-adherence activity was demonstrated using a mouse model, and independent of the iron-scavenging properties, lactoferrin was also able to inhibit *H. pylori* attachment to the stomach mucosa (Wang et al. 2001). \(\beta\)-lactoglobulin extracted from infant formula inhibited *Klebsiella oxytoca* and SfaII-expressing *E. coli* adhesion (Ouwehand et al., 1997 and 1998). Other reports are less consistent. For example, some studies suggest that bovine lactadherin does not inhibit pathogen binding (Kvistgaard et al., 2004), whereas Kanamaru et al., (1999) showed that lactadherin inhibited adherence of human rotavirus. Finally, as noted previously, certain anionic bovine OS have been shown to be effective blocking agents, and our results suggest that even if some of these compounds have anti-adherence properties, the concentration in which they are present would likely not be high enough to significantly inhibit any of the pathogens tested. A particular case that supports our hypothesis is lactoferrin. Quintero (2011) established that a minimum dose of 10 mg/ml of lactoferrin was required to obtain a significant inhibition of *E. sakazakii* to Hep-2 cells (unpublished data). The concentration of lactoferrin in bovine colostrum is up to 1.5 mg/ml (Yekta et al. 2010); even if the microfiltration treatment achieved a partial water removal, lactoferrin concentration would not be close to 10 mg/ml.

In the ultra-filtration step, proteins were removed, and therefore, the UF fraction contains carbohydrates, peptides and small glycoproteins such as GMP. The inhibitory effect of this fraction (UF permeate) can be attributed mainly to OS and GMP. Bovine GMP has been suggested to contain carbohydrate moieties that inhibit bacterial and viral infection. Nakajima et al. (2005) reported that sialylated GMP inhibited adherence of *Salmonella Enteridis* and *E. coli* O157:H7. Specifically, GMP containing the NeuAca2-3Gal41-3GalNAc trisaccharide has been reported to prevent *E. coli* hemaglutination (Parkkipin et al. 1986). Bruk et al (2006) observed that GMP (0.25 mg/ml) reduced association of EPEC and *Salmonella* T. to CaCo-2 cells. Similarly, Halpin et al. (2009) attributed the
ability of several whey commercial products to inhibit the adhesion of *Salmonella* T. *E. coli* O157:H7 and *E. sakazakii* (to CaCo-2 cells) to the activity of GMP. Furthermore, GMP is also able to inhibit virus and non-enteric bacteria. For example, hemagglutination of four different strains of human influenza virus was inhibited by the addition of GMP (Kawasaki et al. 1993). Furthermore, adhesion of carcinogenic bacteria (*Streptococcus mutans*, *S. sanguis*, *S. sobrinus* and *Actinomyces viscosu*) to oral surfaces was inhibited by GMP (Neeser et al. 1994).

Additional interfering compounds present in the UF permeate include lactose and small peptides. Lactose at high concentrations was found to contribute significantly to the inhibition effect for *E. sakazakii* and *Salmonella T*. Naturally occurring free peptides or peptides liberated from other proteins could also have contributed to adherence inhibition. Bruck and collaborators (2006) showed that free peptides liberated from GMP significantly reduced association levels of EPEC. Indeed, it has been suggested that the activity of peptides encrypted within bioactive proteins remains latent until they are subjected to proteolytic action of the enzymes (Sinha et al., 2007). However, the concentration of peptides should be very low in relation with the other active molecules.

The combined activity of OS, GMP and lactose could explain why the UF permeate had higher adherence inhibition than the colostrum carbohydrates fraction. The UF permeate contained a greater diversity of biologically-active glycans that were potentially capable of inhibiting multiple adhesins. GMP-bound glycans contain N-acetylglactosamine, N-acetylglucosamine, fucose and sialic acid, among others (reviewed in Brody, 2000). Thus, GMP has the ability to inhibit adhesins specific for fuscosylated glycan receptors, a property not achieved using bovine OS, exclusively. In the particular case of *E. sakazakii*, UF permeate was an especially effective inhibitor compared to the colostrum carbohydrates. Only 10 mg/ml of UF permeate was necessary to reach significant inhibition whereas 80 mg/ml of colostrum carbohydrates were required. These findings suggest that GMP is a more efficient inhibitor of *E. sakazakii* attachment than OS.

The final filtration was intended to remove lactose and concentrate the bioactive compounds. However, when the obtained retentate was tested it was found to have limited inhibition activity, as only
Salmonella T. was inhibited. There are several possible explanations for this result. The first is that the smallest OS were able to pass through the membrane and were removed along with the lactose. Thus, the amount of inhibitory compounds in the sample decreased substantially. In fact, sialyllactose, the most abundant OS present in bovine colostrum (Tao et al., 2009), has a molecular weight of 633 Da. Therefore, this compound was likely lost during the nanofiltration process, as only an electric repulsion between the acidic OS and the membrane could have prevented the OS from crossing the membrane. The second explanation is that some unidentified compounds present in the fraction could have interfered with the bioactive molecules, reducing their inhibitory effect. In addition, it is possible that some of the glycans had aggregated during the drying operation. The fact that such molecules did not get into solution implies that the bioactive sites were not completely exposed, diminishing their inhibition capacity. Therefore, the drying process must be optimized to increase the powder solubility and prevent the loss of activity due to lack of solubility.

The observation that the NF retentate and the UF permeate inhibited Salmonella with the same effectiveness, suggest that the component responsible for the inhibition of this microorganism was not lost in the filtration process and was present in NF retentate. Based on the previous discussions, this compound is more likely GMP. However, complementary studies have to be done to validate this hypothesis. Interestingly, the soluble portion of the NF concentrate was a less effective inhibitor than the complete fraction. Although precipitation of the insoluble fraction was not observed during the incubation period, it is possible that a microscopic layer of insoluble molecules formed over the cells preventing the bacteria to interact with the receptors.

The results obtained from this study provide evidence to support the concept that bovine milk components can promote consumer health by preventing or reducing enteric infections. These constituents could be used as antimicrobial agents in foods and/or in pharmacological application. In particular, bovine colostrum whey appears to be a suitable source for these compounds. The concentration of glycans in colostrum is higher and more diverse than in mature milk. In addition, the chymosin
treatment increases the biological activity by releasing natural occurring glycan species that are present as part of complex molecules.
References


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<thead>
<tr>
<th>Organism</th>
<th>Primers (5’ - 3’)</th>
<th>Amplification Program</th>
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<tbody>
<tr>
<td>EPEC</td>
<td>eaeFor GGCGATTACGCGAAAGATAC</td>
<td>Initial denaturation at 95°C for 10 min and 40 cycles at 95°C for 15s and 62°C for 1 min for annealing and extension.</td>
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<tr>
<td></td>
<td>eaeRev GATTAACCTATGCCGTTCCA</td>
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<td>ETEC</td>
<td>STa-F GCTAATGGTGCAATTTTTATTTCTGTA STa-R AGGATTACAAACAAAGGTTCCACAGCAGTAA</td>
<td>Initial denaturation at 95°C for 10 min and 40 cycles at 95°C for 15s and 55°C for 10s for annealing and extension.</td>
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<tr>
<td>C. sakazakii</td>
<td>For TATAGGGTTGTCTGCGAAAGCG Rev GTCTTCGTGTCGAGTGGTTG</td>
<td>Initial denaturation at 95°C for 10 s, 45 cycles at 95°C for 5 s, 62°C for 20 s for annealing and extension.</td>
</tr>
<tr>
<td>Salmonella Typhimurium</td>
<td>139 -GTGAAATTATCGCCACGTTGGGCAA 141 -TCATCGCACCCTCAAAGGAACC</td>
<td>Initial denaturation at 94°C for 2 min and 45 cycles at 94°C for 20s and 62°C for 1 min for annealing and extension.</td>
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Table 1. Primers and PCR programs for the different organisms used in this study.
Figure 1. Adherence of a. EPEC, b. ETEC, c. *E. sakazakii*, and d. *Salmonella* T. to HEp-2 cells in the presence of different doses of colostrum carbohydrates and pure lactose.
Figure 2. Adherence of a. EPEC, b. *E. sakazakii*, and c. *Salmonella* T. to HEp-2 cells in the presence of different doses of the micro-filtrated colostrum whey
Figure 3. Adherence of a. EPEC, b. *E. sakazakii*, and c. *Salmonella* T. to HEp-2 cells in the presence of different doses of the ultra-filtration permeate
Figure 4. Adherence of a. EPEC, b. *E. sakazakii*, and c. *Salmonella* T. to HEp-2 cells in the presence of different doses of the nano-filtration retentate
Figure 5. Adherence of a. EPEC, b. *E. sakazakii*, and c. *Salmonella* T. to HEp-2 cells in the presence of different doses of the nano-filtration retentate soluble portion.
Table 2. Summary of the relevant percentage of inhibition obtained during the anti-adherence experiments.

Statistically significant effects compared to control (p<0.05) are indicated by the asterisk.

<table>
<thead>
<tr>
<th></th>
<th>Lactose 40 mg/ml</th>
<th>Lactose 60 mg/ml</th>
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<td>69,10</td>
<td>88,64*</td>
<td>91,80*</td>
<td>98,38*</td>
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<td>-23,83</td>
<td>54,50</td>
<td>77,68*</td>
<td>83,63*</td>
<td>82,46*</td>
</tr>
<tr>
<td>E. sakazakii</td>
<td>84,74*</td>
<td>-338,14</td>
<td>4,96</td>
<td>82,45*</td>
<td>99,08*</td>
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<tr>
<td>Salmonella T.</td>
<td>72,50*</td>
<td>23,41</td>
<td>53,35*</td>
<td>86,32*</td>
<td>85,55*</td>
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<th>Lactose 40 mg/ml</th>
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<th>Lactose 40 mg/ml</th>
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<tr>
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<td>90,19*</td>
<td>90,23*</td>
<td>87,15*</td>
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<td>28,71</td>
<td>74,68*</td>
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Chapter 5

Conclusions
In this study we proposed a simplified model using plant lectins to simulate bacterial adhesins-receptor interactions. The results confirmed our hypothesis that bacterial binding inhibition is caused by exogenous oligosaccharides that are structurally similar to the cognate ligands. In addition, bovine colostrum was demonstrated to be a suitable source of bioactive glycans that are able to inhibit the adherence of enteropathogenic *Escherichia coli*, enterotoxigenic *Escherichia coli*, *Enterobacter sakazakii* and *Salmonella enterica* serovar Typhimurium to epithelial cells. Several methods for the extraction of milk oligosaccharides and degradation or removal of lactose were assessed. Enzymatic hydrolysis and microbial fermentation were effective for degrading lactose, and could be considered as viable approaches for separating lactose from the bioactive oligosaccharides. The major findings of this research are described below.

- Based on lectin-ligand competition assays, the glycan composition of the Hep-2 cells surface was predicted to contain glucose, galactose, α linked mannose, N-Acetyl glucosamine, dimers or oligosaccharides containing N-Acetylgalactosamine and/or glycans with the linkages galactose(β-1,3)N-Acetylglactosamine or galactose (B1-4)N-Acetylglucosamine.

- All the lectins that were able to bind to the HEp-2 cells were inhibited by nearly 100% in the presence of cognate ligands, and when prebiotics were added, inhibition of lectin binding also was observed. The greater the similarity between the prebiotic and the cognate ligands, the greater was the inhibition that was observed.

- GOS significantly inhibited most of the lectins, which support the role of GOS as an efficient anti-adherence agent. In contrast, PDX did not inhibit lectin attachment, suggesting that it is not effective as an adherence inhibition agent.
• Under the evaluated conditions, the physical-chemical separation methods (crystallization, affinity chromatography and nano-filtration) were limited in their ability to enrich for OS from bovine colostrum. However, nano-filtration was a promising option to achieve separation of simple sugars and OS, despite not being able to retain the OS within the retentate fraction.

• Enzymatic hydrolysis and microbial fermentation were effective methods for degrading lactose, although the effect of these treatments on oligosaccharides will need further analysis.

• Bovine colostrum contained fractions that significantly inhibited binding of all four pathogens to HEp-2 tissue culture cells. An ultrafiltration permeate was the most effective inhibitor due, we suggest, to the greater diversity of biologically-active glycans contained in this fraction.

• A nanofiltration retentate had inhibitory properties only against *Salmonella*, and a colostrum whey fraction did not decrease adherence for any of the test organisms.

• The activity of the effective fractions was mainly attributed to the presence of oligosaccharides and/or peptides. However, lactose may have also contributed to the observed adherence inhibition.

• The results obtained from this study provide evidence to support the concept that bovine milk components can promote consumer health by preventing or reducing enteric infections. These constituents could be used as antimicrobial agents in foods and/or in pharmacological applications. In particular, bovinecolostrum whey appears to be a suitable source for these compounds.