Summer 7-2014

Non-digestible Oligosaccharides: Anti-adherence and Other Biological Properties

Maria I. Quintero-Villegas
University of Nebraska-Lincoln, misabelq@gmail.com

NON-DIGESTIBLE OLIGOSACCHARIDES: ANTI-ADHERENCE AND OTHER BIOLOGICAL PROPERTIES

by

Maria Isabel Quintero-Villegas

A DISSERTATION

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Doctor of Philosophy

Major: Food Science & Technology

Under the Supervision of Professor Robert W. Hutkins

Lincoln, Nebraska

July, 2014

NON-DIGESTIBLE OLIGOSACCHARIDES: ANTI-ADHERENCE AND OTHER BIOLOGICAL PROPERTIES

Maria Isabel Quintero-Villegas, Ph.D.

University of Nebraska, 2014

Advisor: Robert W. Hutkins

Bacterial adherence is the first and one of the most important steps in bacterial pathogenesis. Adherence to host cell surfaces requires that the bacteria recognize specific receptors in the surface of epithelial cells. Therefore, agents that act as molecular decoys to interfere with adherence could be useful prophylactic treatments to prevent or mitigate the onset of infections. The goal of this research was to assess the ability of several food grade non-digestible oligosaccharides (NDOs) to act as molecular decoys and prevent bacterial adherence *in vitro* as well as *in vivo*. First, the antiadherence effect of specific species of chitooligosaccharides (CHOS) at different concentrations was tested against enteropathogenic *Escherichia coli* (EPEC). Microscopic evaluation indicated that CHOS reduce adherence of EPEC to HEp-2 cells by more than 90%. Subsequently, the antiadherence activity of lactoferrin, alone and in combination with a 1:1 mix of galactooligosaccharides (GOS) and polydextrose (PDX), was tested against *Cronobacter sakazakii.* Adherence was assessed by quantitative PCR (qPCR) and results indicate that lactoferrin inhibits adherence

of *C. sakazakii* to HEp-2 cells at a minimum concentration of 10mg/ml. Lactoferrin combined with the GOS:PDX mix did not further inhibit adherence, suggesting there is not an additive effect between lactoferrin and GOS:PDX. Moreover, the ability of GOS to reduce adherence was assessed *in vivo* in a *Citrobacter rodentium* mouse model of infection. Conventional and germ free C57Bl/6 mice were supplemented with GOS in the drinking water to receive a dose of 5000mg of GOS/kg of body weight (daily), for two weeks prior to infection and during the course of infection (10 days). At necropsy, adherence and disease severity were assessed in the distal colon. Results indicated that GOS reduces adherence of GOS in conventional mice but not in germ free mice. Nevertheless, the lesions were not improved in mice supplemented with GOS as compared to the control. Finally, the antiadherence effect of yeast derived mannan oligosaccharides (MOSy) was tested against *Vibrio cholerae*. Adherence inhibition was observed at a minimum concentration of 2mg/ml and reached its maximum at 4mg/ml. These results show that different NDOs may be used as a prophylactic treatment to prevent adherence, hence preventing or mitigating infections by enteric pathogens.

Acknowledgements

"Let food be thy medicine, and medicine be thy food" – Hippocrates

As I am approaching the end of my graduate studies, I can only look back and be amazed by how far I've come. I have not only earned a degree, grad school has become my life for the past years, and the lessons I have learned and the experiences I've had will remain in my heart for years to come. The people I met in this journey have all taught me something in one way or another, and they will remain as very special members of one of the most important chapters of my life.

I want to give my most sincere thanks to my advisor Dr. Robert Hutkins. He has not only lead me through my academic path, but he has also supported me through the good and the bad times encountered. To Dr. Rupnow, my deepest thanks for all the support and the opportunity to have grad school as part of my career path. I will always be in debt for all the kindness, support, and encouragement I have received from him; all the beer in the world won't be enough. Many thanks to Dr. Ramer-Tait, she has guided me through the last steps of my research, always teaching me new things and supporting me in every step of my experiments. Many thanks to my lab mates, especially Maria Ximena Maldonado-Gomez and Alejandra Ramirez-Hernandez, working in the lab would not have felt like home without you girls around.

Thanks to my mom, Gloria Ines Villegas-Angel. Without all her support end effort, I would have never come this far. Thank you for your unconditional love and support and for always leading me through my greatest achievements. I love you with my heart and soul.

So many people have crossed my path in this journey, and I will always be grateful for the lessons they taught me. I want to thank my family for being there, always! I love every single one of you, like crazy and unconditionally. My Lincoln friends, thank you for all the happy moments! And for being there in the not so happy ones, you are what makes this town the best place to live. Finally I want to thank my awesome husband-to-be Mauricio Casares. You made me see life with different colors and have lifted me up every time I've needed it. I love you forever and always.

Preface

This thesis is comprised of six chapters. Chapter 1 provides a review of the current literature on the biological properties of non-digestible oligosaccharides. Chapter 2 describes our published (Quintero-Villegas et al. 2013 *Journal of Agricultural Food Chemistry*) results focusing on the antiadhesive effect of chitooligosaccharides (CHOS), against enteropathogenic *Escherichia coli.* Chapter 3 describes our published (Quintero-Villegas et al., 2014 *Current Microbiology*) results obtained when testing the antiadherent effect of lactoferrin alone and in combination with a mix of galactooligosaccharides (GOS) and polydextrose (PDX) against *Cronobacter sakazakii*. Chapter 4 describes results on the antiadherent effect of GOS against *Citrobacter rodentium* in an *in vivo* mouse model (conventional flora and germ free). Chapter 5 describes the results obtained when testing the anti-adherence effect of yeast derived mannan oligosaccharides (MOSy) against *Vibrio cholerae* at different doses, as wells as the results obtained from *in vitro* MOSy fecal fermentations. Finally, Chapter 6 provides a conclusion section that summarizes the major research findings presented within this dissertation, in addition to future experiments proposed.

Table of Contents

Page

List of Tables

Page

Chapter 1:

List of Figures

Page

Chapter 2:

Chapter 1

Non-digestible Oligosaccharides: Food Ingredients Targeted to Host Health

Abstract

Novel food ingredients that contribute to host health, beyond the nutritional value, have now attracted much attention and represent a very evident trend: food ingredients that improve health are now preferred. Non-digestible oligosaccharides (NDOs) are food ingredients that have been shown to have different biological properties, and contribute to host health upon consumption. NDOs have the ability to modulate the gut microbiota, stimulating the growth of beneficial members and hence increasing the production of short chain fatty acids (SCFA); modulate immune responses, either direct or indirect; and serve as anti-adherence agents that are capable of blocking pathogen adherence to the surface of epithelial cells. This review describes the different biological properties that have been shown for some NDOs of interest, focusing mainly on the immunomodulatory potential and anti-adherence effect *in vivo*.

Key words: Non-digestible oligosaccharides, gut microbiota, anti-adherence, adhesins, immune modulation

Introduction

Among the novel food ingredients that have received much research and commercial attention due to their biological properties are the non-digestible oligosaccharides (NDO). NDOs are found naturally in plant material and can be obtained or derived from other natural sources including yeast, bacteria, fungi, shellfish, and milk. Commercially, they are most often derived chemically or enzymatically from the hydrolysis of natural polymers, synthesized from monomers, or extracted directly from natural sources (Boehm and Moro, 2008). The best-studied NDOs include the galactooligosaccharides (GOS), fructooligosaccharides (FOS), inulin, and mannan oligosaccharides (MOS). These are commercially available and some are now being included as prebiotics or supplements for human and animal food applications.

This review provides a short overview of the composition and structure of some NDOs of interest, as well as the biological properties that have been attributed to them. These include the prebiotic activity, in addition to the ability to reduce adherence of pathogens *in vitro* and *in vivo*, and the immune-modulatory potential of some of these NDOs.

Composition and structure of NDOs

In general, NDOs are short-chain, low molecular weight carbohydrates defined by having a degree of polymerization (DP) ranging from 3 – 10 in most cases (Mussatto and Mancilha, 2007). Some oligosaccharides, such as inulin derived

from chicory and other root sources, can have a DP of up to 60, while others like lactulose can be as small as DP2. NDOs are, by definition, resistant to acid hydrolysis and to enzymes present in saliva and the digestive tract, hence they reach the colon intact. The anomeric carbon (C1 or C2) of the monosaccharide units of the NDOs is configured such that the glycosidic bonds are resistant to human digestive enzymes (Roberfroid and Slavin, 2000). Nevertheless, they are potentially hydrolyzed by enzymes produced by colonic bacteria (Swennen et al., 2006). In addition, many have been categorized as functional food ingredients, which are defined as food ingredients that provide a health benefit beyond any nutritional value (Gosling et al., 2010).

Galactooligosaccharides (GOS)

GOS are synthesized enzymatically from lactose by β-galactosidases that have both hydrolytic and transgalactosylating activity. During the transgalactosylation reaction, galactose monomers are added to lactose to produce oligomers of varied DP, ranging from DP2 to DP10, all with a terminal glucose. The resulting linkages include β(1-3), β(1-4), and β(1-6). Structural differences in the GOS molecules can result in different biological properties when assessed *in vitro* and *in vivo* (Gosling et al., 2010).

Inulin and Fructooligosaccharides (FOS)

Inulin and FOS are part of a larger group of carbohydrates called fructans. To be considered a fructan, one or more of the glycosidic bonds should contain

fructosyl-fructose linkages, primarily forming polymers of fructose units. Fructans can be linear or branched, and are often described by the type of glycosidic linkages and DP (Kelly, 2008).

Inulin is a linear fructan with β(2-1) fructosyl-fructose linkages (Roberfroid, 2007; Waterhouse and Chatterton, 1993); some can have a starting glucose molecule. The most common source of inulin for commercial production is chicory root, and chicory inulin configuration usually ranges from DP2 to about DP60; the average DP (DPav) is 12 (Roberfroid, 2005). Only a small percentage is in the range of DP2 to DP4. Inulin can also be synthesized from sucrose. In this case all the resulting molecules start with a glucose unit and the DP ranges from 2 to 4. These are classified as inulin-type fructans called FOS (Kelly, 2008; Roberfroid, 2007). Although the definition of FOS is not consistent in the NDOs literature, FOS can be used to describe short-chain, inulin-type fructan mixes, whereas inulin-type fructans that are derived from the partial hydrolysis of inulin are often referred to as oligofructose. Nonetheless, both compounds must have DP<10 (Kelly, 2008).

Chitooligosaccharides (CHOS)

CHOS are produced enzymatically or chemically from chitosan. The latter is a heteropolymer of glucosamine (GlcN) and N-acetyl glucosamine (GlcNAc), with varying DP (commonly DP<10) (Jeon and Kim, 2000) and degrees of acetylation (F_A); chitosan and its derivatives are β(1-4) linked (Vander, 1998).

The structures of the CHOS rely strongly on the type of enzyme used in the hydrolysis process. The specific cleavage sites of chitinases or chitosanases are determined by the sequences in the heteropolymers of GlcNAc and GlcN (chitosans), hence yielding different combinations of CHOS. These will vary with respect to length and sequence features (Aam et al., 2010); they can be defined by their F_A, DP, and the pattern of N-acetylated sugar residues (Quintero-Villegas et al., 2013).

Pectin oligosaccharides (POS)

Pectin is a structural component in the primary cell walls of plants such as fruits and vegetables. Structurally, it is comprised mainly of three major polysaccharides: homogalacturonan (HG), rhamnogalacturonan-I (RG-I), and rhamnogalacturonan-II (RG-II); these are galacturonic acid-rich polysaccharides (Fanaro et al., 2005; Willats et al., 2001). POS are derived from de-polymerization of pectin rich products or by-products, and are acidic oligosaccharides due to the nature of the polysaccharide from which they are derived. They can be obtained from products such as sugar beet pulp, apple pomace, and citrus peel, by either chemical or enzymatic processing (Gullón et al., 2013; Martínez-Sabajanes et al., 2012).

HG structure is comprised of a galacturonic acid backbone with α(1-4) linkages, containing free or esterified carboxyl groups, which can be partly substituted. RG-I is a branched polymer with alternating units of galacturonic acid and

rhamnose. In addition, branches of arabinan, galactan, and arabinogalactan can be attached. RG-II is a complex branched polymer of galacturonic acid, rhamnose, and galactose, in addition to some unusual sugars (Gullón et al., 2013; Martínez-Sabajanes et al., 2012; Ralet et al., 2005).

Mannan oligosaccharides (MOS)

Yeast cell walls as well as other natural sources, including plant material, are rich in mannan polysaccharides. Yeast cell walls contain an α(1-4) linked mannose monomer backbone, and many carry N-linked glycans with a high-mannose core (Gemmill and Trimble, 1999). There are different structural arrangements of mannans found in nature, and each seem to be characteristic to the organism or source from which it comes (Kocourek and Ballou, 1969). MOSs result from the partial hydrolysis of mannan polysaccharides and are, in general, glucomannan complexes comprised of α(1-4) linked mannose monomers (Dimitroglou et al., 2009).

Human milk oligosaccharides (HMO)

The carbohydrate fraction of human milk (7%) consists of lactose, and a complex mixture of oligosaccharides that has recently been characterized (Coppa et al., 1993; Kunz et al., 2000). Approximately 200 different molecular species have now been identified (Ben et al., 2004; Coppa et al., 1993; Kunz et al., 2000; Zivkovic et al., 2010). This structural diversity is due, in part, to the presence of several functional groups, including fucose and sialic acid residues. Identified

HMOs are comprised of neutral and acidic oligosaccharides, with fucose and sialic acid units at the terminal ends (Ninonuevo et al., 2006) with N-acetyl-lactosamine units (Zivkovic et al., 2010).

Many of these oligosaccharides have no nutritional value, but they have been shown to have other biological properties that confer health benefits upon consumption. In addition, some have similar structures to those found in the surface of intestinal cells (Sharon and Ofek, 2000, 2002), a property that can be protective against pathogen colonization (Kunz et al., 1999; Sharon and Ofek, 2000).

Biological Properties

Important biological properties have been attributed to NDOs. In particular, several NDOs have been classified as prebiotics, due to their selective fermentation by beneficial microorganisms in the gastrointestinal tract. The ability of NDOs to modulate the gut microbiota has been extensively studied *in vitro* as well as in human and animal studies. Despite differences in the monomer content, linkages, and DP, most prebiotic NDOs are able to promote or stimulate the growth of beneficial members of the gut microbiota, including bifidobacteria and lactobacilli (Boehm et al., 2004; Gibson and Roberfroid, 1995; Veereman-Wauters, 2007). Among the studies of NDOs with human subjects, Davis et al., 2010 evaluated the effect of different doses of GOS on the gastrointestinal microbiota of healthy adults. Results indicated that supplementation of 5g of

GOS or higher, on a daily basis, resulted in an increase in the number of bifidobacteria in some, but not all subjects. The efficacy of FOS in modulating the gut microbiota was evaluated by Tuohy et al., 2001. 7 g/day of FOS were delivered in shortbread biscuits and the changes in gut microbiota were assessed by 16s sequencing. Results indicated that FOS conferred a bifidogenic effect upon consumption. Other studies, *in vitro* and *in vivo*, regarding the bifidogenic effect of prebiotics are discussed in more detail in Gibson et al., 2004 and Roberfroid, 2007.

In addition to their prebiotic activity, some NDOs have also been reported to modulate the immune system, either directly or indirectly (Buddington et al., 2007; Meijer et al., 2010). Finally, NDOs have been suggested to act as therapeutic agents for preventing or mitigating bacterial infections, due to their ability to inhibit pathogen binding to the surface of epithelial cells (Quintero et al., 2011; Quintero-Villegas et al., 2013; Shoaf et al., 2006). These latter two properties will be discussed in more detail in this review.

Adherence

The ability of NDOs to inhibit pathogen binding to the surface of epithelial cells has attracted considerable interest for more than 20 years. Bacterial adherence to host cell surfaces is the first and, in many cases, the most important, step in bacterial pathogenesis (Bavington and Page, 2005; Klemm et al., 2010; Shoaf et al., 2006). Adherence is the mechanism by which bacteria are able to avoid the

host's natural displacement mechanisms (peristalsis, acid excretion, flux, etc.). In addition, adherence to the epithelial cell surface provides access to open niches (not colonized by commensal bacteria), as well as access to nutrients located at the epithelial cell surface, hence improving the potential for colonization and infection (Kamada et al., 2013; Ofek et al., 2003a; Sinclair et al., 2009). Bacteria that are not able to express functional adhesins are, in many cases, incapable of initiating infection (Boddicker et al., 2002; Cleary, 2004). Several biological events occur during the interaction between infectious bacteria and the host. In general, physical forces bring the bacteria closer to its target host cell surface (Busscher, 1987), followed by hydrophobic interactions that reversibly bind the bacteria to the target cell receptor (Ofek et al., 2003b). Subsequently, a much stronger interaction takes place. This interaction is formed between specific bacterial adhesins and their complementary host cell surface receptors (Abu-Lail and Camesano, 2003; Cozens and Read, 2012; Pinzón-Arango et al., 2009).

Blocking adherence should reduce the colonization potential of a pathogen. Hence, agents that block pathogen adherence, such as NDOs, have gained considerable interest.

Bacterial Lectins

Many bacterial adhesins are lectin-like components that recognize specific carbohydrate moieties on host cell surfaces; they are usually organized in thread-like organelles called fimbriae or pili (Bavington and Page, 2005; Klemm et al.,

2010; Sharon, 2006). Not surprisingly, a great number of pathogens produce these structures (Foster, 2004; Odenbreit, 2005; Pizarro-Cerdá and Cossart, 2006). Due to their receptor specificity, adhesins are the primary reason why tissue tropism exists among different bacterial species. Their receptor specificity is determined by structural differences between different lectins, which are dependent on the small globular carbohydrate-recognition domains. Slight chemical differences in these domains allow for the selectivity and specificity of each adhesin to its target receptor (Shoaf-Sweeney and Hutkins, 2008; Weis and Drickamer, 1996). Thus, the specific binding of a pathogen to a particular tissue or host is dependent on the particular carbohydrate receptors coating the epithelial cell surface (Klemm and Schembri, 2000; Shoaf-Sweeney and Hutkins, 2008). Certainly, adhesins are considered a primary virulence factor for many bacterial pathogens; they are required for infection of different tissues of the mammalian host (Connell et al., 1997; Klemm et al., 2006, 2007). Bacterial pathogens are capable of expressing more than one adhesin, nevertheless, they are often expressed at different stages of infection (Klemm et al., 2010).

Lectins are identified as the primary type of bacterial adhesin (Sharon and Ofek, 2000). Several adhesins have been identified for different pathogenic bacteria as well as some specific carbohydrate receptors. Nevertheless, due the complexity of the techniques required, the interactions between the adhesin and its specific carbohydrate receptor needs further study (Choudhury, 1999; Dodson et al., 2001).

Fimbrial Architecture and Adhesins

Placing receptor-specific lectins at the cell surface can be a challenge, nevertheless bacteria have developed numerous ways and a variety of adhesins and structures exist (Klemm and Schembri, 2000). As noted above, the majority of bacterial adhesins are arranged in organelles called fimbriae or pili. These are filamentous appendages that are localized on the bacterial surface. In Gram-negative bacteria, they are anchored to the outer membrane and are comprised of several different subunits (Gerlach and Hensel, 2007).

There are several different types of fimbriae among bacterial species. Generally, they are heteropolymers of about 1 µm in length. The fimbrial adhesins are located at the tip of the organelle, where a structural protein displays the adhesin (Klemm and Schembri, 2000; Klemm et al., 2010). Based on structural differences, fimbriae have been classified in different categories. In most cases intestinal and extra-intestinal pathogens use different types of fimbriae and adhesins due to the differences in the tissue to which they adhere. Table 1 describes the main adhesins found in intestinal and extra-intestinal pathogens of interest.

Anti-adherence

Bacterial adhesins recognize specific carbohydrate moieties on the surface of the epithelium, to which they tightly bind. Some of these receptors that are recognized by different pathogens have been identified and their structures are

known (Table 2). Interestingly, several NDOs have been shown to have similar structures to the carbohydrate moieties that are located on the surface of epithelial cells and that are recognized as pathogen binding sites. Hence, these NDOs could act as molecular decoys that can prevent the initial binding of pathogens to the surface of cells (Sharon and Ofek, 2000, 2002; Shoaf-Sweeney and Hutkins, 2008). This binding interference could therefore result in the prevention or mitigation of the onset of an infection. Many *in vitro* studies have shown the ability of NDOs to inhibit pathogen adherence to tissue culture cells (Table 3)**.**

Several *in vivo* models have also been used to assess the anti-adherence effect of NDOs. Manthey et al., 2014 used a suckling mice model to test the role of HMOs and GOS in preventing enteric infections; enteropathogenic *Escherichia coli* (EPEC) was used as a model of infection. Newborn mice were infected orally with EPEC with or without pre-incubation with HMOs or GOS. In addition some groups were gavaged with GOS, HMO, or PBS before and after infection. Results showed that HMOs were effective at reducing EPEC colonization in suckling mice, when administered at the day of infection and throughout the infection period. Interestingly, GOS did not show any significant differences in EPEC colonization rates as compared to the PBS control mice.

Liu et al., 2010 used a pig model to assess the ability of CHOS to reduce clinical signs associated with *E. coli* K88 infection. Weaned pigs were fed either a corn-

soybean meal diet or the same diet supplemented with CHOS, and both groups were then challenged with *E. coli* K88. Pigs supplemented with CHOS had decreased incidence of diarrhea and lower *E. coli* K88 counts in the small and large intestine, compared to the un-supplemented diets. Nevertheless, the growth performance of *E. coli* K88-challenged pigs supplemented with CHOS was not significantly different from pigs that were not given CHOS. Hence, although CHOS was able to reduce *E. coli* colonization, it did not appear to be a suitable growth promoter.

Searle et al., 2009 studied the ability of GOS to protect from a *Salmonella enterica* serovar Typhimurium infection a BALB/c mouse model. GOS, or saline solution as a control, was gavaged prior to challenge with *S.* Typhimurium. The delivery of GOS prior to challenge resulted in significant reductions in colonization. Additionally, they showed that the presence of GOS in a murine ligated ileal loop model prevented colonization by *S.* Typhimurium and its associated pathology. In a subsequent study, Searle et al., 2010 confirmed the results from the previous study by assessing the effect of the basal ingredients of GOS in the same mouse model. Results indicated that only the groups that were supplemented with GOS prior to challenge with *S.* Typhimurium showed reduced colonization as compared to the control.

Ruiz-Palacios et al., 2003 tested the efficacy of HMOs to inhibit colonization by *Campylobacter jejuni* in BALB/c mice. Mice were challenged with either 10^4 or

10^8 CFU of *C. jejuni,* and were supplemented with a total of 6 mg of neutral HMOs delivered in three doses: 2 hours before, during, and after oral challenge with *C. jejuni.* In addition, they tested *C. jejuni* colonization in transgenic mice pups that carried the human fucosyltransferase gene (FUT1), which results in expression fucosyl α(1-2) ligand in the mammary gland tissue of lactating mice. As a result, the milk of the lactating mice contains H-2 group blood antigen. Transgenic pups were inoculated with three different doses of *C. jejuni*, as were pups from conventional (wild-type) mice as a control. Results indicated that mice supplemented with HMOs were colonized at a significantly lower level by *C. jejuni.* In the transgenic mouse model, transgenic mice pups cleared the *C. jejuni* infection quicker than conventional (wild-type) mice over the course of study. For each inoculum, colonization levels in transgenic mice were significantly lower than those in conventional (wild-type) mice.

Umar et al., 2003 tested the ability of 6% pectin in the diet, compared to a fiber free diet, to ameliorate *C. rodentium* infection in Swiss-Webster mice. Signs of colonic hyperplasia, such as crypt length, were assessed. Results indicated that pectin supplementation in the diet ameliorated some of the disease pathology. Pectin reduced increases in cell proliferation and crypt length, as well as it reduced β-catenin, cyclin D1, and c-myc levels, which are molecules associated with *C. rodentium* infection.

Wang et al., 2001 used BALB/c mice to evaluate the efficacy of bovine milk glycoconjugates to inhibit adherence of *Helicobacter pylori* 317p. Bovine milk glycoconjugates were delivered in a dose of 4000mg/kg of body weight. Results indicated that mice that were given the glycoconjugates had reduced rates of *H. pylori* gastric colonization as well as lower inflammation scores as compared with the *H. pylori*-infected control mice.

Mysore et al., 1999 used 3'sialyllactose (3'SL), an oligosaccharide that occurs naturally in bovine colostrum and human milk, to treat *H. pylori*-infected rhesus monkeys. 3'SL was supplemented alone or in combination with proton pump inhibitors (PMP) to determine if these would increase the efficacy. In a portion of the monkeys treated with 3'SL, *H. pylori* infection was not detected, nevertheless, the addition of PMP did not seem to play a role in helping eradicate *H. pylori* from the monkeys.

A rabbit model of pneumonia was used by Idänpään-Heikkilä et al., 1997 to evaluate the efficacy of different oligosaccharides to inhibit adherence of *Streptococcus pneumoniae* when administered together via intra-tracheal instillation. Bacterial quantitation was obtained from bronchoalveolar lavage fluid (BAL) 48 hours after challenge. Oligosaccharides tested included: LNnT (Gal β(1–4) GlcNAc β(1–3) Gal β(1–4) Glc), LSTc (Neu5Ac α(2–6) Gal β(1–4) GlcNAc β(1–3) Gal β(1–4) Glc), 3'SLNnT (Neu5Ac α(2–3) Gal β(1–4) GlcNAc β(1–3) Gal β(1–4) Glc), 3'SLn (Neu5Ac α(2–3) Gal β(1–4) GlcNAc), 3'GL (GalNAc β(1–3)

Gal β(1–4) Glc); these were chosen as a representative range of specific adherence moieties of *S. pneumoniae* on respiratory tract cells. All but 3'SLn and 3'GL reduced *S. pneumoniae* counts of BAL as compared to the control group, protecting the rabbits from bacteremia. A rat nasopharyngeal colonization model was included in this study. Infant Sprague-Dawley rats were challenged with 10^6 *S. pneumoniae*, intranasally. Intranasal supplementation of oligosaccharides, along with the bacteria, prevented colonization of the nasopharynx.

Mouricout et al., 1990 tested glycoprotein glycans in the ability to reduce adherence of enterotoxigenic *E. coli* (ETEC) to the intestinal cells of newborn calves that had been colostrum deprived. Glycoprotein glycans were obtained from bovine plasma, and the glycan moieties used were shown to mimic the oligosaccharide moieties recognized by the *E. coli* K99 pili. Newborn calves were inoculated with approximately 10^{10} ETEC and supplemented with more than 500mg of glycan preparation per day. Results indicated that calves treated with glycans had fewer ETEC cells adhered to the intestines, specifically to duodenum, jejunum, and ileum sections.

Aronson et al., 1979 tested methyl α-D-mannopyranoside (αMM), known to interfere with mannose binding in *E. coli*, for its ability to prevent urinary tract infections in a mouse model. αMM was injected along with the bacterium. As a

result, mice treated with αMM exhibited a reduction in the number of mice with bacteremia.

In vitro and *in vivo* studies support the idea that different NDOs can block pathogen attachment to the surface of epithelial cells. Although further studies are required that involve different pathogens, and a variety of NDOs, these are the first approaches to alternative prophylactic methods to prevent an onset of infection.

Although it has been shown that NDOs can prevent and/or reduce bacterial adherence in animal models, only two studies using humans as a subject have been reported. In spite of results from animal models being indeed very promising, the human trials have not proven as successful. In a clinical trial by Ukkonen et al., 2000, more than 500 children 10 to 24 months old received 3-sialyllacto-N-neotetraose (NeuAc2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc) as a prophylactic treatment to reduce nasopharyngeal colonization and disease caused by *Streptococcus pneumoniae, Moraxella catarrhalis* and *Haemophilus influenzae*, as well as the incidence of acute otitis media. The treatment was administered by nasal spray (2mg in each nostril per day) for 3 months. The treatment did not reduce carriage of any of the three pathogens of interest, and did not show any beneficial effect on the occurrence of acute otitis media. In another trial by Parente et al., 2003, 3'sialyllactose (10 or 20 g/day) was administered to 61 patients to reduce colonization of *H. pylori.* Although the

treatment was well-tolerated and did not present any side effects, it was not effective at clearing off patients from *H. pylori* colonization. The study by Mysore et al., 1999 with rhesus monkeys showed that although some of the subjects were cleared off *H. pylori* colonization, the treatment was not effective in all of the monkeys that were treated.

Immune modulation

Several NDOs have been reported to modulate the immune system (Buddington et al., 2007; Guigoz et al., 2002; Meijer et al., 2010; Watzl et al., 2007). In general, immune modulation occurs indirectly and is mediated via selective stimulation of specific members of the gut microbiota, in particular, species of *Bifidobacterium* and *Lactobacillus*. Many of these organisms ferment NDOs and can produce short chain fatty acids (SCFA), such as acetate, butyrate, and propionate, which play an important role in the gut. Butyrate, for example, has been extensively studied and has been identified as an important immune modulator (Gourbeyre et al., 2011). SCFAs are absorbed from the colonic lumen and serve as an energy source for colonic epithelial cells. A fraction of the produced SCFAs are released into the bloodstream (Meijer et al., 2010).

Immune cells carry receptors that are believed to be responsible for the interactions with SCFAs; these are the G protein-coupled receptors (GPR). GPR41 and GPR43 are recognized as the main SCFA receptors; both are expressed on immune cells (Le Poul et al., 2003). Receptors are activated by

different SCFAs, and the efficacy of the activation varies depending on the SCFA. For example, GPR41 will be activated by propionate, butyrate, and acetate, with the activation by propionate being 100 fold more potent than that of acetate (Le Poul et al., 2003).

Cytokine production derived from the stimulation of leukocytes by SCFAs, especially butyrate, has been reported (Hamer et al., 2008; Säemann et al., 2000). Butyrate has been shown to induce a shift from a pro-inflammatory Th1-like response to an anti-inflammatory profile, upon stimulation with agents that induce inflammation such as lipopolysaccharide (LPS) (Maa et al., 2010; Park et al., 2007; Säemann et al., 2000). Investigators have suggested that one of the mechanisms by which butyrate exerts an anti-inflammatory profile is mediated by signaling pathways such as NF-κB (Park et al., 2007; Segain, 2000; Usami et al., 2008). Propionate and acetate have also been shown to inhibit NF-κB reporter activity in a dose dependent manner as well as to decrease TNF-α release from neutrophils, when stimulated with LPS (Tedelind et al., 2007).

Another effect of SCFA in immune modulation is related to chemotaxis. Acetate, butyrate, and propionate have been shown to increase the chemotactic response of neutrophils, as well as an increased expression of L-selectin in the surface of the latter. The concentration of the different SCFA is important, since it was shown that higher concentrations do not exhibit any chemotactic effect as compared with the lower concentrations used in the studies (M et al., 2009; Sina

et al., 2009). As expected, the effect of SCFA in chemotaxis depends on the type of SCFA and the concentration at which it is tested. In addition, different immune cell types will exhibit a different response upon stimulation with SCFAs (Meijer et al., 2010).

The immunomodulatory potential of NDOs does not rely solely on the modulation of the gut microbiota resulting in the increased production of SCFA. Other studies have been reported in which an immunomodulatory potential of NDOs is shown without necessarily being linked to SCFA production. Some of the most recent *in vivo* animal studies are summarized in Table 4.

Conclusion

NDOs have been shown to have different biological properties beneficial to the host upon consumption. Whether direct or indirect, the beneficial effects reported have raised considerable interest towards NDOs as functional food ingredients. The modulation of the gut microbiota, the use as anti-adherence agents with prophylactic purpose, and their immunomodulatory potential are desirable traits in a functional food ingredient. Although further research is needed, especially to determine if they could be used as prophylactic agents to prevent bacterial infections, promising results have already been shown that serve as a basis to advance in the field. A profound characterization of the factors affecting expression of adhesins during the course of an infection, and identifying the types of adhesins, should lead to a better understanding of bacterial adherence

and the development of anti-adherence agents that could be applied to prevent infections.

In conclusion, there is enough evidence that NDOs can confer benefits to the host upon consumption. As the field advances, NDOs can be recognized as promising therapeutic agents to prevent bacterial infections and promote health.

References

Aam, B.B., Heggset, E.B., Norberg, A.L., Sørlie, M., Vårum, K.M., and Eijsink, V.G.H. (2010). Production of chitooligosaccharides and their potential applications in medicine. Mar. Drugs 8, 1482–1517.

Abu-Lail, N.I. and Camesano, T.A. (2003). Role of ionic strength on the relationship of biopolymer conformation, DLVO contributions, and steric interactions to bioadhesion of *Pseudomonas putida* KT2442. Biomacromolecules 4, 1000–1012.

Andersson, B., Porras, O., Hanson, L.Å., Lagergård, T., and Svanborg-Edén, C. (1986). Inhibition of Attachment of *Streptococcus pneumoniae* and *Haemophilus influenzae* by Human Milk and Receptor Oligosaccharides. J. Infect. Dis. 153, 232 – 237.

Aronson, M., Medalia, O., Schori, L., Mirelman, D., Sharon, N., and Ofek, I. (1979). Prevention of colonization of the urinary tract of mice with *Escherichia*

coli by blocking of bacterial adherence with methyl-D-mannopyranoside. J. Infect. Dis. 139, 329–332.

Barthelson, R., Mobasseri, A., Zopf, D., and Simon, P. (1998). Adherence of *Streptococcus pneumoniae* to respiratory epithelial cells is inhibited by sialylated oligosaccharides. Infect. Immun. 66, 1439–1444.

Baumler, A.J., Tsolis, R.M., and Heffron, F. (1996). The lpf fimbrial operon mediates adhesion of *Salmonella typhimurium* to murine peyer's patches. PNAS *93*, 279–283.

Bäumler, A.J., Tsolis, R.M., and Heffron, F. (1997). Fimbrial adhesins of *Salmonella typhimurium*. Role in bacterial interactions with epithelial cells. Adv. Exp. Med. Biol. 412, 149–158.

Bavington, C., and Page, C. (2005). Stopping bacterial adhesion: a novel approach to treating infections. Respiration. 72, 335–344.

Ben, X.M., Zhou, X.Y., Zhao, W.H. Yu, W.L., Pan, W., Zhang, W.L., Wu, S.M., Christien, M.V.B., and Anne, S. (2004). Supplementation of milk formula with galacto-oligosaccharides improves intestinal micro-flora and fermentation in term infants. Chin. Med. J. 117, 927–931.

Boddicker, J.D., Ledeboer, N.A., Jagnow, J., Jones, B.D., and Clegg, S. (2002). Differential binding to and biofilm formation on, HEp-2 cells by *Salmonella*

enterica serovar Typhimurium is dependent upon allelic variation in the fimH gene of the fim gene cluster. Mol. Microbiol. 45, 1255–1265.

Boehm, G., and Moro, G. (2008). Structural and functional aspects of prebiotics used in infant nutrition. J. Nutr. 138, S1818–S1828.

Boehm, G., Jelinek, J., Stahl, B., van Laere, K., Knol, J., Fanaro, S., Moro, G., and Vigi, V. (2004). Prebiotics in infant formulas. J. Clin. Gastroenterol. 38, S76–S79.

Buddington, R.K., Kelly-Quagliana, K., Buddington, K.K., and Kimura, Y. (2007). Non–digestible oligosaccharides and defense functions: lessons learned from animal models. Br. J. Nutr. 87, S231-S239.

Busscher, H. (1987). Specific and non-specific interactions in bacterial adhesion to solid substrata. FEMS Microbiol. Lett. 46, 165–173.

Buts, L., Bouckaert, J., De Genst, E., Loris, R., Oscarson, S., Lahmann, M., Messens, J., Brosens, E., Wyns, L., and De Greve, H. (2004). The fimbrial adhesin F17-G of enterotoxigenic *Escherichia coli* has an immunoglobulin-like lectin domain that binds N-acetylglucosamine. Mol. Microbiol. 49, 705–715.

Choudhury, D. (1999). X-ray Structure of the FimC-FimH Chaperone-Adhesin Complex from uropathogenic *Escherichia coli*. Science. 285, 1061–1066.

Cleary, J. (2004). Enteropathogenic *Escherichia coli* (EPEC) adhesion to intestinal epithelial cells: role of bundle-forming pili (BFP), EspA filaments and intimin. Microbiol. 150, 527–538.

Collinson, S.K., Emody, L., Muller, K.H., Trust, T.J., and Kay, W.W. (1991). Purification and characterization of thin, aggregative fimbriae from *Salmonella enteritidis*. J. Bacteriol. 173, 4773–4781.

Collinson, S.K., Doig, P.C., Doran, J.L., Clouthier, S., Trust, T.J., and Kay, W.W. (1993). Thin, aggregative fimbriae mediate binding of *Salmonella enteritidis* to fibronectin. J. Bacteriol. 175, 12–18.

Connell, H., Hedlund, M., Agace, W., and Svanborg, C. (1997). Bacterial attachment to uro-epithelial cells: mechanisms and consequences. Adv. Dent. Res. 11, 50–58.

Coppa, G., Bruni, S., and Zampini, L. (2003). Oligosaccharides of human milk inhibit the adhesion of *Listeria monocytogenes* to Caco-2 cells. Ital. J. Pediatr. 29, 61-68.

Coppa, G. V, Zampini, L., Galeazzi, T., Facinelli, B., Ferrante, L., Capretti, R., and Orazio, G. (2006). Human milk oligosaccharides inhibit the adhesion to Caco-2 cells of diarrheal pathogens: *Escherichia coli, Vibrio cholerae, and Salmonella fyris*. Pediatr. Res. 59, 377–382.

Coppa, G. V., Gabrielli, O., Pierani, P., Catassi, C., Carlucci, A., and Giorgi, P.L. (1993). Changes in carbohydrate composition in human milk over 4 months of lactation. Pediatr. 91, 637–641.

Cozens, D., and Read, R.C. (2012). Anti-adhesion methods as novel therapeutics for bacterial infections. Expert Rev. Anti. Infect. Ther. 10, 1457–1468.

Craig, L., Pique, M.E., and Tainer, J.A. (2004). Type IV pilus structure and bacterial pathogenicity. Nat. Rev. Microbiol. *2,* 363–378.

Davis, L.M.G., Martínez, I., Walter, J., and Hutkins, R. (2010). A dose dependent impact of prebiotic galactooligosaccharides on the intestinal microbiota of healthy adults. Int. J. Food Microbiol. 144, 285–292.

Dimitroglou, A., Merrifield, D.L., Moate, R., Davies, S.J., Spring, P., Sweetman, J., and Bradley, G. (2009). Dietary mannan oligosaccharide supplementation modulates intestinal microbial ecology and improves gut morphology of rainbow trout, *Oncorhynchus mykiss* (Walbaum). J. Anim. Sci. 87, 3226–3234.

Dodson, K.W., Pinkner, J.S., Rose, T., Magnusson, G., Hultgren, S.J., and Waksman, G. (2001). Structural basis of the interaction of the pyelonephritic *E. coli* adhesin to its human kidney receptor. Cell 105, 733–743.

Donnenberg, M.S., Girón, J.A., Nataro, J.P., and Kaper, J.B. (1992). A plasmid-encoded type IV fimbrial gene of enteropathogenic *Escherichia coli* associated with localized adherence. Mol. Microbiol. 6, 3427–3437.

El-Hawiet, A., Kitova, E.N., Kitov, P.I., Eugenio, L., Ng, K.K.S., Mulvey, G.L., Dingle, T.C., Szpacenko, A., Armstrong, G.D., and Klassen, J.S. (2011). Binding of *Clostridium difficile* toxins to human milk oligosaccharides. Glycobiol. 21, 1217–1227.

Ernst, B., and Magnani, J.L. (2009). From carbohydrate leads to glycomimetic drugs. Nat. Rev. Drug Discov. 8, 661–677.

Fanaro, S., Jelinek, J., Stahl, B., Boehm, G., Kock, R., and Vigi, V. (2005). Acidic oligosaccharides from pectin hydrolysate as new component for infant formulae: effect on intestinal flora, stool characteristics, and pH. J. Pediatr. Gastroenterol. Nutr. 41, 186–190.

Foster, T.J. (2004). The *Staphylococcus aureus* “superbug”. J. Clin. Invest. *114*, 1693–1696.

Ganan, M., Collins, M., Rastall, R., Hotchkiss, A.T., Chau, H.K., Carrascosa, A. V, and Martinez-Rodriguez, A.J. (2010). Inhibition by pectic oligosaccharides of the invasion of undifferentiated and differentiated Caco-2 cells by *Campylobacter jejuni*. Int. J. Food Microbiol. 137, 181–185.

Gemmill, T., and Trimble, R. (1999). Overview of *N* and *O*-linked oligosaccharide structures found in various yeast species. Biochim. Biophys. Acta 1426, 227-237.

Gerlach, R.G., and Hensel, M. (2007). Protein secretion systems and adhesins: the molecular armory of Gram-negative pathogens. Int. J. Med. Microbiol. 297, 401–415.

Gibson, G.R., and Roberfroid, M.B. (1995). Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J. Nutr. 125, 1401–1412.

Gibson, G.R., Probert, H.M., Loo, J. Van, Rastall, R.A., and Roberfroid, M.B. (2004). Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. Nutr. Res. Rev. 17, 259–275.

Giron, J., Ho, A., and Schoolnik, G. (1991). An inducible bundle-forming pilus of enteropathogenic *Escherichia coli.* Science. 254, 710–713.

Gopalakrishnan, A., Clinthorne, J.F., Rondini, E.A., McCaskey, S.J., Gurzell, E.A., Langohr, I.M., Gardner, E.M., and Fenton, J.I. (2012). Supplementation with galacto-oligosaccharides increases the percentage of NK cells and reduces colitis severity in Smad3-deficient mice. J. Nutr. 142, 1336–1342.

Gosling, A., Stevens, G.W., Barber, A.R., Kentish, S.E., and Gras, S.L. (2010). Recent advances refining galactooligosaccharide production from lactose. Food Chem. 121, 307–318.

Gourbeyre, P., Denery, S., and Bodinier, M. (2011). Probiotics, prebiotics, and synbiotics: impact on the gut immune system and allergic reactions. J. Leukoc. Biol. 89, 685–695.

Guigoz, Y., Rochat, F., Perruisseau-Carrier, G., Rochat, I., and Schiffrin, E.. (2002). Effects of oligosaccharide on the faecal flora and non-specific immune system in elderly people. Nutr. Res. 22, 13–25.

Gullón, B., Gómez, B., Martínez-Sabajanes, M., Yáñez, R., Parajó, J.C., and Alonso, J.L. (2013). Pectic oligosaccharides: manufacture and functional properties. Trends Food Sci. Technol. 30, 153–161.

Hamer, H.M., Jonkers, D., Venema, K., Vanhoutvin, S., Troost, F.J., and Brummer, R.-J. (2008). Review article: the role of butyrate on colonic function. Aliment. Pharmacol. Ther. 27, 104–119.

Idänpään-Heikkilä, I., Simon, P.M., Zopf, D., Vullo, T., Cahill, P., Sokol, K., and Tuomanen, E. (1997). Oligosaccharides interfere with the establishment and progression of experimental pneumococcal pneumonia. J. Infect. Dis. 176, 704–712.

Janardhana, V., Broadway, M.M., Bruce, M.P., Lowenthal, J.W., Geier, M.S., Hughes, R.J., and Bean, A.G.D. (2009). Prebiotics modulate immune responses in the gut-associated lymphoid tissue of chickens. J. Nutr. 139, 1404–1409.

Jeon, Y.J., and Kim, S.K. (2000). Production of chitooligosaccharides using an ultrafiltration membrane reactor and their antibacterial activity. Carbohydr. Polym. 41, 133–141.

Jones, C.H. (1995). FimH Adhesin of Type 1 pili is assembled into a fibrillar tip structure in the *Enterobacteriaceae*. PNAS 92, 2081–2085.

Kamada, N., Chen, G.Y., Inohara, N., and Núñez, G. (2013). Control of pathogens and pathobionts by the gut microbiota. Nat. Immunol. 14, 685–690.

Kelly, G. (2008) Inulin-type prebiotics - a review: part 1. Altern. Med. Rev. 13, 315-329.

Klemm, P., and Schembri, M.A. (2000). Bacterial adhesins: function and structure. Int. J. Med. Microbiol. 290, 27–35.

Klemm, P., Roos, V., Ulett, G.C., Svanborg, C., and Schembri, M.A. (2006). Molecular characterization of the *Escherichia coli* asymptomatic bacteriuria strain 83972: the taming of a pathogen. Infect. Immun. 74, 781–785.

Klemm, P., Hancock, V., and Schembri, M.A. (2007). Mellowing out: adaptation to commensalism by *Escherichia coli* asymptomatic bacteriuria strain 83972. Infect. Immun. 75, 3688–3695.

Klemm, P., Vejborg, R.M., and Hancock, V. (2010). Prevention of bacterial adhesion. Appl. Microbiol. Biotechnol. 88, 451–459.

Kocourek, J., and Ballou, C. (1969). Method for fingerprinting yeast cell wall mannans. J. Bacteriol. 100, 1175-1181.

Kovacs-Nolan, J., Kanatani, H., Nakamura, A., Ibuki, M., and Mine, Y. (2013). β-1,4-mannobiose stimulates innate immune responses and induces TLR4-dependent activation of mouse macrophages but reduces severity of inflammation during endotoxemia in mice. J. Nutr. 143, 384–391.

Krogfelt, K.A., Bergmans, H., and Klemm, P. (1990). Direct evidence that the FimH protein is the mannose-specific adhesin of *Escherichia coli* type 1 fimbriae. Infect. Immun. 58, 1995–1998.

Kunz, C., Rodriguez-Palmero, M., Koletzko, B., and Jensen, R. (1999). Nutritional and biochemical properties of human milk, Part I: General aspects, proteins, and carbohydrates. Clin. Perinatol. 26, 307–333.

Kunz, C., Rudloff, S., Baier, W., Klein, N., and Strobel, S. (2000). Oligosaccharides in human milk: structural, functional, and metabolic aspects. Annu. Rev. Nutr. 20, 699–722.

Lara-Villoslada, F., Debras, E., Nieto, A., Concha, A., Gálvez, J., López-Huertas, E., Boza, J., Obled, C., and Xaus, J. (2006). Oligosaccharides isolated from goat milk reduce intestinal inflammation in a rat model of dextran sodium sulfate-induced colitis. Clin. Nutr. 25*,* 477–488.

Liu, P., Piao, X.S., Thacker, P. a, Zeng, Z.K., Li, P.F., Wang, D., and Kim, S.W. (2010). Chito-oligosaccharide reduces diarrhea incidence and attenuates the immune response of weaned pigs challenged with *Escherichia coli* K88. J. Anim. Sci. 88, 3871–3879.

Vinolo, M.A., Rodrigues, H.G., Hatanaka, E., Hebeda, C.B., Farsky, S.H., and Curi, R. (2009). Short-chain fatty acids stimulate the migration of neutrophils to inflammatory sites. Clin. Sci. (Lond). 117, 331-338.

Maa, M.C., Chang, M.Y., Hsieh, M.Y., Chen, Y.J., Yang, C.J., Chen, Z.C., Li, Y.K., Yen, C.K., Wu, R.R., and Leu, T.H. (2010). Butyrate reduced lipopolysaccharide-mediated macrophage migration by suppression of Src enhancement and focal adhesion kinase activity. J. Nutr. Biochem. 21, 1186–1192.

Manhart, N., Spittler, A., Bergmeister, H., Mittlböck, M., and Roth, E. (2003). Influence of fructooligosaccharides on peyer's patch lymphocyte numbers in healthy and endotoxemic mice. Nutr. 19, 657–660.

Manthey, C.F., Autran, C.A., Eckmann, L., and Bode, L. (2014). Human milk oligosaccharides protect against enteropathogenic *Escherichia coli* attachment *in vitro* and EPEC colonization in suckling mice. J. Pediatr. Gastroenterol. Nutr. 58, 167–170.

Martínez Sabajanes, M., Yáñez, R., Alonso, J.L., and Parajó, J.C. (2012). Pectic oligosaccharides production from orange peel waste by enzymatic hydrolysis. Int. J. Food Sci. Technol. 47, 747–754.

Meijer, K., Vos, P., and Priebe, M.G. (2010). Butyrate and other short-chain fatyy acids as modulators of immunity: what relevance for health? Curr. Opin. Clin. Nutr. Metab. Care 13, 715–721.

Mouricout, M., Petit, J.M., Carias, J.R., and Julien, R. (1990). Glycoprotein glycans that inhibit adhesion of *Escherichia coli* mediated by K99 fimbriae: treatment of experimental colibacillosis. Infect. Immun. 58, 98–106.

Mussatto, S.I., and Mancilha, I.M. (2007). Non-digestible oligosaccharides: a review. Carbohydr. Polym. 68, 587–597.

Mysore, J. V., Wigginton, T., Simon, P.M., Zopf, D., Heman-Ackah, L.M., and Dubois, A. (1999). Treatment of Helicobacter pylori infection in rhesus monkeys using a novel antiadhesion compound. Gastroenterology 117, 1316–1325.

Nakamura, Y., Nosaka, S., Suzuki, M., Nagafuchi, S., Takahashi, T., Yajima, T., Takenouchi-Ohkubo, N., Iwase, T., and Moro, I. (2004). Dietary fructooligosaccharides up-regulate immunoglobulin A response and polymeric immunoglobulin receptor expression in intestines of infant mice. Clin. Exp. Immunol. 137, 52–58.

Nasr, A., Olsén, A., Sjöbring, U., Müller-Esterl, W., and Björck, L. (1996). Assembly of human contact phase proteins and release of bradykinin at the surface of curli-expressing *Escherichia coli.* Mol. Microbiol. 20, 927–935.

Ninonuevo, M.R., Park, Y., Yin, H., Zhang, J., Ward, R.E., Clowers, B.H., German, J.B., Freeman, S.L., Killeen, K., Grimm, R., et al. (2006). A strategy for annotating the human milk glycome. J. Agric. Food Chem. 54, 7471–7480.

Odenbreit, S. (2005). Adherence properties of *Helicobacter pylori*: impact on pathogenesis and adaptation to the host. Int. J. Med. Microbiol. 295, 317–324.

Ofek, I., Hasty, D.L., and Sharon, N. (2003a). Anti-adhesion therapy of bacterial diseases: prospects and problems. FEMS Immunol. Med. Microbiol. 38, 181–191.

Ofek, I., Hasty, D.L., and Doyle, R.J. (2003b). Bacterial adhesion to animal cells and tissues. ASM Press, Washington DC, USA.

Olano-Martin, E., Williams, M.R., Gibson, G.R., and Rastall, R.A. (2003). Pectins and pectic-oligosaccharides inhibit *Escherichia coli* O157:H7 Shiga toxin as directed towards the human colonic cell line HT29. FEMS Microbiol. Lett. 218, 101–105.

Olsen, A., Wick, M.J., Morgelin, M., and Bjorck, L. (1998). Curli, fibrous surface proteins of *Escherichia coli*, interact with major histocompatibility complex class I molecules. Infect. Immun. 66, 944–949.

Parente, F., Cucino, C., Anderloni, A., Grandinetti, G., and Porro, G.B. (2003). Treatment of *Helicobacter Pylori* infection using a novel antiadhesion compound (3'sialyllactose sodium salt). A double blind, placebo-controlled clinical study. Helicobacter 8, 252–256.

Park, J.S., Lee, E.J., Lee, J.C., Kim, W.K., and Kim, H.S. (2007). Anti-inflammatory effects of short chain fatty acids in IFN-gamma-stimulated RAW 264.7 murine macrophage cells: involvement of NF-kappaB and ERK signaling pathways. Int. Immunopharmacol. 7, 70–77.

Pinzón-Arango, P.A., Liu, Y., and Camesano, T.A. (2009). Role of cranberry on bacterial adhesion forces and implications for *Escherichia coli*-uroepithelial cell attachment. J. Med. Food 12, 259–270.

Pizarro-Cerdá, J., and Cossart, P. (2006). Bacterial adhesion and entry into host cells. Cell 124, 715–727.

Le Poul, E., Loison, C., Struyf, S., Springael, J.Y., Lannoy, V., Decobecq, M.E., Brezillon, S., Dupriez, V., Vassart, G., Van Damme, J., et al. (2003). Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation. J. Biol. Chem. 278, 25481–25489.

Qiao, Y., Bai, X.F., and Du, Y.G. (2011). Chitosan oligosaccharides protect mice from LPS challenge by attenuation of inflammation and oxidative stress. Int. Immunopharmacol. 11, 121–127.

Quintero, M., Maldonado, M., Perez-Munoz, M., Jimenez, R., Fangman, T., Rupnow, J., Wittke, A., Russell, M., and Hutkins, R. (2011). Adherence Inhibition of Cronobacter sakazakii to Intestinal Epithelial Cells by Prebiotic Oligosaccharides. Curr. Microbiol. 62, 1448-1454.

Quintero-Villegas, M.I., Aam, B.B., Rupnow, J., Sorile, M., Eijsink, V.G.H., and Hutkins, R.W. (2013). Adherence inhibition of enteropathogenic Escherichia coli by chitooligosaccharides with specific degrees of acetylation and polymerization. J. Agric. Food Chem. 61, 2748-2754.

Ralet, M.C., Cabrera, J.C., Bonnin, E., Quéméner, B., Hellìn, P., and Thibault, J.F. (2005). Mapping sugar beet pectin acetylation pattern. Phytochemistry 66, 1832–1843.

Roberfroid, M. (2005). Introducing inulin-type fructans. Br. J. Nutr. S1, S13-S25.

Roberfroid, M. (2007). Prebiotics: The Concept Revisited. J. Nutr. 137, S830–S837.

Roberfroid, M., and Slavin, J. (2000). Nondigestible oligosaccharides. Crit. Rev. Food Sci. Nutr. 40, 461–480.

Robinson, L.S., Ashman, E.M., Hultgren, S.J., and Chapman, M.R. (2006). Secretion of curli fibre subunits is mediated by the outer membrane-localized CsgG protein. Mol. Microbiol. 59, 870–881.

Ruiz-Palacios, G.M., Cervantes, L.E., Ramos, P., Chavez-Munguia, B., and Newburg, D.S. (2003). *Campylobacter jejuni* binds intestinal H(O) antigen (Fuc alpha 1, 2Gal beta 1, 4GlcNAc), and fucosyloligosaccharides of human milk inhibit its binding and infection. J. Biol. Chem. 278, 14112–14120.

Säemann, M.D., Böhmig, G.A., Osterreicher, C.H., Burtscher, H., Parolini, O., Diakos, C., Stöckl, J., Hörl, W.H., and Zlabinger, G.J. (2000). Anti-inflammatory effects of sodium butyrate on human monocytes: potent inhibition of IL-12 and up-regulation of IL-10 production. FASEB J. 14, 2380–2382.

Saulino, E.T., Bullitt, E., and Hultgren, S.J. (2000). Snapshots of usher-mediated protein secretion and ordered pilus assembly. PNAS. 97, 9240–9245.

Schoeni, J.L., and Wong, A.C. (1994). Inhibition of *Campylobacter jejuni* colonization in chicks by defined competitive exclusion bacteria. Appl. Envir. Microbiol. 60, 1191–1197.

Searle, L.E.J., Best, A., Nunez, A., Salguero, F.J., Johnson, L., Weyer, U., Dugdale, A.H., Cooley, W.A., Carter, B., Jones, G., et al. (2009). A mixture containing galactooligosaccharide, produced by the enzymic activity of *Bifidobacterium bifidum*, reduces *Salmonella enterica* serovar Typhimurium infection in mice. J. Med. Microbiol. 58, 37–48.

Searle, L.E.J., Cooley, W.A., Jones, G., Nunez, A., Crudgington, B., Weyer, U., Dugdale, A.H., Tzortzis, G., Collins, J.W., Woodward, M.J., et al. (2010). Purified galactooligosaccharide, derived from a mixture produced by the enzymic activity of *Bifidobacterium bifidum*, reduces *Salmonella enterica* serovar Typhimurium adhesion and invasion *in vitro* and *in vivo*. J. Med. Microbiol. 59, 1428–1439.

Segain, J.P. (2000). Butyrate inhibits inflammatory responses through NFkappa B inhibition: implications for Crohn's disease. Gut *47*, 397–403.

Servin, A.L. (2005). Pathogenesis of Afa/Dr diffusely adhering *Escherichia coli*. Clin. Microbiol. Rev. 18, 264–292.

Sharon, N. (2006). Carbohydrates as future anti-adhesion drugs for infectious diseases. Biochim. Biophys. Acta 1760, 527–537.

Sharon, N., and Ofek, I. (2000). Safe as mother's milk: Carbohydrates as future anti-adhesion drugs for bacterial diseases. Glycononjugate J. 17, 659-664.

Sharon, N., and Ofek, I. (2002). Fighting infectious diseases with inhibitors of microbial adhesion to host tissues. Crit. Rev. Food Sci. Nutr. 42, 267–272.

Shoaf, K., Mulvey, G.L., Armstrong, G.D., and Hutkins, R.W. (2006). Prebiotic galactooligosaccharides reduce adherence of enteropathogenic *Escherichia coli* to tissue culture cells. Infect. Immun. *74*, 6920–6928.

Shoaf-Sweeney, K.D., and Hutkins, R.W. (2008). Adherence, anti-adherence, and oligosaccharides: preventing pathogens from sticking to the host. Adv. Food Nutr. Res. 55, 101–161.

Simon, P., Goode, P., Mobasseri, A., and Zopf, D. (1997). Inhibition of *Helicobacter pylori* binding to gastrointestinal epithelial cells by sialic acid-containing oligosaccharides. Infect. Immun. 65, 750–757.

Sina, C., Gavrilova, O., Förster, M., Till, A., Derer, S., Hildebrand, F., Raabe, B., Chalaris, A., Scheller, J., Rehmann, A., et al. (2009). G protein-coupled receptor 43 is essential for neutrophil recruitment during intestinal inflammation. J. Immunol. 183, 7514–7522.

Sinclair, H.R., de Slegte, J., Gibson, G.R., and Rastall, R.A. (2009). Galactooligosaccharides (GOS) inhibit *Vibrio cholerae* toxin binding to its GM1 receptor. J. Agric. Food Chem. 57, 3113–3119.

Smyth, C.J., Marron, M.B., Twohig, J.M.G.J., and Smith, S.G.J. (1996). Fimbrial adhesins: similarities and variations in structure and biogenesis. FEMS Immunol. Med. Microbiol. 16, 127–139.

Soto, G.E., and Hultgren, S.J. (1999). Bacterial adhesins: common themes and variations in architecture and assembly. J. Bacteriol. 181, 1059–1071.

Strömberg, N., Marklund, B.I., Lund, B., Ilver, D., Hamers, A., Gaastra, W., Karlsson, K.A., and Normark, S. (1990). Host-specificity of uropathogenic *Escherichia coli* depends on differences in binding specificity to Gal alpha 1-4Gal-containing isoreceptors. EMBO J. 9, 2001–2010.

Swanson, K.S., Grieshop, C.M., Flickinger, E.A., Bauer, L.L., Healy, H.-P., Dawson, K.A., Merchen, N.R., and Fahey, G.C. (2002). Supplemental fructooligosaccharides and mannanoligosaccharides influence immune function, ileal and total tract nutrient digestibilities, microbial populations and concentrations of protein catabolites in the large bowel of dogs. J. Nutr. 132, 980–989.

Swennen, K., Courtin, C.M., and Delcour, J.A. (2006). Non-digestible oligosaccharides with prebiotic properties. Crit. Rev. Food Sci. Nutr. 46, 459–471.

Tedelind, S., Westberg, F., Kjerrulf, M., and Vidal, A. (2007). Anti-inflammatory properties of the short-chain fatty acids acetate and propionate: a study with relevance to inflammatory bowel disease. World J. Gastroenterol. 13, 2826–2832.

Tennent, J.M., and Mattick, J.S. (1994). Type 4 fimbriae. In Adhesion, genetics, biogenesis, and vaccines, P. Klemm, ed. CRC Press, Inc, Boca Raton FL. 127–146.

Tobe, T., and Sasakawa, C. (2001). Role of bundle-forming pilus of enteropathogenic *Escherichia coli* in host cell adherence and in microcolony development. Cell. Microbiol. 3, 579–585.

Tuohy, K.M., Kolida, S., Lustenberger, A.M., and Gibson, G.R. (2001). The prebiotic effects of biscuits containing partially hydrolysed guar gum and fructo-oligosaccharides-a human volunteer study. Br. J. Nutr. 86, 341–348.

Ukkonen, P., Varis, K., Jernfors, M., Herva, E., Jokinen, J., Ruokokoski, E., Zopf, D., and Kilpi, T. (2000). Treatment of acute otitis media with an antiadhesive oligosaccharide: a randomised, double-blind, placebo-controlled trial. Lancet 356, 1398–1402.

Umar, S., Morris, A.P., Kourouma, F., and Sellin, J.H. (2003). Dietary pectin and calcium inhibit colonic proliferation *in vivo* by differing mechanisms. Cell Prolif. 36, 361–375.

Usami, M., Kishimoto, K., Ohata, A., Miyoshi, M., Aoyama, M., Fueda, Y., and Kotani, J. (2008). Butyrate and trichostatin A attenuate nuclear factor kappaB activation and tumor necrosis factor alpha secretion and increase prostaglandin E2 secretion in human peripheral blood mononuclear cells. Nutr. Res. 28, 321–328.

Vander, P. (1998). Comparison of the ability of partially N-Acetylated chitosans and chitooligosaccharides to elicit resistance reactions in wheat leaves. Plant Physiol. 118, 1353–1359.

Veereman-Wauters, G. (2007). Application of prebiotics in infant foods. Br. J. Nutr. 93, S57-S60.

Vetsch, M., Puorger, C., Spirig, T., Grauschopf, U., Weber-Ban, E.U., and Glockshuber, R. (2004). Pilus chaperones represent a new type of protein-folding catalyst. Nature 431, 329–333.

Vos, A.P., Haarman, M., Buco, A., Govers, M., Knol, J., Garssen, J., Stahl, B., Boehm, G., and M'Rabet, L. (2006). A specific prebiotic oligosaccharide mixture stimulates delayed-type hypersensitivity in a murine influenza vaccination model. Int. Immunopharmacol. 6, 1277–1286.

Vos, A.P., Haarman, M., van Ginkel, J.-W.H., Knol, J., Garssen, J., Stahl, B., Boehm, G., and M'Rabet, L. (2007a). Dietary supplementation of neutral and acidic oligosaccharides enhances Th1-dependent vaccination responses in mice. Pediatr. Allergy Immunol. 18, 304–312.

Vos, A.P., van Esch, B.C., Stahl, B., M'Rabet, L., Folkerts, G., Nijkamp, F.P., and Garssen, J. (2007b). Dietary supplementation with specific oligosaccharide mixtures decreases parameters of allergic asthma in mice. Int. Immunopharmacol. *7*, 1582–1587.

Wang, X., Hirmo, S., Willen, R., and WAdstrom, T. (2001). Inhibition of *Helicobacter pylori* infection by bovine milk glycoconjugates in a BALB/cA mouse model. J. Med. Microbiol. 50, 430–435.

Waterhouse, A., and Chatterton, N. (1993). Glossary of fructan terms. Sci. Technol. Fructans. 1-7.

Watzl, B., Girrbach, S., and Roller, M. (2007). Inulin, oligofructose and immunomodulation. Br. J. Nutr. 93, S49.

Weis, W.I., and Drickamer, K. (1996). Structural basis of lectin-carbohydrate recognition. Annu. Rev. Biochem. 65, 441–473.

Willats, W.G., McCartney, L., Mackie, W., and Knox, J.P. (2001). Pectin: cell biology and prospects for functional analysis. Plant Mol. Biol. 47, 9–27.

Wu, X.R. (1996). *In vitro* binding of type 1-fimbriated *Escherichia coli* to uroplakins Ia and Ib: Relation to urinary tract infections. PNAS. 93, 9630–9635.

Xia, Y., Gally, D., Forsman-Semb, K., and Uhlin, B.E. (2000). Regulatory cross-talk between adhesin operons in *Escherichia coli*: inhibition of type 1 fimbriae expression by the PapB protein. EMBO J. *19*, 1450–1457.

Yin, Y.L. Tang, Z.R., Sun, Z.H., Liu, Z.Q., Li, T.J., Huang, R.L., Ruan, Z., Deng, Z.Y., Gao, B., Chen, L.X., et al. (2008) Effect of galacto-mannan-oligosaccharides or chitosan supplementation on cytoimmunity and humoral immunity in early-weaned piglets. Asian-Australasian J. Anim. Sci. 21, 723–731.

Zhou, G., Mo, W.J., Sebbel, P., Min, G., Neubert, T.A., Glockshuber, R., Wu, X.R., Sun, T.T., and Kong, X.P. (2001). Uroplakin Ia is the urothelial receptor for uropathogenic *Escherichia coli*: evidence from *in vitro* FimH binding. J. Cell Sci. 114, 4095–4103.

Zivkovic, A.M., German, J.B., Lebrilla, C.B., and Mills, D.A. (2010). Human milk glycobiome and its impact on the infant gastrointestinal microbiota. PNAS. 108, 4653–4658.

Table 1. Fimbrial adhesins of intestinal and extra-intestinal pathogens. Description of architecture and fimbrial adhesin characteristics.

Fimbrial Adhesins	Architecture and Characteristics	Present in	References
Pyelonephritis Associated Fimbriae (P)	PapG adhesin Outer Membrane Assembled via Chaperone Usher (CU) Pathway Encoded in the *pap* operon	*Escherichia coli*	(Dodson et al., 2001; Gerlach and Hensel, 2007; Saulino et al., 2000; Strömberg et al., 1990; Vetsch et al., 2004)
Type I Fimbriae	Encoded in *fim* operon FimH is the fimbrial adhesin FimH main binding receptor: uroplakin Assembled via CU pathway	*Escherichia coli* *Salmonella* Typhimurium *Salmonella* Enteritidis *Klebsiella pneumoniae*	(Gerlach and Hensel, 2007; Jones, 1995; Krogfelt et al., 1990; Shoaf-Sweeney and Hutkins, 2008; Wu, 1996; Xia et al., 2000; Zhou et al., 2001)
CS Fimbriae	Assembled via alternate CU pathway	Enterotoxigenic *Escherichia coli* (ETEC)	(Gerlach and Hensel, 2007; Soto and Hultgren, 1999)

Type IV Fimbriae	Pilin protein adhesin Polymerization of subunits in cytoplasmic membrane Two sub-groups: type IVa and type IVb (based on amino acid sequence) Differ in diameter and helical structure	*Neisseria gonorrhoeae* *Neisseria meningitides* *Moraxella bovis* *Pseudomonas aeruginosa* *Vibrio cholerae*	(Craig et al., 2004; Smyth et al., 1996; Tennent and Mattick, 1994)
Curli	Thin coiled fibers Biofilm formation Adherence to human matrix and plasma proteins and major histocompatibility complex (MHC) class I molecules Outer membrane protein CsgA essential for optimal curli assembly Assembled via extracellular nucleation-precipitation pathway	*Salmonella* spp. *Escherichia coli* spp.	(Nasr et al., 1996; Olsen et al., 1998; Robinson et al., 2006)

Long Polar Fimbriae (Lpf)	Adhesion to Peyer's patches Assembled via CU pathway	*Salmonella* Typhimurium *Salmonella* Enteritidis	(Baumler et al., 1996; Bäumler et al., 1997)
Thin Aggregative Fimbriae (Tafi)	Assembled via the nucleation precipitation pathway Adhesins with a diameter of 3-4 nm Used for auto-aggregation Expressed in response to nutrient limitation, low osmolarity and temperature	*Salmonella* spp.	(Collinson et al., 1991, 1993)
Bundle forming pili (BFP)	Type IV pilus Encoded on Enteropathogenic *Escherichia coli* (EPEC) adherence factor (EAF) plasmid Bacteria-bacteria interaction and microcolony formation Initial EPEC binding to epithelial cells	EPEC	(Donnenberg et al., 1992; Giron et al., 1991; Tobe and Sasakawa, 2001)

F17 Fimbriae	3 nm wide Major pilin subunit: F17-A F17-G expressed on the tip Mediate binding to N-acetylglucosamine receptors on microvilli of ruminants' intestinal epithelia	Enterotoxigenic *E. coli* (ETEC)	(Buts et al., 2004; Sharon, 2006)
Diffuse Adherence Fibrillar Adhesin (Afa/Dr)	Assembled via CU pathway	Diffusely adhering *Escherichia coli* (DAEC)	(Gerlach and Hensel, 2007; Servin, 2005)

Table 2. Carbohydrate moieties identified as pathogen binding sites on animal tissues. Adapted from Ernst and Magnani, 2009; Ofek et al., 2003b; Sharon, 2006.

Organism /Fimbriae	**Carbohydrate Moieties**
Campylobacter jejuni	Fucα2GalβGlcNAc
Escherichia coli	
Type 1	Manα3Manα6Man
P Fimbriae	Galα4Gal
S Fimbriae	NeuAc (α2–3)Galβ3GalNAc
CFA/1	NeuAc (α2–8)
F1C	GalNAcβ4Galβ
F17	GlcNAc
K1	GlcNAcβ4GlcNAc
K99	NeuAc(α2–3)Galβ4Glc
Haemophilus influenzae	[NeuAc(α2–3)]0,1 Galβ4GlcNAcβ3Galβ4GlcNAc
Helicobacter pylori	NeuAc(α2–3)Galβ4GlcNAc Fucα2Galβ3(Fucα4)Gal
Klebsiella pneumoniae	Man
Neisseria gonorrhea	Galβ4Glc(Nac)
Neisseria meningitidis	[NeuAc(α2–3)]0,1 Galβ4GlcNAcβ3Galβ4GlcNAc
Pseudomonas aeruginosa	L-Fuc Galβ3Glc(Nac)β3Galβ4Glc
Salmonella Typhimurium	Man
Streptococcus pneumoniae	[NeuAc(α2–3)]0,1 Galβ4GlcNAcβ3Galβ4GlcNAc
Streptococcus suis	Galα4Galβ4Glc

Table 3 Inhibition of bacterial pathogens to the surface of epithelial cells *in vitro* by non-digestible oligosaccharides.

Non-digestible Oligosaccharide	Cell Line	Adherence Inhibition of:	References
HMO	Caco-2	*Escherichia coli* *Vibrio cholerae* *Salmonella* Fyris	(Coppa et al., 2006)
HMO		*Listeria monocytogenes*	(Coppa et al., 2003)
HMO	HEp-2	*Campylobacter jejuni*	(Ruiz-Palacios et al., 2003)
HMO		*Streptococcus pneumoniae* *Haemophilus influenzae*	(Andersson et al., 1986)
HMO	HeLa HEp-2 T84	Enteropathogenic *E. coli* (EPEC)	(Manthey et al., 2014)
GOS	HEp-2	EPEC	(Shoaf et al., 2006)
GOS	HEp-2	*Cronobacter sakazakii*	(Quintero et al., 2011)
GOS	HT-29	*Salmonella enterica* serovar Typhimurium	(Searle et al., 2009)
Yeast-derived mannoproteins	Caco-2	*Campylobacter jejuni*	(Schoeni and Wong, 1994)
POS	Caco-2	*Campylobacter jejuni*	(Ganan et al., 2010)

CHOS	HEp-2	EPEC	(Quintero-Villegas et al., 2013)
Glycoprotein glycans	Erythrocyte glycoconjugates	*E. coli* K99	(Mouricout et al., 1990)
3'sialyllactose	HuTu-80 HEp-2	*Helicobacter pylori*	(Simon et al., 1997)
Lacto-*N*-neotetraose	Type II lung cell line A549	*Streptococcus pneumoniae*	(Idänpään-Heikkilä et al., 1997)
Lacto-*N*-neotetraose NeuAcα2-6Galβ1-4GlcNAc GalNAcβ1-3LacNAc	Chang cells	*Streptococcus pneumoniae*	(Barthelson et al., 1998)
POS	HT29	Enterohemorrhagic *E. coli* (EHEC) O157:H7 Shiga toxins	(Olano-Martin et al., 2003)
GOS	GM1 (cell surface toxin receptor)	*Vibrio cholerae* toxin	(Sinclair et al., 2009)
HMO		*Clostridium difficile* toxins A and B (TcdA and TcdB)	(El-Hawiet et al., 2011)

Table 4. Studies of immune modulation by Non-digestible oligosaccharides *in vivo*

Non-digestible Oligosaccharide	**Model**	**Findings**	**References**
GOS **5000mg GOS/kg body weight**	Colitis development in *Smad3*-deficient mice treated with *Helicobacter hepaticus*	- Colitis severity reduced - Increase of NK cells in spleen - Increase in MsLN - Increased expression of CCR9 in blood, spleen and MsLN IL-15 production stimulated	(Gopalakrishnan et al., 2012)
FOS **5% w/w in diet**	2-week- old BALB/c mice	- Increase in intestinal IgA (ileum, jejunum, and colon) - % of B220+IgA+ cells in Peyer's patches (PP) increased	(Nakamura et al., 2004)
FOS **10% w/w in diet**	6-week-old female BALB/c mice (healthy and endotoxemic) Endotoxemia induced by lipopolysaccharide (LPS) intraperitoneally	- FOS increased total immune cell yield in both groups - B cells were increased in both groups - T lymphocytes unaltered in healthy mice - T lymphocytes increased in endotoxemic mice	(Manhart et al., 2003)

FOS and/or MOS **2grs/day**	Adult female Dogs	- Ileal IgA increased in dogs fed FOS + MOS - Lymphocytes greater in dogs fed MOS - Serum IgA greater in dogs fed MOS	(Swanson et al., 2002)
POS **1-5% (w/w) in diet** POS + (GOS:FOS) (9:1 mixture) **2% (w/w) in diet**	6-week-old female C57Bl/6 mice Influenza Vaccination Model	- POS enhanced vaccine-specific delayed-type hypersensitivity (DTH) (dose dependent) - Reduction in T-helper 2 (Th2) cytokine production in splenocytes - POS + (GOS:FOS) was more effective in enhancing DTH (Vos et al., 2007a)	
β-1,4 Mannobiose (MNB) **0, 5, 10, or 25 mg/kg**	6-8-week-old female BALB/c mice (healthy and endotoxemic mice)	In healthy mice: - MNB induced expression of Th1 and Th2-type cytokines in ileum - Increased fecal IgA production - Increased splenic NK cell activity In endotoxemic mice: - Reduced expression of TNF-α, IL-6, iNOS, and IL-10 - Increased IL-12p40, IFN-γ, and IFN-α expression and NK cell activity	(Kovacs-Nolan et al., 2013)
Galacto-mannan-oligosaccharides (GMOS) **0.2%**	15-day-old piglets	GMOS and CHOS - Enhanced IL-1β gene expression in jejunal mucosa and lymph nodes - Enhanced serum levels of IL-1β, IL-	(Yin et al., 2008)

CHOS **250mg/kg**		2, IL-6, IgA, IgG, and IgM	
MOS or FOS **5 g/kg**	Day-old Cobb male broilers	FOS and MOS supplementation - Significant reduction in proportion of B cells and mitogen responsiveness of lymphocytes in cecal tonsils (CT) FOS - Enhanced IgM and IgG in plasma	(Janardhana et al., 2009)
Goat milk oligosaccharides (GMO) **5% (w/w) in diet (20 g/kg of diet)**	Male Sprague-Dawley rats DSS induced inflammation	GMO supplementation - Protected from weight loss - Ameliorated acute colonic inflammatory process - Less severe colonic lesions	(Lara-Villoslada et al., 2006)

GOS:FOS (9:1) **Between 1% and 10% (w/w) in diet**	6-8 week old C57Bl/6 mice Influenza Vaccination Model	DTH response used as a marker of Th1 immunity - GOS:FOS enhanced DTH response in a dose dependent manner - No significant differences on splenocyte proliferation - No significant difference on vaccine-specific serum antibody concentrations	(Vos et al., 2006)
GOS:FOS **1% (w/w) in diet** GOS:FOS (83%) + POS (17%) **1% (w/w) in diet**	5-8 week old BALB/c mice	Mice sensitized with ovalbumin (OVA) GOS:FOS - suppressed OVA-induced airway inflammation and hyper-responsiveness - OVA specific IgE decreased - enhanced Th1 and suppressed Th2 parameters GOS:FOS + POS - suppressed OVA-induced airway inflammation and hyper-responsiveness - OVA specific IgE decreased - enhanced Th1 and suppressed Th2 parameters	(Vos et al., 2007b)

CHOS **100 mg/kg intraperitoneally (i.p)**	8-10 week old BALB/c mice LPS-induced sepsis	CHOS treated mice - attenuated organ dysfunction -improved survival rate - TNF-α and IL-1β reduced in serum - neutrophil infiltration in organs attenuated - protected from redox imbalance (attenuated reduction of glutathione and catalase and increase of malondialdehyde) -c-Jun NH_2-terminal kinase activation attenuated - p38 mitogen-activated protein kinase signal activation attenuated	(Qiao et al., 2011)

Chapter 2

Adherence Inhibition of Enteropathogenic *Escherichia coli* by Chitooligosaccharides with Specific Degrees of Acetylation and Polymerization

Adherence inhibition of enteropathogenic *Escherichia coli* by chitooligosaccharides with specific degrees of acetylation and polymerization

Maria I. Quintero-Villegas[1], Berit B. Aam[2], John Rupnow[1], Morten Sørlie[2], Vincent G.H. Eijsink[2], Robert W. Hutkins[1*]

[1]Department of Food Science and Technology, University of Nebraska, Lincoln, NE 68583-0919, USA.

[2] Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, P.O. Box 5003, N-1432 Aas, Norway

*Correspondence to Robert Hutkins, Department of Food Science and Technology, University of Nebraska, 338 Food Industry Complex, Lincoln, NE 68583-0919, USA.

Phone: (402) 472-2820; Fax (402) 472-1693; E-mail: rhutkins1@unl.edu

Abstract

Some oligosaccharides are known to act as molecular decoys by inhibiting pathogen adherence to epithelial cells. The present study was aimed at analyzing whether chitooligosaccharides (CHOS), i.e. oligomers of D-glucosamine and *N*-acetyl-D-glucosamine, have such anti-adherence activity. CHOS of varied degree of polymerization (DP) and fraction of acetylation (F_A) were produced. Adherence of enteropathogenic *Escherichia coli* (EPEC) to the surface of a human HEp-2 cell line was determined in the absence or presence of the various CHOS fractions. Adherence was assessed by microscopic counting and image analysis of bacterial clusters and cells. The results showed that all CHOS fractions inhibited adherence of EPEC to HEp-2 cells. Hydrolysates with lower F_A were more effective at reducing adherence. This effect is greater than that obtained with other oligosaccharides, such as galactooligosaccharides, applied at the same concentrations.

Keywords

Oligosaccharides, adherence, chitooligosaccharides, chitosan, chitinase, chitosanase

Introduction

Oligosaccharides have long been known to have a variety of biological activities, although the full diversity of their functions is not yet fully understood[1,2]. In particular, they are known to serve as ligands and participate in binding interactions with specific lectins [2]. Recently, it has been suggested that some food grade oligosaccharides can protect host tissue from pathogen adherence [3]. Specifically, galactooligosaccharides (GOS), mannan oligosaccharides, and pectic oligosaccharides have been shown to be effective in inhibiting pathogen binding to the surface of tissue culture cells[4–7].

For most microbial enteric pathogens, the first step in the infection process is adherence to the epithelial cells that line the intestinal tract. Adherence is generally mediated in these bacteria via expression of lectin-like adhesins that recognize carbohydrate-containing receptor sites on the surfaces of host epithelial cells [8,9]. Accordingly, adherence inhibition may occur in the presence of substances that interfere with the lectin-receptor interaction, for example, by anti-adherence oligosaccharides that resemble the glyco-moieties of the host receptor sites. Thus, strategies based on preventing or inhibiting pathogen adherence could be effective at reducing infections and the subsequent onset of disease [7,10,11].

One group of oligosaccharides that has attracted considerable research and commercial interest due to their biological properties are the chitooligosaccharides (CHOS). CHOS are produced enzymatically or chemically

from chitosan; linear heteropolymers of *β* (1→4) linked *N*-acetyl-D-glucosamine (GlcNAc) and its deacetylated counterpart D-glucosamine (GlcN). Chitosans may have varying compositions, usually indicated by the fraction of acetylated sugar residues (F_A). Soluble chitosans are produced from insoluble chitin by partial or complete *N*-deacetylation, either by homogenous [12] or by heterogeneous deacetylation [13]. Chitin is an abundant natural product found in nature as a structural component of the cell wall of fungi and yeasts and in the exoskeletons of insects and arthropods (e.g., crabs, lobsters and shrimps). Chitosan has a wide range of applications[14–16], including its use as an antimicrobial agent[17–22].

Chitosan can be hydrolyzed by chitinases or chitosanases to give CHOS [23–26]. These hydrolytic enzymes vary with respect to their specific cleavage sites, which are determined by sequences in heteropolymers of GlcNAc and GlcN. Thus, different combinations of chitosans (varying in F_A) and hydrolytic enzymes (varying in sequence specificity) will yield CHOS differing in both length and sequence features [27]. The resulting CHOS are defined by their F_A, their average degree of polymerization (DP_n) and their sequence, i.e. the pattern of *N*-acetylated sugar residues (P_A). The DP_n is related to α, a parameter that indicates the degree of scission, where $\alpha = 1/DP_n$. Complete conversion of chitosan to dimers ($DP_n = 2$) would yield an α value of 0.50. Methods exist to separate CHOS by DP (e.g, size exclusion chromatography [26]) and by charge

(e.g., cation exchange chromatography [28]). The latter is based on the fraction of deacetylated residues.

CHOS possess a wide range of bioactivities and are used for their anti-angiogenesis effects, as well as for wound healing and as vectors in gene therapy [27,29–32]. Chitosan and CHOS are biodegradable and are considered non-toxic [33]; therefore, these compounds have a variety of potential applications in food [34]. There is evidence that CHOS (DP < 30, F_A 0.01 - 0.12) may be prebiotic, enhancing growth of *Bifidobacterium a*nd *Lactobacillus* strains in cell cultures [35,36]. This effect is apparently dependent on F_A, since Fernandes *et* al. [37] reported that CHOS with similar DP but higher F_A (F_A 0.35) did not stimulate growth of selected strains of bifidobacteria and lactobacilli.

Although pathogen adherence by CHOS has received relatively little attention, one previous study showed that a non-defined CHOS mixture of F_A0.03 and DP_n ~ 4 inhibited adherence of three different strains of enteropathogenic *Escherichia coli* (EPEC) on HT-29 cells [38]. In additional, Liu et al. showed that weaned pigs that were fed CHOS exhibited reduced incidence of diarrhea after being challenged with *E. coli* K88 [39]. It is now possible, however, to produce more defined CHOS fractions and to assess these fractions for anti-adherence activity. Thus, the main objective of the present study was to test CHOS with different F_A and DP for their ability to inhibit adherence of EPEC, a widely recognized enteric pathogen, on tissue culture cells.

Materials and Methods

Preparation of CHOS. Three chitosans with different F_A were enzymatically hydrolyzed. A chitosan with F_A0.15 (KitoNor from Norwegian Chitosan, Gardermoen, Norway) and a chitosan with F_A0.3 (Heppe Medical Chitosan GmbH, Halle, Germany) were hydrolyzed with purified recombinant chitosanase ScCsn46A from *Streptomyces coelicolor A3(2)* [23]. A F_A 0.65 chitosan was prepared by homogenous deacetylation of chitin from shrimp shells[12] (Chitinor, Senjahopen, Norway). This F_A0.65 chitosan was hydrolysed with purified recombinant ChiB from *Serratia marcescens* [26,40]. The F_A of the chitosans before enzymatic hydrolysis, and the degree of scission (α) after degradation were determined by ^{1}H-NMR using a Varian Gemini instrument at 300 MHz [26,41].

The F_A 0.65 chitosan was soluble in water, whereas the F_A 0.15 and F_A 0.3 chitosans required 0.5 % acid to dissolve. All three chitosans were dissolved/suspended in buffer (40 mM NaAc, 100 mM NaCl, pH 5.5) to a concentration of 10 mg/mL. Then, 0.5 % (v/v) 12 M HCl was added to the F_A 0.15 and F_A 0.3 chitosan samples, and after the chitosan was dissolved, the pH was adjusted to 5.5 with 6 M NaOH. Enzymes were added to pre-warmed chitosan solutions to a final concentration of 0.5 µg/mg chitosan and the reactions, with a final chitosan concentration of approximately 9.8 mg/mL, were incubated at 37°C with shaking (225 rpm). Reactions were stopped by decreasing the pH to 2.5 with HCl. The CHOS samples were filtered through

Filtropur S 0.2 µm sterile filters (Sarstedt, Germany), lyophilized and resuspended in the size exclusion chromatography (SEC) mobile phase to a concentration of 20 mg/mL prior to separation on SEC.

Separation of CHOS. The CHOS were separated by size exclusion chromatography (SEC) on three XK 26 columns packed with Superdex^{TM} 30 prep grade (GE Healthcare) coupled in series with an overall dimension of 2.6 cm × 180 cm. The mobile phase (150 mM NH_4Ac, pH 4.6) was run at a constant flow of 0.8 mL/min [26]. The column eluent was monitored using an RI detector (Gilson model 133). In each run 100 mg of chitosan hydrolysate was applied (i.e. 5 mL) and 3.2 mL fractions were collected. Identification of oligomers in the fractions was performed with MALDI-TOF-MS. The fractions were dialyzed with Float-A-Lyzers (MWCO 100-500 Da, SpectrumLabs) to remove salts, sterile filtrated and lyophilized. Prior to use, the CHOS were dissolved in sterile distilled water.

To limit the number of assays, initial experiments were done with chitosan hydrolysates containing mixtures of CHOS. In this case, dried material was resuspended in sterile water to a final volume of 1 mL (final concentration varied according to the amount available of each sample). For other experiments, samples were diluted to a final concentration of 16 mg/mL.

Strains and Organisms. EPEC strain E2348/69 (O127:H6) was obtained from M. Donnenberg (University of Maryland School of Medicine, Baltimore) and was used as a model organism for the anti-adherence experiments. Before each experiment, cells from frozen stocks were plated on tryptic soy agar (TSA; Difco,

Sparks, USA) and grown overnight at 37°C, as described previously (Shoaf et al., 2006). A single colony was then inoculated into 10 mL of tryptic soy broth (TSB; Difco) and incubated overnight at 37°C without shaking. Overnight cultures were used to inoculate (1% v/v) minimal essential medium (MEM; Hyclone, Logan, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Hyclone, Logan, USA). MEM was pre-equilibrated overnight at tissue culture conditions (5% CO_2, 95% relative humidity, 37°C). The cells were then incubated for 80 min at 37°C, aerobically, prior to the start of the experiment.

Tissue Culture Cells. HEp-2 (CCL-23) cells were obtained from the American Type Culture Collection (Manassas, Virginia). This cell line was used to assess bacterial adherence to epithelial cells; they are a widely used cell line for studies involving bacterial intestinal adherence [42–44]. HEp-2 cells were grown as described previously (Shoaf et al., 2006). Briefly, cells were grown in 75 cm^2 tissue culture flasks containing 25 mL of MEM (pH 7.4) supplemented with 10% FBS in a CO_2 incubator at tissue culture conditions. Confluent HEp-2 cells were harvested by removing MEM and washing the cells once with PBS. Subsequently, 0.5 mL of a 0.25% Trypsin-EDTA solution was added followed by a 10 minute incubation at tissue culture conditions. After incubation, 0.5 mL of FBS was added to inactivate the trypsin. Cells were then seeded onto 12 mm diameter glass coverslips in 24-well tissue culture plates at approximately 3.6×10^5 cells per well, and 500 µl of MEM supplemented with 10% FBS was added to each well. Plates were incubated under tissue culture conditions for about 20

hours prior to the start of each experiment. Cells were checked before the experiment under an inverted microscope to make sure they had reached about 70% confluency.

Anti-adherence Assays. CHOS were dissolved in sterile water and mixed with bacterial cultures (approx. 10^8 cells/mL in MEM supplemented with 10% FBS) to final concentrations of 16 mg/mL, prior to addition to the tissue culture cells. A total of 14 fractions were analyzed - three CHOS mixtures resulting from enzymatic hydrolysis of three different chitosans (F_A 0.15, F_A 0.3, and F_A 0.65) and 11 fractions derived from hydrolyzed F_A 0.15 chitosan by size exclusion. In addition, a mixture of *N*-acetyl-D-glucosamine (Sigma) and D-glucosamine (Sigma) (15:85) was used as a control. It was not possible to test the non-hydrolyzed chitosans, due to the viscous nature of the chitosan solutions. Sterile water was also used as a control. The standard CHOS concentration of 16 mg/mL was used because previous experiments with GOS had shown this concentration to be effective in inhibiting adherence of EPEC [7]. However, for some of the fractions the amount of material was limited, and the concentration used was significantly lower. In addition, dose responses of the fractions F_A 0.15 and F_A 0.3 were performed to determine the effect of concentration on adherence inhibition.

After addition of the bacteria/CHOS mixtures to tissue culture cells, the plates were incubated for 30 minutes at tissue culture conditions (as described above). The wells were then washed five times with PBS to remove non-

adhered bacteria. Cells were then fixed with 100% methanol and stained with 10% Geimsa. Glass coverslips with stained cells were mounted on microscope slides and analyzed by phase contrast microscopy (100x) with an attached camera. A predetermined horizontal and vertical pattern was established to obtain fifteen images of each cover slip. Bacterial clusters (defined as bundles of 4 or more bacteria) and HEp-2 cells were counted using ImageJ software to obtain a ratio of bacterial clusters/100 HEp-2 cells. Single concentration experiments were replicated once (n=2) and dose responses were replicated five times (n=5). The % inhibition was calculated as the number of adhered clusters in the control minus the number of adhered clusters in the treatment, all divided by the number of adhered clusters in the control. Thus, 0% inhibition would refer to the control containing only water. Because EPEC cluster formation occurs via bundles of 4 or more bacteria, microscopy is the preferred method for quantifying adherence of this organism. It is also used to assess the manner of cell attachment of other bacteria [7,45].

Results

Production, separation and characterization of CHOS. In the initial experiments, chitosans with F_A 0.15 and F_A 0.3 were enzymatically hydrolyzed with chitosanase SnCsn46A from *Streptomyces coelicolor A3(2)* to α = 0.22 and 0.25, respectively (corresponding to DP_n values of 4.5 and 4.0, respectively). The F_A 0.65 chitosan was enzymatically hydrolysed by ChiB from *Serratia*

marcescens to α = 0.19 (DP_n 5.3). The α values were determined by ^{1}H-NMR as described previously [31] and SEC chromatograms of the samples (not shown) confirmed that, as expected, the majority of the CHOS was in the DP 2 - 20 range.

Since the F_A 0.15 sample was the most inhibitory in the initial experiments (see below), a new hydrolysis reaction was set up (α = 0.16, DP_n 6.3) and the CHOS were separated into single fractions (DP 3 – DP12) and one fraction with DP > 12 and a DP_n of 25 (Figure 1A). The MALDI-TOF-MS spectra of the individual DP3 - DP12 fractions demonstrate that the various fractions were generally homogeneous with respect to DP (Figure 1B and 1C). The mass spectra also give an impression of the F_A distributions within the samples. For example, the DP4 fraction contains primarily D_4 and D_3A_1, the DP6 fraction contains D_6, D_5A_1 and D_4A_2, and the DP12 fraction contains D_{12}, $D_{11}A_1$, $D_{10}A_2$, D_9A_3 and D_8A_4.

Inhibition of EPEC adherence by CHOS mixtures with F_A 0.15, F_A 0.3 and F_A 0.65. The non-separated hydrolysates of chitosans with F_A 0.15, F_A 0.3 and F_A 0.65, were tested for their ability to inhibit EPEC adherence at a concentration of 16 mg/mL, a concentration used in previous studies for other prebiotic oligosaccharides [7,45]. In addition, the adherence inhibition activity of fractions F_A 0.15 and F_A 0.3 was also assessed over a range of concentrations (0, 0.5, 1, 5, 10, and 16 mg/mL). Microscopic analysis revealed that EPEC adherence was reduced by the F_A 0.15 fraction (Figure 2), and by image

analysis, all three hydrolysates significantly inhibited adherence (Figure 3C). For both fractions F_A 0.15 and F_A 0.3, a dose- dependent trend was observed (Figure 3A and 3B). Comparison of CHOS based on F_A revealed that the F_A 0.15 fraction gave the highest inhibition (92%) as compared to the F_A 0.65 sample (75%) and the F_A 0.3 sample (84%). It was not possible, however, to assess the activity of non-hydrolyzed chitosans because the high viscosity of the chitosan solutions interfered with adherence experiments. Bacterial motility is reduced in highly viscous solutions, resulting in obstruction of bacteria from coming in contact with the tissue culture cells.

Inhibition of EPEC adherence by purified CHOS fractions. Single fractions of CHOS purified from hydrolyzed F_A 0.15 chitosan as described above (Figure 1) were then tested in the same EPEC adherence assay. The fractions tested were single fractions of DP3 to DP12, and a mixture with DP>12 and DP_n = 25. All CHOS fractions significantly inhibited adherence compared to the control, reaching inhibition levels of close to 100% (Figure 4). Notably, adherence was not inhibited by addition of a 15:85 mixture of the monomers, GlcNAc and GlcN (DP1 in Figure 4), indicating that the oligomeric nature of the sugars is essential for the inhibitory effect.

Growth of EPEC in the presence of CHOS, GlcNAc and GlcN. EPEC was grown in TSB medium containing monomers of GlcNAc, GlcN, and a 15:85 mixture of these sugars, all at a concentration of 16 mg/mL (i.e. the same concentration used in the CHOS anti-adherence assays). Growth of EPEC was

not impaired by the presence of any of these monomers or the mixture (Figure 5). A similar experiment with one of the CHOS fractions also showed that growth of EPEC was unaffected by CHOS in the media. In addition, the bacterial inoculum was enumerated before and after incubation to assure there was no reduction due to a potential bactericidal effect of the CHOS (data not shown).

Discussion

The use of molecular decoys as anti-adherence agents was proposed more than a decade ago [8,46–48]. In recent years, several food grade prebiotic oligosaccharides and plant extracts have been tested for their ability to inhibit pathogen adherence to the surface of intestinal epithelial cells. In a previous study, we showed that GOS inhibited EPEC adherence by up to 65% under conditions similar to those used in the present study [7]. Interestingly, the results indicate that CHOS, especially those with low F_A, are more effective inhibitors of EPEC adherence than GOS, given that inhibition reached almost 100% for some of the fractions tested.

Our results also showed that chitooligosaccharides with different $F_{A,}$ but similar DP_n (4.0 – 5.3) had comparable adherence inhibition activities, with low F_A being the most effective (Figure 3C). Thus, the glucosamine content, which affects charge density due to the titratable amino group of this sugar, appears to affect the activity of CHOS against EPEC adherence. Furthermore, a dose-dependent anti-adherence effect was observed, as greater adherence inhibition occurred at the higher concentrations, until a plateau was reached. In addition,

the results showed that hydrolysates with different F_A require different concentrations to reach the same level of inhibition (Figure 3A and 3B). Adherence inhibition, however, did not appear to be related to DP, as CHOS fractions purified from a hydrolyzed F_A 0.15 chitosan, but with DP's ranging from 3 to greater than 12, all inhibited adherence by up to 99%. Nevertheless, it should be noted that CHOS with different DPs will have different molecular weights, hence contributing a different number of target molecules despite being used at the same concentration.

The anti-adherence property of oligosaccharides has been attributed to the similarity between the oligosaccharide structure and cell surface receptor to which bacteria attach prior to colonization. Via a phenomenon known as phase variation [49], bacteria can modulate adhesin expression, depending, in part, on the available receptors expressed by the host cells. This may account for why some oligosaccharides are effective in inhibiting adherence of particular pathogens whereas other pathogens are not affected. Moreover, the molecular interaction between oligosaccharides and bacterial adhesins varies among different pathogens, and in some cases among different strains [7,45].

Recently, it was suggested that pathogen adherence tropism is dependent on three key elements: expression of adhesins; adhesion specificity; and the presence of cognate receptors on the surface of specific tissue culture cells [50]. Although the precise mechanism for how CHOS prevent adherence of EPEC to epithelial cells will require further investigation, we suggest that CHOS interferes

with adhesion attachment to the cognate ligands. In particular, one of the monomers of CHOS is GlcNAc, which is a common constituent of receptor ligands for many bacterial lectins [51–53]. However, the occurrence of non-acetylated glucosamines as a target ligand on the surface of epithelial cells has not been reported. In addition, the present data clearly shows that inhibition of adherence requires an oligomeric carbohydrate (Figure 4), as free monomeric sugars had no effect on adherence.

The ability of chitosan polymers to inhibit growth of *E. coli* has been reported previously[19,54,55], although this effect was observed only for chitosans of higher DP than that of the CHOS used in the present study. Other studies have shown that shorter CHOS, at DP < 20 do not kill *E. coli* [20,55]. Indeed, growth of EPEC was not impaired by the CHOS used in this study, indicating that reduced adherence of EPEC was not due to growth inhibition or cell killing. Thus, it seems that the anti-adherence effect of CHOS is independent of the other biological effects of CHOS and chitosan.

In summary, our results show that different fractions of CHOS inhibit adherence of EPEC to the surface of tissue culture cells. Further research is needed to identify the specific CHOS species responsible for the observed inhibition and to assess these effects *in vivo*, i.e., on pathogen adherence in the animal gastrointestinal tract. Finally, other potential biological activities of CHOS, including their possible impact on the intestinal commensal microbiota, should also be considered. Certainly, non-pathogenic strains of *E. coli* may also

express adhesins and bind to CHOS or other molecular decoys. However, provided the concentration of the anti-adherence agents is sufficient, inhibition of targeted pathogens would still be expected to occur.

Abbreviations

Chitooligosaccharides (CHOS), degree of polymerization (DP), enteropathogenic *Escherichia coli* (EPEC), fraction of acetylation (FA), tryptic soy broth (TSB), tryptic soy agar (TSA), minimal essential medium (MEM)

Acknowledgements

This work was financed in part by Norwegian Research Council grant number 197388 to BBA.

References

1. Thomas, R. J. Receptor mimicry as novel therapeutic treatment for biothreat agents. *Bioeng. Bugs* **2010**, *1*, 17–30.

2. Varki, A. Biological roles of oligosaccharides: all of the theories are correct. *Glycobiology* **1993**, *3*, 97–130.

3. Shoaf-Sweeney, K. D.; Hutkins, R. W. Adherence, anti-adherence, and oligosaccharides: preventing pathogens from sticking to the host. *Adv. Food Nutr. Res.* **2008**, 101–161.

4. Ganan, M.; Collins, M.; Rastall, R.; Hotchkiss, A. T.; Chau, H. K.; Carrascosa, A. V; Martinez-Rodriguez, A. J. Inhibition by pectic oligosaccharides of the invasion of undifferentiated and differentiated Caco-2 cells by *Campylobacter jejuni*. *Int. J. Food. Microbiol.* **2010**, *137*, 181–185.

5. Ghosh, S.; Mehla, R. K. Influence of dietary supplementation of prebiotics (mannanoligosaccharide) on the performance of crossbred calves. *Trop. Anim. Health Pro.* **2012**, *44*, 617–622.

6. Kunz, C.; Rudloff, S.; Baier, W.; Klein, N.; Strobel, S. Oligosaccharides in human milk: structural, functional, and metabolic aspects. *Annu.Rev. Nutr.* **2000**, *20*, 699–722.

7. Shoaf, K.; Mulvey, G. L.; Armstrong, G. D.; Hutkins, R. W. Prebiotic galactooligosaccharides reduce adherence of enteropathogenic *Escherichia coli* to tissue culture cells. *Infect. Immun.* **2006**, *74*, 6920–6928.

8. Ofek, I.; Beachey, E. H. Mannose Binding and Epithelial Cell Adherence of *Escherichia coli*. *Infect. Immun.* **1978**, *22*, 247–254.

9. Ofek, I.; Hasty, D. L.; Doyle, R. J. *Bacterial adhesion to animal cells and tissues*; ASM Press, Washington DC, USA, **2003**.

10. Bavington, C.; Page, C. Stopping bacterial adhesion: a novel approach to treating infections. *Respiration* **2005**, *72*, 335–344.

11. Klemm, P.; Vejborg, R. M.; Hancock, V. Prevention of bacterial adhesion. *Appl. Microbiol. Biot.* **2010**, *88*, 451–459.

12. Sannan, T.; Kurita, K.; Iwakura, Y. Studies on chitin, 1. Solubility change by alkaline treatment and film casting. *Makromol.Chem.* **1975**, *176*, 1191–1195.

13. Rigby, G. W. Substantially undegraded deacetylated chitin and process for producing the same, U.S. Paent 2,040,879. **1934**.

14. Alishahi, A.; Aïder, M. Applications of Chitosan in the Seafood Industry and Aquaculture: A Review. *Food Bioprocess Technol.* **2011**, *5*, 817–830.

15. Jayakumar, R.; Menon, D.; Manzoor, K.; Nair, S. V.; Tamura, H. Biomedical applications of chitin and chitosan based nanomaterials—a short review. *Carbohydr. Polym.* **2010**, *82*, 227–232.

16. Muzzarelli, R. Chitosan-based dietary foods. *Carbohydr. Polym.* **1996**, *29*, 309–316.

17. Devlieghere, F.; Vermeulen, a; Debevere, J. Chitosan: antimicrobial activity, interactions with food components and applicability as a coating on fruit and vegetables. *Food Microbiol.* **2004**, *21*, 703–714.

18. Helander, I. M.; Nurmiaho-Lassila, E. L.; Ahvenainen, R.; Rhoades, J.; Roller, S. Chitosan disrupts the barrier properties of the outer membrane of gram-negative bacteria. *Int. J. Food. Microbiol.* **2001**, *71*, 235–244.

19. Liu, H.; Du, Y.; Wang, X.; Sun, L. Chitosan kills bacteria through cell membrane damage. *Int. J. Food Microbiol.* **2004**, *95*, 147–155.

20. Mellegård, H.; Strand, S. P.; Christensen, B. E.; Granum, P. E.; Hardy, S. P. Antibacterial activity of chemically defined chitosans: influence of molecular weight, degree of acetylation and test organism. *Int. J. Food. Microbiol.* **2011**, *148*, 48–54.

21. Rabea, E. I.; Badawy, M. E.-T.; Stevens, C. V; Smagghe, G.; Steurbaut, W. Chitosan as antimicrobial agent: applications and mode of action. *Biomacromolecules* **2003**, *4*, 1457–1465.

22. Tsai, G.-J.; Su, W.-H.; Chen, H.-C.; Pan, C.-L. Antimicrobial activity of shrimp chitin and chitosan from different treatments and applications of fish preservation. *Fish. Sci.* **2002**, *68*, 170–177.

23. Heggset, E. B.; Dybvik, A. I.; Hoell, I. A.; Norberg, A. L.; Sørlie, M.; Eijsink, V. G. H.; Vårum, K. M. Degradation of chitosans with a family 46 chitosanase from *Streptomyces coelicolor* A3(2). *Biomacromolecules* **2010**, *11*, 2487–2497.

24. Heggset, E. B.; Tuveng, T. R.; Hoell, I. A.; Liu, Z.; Eijsink, V. G. H.; Vårum, K. M. Mode of action of a family 75 chitosanase from *Streptomyces avermitilis*. *Biomacromolecules* **2012**, *13*, 1733–1741.

25. Heggset, E. B.; Hoell, I. A.; Kristoffersen, M.; Eijsink, V. G. H.; Vårum, K. M. Degradation of chitosans with chitinase G from *Streptomyces coelicolor* A3(2): production of chito-oligosaccharides and insight into subsite specificities. *Biomacromolecules* **2009**, *10*, 892–899.

26. Sørbotten, A.; Horn, S. J.; Eijsink, V. G. H.; Vårum, K. M. Degradation of chitosans with chitinase B from *Serratia marcescens*. Production of chito-oligosaccharides and insight into enzyme processivity. *FEBS J.* **2005**, *272*, 538–549.

27. Aam, B. B.; Heggset, E. B.; Norberg, A. L.; Sørlie, M.; Vårum, K. M.; Eijsink, V. G. H. Production of chitooligosaccharides and their potential applications in medicine. *Mar. Drugs* **2010**, *8*, 1482–1517.

28. Haebel, S.; Bahrke, S.; Peter, M. G. Quantitative sequencing of complex mixtures of heterochitooligosaccharides by vMALDI-linear ion trap mass spectrometry. *Anal. Chem.* **2007**, *79*, 5557–5566.

29. Benhabiles, M. S.; Salah, R.; Lounici, H.; Drouiche, N.; Goosen, M. F. A.; Mameri, N. Antibacterial activity of chitin, chitosan and its oligomers prepared from shrimp shell waste. *Food Hydrocolloid.* **2012**, *29*, 48–56.

30. Muzzarelli, R. A. A. . M. C. Chitosan chemistry: relevance to the biomedical sciences. *Adv. Polym. Sci.* **2005,** 151-209.

31. Wu, H.; Aam, B. B.; Wang, W.; Norberg, A. L.; Sørlie, M.; Eijsink, V. G. H.; Du, Y. Inhibition of angiogenesis by chitooligosaccharides with specific degrees of acetylation and polymerization. *Carbohydr. Polym.* **2012**, *89*, 511–518.

32. Xia, W.; Liu, P.; Zhang, J.; Chen, J. Biological activities of chitosan and chitooligosaccharides. *Food Hydrocolloid.* **2011**, *25*, 170–179.

33. Kean, T.; Thanou, M. Biodegradation, biodistribution and toxicity of chitosan. *Adv. Drug Deliv. Rev.* **2010**, *62*, 3–11.

34. No, H. K.; Meyers, S. P.; Prinyawiwatkul, W.; Xu, Z. Applications of chitosan for improvement of quality and shelf life of foods: a review. *J. Food Sci.* **2007**, *72*, 87–100.

35. Lee, H.-W.; Park, Y.-S.; Jung, J.-S.; Shin, W.-S. Chitosan oligosaccharides, dp 2-8, have prebiotic effect on the *Bifidobacterium bifidium* and *Lactobacillus sp*. *Anaerobe* **2002**, *8*, 319–324.

36. Simůnek, J.; Koppová, I.; Filip, L.; Tishchenko, G.; Bełzecki, G. The antimicrobial action of low-molar-mass chitosan, chitosan derivatives and chitooligosaccharides on bifidobacteria. *Folia Microiol.* **2010**, *55*, 379–382.

37. Fernandes, J. C.; Eaton, P.; Franco, I.; Ramos, O. S.; Sousa, S.; Nascimento, H.; Gomes, A.; Santos-Silva, A.; Malcata, F. X.; Pintado, M. E. Evaluation of chitoligosaccharides effect upon probiotic bacteria. *Int.J. Biol. Macromol.* **2012**, *50*, 148–152.

38. Rhoades, J.; Gibson, G.; Formentin, K.; Beer, M.; Rastall, R. Inhibition of the adhesion of enteropathogenic *Escherichia coli* strains to HT-29 cells in culture by chito-oligosaccharides. *Carbohydr. Polym.* **2006**, *64*, 57–59.

39. Liu, P.; Piao, X. S.; Thacker, P. a; Zeng, Z. K.; Li, P. F.; Wang, D.; Kim, S. W. Chito-oligosaccharide reduces diarrhea incidence and attenuates the immune response of weaned pigs challenged with *Escherichia coli* K88. *J.Anim. Sci.* **2010**, *88*, 3871–3879.

40. Bruberg May B., Eijsink Vincent G., Haandrikman Alfred J., Venema Gerard, N. I. F. Chitinase B from *Serratia marcescens* BJL200 is exported to the periplasm without processing. *Microbiology* **1995**, *141*, 123–131.

41. Vårum, K. M.; Anthonsen, M. W.; Grasdalen, H.; Smidsrød, O. Determination of the degree of N-acetylation and the distribution of N-acetyl groups in partially N-deacetylated chitins (chitosans) by high-field n.m.r. spectroscopy. *Carbohydr. Res.***1991**, *211*, 17–23.

42. Kudva, I. T.; Dean-Nystrom, E. Bovine recto-anal junction squamous epithelial (RSE) cell adhesion assay for studying *Escherichia coli* O157 adherence. *J.Appl. Microbiol.* **2011**, *111*, 1283–1294.

43. Rendón, M. a; Saldaña, Z.; Erdem, A. L.; Monteiro-Neto, V.; Vázquez, A.; Kaper, J. B.; Puente, J. L.; Girón, J. Commensal and pathogenic *Escherichia coli* use a common pilus adherence factor for epithelial cell colonization. *Proc.Natl. Acad. Sci. USA* **2007**, *104*, 10637–10642.

44. Baldi, D. L.; Higginson, E. E.; Hocking, D. M.; Praszkier, J.; Cavaliere, R.; James, C. E.; Bennett-Wood, V.; Azzopardi, K. I.; Turnbull, L.; Lithgow, T.; Robins-Browne, R. M.; Whitchurch, C. B.; Tauschek, M. The type II secretion system and its ubiquitous lipoprotein substrate, SslE, are required for biofilm formation and virulence of enteropathogenic *Escherichia col*i. *Infect. Immun.* **2012**, *80*, 2042–2052.

45. Quintero, M.; Maldonado, M.; Perez-Munoz, M.; Jimenez, R.; Fangman, T.; Rupnow, J.; Wittke, A.; Russell, M.; Hutkins, R. Adherence inhibition of C*ronobacter sakazakii* to intestinal epithelial cells by prebiotic oligosaccharides. *Curr. Microbiol.* **2011**, *5,* 1448-1454.

46. Andersson, B.; Porras, O.; Hanson, L. A.; Lagergard, T.; Svanborg-Eden, C. Inhibition of attachment of *Streptococcus pneumoniae* and *Haemophilus*

influenzae by human milk and receptor oligosaccharides. *J.Infect. Dis.***1986**, *153*, 232–237.

47. Cravioto, A.; Tello, A.; Villafan, H.; Ruiz,, J.; Del Vedovo, S.; Neeser, J.-R. Inhibition of localized adhesion of enteropathogenic *Escherichia coli* to HEp-2 cells by immunoglobulin and oligosaccharide fractions of human colostrum and breast milk. *J. Infect. Dis.* **1991**, *163*, 1247–1255.

48. Ebrahim, G. J. Editorial. Breastmilk oligosaccharides point the way to new therapeutic strategies. *J. Trop. Pediatrics* **1997**, *43*, 2–3.

49. Thanassi, D. G. The long and the short of bacterial adhesion regulation. *Journal of bacteriology* **2011**, *193*, 327–8.

50. Korea, C.-G.; Ghigo, J.-M.; Beloin, C. The sweet connection: Solving the riddle of multiple sugar-binding fimbrial adhesins in *Escherichia coli*: Multiple *E. coli* fimbriae form a versatile arsenal of sugar-binding lectins potentially involved in surface-colonisation and tissue tropism. *BioEssay* **2011**, *33*, 300–311.

51. Buts, L.; Bouckaert, J.; De Genst, E.; Loris, R.; Oscarson, S.; Lahmann, M.; Messens, J.; Brosens, E.; Wyns, L.; De Greve, H. The fimbrial adhesin F17-G of enterotoxigenic *Escherichia coli* has an immunoglobulin-like lectin domain that binds N-acetylglucosamine. *Mol. Microbiol.* **2004**, *49*, 705–715.

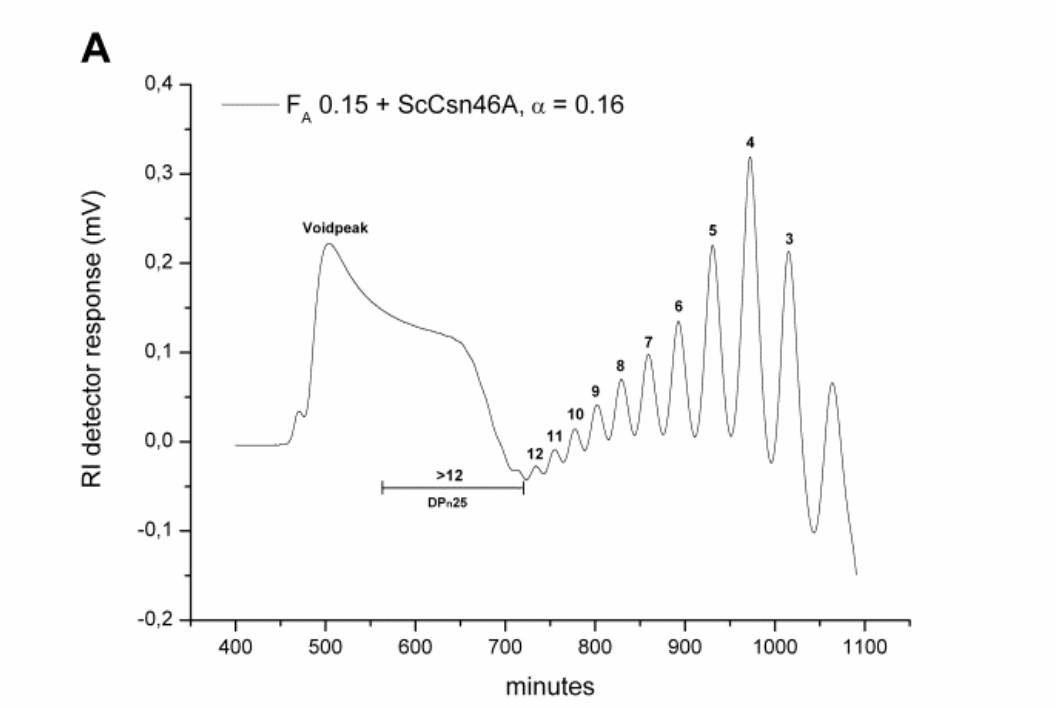
52. Sharon, N. Bacterial lectins, cell-cell recognition and infectious disease. *FEBS Lett.* **1987**, *217*, 145–157.

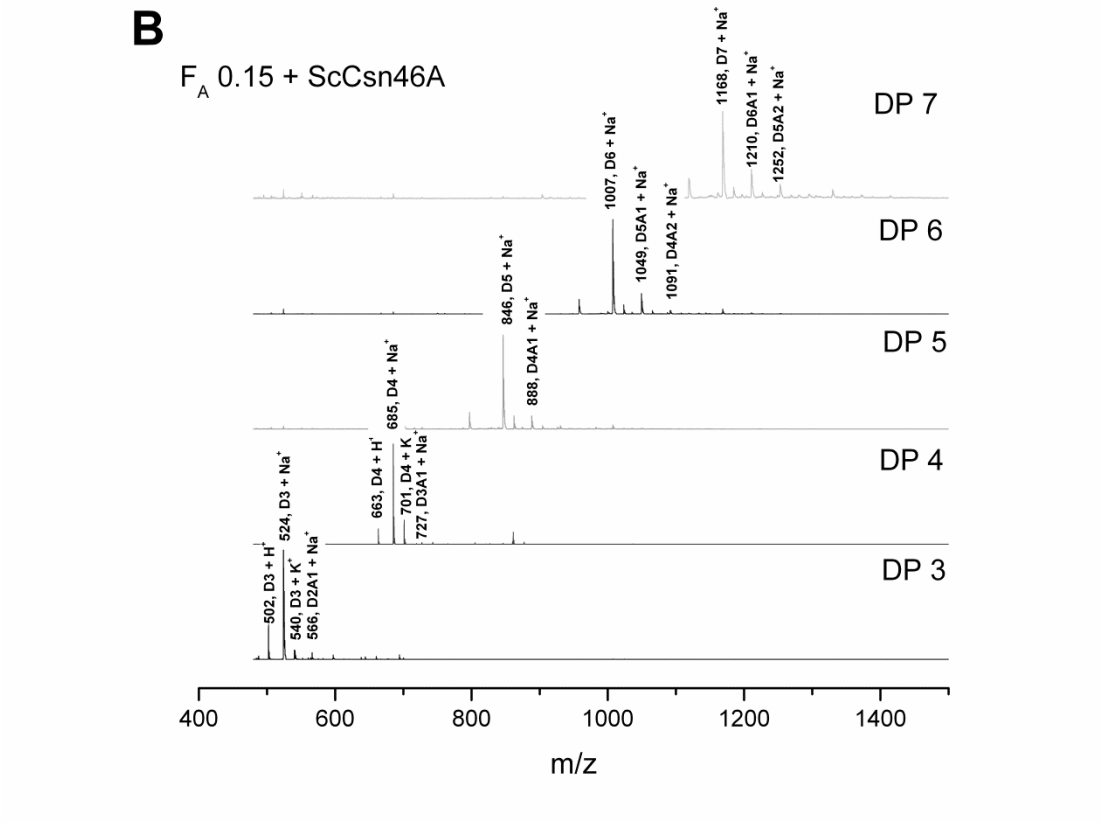
53. Sharon, N. Carbohydrates as future anti-adhesion drugs for infectious diseases. *Biochim. Biopys. Acta* **2006**, *1760*, 527–537.

54. Eaton, P.; Fernandes, J. C.; Pereira, E.; Pintado, M. E.; Xavier Malcata, F. Atomic force microscopy study of the antibacterial effects of chitosans on *Escherichia coli* and *Staphylococcus aureus*. *Ultramicroscopy* **2008**, *108*, 1128–1134.

55. Li, X.; Feng, X.; Yang, S.; Fu, G.; Wang, T.; Su, Z. Chitosan kills *Escherichia coli* through damage to be of cell membrane mechanism. *Carbohydr. Polym.* **2010**, *79*, 493–499.

Figure 1. SEC and MALDI-TOF analysis of hydrolyzed F_A0.15 chitosan. Size exclusion chromatogram (SEC) of CHOS obtained by enzymatic hydrolysis of the F_A0.15 chitosan with ScCsn46A from *Streptomyces coelicolor A3(2)* (A). Peaks are labeled by the DP of the oligomers they contain; the region labeled “>12” and “DP_n25” was collected and tested as one (mixed) fraction. MALDI-TOF-MS analysis was performed on the different SEC fractions. The DP 3-7 fractions (B) and DP 8-12 fractions (C) are shown. Major signals are labeled by mass, sugar composition (A, GlcNAc; D, GlcN) and adduct type (H^+, Na^+ or K^+).


A
F_A 0.15 + ScCsn46A, α = 0.16
RI detector response (mV)
minutes
Voidpeak
>12
DPn25
3
4
5
6
7
8
9
10
11
12


B
F_A 0.15 + ScCsn46A
DP 7
DP 6
DP 5
DP 4
DP 3
1168, D7 + Na+
1210, D6A1 + Na+
1252, D5A2 + Na+
1007, D6 + Na+
1049, D5A1 + Na+
1091, D4A2 + Na+
846, D5 + Na+
888, D4A1 + Na+
685, D4 + Na+
663, D4 + H+
701, D4 + K+
727, D3A1 + Na+
502, D3 + H+
524, D3 + Na+
540, D3 + K+
566, D2A1 + Na+
m/z

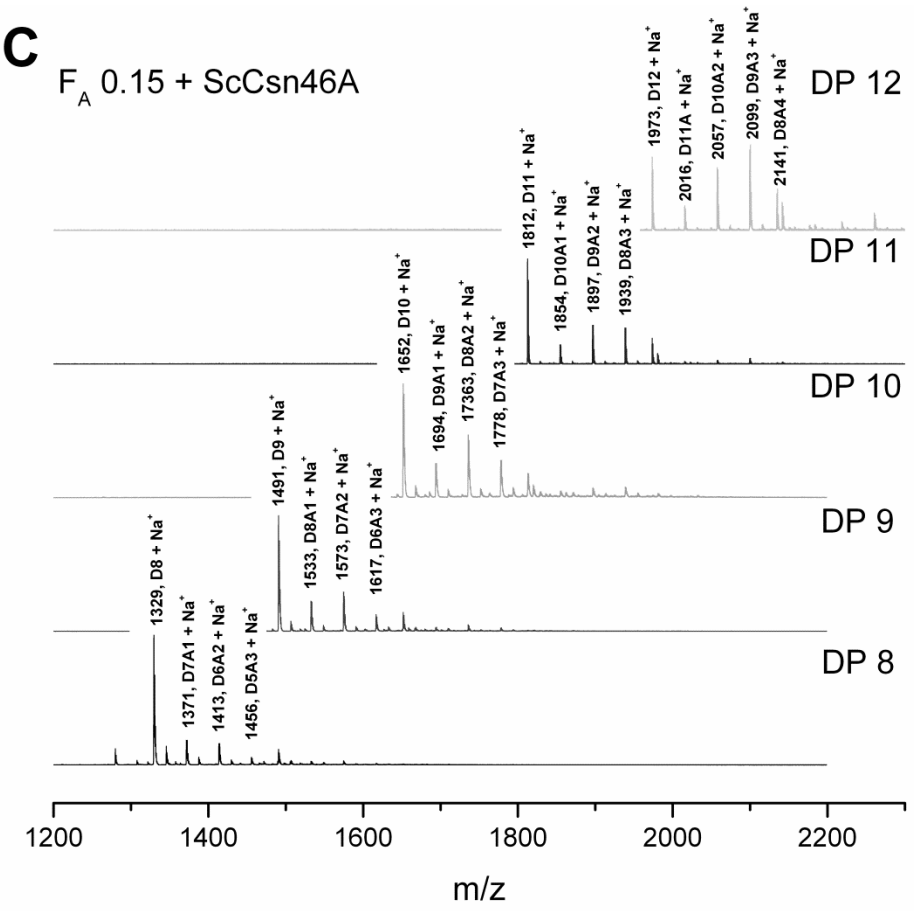
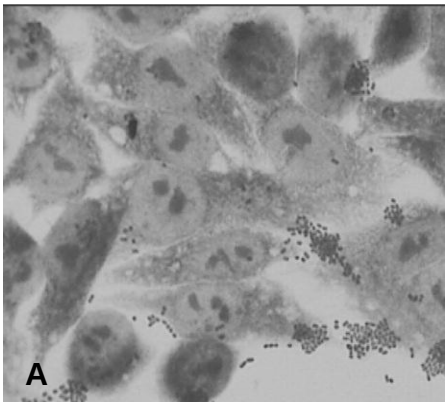

C
F_A 0.15 + ScCsn46A
DP 12
DP 11
DP 10
DP 9
DP 8
1973, D12 + Na+
2016, D11A + Na+
2057, D10A2 + Na+
2099, D9A3 + Na+
2141, D8A4 + Na+
1812, D11 + Na+
1854, D10A1 + Na+
1897, D9A2 + Na+
1939, D8A3 + Na+
1652, D10 + Na+
1694, D9A1 + Na+
17363, D8A2 + Na+
1778, D7A3 + Na+
1491, D9 + Na+
1533, D8A1 + Na+
1573, D7A2 + Na+
1617, D6A3 + Na+
1329, D8 + Na+
1371, D7A1 + Na+
1413, D6A2 + Na+
1456, D5A3 + Na+
1200
1400
1600
1800
2000
2200
m/z

Figure 2. Micrographs (100X magnification) of EPEC adherence to HEp-2 cells in the absence (A) and presence (B) of CHOS with F_A 0.15 at a concentration of 16 mg/ml.


A

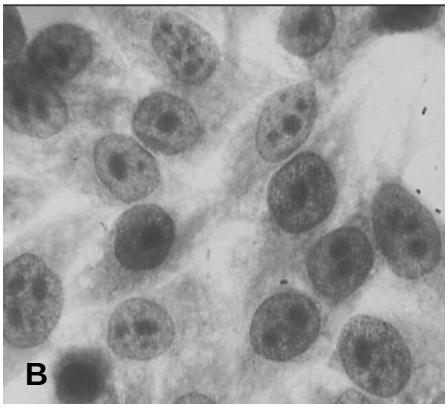
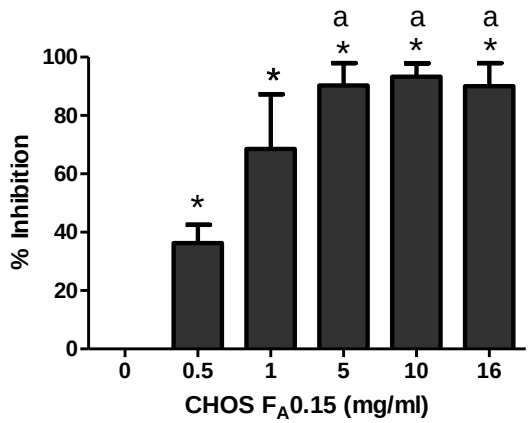
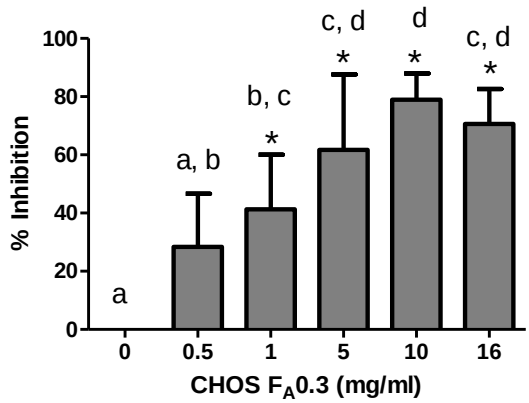

B

Figure 3. Inhibition of EPEC adherence to HEp-2 cells by CHOS mixtures with different F_A. The % Inhibition was calculated as described in the text. Statistical analysis was performed by Analysis of Variance (ANOVA) to determine statistical differences from the control, and Tukey's test was used to determine significant differences among the treatments. Values sharing the same letter are not significantly different from each other ($p < 0.05$) For F_A 0.15 and F_A 0.3 dose experiments (A and B, respectively), n = 5; for comparison between F_A 0.15, F_A 0.3, and F_A 0.65 (C), n = 2

A


a
a
a
*
*
*
*
*
*
100
80
60
40
20
0
% Inhibition
0
0.5
1
5
10
16
CHOS F_A0.15 (mg/ml)

B


c, d
d
c, d
b, c
a, b
a
*
*
*
*
*
100
80
60
40
20
0
% Inhibition
0
0.5
1
5
10
16
CHOS F_A0.3 (mg/ml)

C

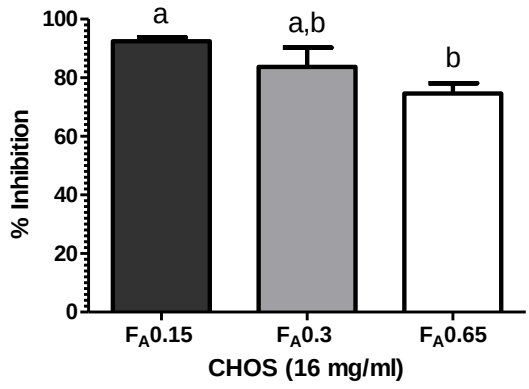

a
a,b
b
100
80
60
40
20
0
% Inhibition
F_A0.15
F_A0.3
F_A0.65
CHOS (16 mg/ml)

Figure 4. Inhibition of EPEC adherence to HEp-2 cells by purified CHOS fractions with different DP. Statistical analysis was performed by Analysis of Variance (ANOVA) to determine statistical differences from the control, and Tukey's test was used to determine significant differences among the treatments (n = 2). Groups sharing the same letter are not significantly different from each other ($p < 0.05$).

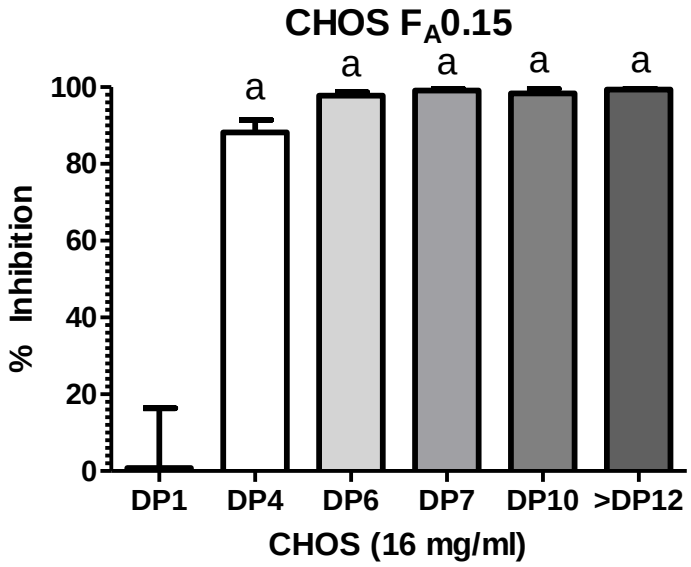
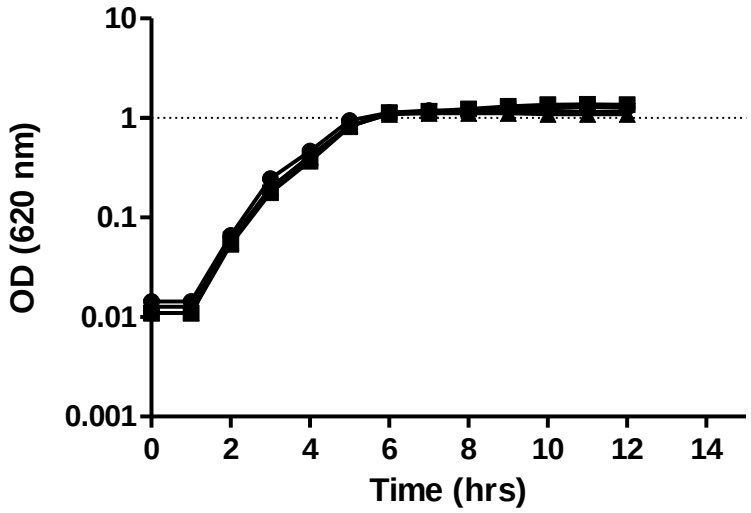

CHOS F$_A$0.15
% Inhibition
100
80
60
40
20
0
a
a
a
a
a
DP1
DP4
DP6
DP7
DP10
>DP12
CHOS (16 mg/ml)

Figure 5. Growth of EPEC in the presence and absence of GlcN and GlcNAc. Growth of EPEC in TSB at 37°C was measured in the absence (●) or in the presence of added sugars. Sugars tested were GlcN (■), GlcNAc (▲) and a 85:15 mix of GlcN: GlcNAc (▼), all at a total sugar concentration of 16 mg/ml.


10
1
0.1
0.01
0.001
OD (620 nm)
0
2
4
6
8
10
12
14
Time (hrs)

Chapter 3

Adherence Inhibition of *Cronobacter sakazakii* to Intestinal Epithelial Cells by Lactoferrin

Adherence inhibition of *Cronobacter sakazakii* to intestinal epithelial cells by lactoferrin

Maria I. Quintero-Villegas[1], Anja Wittke[2], and Robert W. Hutkins [1*]

[1]Department of Food Science and Technology, University of Nebraska, Lincoln, NE 68583-0919, United States

[2]Mead Johnson Pediatric Nutrition Institute, 2400 West Lloyd Expressway, Evansville, Indiana 47721, United States

*Correspondence to:

Robert W. Hutkins PhD

E-mail address: rhutkins1@unl.edu

Abstract

Cronobacter sakazakii is now recognized as an opportunistic pathogen and has been implicated in rare but severe cases of necrotizing enterocolitis, meningitis, and sepsis in neonates. The first step in bacterial pathogenesis requires that the organism adhere to host cells surfaces; therefore, agents that inhibit adherence might be useful for preventing infections. Lactoferrin, an iron binding protein found in milk, has been shown to inhibit bacterial adherence by direct interaction and disruption of bacterial surfaces. Therefore, the goal of this research was to assess the ability of two different types of bovine lactoferrin, alone and in combination with a 1:1 blend of galactooligosaccharides and polydextrose, to inhibit adherence of *Cronobacter sakazakii* to a HEp-2 human cell line. Results showed that the adherence of *C. sakazakii* was significantly reduced at a minimum lactoferrin concentration of 10 mg/ml. However, in combination with the oligosaccharide blend, no synergistic effect was observed in adherence inhibition. These results suggest that lactoferrin might interact with *C. sakazakii* and directly inhibit adhesion to tissue culture cells.

Key Words: Lactoferrin, Galactooligosaccharides, Polydextrose, Antiadherence

Background

Cronobacter sakazakii is now considered an opportunistic pathogen that has been implicated in bacteremia, necrotizing enterocolitis, and neonatal meningitis [19]. Although *C. sakazakii* infections are more common in neonates and premature babies, it has been reported that immune compromised adults are also susceptible to infections caused by this bacterium [19]. The infant mortality rate associated with *C. sakazakii* infections ranges from 40 to 80% [7]. In addition, survivors often develop serious long-term neurological complications [26]. The bacterium has been isolated from a range of food sources including dairy-based foods, dried meats, water, and rice [6, 44]. In particular, powdered infant formula has been epidemiologically linked with many of the infections reported [54]. Despite the relatively low incidence of disease, the severity of these diseases has led to considerable interest in developing strategies for preventing or mitigating infections.

Lactoferrin (Lf) is an 80 kDa iron binding glycoprotein, belonging to the transferrin family. It is found in high concentrations, up to 10 mg/ml in human colostrum [1] and up to 1.5 mg/ml in bovine colostrum [55]. The concentration of Lf in mature milk varies over time and is dependent on the stage of lactation [14].

Several biological functions have been attributed to Lf [30]. It has been reported to have bactericidal and bacteriostatic properties, as well as anti-inflammatory and immunomodulatory activities, based on both *in vitro* and *in vivo*

models [16]. In particular, Lf is known for its antimicrobial activity, for which at least two different mechanisms have been proposed. The first model is based on the ability of Lf to sequester iron, depriving pathogens from this essential nutrient and inhibiting their growth [14, 21]. This antimicrobial activity is reduced upon iron saturation of the molecule, suggesting that Lf is bacteriostatic rather than bactericidal [4, 14, 22, 50, 53]. A second means by which Lf exhibits antimicrobial activity is via an iron-independent mechanism. According to this model, Lf inhibits bacterial pathogens by a direct interaction mediated by binding of the Lipid A portion of the lipopolysaccharide (LPS) of Gram negative bacteria [2, 8, 43].

There is now evidence that Lf can also inhibit bacterial adherence to host cell surfaces - the first step for bacterial pathogenesis. Lf isolated from human milk was reported to inhibit adherence of enteropathogenic *Escherichia coli* (EPEC) to HeLa cells [3]. Similarly, bovine Lf was shown to significantly decrease adherence of enteroaggregative *E. coli* (EAEC) to HEp-2 cells and enterotoxigenic *E. coli* (ETEC) adherence to JTC-17 cells [23, 32]. In addition, it was reported that Lf inhibits adherence of *E. coli* O157:H7 to Caco-2 cells in a dose dependent manner, mainly due to disruption of the Type III Secretory System [55]. Other studies have shown that human Lf has proteolytic activity, providing an additional mechanism by which it can exhibit anti-adherent activity [38, 50].

It was previously reported that galactooligosaccharides (GOS) have an inhibitory effect on the adherence of *C. sakazakii* to intestinal-derived cells in tissue culture experiments [40]. In addition, polydextrose (PDX), an oligosaccharide chemically synthesized from glucose, exhibited anti-adherence activity, but to a somewhat lesser extent [39]. The main goal of this study was to assess the ability of bovine Lf, alone and in combination with a prebiotic blend of GOS and PDX, to inhibit *C. sakazakii* adherence. EPEC was used as a positive control, as it has been previously reported that Lf inhibits EPEC adherence to epithelial cells [3].

Materials and Methods

Organisms and growth conditions.

C. sakazakii 4603 (milk powder – infant formula isolate) used in this study was obtained from K. Venkitanarayanan (Department of Animal Science, University of Connecticut) and conditions for its growth have been previously described [39]. Briefly, frozen stock cultures of the organism were thawed, plated onto Tryptic Soy Agar (TSA; Difco) and grown overnight at 37°C. A single colony was inoculated into 10 ml of Tryptic Soy Broth (TSB; Difco) and incubated aerobically, without shaking. A 1% inoculum was transferred to fresh TSB media and incubated for 4 hours at 37°C without shaking. Cultures were harvested by centrifugation (3,184 x *g* for 8 minutes), washed once with phosphate-buffered saline (PBS) and re-suspended in minimal essential medium (MEM; Hyclone,

Logan, Utah), supplemented with 10% fetal bovine serum (FBS; Hyclone). Minimal essential medium was pre-equilibrated at tissue culture conditions (5% CO2, 95% relative humidity, 37°C).

EPEC strain E2348/69 (O127:H6) was obtained from M. Donnenberg (University of Maryland School of Medicine, Baltimore). Before each experiment, frozen stock cultures were plated onto TSA and grown overnight at 37°C. A single colony was inoculated into 10 ml of TSB and incubated overnight at 37°C without shaking. Overnight cultures were then inoculated at 1% (vol/vol) into MEM supplemented with 10% FBS that was pre-equilibrated at tissue culture conditions. Cells were then incubated in MEM for 80 min at 37°C prior to the start of the experiment.

Lactoferrin. Two different types of bovine Lf were used in these experiments. Lf-A was a spray-dried product from FrieslandCampina Domo (Amersfoort, Netherlands) and Lf-B was a freeze-dried product from Fonterra (New Zealand). Purity (> 90%) and iron saturation levels were similar for both products. Both Lf were prepared as concentrated stock solutions with distilled sterile water to give a final concentration of 100 mg/ml. The solutions were filter sterilized using 0.22 µm membrane filters.

Tissue culture cells. HEp-2 cells, a widely-used cell-line for adherence experiments, were obtained from the American Type Culture Collection (ATCC; Manassas, Virginia). Cells were grown in 75 cm^2 tissue culture flasks (Corning)

containing 25 ml of MEM supplemented with 10% FBS in a CO_2 incubator at tissue culture conditions. Confluent Hep-2 cells were harvested by adding 0.5 ml of 0.25% Trypsin-EDTA Solution (Sigma) and incubating for 15 minutes at tissue culture conditions. Trypsin was inactivated with 0.5 ml of FBS. Cells were then seeded onto 12-mm diameter glass coverslips in 24-well tissue culture plates (Falcon) at approximately 3.6×10^5 viable cells per well, and 500 µl of MEM supplemented with 10% FBS was added to each well. Plates were incubated under tissue culture conditions 30 hours prior to the start of each experiment, or until confluency was reached.

Adherence Assays. Cell suspensions of *C. sakazakii* 4603 and EPEC were prepared as described above. Lf was mixed with bacterial cultures at a concentration of 10^8 CFU/ml (in MEM supplemented with FBS) prior to addition to the tissue culture cells. The plates were incubated for 30 minutes at tissue culture conditions. Preliminary experiments indicated that 30 minutes of incubation were optimal for adherence of the bacterial strains to the HEp-2 tissue culture. Assays were done in duplicate and replicated 3 times (n=6). ANOVA with Tukey's test was used for statistical analysis using GraphPad Prism5 software.

Quantitative PCR (qPCR). qPCR was used for enumeration of adhered *C. sakazakii* 4603 cells. Bacterial cells from adherence assays were detached by the addition of 1 ml of 0.1% Triton X-100 for 30 minutes at room temperature.

Bacterial cells were harvested and centrifuged for 5 min at 10,000 x g, supernatants were discarded, and pellets were resuspended 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.

Species-specific primers described by Liu et al. [29] (forward primer: 5'-TATAGGTTGTCTGCGAAAGCG – 3'; reverse primer: 5'-GTCTTCGTGCTGCGAGTTTG – 3') were used. A standard curve was prepared from overnight *C. sakazakii* 4603 cultures with known cell counts. DNA was extracted as previously described. The qPCR was run 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 1µl of DNA template [31]. The program used consisted in an initial denaturation at 95°C for 10s, and 45 cycles of denaturation at 95°C for 5s and 62°C for 20s for annealing and extension. Detection limit was determined by running DNA samples of known *C. sakazakii* 4603 concentration.

Results

Lf-A and Lf-B, alone and in combination with a mix of GOS-PDX, inhibit adherence of *C. sakazakii* to HEp-2 cells.

Adherence assays were performed with *C. sakazakii* on a HEp-2 cell line. Strain 4603 has been previously reported to be a suitable model for anti-adherence experiments [39], and was used for all experiments in this study. Adherence of *C. sakazakii* to HEp-2 cells in the presence and absence of Lf by itself or in combination with GOS-PDX was measured by qPCR. Initial Lf doses used ranged from 0.1 to 1.0 mg/ml, however, no inhibition was observed at these concentrations (data not shown). Subsequently, adherence of *C. sakazakii* 4603 in the presence of Lf-A and Lf-B, in doses ranging from 2 to 50 mg/ml, was assessed. Adherence inhibition (80 – 99%) was observed at a minimum concentration of 10 mg/ml of Lf-A and Lf-B, respectively. Higher concentrations, however, did not show any additional significant anti-adhesive effects (data not shown). Lf was used at a concentration of 10 mg/ml for all subsequent anti-adherence assays with *C. sakazakii.*

Next, adherence of *C. sakazakii* 4603 was measured in the presence of Lf, in combination with a mixture of GOS-PDX (16 mg/ml). However, no additional adherence inhibition was observed in the treatment samples that contained Lf and GOS-PDX, indicating there was not a synergistic or additive effect when combining these agents (Fig. 1).

Lf-A and Lf-B, alone and in combination with a mix of GOS-PDX, inhibit adherence of EPEC to HEp-2 cells.

EPEC adherence was measured in the presence and absence of Lf-A and Lf-B, alone and in combination with a mix of GOS-PDX, by qPCR. First, EPEC adherence was assessed in the presence and absence of Lf-A and Lf-B at concentrations ranging from 2 to 50 mg/ml. The results indicated that a minimum Lf-A and Lf-B concentration of 10 mg/ml was required to inhibit adherence of EPEC to HEp-2 cells. However, as Lf-A and Lf-B concentrations increased, higher adherence inhibition was observed, suggesting that the effect on EPEC adherence varies in a dose dependent manner (data not shown). Subsequently, EPEC adherence was assessed in the presence and absence of Lf-A and Lf-B at a concentration of 10 mg/ml, alone and in combination with a mix of GOS-PDX at a concentration of 16 mg/ml. Lf-A and Lf-B inhibited adherence of EPEC to HEp-2 cells, alone and in combination with GOS-PDX. However, as for *C. sakazakii,* no significant increase in adherence inhibition was observed when both treatments were combined. This suggests that there is no synergistic effect by combining both treatments (Fig. 2).

Lf-A and Lf-B do not impair growth of *C. sakazakii* 4603.

C. sakazakii 4603 was grown in TSB containing Lf-A or Lf-B at a concentration of 10 mg/ml (i.e. the same concentration used in the anti-adherence assays) (Fig. 3). Growth of *C. sakazakii* 4603 was not impaired by the presence of Lf-A and Lf-B in the growth medium, suggesting that the reduction in adherence was not due to a bactericidal effect against *C. sakazakii*. Additionally, Lf had no effect on

growth of EPEC, indicating that reduction of adherence was not due to a bactericidal effect (data not shown).

Moreover, *C. sakazakii* 4603 and EPEC were grown in TSB containing Lf-A or Lf-B (10 mg/ml) combined with GOS-PDX (16 mg/ml). Growth was not impaired by the presence of Lf-A and Lf-B combined with GOS-PDX in the growth medium (data not shown).

Discussion

Preventing pathogen adherence to host cell surfaces has been recognized as a novel approach to prevent or mitigate bacterial infections [11, 46, 47]. Adherence to host cell surfaces requires recognition of specific host cell receptors by the bacterial pathogen, and it is the first, and one of the main, steps in bacterial pathogenesis [36]. This interaction can be mediated by different mechanisms including bacterial lectins and host glycoproteins, bacterial lectins and host glycolipids, bacterial lipopolysaccharide and host lectins, hydrophobin – protein interactions, and protein – protein interactions [36]. Compounds with similar structures to those found in the surface of cells may act as molecular decoys that interfere with the different mechanisms of pathogen binding [45, 46, 49]. Among these compounds, non-digestible oligosaccharides, such as GOS, have been shown to inhibit pathogen adherence to epithelial cells *in vitro* and *in vivo* [24, 32, 33]. Other compounds, such as bovine Lf, have attracted much attention for their biological properties, including anti-adherence.

Among the biological functions attributed to Lf are antimicrobial, anti-inflammatory, and immunomodulatory activities [1, 9, 25, 27, 52]. The mechanisms that account for the antimicrobial properties have been reported to be iron-dependent and iron-independent [4, 10, 12, 37]; the latter implying direct interaction of Lf with the bacterial cell surface [2, 8, 43]. This direct interaction can result in the inability of the bacterium to adhere to host cell surfaces [13, 18, 21, 34, 38]. Previous studies have shown that Lf prevents colonization of *Haemophilus influenzae,* degrades virulence proteins secreted by *Shigella flexeneri,* and inhibits adherence of *Salmonella Typhimurium.* In addition, Lf has been shown to interfere with the Type III Secretory System present in many pathogenic bacteria [5, 13, 16–18, 21, 34, 38].

This is the first study that provides evidence that Lf inhibits adherence of *C. sakazakii* to human epithelial cells *in vitro*. Our results show that two different types of Lf inhibited adherence of *C. sakazakii* and EPEC, alone and in combination with a mix of GOS-PDX. It was previously reported that a mix of GOS-PDX inhibits adherence of *C. sakazakii* to HEp-2 cells at a concentration of 16 mg/ml [39]. However, no synergistic effect was observed when using this same prebiotic blend in combination with Lf. Despite lacking a synergistic effect, Lf was shown to inhibit adherence of *C. sakazakii* at a minimum concentration of 10 mg/ml. Interestingly, the minimal effective concentration of Lf to inhibit adherence of *C. sakazakii* and EPEC corresponds to the Lf levels found in human colostrum [40, 41]. At this same concentration (10 mg/ml), neither of the

lactoferrins were able to inhibit growth of *C. sakazakii* (Fig. 3), nor was a bactericidal effect evident when EPEC was incubated in the presence of Lf. In contrast, Wakabayashi et al. [51] reported that bovine lactoferrin exhibited antimicrobial activity against *C. sakazakii* and *E. coli*.

The antimicrobial effect of native, partially hydrolyzed, and heated lactoferrin has been well studied. Although iron sequestration may contribute, in part, to microbial inhibition, especially in low iron environments, other factors are likely involved [21]. In particular, it has also been shown that the positively charged N-terminal regions of lactoferrin interact with the lipid A component of the bacterial membrane, resulting in membrane disruption [2, 15]. However, other studies have shown no antimicrobial effect of bovine Lf on EPEC. For example, Ochoa et al. [33, 34] did not observe any bactericidal effect when EPEC was grown in DMEM in the presence of bovine Lf. In addition, the ability of Lf to degrade EPEC virulence proteins has been reported, independent from any bactericidal effect [35].

The pathogenesis of *C. sakazakii,* its mode of adherence to host cell surfaces and expression of adhesins have not been well studied, although it has been established that an outer membrane protein A (OmpA) might play an important role in the attachment and invasion of Caco-2 cells [24]. Following cell attachment, human isolates of *C. sakazakii* have been shown to disrupt cell tight junctions and increase enterocyte permeability [28]. The mechanism by which Lf

inhibits adherence of *C. sakazakii* will require further investigation. For *E. coli* O157:H7, Lf-mediated adherence inhibition to Caco-2 cells was suggested to be due, in part, to disruption of the Type III Secretory System [55]. However, it is not clear that this effect is strictly dependent on the direct interaction of the protein with the bacterial cell surface.

Collectively, these results show that Lf-A and Lf-B, at a concentration of 10 mg/ml, inhibit adherence of *C. sakazakii* 4603 and EPEC to HEp-2 cells. However, a synergistic effect was not observed when combining Lf with a mixture of GOS-PDX. Novel ingredients that are able to prevent binding of pathogens to the surface of cells have attracted great interest, as they have the potential to be used as prophylactic treatments to prevent or mitigate infections. Preventative approaches are especially warranted in the case of *Cronobacter*-induced diseases, due to the high mortality and limited post-infection therapies [20].

Acknowledgements

This work was funded by Mead Johnson Nutrition. We thank Dr. K. Venkitanarayanan, Department of Animal Science, University of Connecticut, for providing strains of *C. sakazakii.*

References

1. Actor JK, Hwang SA, Kruzel ML. (2009) Lactoferrin as a natural immune modulator. Curr Pharm Des 15:1956–1973.

2. Appelmelk BJ, An YQ, Geerts M, Thijs BG, Boer HA et al (1994) Lactoferrin is a lipid A-binding protein. Infect Immun 62: 2628–2632 .

3. Araújo AN., Giugliano LG (2001) Lactoferrin and free secretory component of human milk inhibit the adhesion of enteropathogenic *Escherichia coli* to HeLa cells. BMC Microbiol. 1: 25.

4. Arnold RR, Brewer M, Gauthier JJ (1980) Bactericidal activity of human lactoferrin: sensitivity of a variety of microorganisms. Infect Immun 28:893–898.

5. Bessler HC, Oliveira IR, Giugliano LG (2006) Human milk glycoproteins inhibit the adherence of *Salmonella typhimurium* to HeLa cells. Microbiol Immunol 50:877–882.

6. Beuchat LR, Kim H, Gurtler JB, Lin LC, Ryu JH et al (2009) *Cronobacter sakazakii* in foods and factors affecting its survival, growth, and inactivation. Int J Food Microbiol 136:204–213.

7. Bowen AB, Braden CR (2006) Invasive *Enterobacter sakazakii* disease in infants. Emerg Infect Dis 12: 1185–11893.

8. Brandenburg K, Jürgens G, Müller M, Fukuoka S, Koch MH (2001) Biophysical characterization of lipopolysaccharide and lipid A inactivation by lactoferrin. Biol Chem 382:1215–1225.

9. Brock JH (2002) The Physiology of Lactoferrin. Biochem Cell Biol 80:1 – 6.

10. Bullen JJ, Rogers HJ, Leigh L (1972) Iron-binding proteins in milk and resistance to *Escherichia coli* infection in infants. BMJ. 1:69–75.

11. Cozens D, Read RC (2012) Anti-adhesion methods as novel therapeutics for bacterial infections. Expert Rev Anti-infect Ther 10:1457–1468.

12. Dalmastri C, Valenti P, Visca P, Vittorioso P, Orsi N (1998) Enhanced antimicrobial activity of lactoferrin by binding to the bacterial surface. Microbiologica 11: 225–230.

13. DeVinney R, Gauthier A, Abe A, Finlay BB (1999) Enteropathogenic *Escherichia coli*: a pathogen that inserts its own receptor into host cells. Cell Mol Life Sci 55: 961–976.

14. Farnaud S (2003) Lactoferrin—a multifunctional protein with antimicrobial properties. Mol Immunol 40:395–405.

15. Flores-Villaseñor H, Canizalez-Roman A, Reyes-Lopez M, Nazmi K, de la Garza M et al (2010) Bactericidal effect of bovine lactoferrin, LFcin, LFampin and LFchimera on antibiotic-resistant *Staphylococcus aureus* and *Escherichia coli*. Biometals 23:569–578.

16. Gomez HF, Ochoa TJ, Carlin LG, Cleary TG (2003) Human lactoferrin impairs virulence of *Shigella flexneri*. J Infect Dis 187: 87 – 95.

17. Gomez HF, Herrera-Insua I, Siddiqui MM, Diaz-Gonzalez VA, Caceres E et al (2001) Protective role of human lactoferrin against invasion of *Shigella flexneri* M90T. Adv Exp Med Biol 501: 457–467.

18. Goosney DL, Knoechel DG, Finlay BB (1999): Enteropathogenic *E. coli, Salmonella*, and *Shigella*: masters of host cell cytoskeletal exploitation. Emerg Infect Dis 5:216–223.

19. Healy B, Cooney S, O'Brien S, Iversen C, Whyte P et al (2010) *Cronobacter* (*Enterobacter sakazakii*): an opportunistic foodborne pathogen. Foodborne Pathog Dis 7:339–350.

20. Hunter CJ, Bean JF (2013) *Cronobacter*: an emerging opportunistic pathogen associated with neonatal meningitis, sepsis and necrotizing enterocolitis. J Perinatol 33:581–585.

21. Jenssen H, Hancock REW (2009) Antimicrobial properties of lactoferrin. Biochimie 91:19–29.

22. Kalmar JR, Arnold RR (1998) Killing of *Actinobacillus actinomycetemcomitans* by human lactoferrin. Infect Immun 56:2552–2557.

23. Kawasaki Y, Tazume S, Shimizu K, Matsuzawa H, Dosako S et al (2000) Inhibitory effects of bovine lactoferrin on the adherence of enterotoxigenic *Escherichia coli* to host cells. Biosci Biotechnol Biochem 64:348–354.

24. Kim KP, Loessner MJ (2008) *Enterobacter sakazakii* invasion in human intestinal Caco-2 cells requires the host cell cytoskeleton and is enhanced by disruption of tight junction. Infect Immun 76:562–570.

25. Kruzel ML, Harari Y, Mailman D, Actor JK (2002) Differential effects of prophylactic, concurrent and therapeutic lactoferrin treatment on LPS-induced inflammatory responses in mice. Clin Exp Immunol 130:25–31.

26. Lai KK (2001) *Enterobacter sakazakii* infections among neonates, infants, children, and adults. Case reports and a review of the literature. Medicine 80:113–122.

27. Legrand D, Pierce A, Elass E, Carpentier M, Mariller C et al (2008) Lactoferrin structure and functions. Adv Exp Med Biol 606:163–194.

28. Liu Q, Mittal R, Emami CN, Iversen C, Ford HR et al (2012) Human isolates of *Cronobacter sakazakii* bind efficiently to intestinal epithelial cells *in vitro* to induce monolayer permeability and apoptosis. J Surg Res 176:437–447.

29. Liu Y, Cai X, Zhang X, Gao Q, Yang X et al (2006) Real time PCR TaqMan and SYBR Green for detection of *Enterobacter sakazakii* in infant formula. J. Micriobiol. Methods 65:21-31.

30. Lonnerdal B, Suzuki YA (2013) Lactoferrin. In: McSweeney PLH, Fox PX (eds) Advanced Dairy Chemistry: Volume 1A: Proteins: Basic Aspects 4th edn. Springer, pp 295–316.

31. Martínez I, Wallace G, Zhang C, Legge R, Benson AK et al (2009) Diet-induced metabolic improvements in a hamster model of hypercholesterolemia are strongly linked to alterations of the gut microbiota. Appl Environ Microbiol 75:4175–4184.

32. Ochoa TJ, Brown EL, Guion CE, Chen JZ, McMahon RJ et al (2006) Effect of lactoferrin on enteroaggregative *E . coli* (EAEC)1. Biochem Cell Biol 84:369–376.

33. Ochoa TJ, Noguera-Obenza M, Cleary TG (2004) Lactoferrin blocks the initial host cell attachment mechanism of enteropathogenic *E. coli* (EPEC). Adv Exp Med Biol 554:463–466.

34. Ochoa TJ, Noguera-Obenza M, Ebel F, Guzman CA, Gomez HF et al (2003) Lactoferrin impairs type III secretory system function in enteropathogenic *Escherichia coli.* Infect Immun 71:5149–5155.

35. Ochoa TJ, Cleary TG (2009) Effect of lactoferrin on enteric pathogens. Biochimie 91:30–34.

36. Ofek I, Hasty DL, Doyle RJ (2003) Bacterial adhesion to animal cells and tissues. ASM Press

37. Orsi N (2004)The antimicrobial activity of lactoferrin: Current status and perspectives. BioMetals 17:189–196.

38. Qiu J (1998) Human milk lactoferrin inactivates two putative colonization factors expressed by *Haemophilus influenzae*. Proc Natl Acad Sci 95:12641–12646.

39. Quintero M, Maldonado M, Perez-Munoz M, Jimenez R, Fangman T et al (2011) Adherence Inhibition of *Cronobacter sakazakii* to intestinal epithelial cells by prebiotic oligosaccharides. Curr Microbiol 62:1448-1454.

40. Rai D, Adekman AS, Zhuang W, Rai GP, Boettcher J et al (2011) Longitudinal changes in lactoferrin concentrations in human milk - a global systematic review. Crit Rev Food Sci Nutr. doi:10.1080/10408398.2011.642422

41. Ronayne de Ferrer PA, Baroni A, Sambucetti ME, Lopez NE, Ceriani-Cernadas JM (2000) Lactoferrin levels in term and preterm milk. J Am Coll Nutr 19:370–373.

42. Searle LEJ, Best A, Nunez A, Salguero FJ, Johnson L et al A mixture containing galactooligosaccharide, produced by the enzymic activity of *Bifidobacterium bifidum*, reduces *Salmonella enterica* serovar Typhimurium infection in mice. J Med Microbiol 58:37–48.

43. Shahriar F, Gordon JR, Simko E (2006) Identification of lipopolysaccharide-binding proteins in porcine milk. Can J Vet Res 70:243–250.

44. Shaker R, Osaili T, Al-Omary W, Jaradat Z, Al-Zuby M (2007) Isolation of *Enterobacter sakazakii* and other *Enterobacter* sp. from food and food production environments. Food Control 18:1241–1245.

45. Sharon N (2006) Carbohydrates as future anti-adhesion drugs for infectious diseases. Biochim Biophys Acta 1760:527–537.

46. Sharon N, Ofek I (2002) Fighting infectious diseases with inhibitors of microbial adhesion to host tissues. Crit Rev Food Sci Nutr 42: 267–272.

47. Sharon N, Ofek I (2000) Safe as mother's milk: Carbohydrates as future anti-adhesion drugs for bacterial diseases. Glycoconjugate J 17:659-664.

48. Shoaf K, Mulvey GL, Armstrong GD, Hutkins RW (2006) Prebiotic galactooligosaccharides reduce adherence of enteropathogenic *Escherichia coli* to tissue culture cells. Infect Immun 74:6920–6928.

49. Shoaf-Sweeney K.D, Hutkins RW (2008) Adherence, anti-adherence, and oligosaccharides: preventing pathogens from sticking to the host. Adv Food Nutr Res 101–161.

50. Valenti P, Antonini G (2005) Lactoferrin: an important host defence against microbial and viral attack. Cell Mol Life Sci 62:2576–2587.

51. Wakabayashi H, Yamauchi K, Takase M (2008) Inhibitory effects of bovine lactoferrin and lactoferricin B on *Enterobacter sakazakii*. Biocontrol Sci 13:29–32.

52. Ward PP, Uribe-Luna S (2002) Lactoferrin and host defense. Biochem Cell Biol 80:95–102.

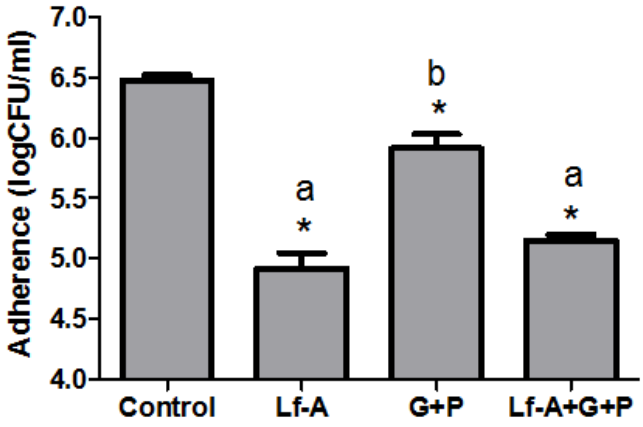
53. Yamauchi K, Tomita M, Giehl TJ, Ellison RT (1993) Antibacterial activity of lactoferrin and a pepsin-derived lactoferrin peptide fragment. Infect Immun 61:719–728.

54. Yan QQ, Condell O, Power K, Butler F, Tall BD et al (2012) *Cronobacter* species (formerly known as *Enterobacter sakazakii*) in powdered infant formula: a review of our current understanding of the biology of this bacterium. J Appl Microbiol 113:1–15.

55. Yekta MA, Verdonck F, Broeck WVD, Goddeerins BM, Cox E et al (2010) Lactoferrin inhibits *E . coli* O157 : H7 growth and attachment to intestinal epithelial cells. Children. 2010:359–368**.**

Fig. 1 Lf-A and Lf-B, in combination with 16 mg/ml GOS-PDX, inhibit adherence of *C. sakazakii* to HEp-2 cells

Adherence of *C. sakazakii* in the presence and absence of Lf-A and Lf-B and a GOS-PDX mix was measured by qPCR. Adherence inhibition was observed at a concentration of 10 mg/ml Lf combined with 16 mg/ml PDX-GOS. Groups significantly different from the control are indicated by an *. Groups with different letters are significantly different from each other ($\alpha < 0.05$).


Adherence (logCFU/ml)
7.0
6.5
6.0
5.5
5.0
4.5
4.0
a
*
b
*
a
*
Control
Lf-A
G+P
Lf-A+G+P

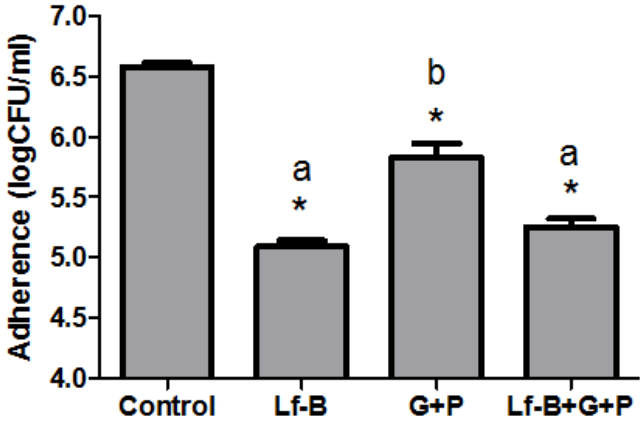
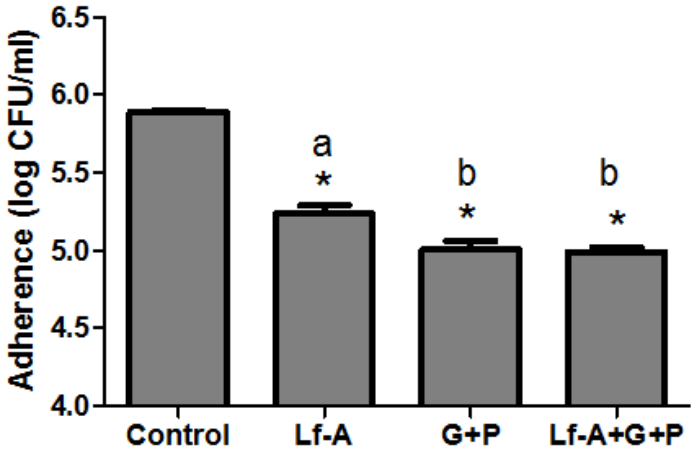

Adherence (logCFU/ml)
7.0
6.5
6.0
5.5
5.0
4.5
4.0
a
*
b
*
a
*
Control
Lf-B
G+P
Lf-B+G+P

Fig. 2 Lf-A and Lf-B, in combination with 16 mg/ml GOS-PDX, inhibit adherence of EPEC to HEp-2 cells

Adherence of EPEC in the presence and absence of Lf-A and Lf-B and a GOS-PDX mix was measured by qPCR. Adherence inhibition was observed at a concentration of 10 mg/ml Lf combined with 16mg/ml PDX-GOS. There is not a synergistic effect observed when combining both treatments. Groups significantly different from the control are indicated by an *. Groups with different letters are significantly different from each other ($\alpha < 0.05$).


Adherence (log CFU/ml)
6.5
6.0
5.5
5.0
4.5
4.0
a
*
b
*
b
*
Control
Lf-A
G+P
Lf-A+G+P

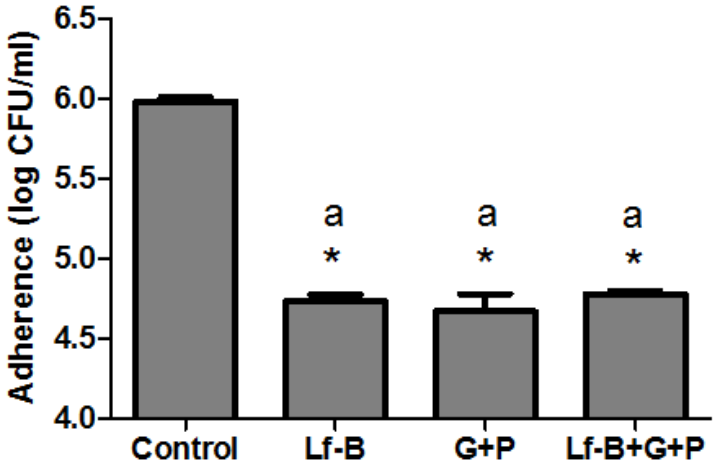
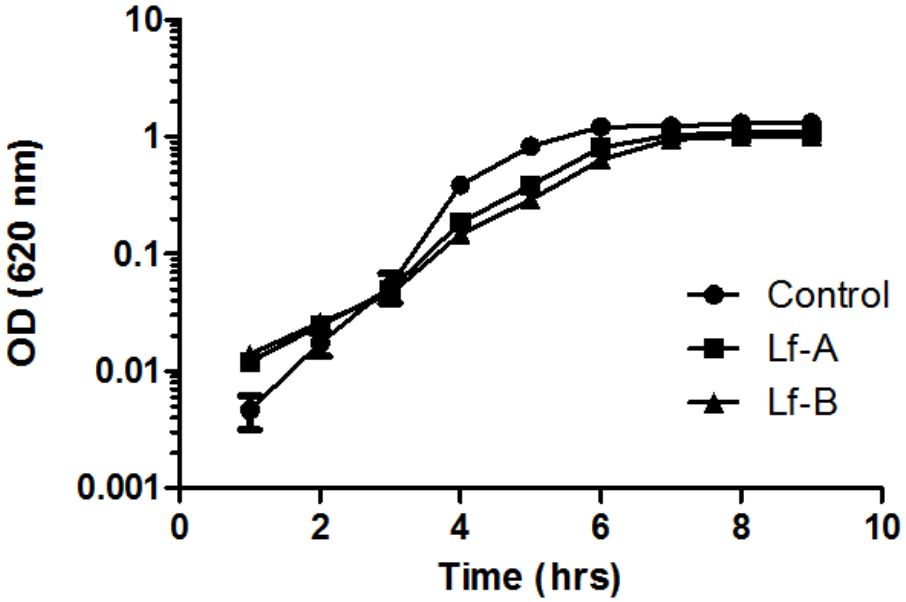

Adherence (log CFU/ml)
6.5
6.0
5.5
5.0
4.5
4.0
a
*
a
*
a
*
Control
Lf-B
G+P
Lf-B+G+P

Fig. 3 Growth of *C. sakazakii* 4603 in the presence and absence of Lf-A and Lf-B

Growth of *C. sakazakii* 4603 in TSB at 37°C was measured in the presence (■, ▲) or absence (●) of added Lf. Total Lf concentration tested was 10mg/ml.


10
1
0.1
0.01
0.001
OD (620 nm)
0
2
4
6
8
10
Time (hrs)
Control
Lf-A
Lf-B

Chapter 4

Impact of Galactooligosaccharide Supplementation on Susceptibility to Enteric Bacterial Pathogens

Abstract

In addition to their ability to modulate the gastrointestinal microbiota, some prebiotic oligosaccharides have other biological properties. Specifically, galactooligosaccharides (GOS) and other prebiotics have also been shown to inhibit adherence of pathogens to the surface of epithelial cells. In this study, GOS was tested for the ability to reduce adherence of *Citrobacter rodentium* to the surface of epithelial cells *in vitro*, on a HEp-2 tissue culture cell line, and *in vivo*, in conventional flora and germ free C57Bl/6 mice. *C. rodentium* is an attaching and effacing (A/E) mouse pathogen used as a surrogate model to study enteropathogenic *Escherichia coli* (EPEC) and enterohemorragic *E. coli* (EHEC) in mice, and was used as the model organism. For the *in vitro*experiments, HEp-2 cells were incubated with *C. rodentium* DBS100Nal^r with and without GOS (10 - 50 mg/ml). Bacterial adherence was quantified by serial dilutions and plating. For *in vivo* experiments, germ free and conventional flora mice were supplemented with GOS for two weeks prior to inoculation with *C. rodentium* DBS100Nal^r. Bacterial adherence to the distal colon was quantified by plating and histopathology was performed to assess the severity of the lesions. Results indicated that GOS reduced adherence of *C. rodentium* DBS100Nal^r *in vitro* on HEp-2 cells. When mice were supplemented with GOS at a concentration of 5000 mg/kg of body weight, adherence of *C. rodentium* to the mouse distal colon was reduced in conventional flora mice but not in germ free mice. Histological scores in conventional flora mice indicated profound lesions in the mice infected

with *C. rodentium*, and no significant difference was observed in the GOS supplemented group as compared to the infected control.

Key Words: galactooligosaccharides, antiadherence, *Citrobacter rodentium*, conventional flora mice, germ free mice.

Introduction

Prebiotic oligosaccharides are currently defined as selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microbiota, that confer benefits upon host well-being and health (Roberfroid, 2007). Although this definition has evolved since it was first stated nearly 20 years ago (Gibson and Roberfroid, 1995), to be considered as a prebiotic the substance must still be able to modulate the gut microbiota. Among the specific colonic microbial species that selectively ferment prebiotic oligosaccharides are members of the genus *Bifidobacterium*. These colonic bacteria ferment prebiotic substrates and produce short chain fatty acids (SCFA) that are beneficial to the host (Nicholson et al., 2012; Wong et al., 2006) and inhibitory against opportunistic pathogens (Defoirdt et al., 2006; Van Immerseel, 2003; Tejero-Sariñena et al., 2012).

In addition to their role as fermentation substrates, some prebiotic oligosaccharides have been shown to possess another important biological activity. Specifically, they can act as molecular decoys that competitively inhibit adherence of pathogens to host cells (Quintero et al., 2011; Quintero-Villegas et al., 2013; Shoaf et al., 2006; Shoaf-Sweeney and Hutkins, 2008). This adherence inhibition property is due to the structural similarity between prebiotic oligosaccharides and the carbohydrate moieties located on the surface of epithelial cells. For many enteric pathogens, these surface carbohydrates serve as binding sites for specific bacterial adhesins. Accordingly, the bacterial

adhesins will bind to the exogenous oligosaccharides rather than to the surface of the cell. Reduced bacterial adherence may ultimately decrease infection.

Several oligosaccharides have been reported to inhibit pathogen adherence. Mannan oligosaccharides have long been known to inhibit Type-1 fimbriae-mediated adherence of *Escherichia coli* and *Salmonella* spp. (Firon, 1982; Kisiela et al., 2006; Neeser et al., 1986). Galactooligosaccharides (GOS) were shown to inhibit adherence of enteropathogenic *E. coli* (EPEC), as well as the neonatal opportunistic pathogen, *Cronobacter sakazakii*. Pectin oligosaccharides have been reported to reduce invasion of *Campylobacter jejuni* to Caco-2 cells (Ganan et al., 2010) as well as neutralize *E. coli* Shiga toxins (Olano-Martin et al., 2003). Oligosaccharides derived from human and bovine milk have also been shown to inhibit adherence of *E. coli*, *Vibrio cholerae*, and *Salmonella fyris* in various cell lines (Coppa et al., 2006), among others.

Although the anti-adherence activity of several of these oligosaccharides against enteric pathogens has been assessed *in vitro*, testing biological activity in animal models requires that the pathogen is capable of colonizing and causing infection in the selected host. While EPEC has been commonly used for *in vitro* adherence studies due to its ability to express multiple oligosaccharide binding adhesins, infection of conventional flora mice with EPEC strains does not result in high levels of EPEC colonization and shedding (Mundy et al., 2006). Therefore, the *Citrobacter rodentium* mouse model is now widely used as a surrogate to study EPEC and enterohemorrhagic *E. coli* (EHEC) pathogenesis in mice (Bishop et al., 2007; Mundy et al., 2005). *C. rodentium* is an attaching and

effacing (A/E) mouse pathogen whose virulence genes are encoded on a pathogenicity island known as the locus of enterocyte effacement (LEE), with very similar arrangements as the LEE found in EPEC and EHEC. Thus, they share many virulence factors and colonization mechanisms (Deng et al., 2001; Mundy et al., 2005).

In this study, we evaluated the ability of GOS to reduce adherence of *C. rodentium,* first*, in vitro* using a HEp-2 cell line, and then *in vivo*, in conventional flora and germ free C57Bl/6 mice. We also assessed the ability of GOS to prevent or ameliorate the severity of disease pathologies in these mice.

Materials and Methods

Organisms and growth conditions *Citrobacter rodentium* DBS100Nal^r (nalidixic acid resistant) was kindly provided by Dr. Allen Smith (USDA, ARS, Baltimore). Prior to each experiment, frozen stock cultures were plated on Luria Bertani (LB; Difco) agar containing 100 µg/ml of nalidixic acid (Sigma) (LB-Nal) and grown overnight at 37°C. A single colony was inoculated into LB Broth and incubated aerobically at 37°C for 18 hours. For *in vitro* antiadherence experiments, cells were harvested by centrifugation (3,184 x *g* for 8 minutes), washed once with phosphate buffered saline (PBS) pH 7.0, and re-suspended in minimal essential medium (MEM; Hyclone, Logan, Utah), supplemented with 10% fetal bovine serum (FBS; Hyclone). MEM was pre-equilibrated overnight at tissue culture conditions (5% CO_2, 95% relative humidity, 37°C).

For mouse experiments, a single colony was grown overnight at 37°C in LB broth. A 1% inoculum was transferred to fresh LB broth and incubated for 5 hours at 37°C, shaking at 200 rpm. Preliminary experiments show that *C. rodentium* DBS100 Nal^r reaches mid to late log phase after 5 to 6 hour incubation and achieves a cell density of about 2.0 x 10^8 CFU/ml. Bacteria were concentrated by centrifugation to deliver 2.5 x 10^8 *C. rodentium* CFU in 100 µl of PBS.

Animals 8-week old conventional flora (CF) C57Bl/6 female mice were obtained from Charles River. Thirty-six 8-week old germ free (GF) C57BL/6 mice were bred and maintained at the GF facility in the Life Sciences Annex facility at the University of Nebraska – Lincoln (UNL). Water and food were provided *ad libitum*. All animal studies were performed according to approved protocols by the Institutional Animal Care and Use Committee (IACUC) at UNL.

Tissue culture cells HEp-2 cells were obtained from ATCC. Cells were grown in 75 cm^2 tissue culture flasks containing 25 ml of MEM supplemented with 10% FBS in a CO_2 incubator at tissue culture conditions. Confluent HEp-2 cells were harvested by removing MEM and washing the cells once with PBS. Subsequently, 0.5 ml of a 0.25% Trypsin-EDTA solution was added followed by 10 minute incubation at tissue culture conditions. After incubation, 0.5 ml of FBS were added to inactivate the trypsin. Cells were then seeded onto 12 mm diameter glass coverslips in 24-well tissue culture plates at approximately 3.6 × 10^5 cells per well, and 500 µl of MEM supplemented with 10% FBS was added to each well. Plates were incubated under tissue culture conditions for about 20 hours prior to the start of each experiment. Before each experiment, cells were

viewed using an inverted microscope to establish that they had reached about 70% confluency.

Galactooligosaccharides (GOS) Purimune GOS (formerly GTC Nutrition) was prepared as a stock solution and filter sterilized through a 0.2µm membrane filter. For *in vitro* experiments, a stock solution of GOS was prepared at 100 mg/ml.

Adherence Assays *C. rodentium* DBS100 Nal^r was prepared as previously described. GOS at concentrations ranging from 10 to 50 mg/ml was mixed with bacterial cultures (10^8 CFU/ml) prior to addition to tissue culture cells. Autoclaved distilled water instead of GOS was used as a control. Plates were incubated for 90 minutes at tissue culture conditions to allow for bacteria to adhere. Assays were done in duplicate and replicated 3 times (n=6). ANOVA with Tukey's test was used for statistical analysis using GraphPad Prism5 Software.

Culture Enumeration After incubation, cells were detached by addition of 0.1% Triton X-100, serially diluted and plated on LB-Nal and incubated overnight at 37°C.

GOS Tolerance and Protection Assay (CF Mice) A GOS tolerance assay was performed to determine if any GOS concentration would cause signs of distress in the mice. 25 mice were randomly divided into 5 groups (A,B,C,D,E) (5 mice per cage) and housed with food and water provided *ad libitum.* On average, a mouse consumes around 5 ml of water per day. Hence, GOS was provided in the drinking water to deliver the following doses of GOS based on 5 ml water consumption per mouse, per day: 140 mg GOS/kg body weight, 2500 mg

GOS/kg body weight, and 5000 mg/kg body weight, two weeks prior to inoculation with *C. rodentium* and during the course of infection (10 days). Treatment groups were divided as follows: A) Control (non-treated group); B) Infected control; C) 140 mg GOS/kg body weight; D) 2500 mg GOS/kg body weight E) 5000 mg GOS/kg body weight. Groups C, D, and E were dosed by oral gavage with 2.5 x 10^8 CFU of *C. rodentium* (DBS100Nal^r) in 100 µl of PBS after two weeks of GOS supplementation.

Fecal pellets were collected every other day starting on day one of GOS supplementation. Briefly, mice pellets were collected in sterile brown paper bags and transferred into sterile microcentrifuge tubes. To quantify *C. rodentium* shedding, pellets were weighed and homogenized in PBS, serially diluted, and plated on LB-Nal Agar.

Necropsy was performed at day 10 post infection. Colon length was measured and 0.5 cm of the most distal portion was saved in 10% formalin for histopathology. To assess adherence to intestinal tissue, the next 3 cm distal sections were cut and washed with PBS, homogenized in a gentleMACS dissociator (Miltenyi Biotec; San Diego, CA) with 0.1% Triton x-100 in PBS, serially diluted and plated in LB-Nal Agar for quantification of adhered *C. rodentium*. Cecal contents were collected in 2 ml freezing vials and kept at -80°C.

GOS Protection Assay (CF and GF Mice) Mice were randomly divided into 4 groups (A, B, C, D), with 4 mice per cage and two cages per group. Mice were provided with food and water provided *ad libitum*. GOS was provided in the

drinking water to deliver 5000 mg/kg of body weight per mouse, based on 5 ml water consumption per mouse, per day, two weeks prior to inoculation with *C. rodentium* and during the course of infection (10 days). Treatment groups were divided as follows: A) Control (non-treated group); B) Infected control (*C. rodentium* infected); C) GOS control (non-infected, GOS supplemented); D) GOS treatment (GOS supplemented, *C. rodentium* infected). Groups B and D were dosed by oral gavage with 2.5×10^8 CFU of *C. rodentium* (DBS100Nal^r) in 100 µl of PBS.

Fecal pellets were collected every other day starting on day one of GOS supplementation and *C. rodentium* shedding was quantified as above.

Necropsy was performed at day 10 post infection as above. Additionally, 3 cm of proximal colon were saved for tissue explants to evaluate pro and anti-inflammatory cytokine expression.

For the GF experiments, the same procedure was followed. Fecal collection was not performed every other day to assess bacterial shedding, only at day 10 post infection.

Histopathology Colonic tissue was fixed in 10% formalin. Tissues were paraffin embedded, sectioned, and stained with hematoxylin and eosin (H&E). To assess pathology, a scoring system was adapted from Gibson et al., 2008. Tissue scoring was performed by a blinded observer as following: sub-mucosal edema (0=no change; 1=mild; 2=moderate; 3=profound), goblet cell depletion (number of goblet cells on 5 microscopic fields averaged, 400x magnification); epithelial

hyperplasia (as compared to normal height of a control / 0=no change; 1=1-50%; 2=51-100%; 3=>100%), epithelial integrity (0=no change; 1=<10 epithelial cells shedding; 2=11-20 epithelial cells shedding; 3=ulceration of epithelium; 4=ulceration accompanied with severe crypt destruction), neutrophil and mononuclear cell infiltration (0=none; 1=mild; 2=moderate; 3=severe). Adding up scores for each parameter gives the total pathology score, which can reach a maximum of 16. *C. rodentium* infected mice would elicit a pathology score around 6 to 8; scores greater than 8 indicate profound tissue damage due to infection.

GraphPad Prism5 software was used for statistical analysis of the data. A Kruskal-Wallis test was performed with Dunns post-test to compare all pairs of columns; significant difference between treatments with $p<0.05$.

Results

GOS reduces adherence of *C. rodentium* DBS100 Nal^r to HEp-2 cells

Adherence of *C. rodentium* to HEp-2 cells was assessed in the presence and absence of GOS at concentrations ranging from 10 to 50 mg/ml. Adherence inhibition occurred at all concentrations in a dose-dependent manner. At the lowest dose (10 mg/ml), a one log reduction in adherence was observed. As the GOS concentration increased, adherence was reduced nearly two logs with the highest concentration tested (50 mg/ml) (Fig. 1).

GOS reduces adherence of *C. rodentium* DBS100Nal^r to the colon of CF mice

A GOS tolerance study was performed to evaluate mice tolerance to the different GOS doses, as well as to determine the effective GOS protective dose for subsequent experiments. Mice provided with or without GOS for two weeks were infected with *C. rodentium.* After 10 days, mice were necropsied and adherence was assessed in 3 cm sections from the distal colon. Colon sections were homogenized, serially diluted, and plated in LB-Nal agar for bacterial enumeration. Results revealed that supplementation with GOS reduced adherence of *C. rodentium* to the surface of the proximal colon compared to the control (Fig. 2A). Adherence inhibition was achieved when mice were supplemented with 2,500 mg GOS/kg body weight and 5,000 mg GOS/kg body weight, but not with the lowest dose of GOS tested, 140 mg GOS/kg body weight (Fig. 2A). Shedding of *C. rodentium* in feces was assessed by serially diluting and plating samples on LB-Nal agar. Shedding levels were the same for both the GOS-treated and untreated groups (data not shown).

For the subsequent CF mouse experiment, a dose of 5,000 mg GOS/kg body weight was supplemented to the GOS treated mice. Adherence of *C. rodentium* was assessed in a 3 cm portion of the distal colon. The colon was homogenized, serially diluted and plated in LB-Nal agar for bacterial enumeration. Lower bacterial adherence was observed in the group supplemented with GOS as compared to the infected control (Fig. 2B). No difference in *C. rodentium* shedding in feces was observed between the infected groups (data not shown).

In addition, GOS supplementation resulted in ameliorated colon shortening in the GOS treated group (data not shown).

GOS does not reduce adherence of *C. rodentium* DBS100 Nal^r in GF mice

Mice provided with or without GOS for two weeks were infected with *C. rodentium.* After 10 days, mice were necropsied and adherence was assessed in 3 cm sections from the distal colon. Colon sections were homogenized, serially diluted, and plated in LB-Nal agar for bacterial enumeration. Adherence of *C. rodentium* was not reduced in the GOS supplemented group as compared to the infected control (Fig. 2C). There was no evidence of colon shortening in either the infected control, or the GOS-*C. rodentium* infected group, as compared to the non-treated group.

GOS does not elicit protection in CF mice against *C. rodentium* DBS100Nal^r associated disease

At necropsy, a 0.5 cm section of the distal colon was fixed in 10% formalin, paraffin embedded, and H&E stained. Slides were assessed for lesions associated with *C. rodentium* infection. Parameters used to characterize the severity of disease included sub-mucosal edema, epithelial hyperplasia, epithelial integrity, goblet cell depletion, and neutrophil and mononuclear cell infiltration. The non-infected control groups did not show any signs of disease, as was expected. *C. rodentium*-infected groups showed typical signs of infection. Each parameter was evaluated independently, and significant differences were detected between the infected control and GOS supplemented – infected group. In addition, scores for all parameters were added up to obtain a total pathology score. No significant difference between the two groups was found, indicating

that GOS supplementation does not significantly reduce or prevent lesions caused by *C. rodentium* infection (Fig 3).

Discussion

In this study, we evaluated the ability of GOS to reduce adherence of *C. rodentium* DBS100Nal^r *in vitro* and *in vivo* in CF and GF C57Bl/6 mice. In addition, we assessed the ability of GOS to prevent or mitigate the lesions associated with *C. rodentium* infection in mice.

The results from the *in vitro* adherence experiments indicated that GOS inhibits adherence of *C. rodentium* DBS100Nal^r to HEp-2 cells in a dose dependent manner. These results are consistent with previous studies showing that GOS inhibits adherence of EPEC, *C. sakazakii*, and *S.* Typhimurium to the surface of epithelial cells *in vitro* in a similar dose-dependent manner (Shoaf et al., 2006; Quintero et al. 2011; Searle et al., 2009, 2010). For *C. rodentium*, as well as EPEC, virulence factors are encoded on a LEE pathogenicity island, but in a slightly different arrangement (Deng et al., 2001). Although the *C. rodentium* adherence machinery has not been clearly established, LEE virulence factors are required for infection in CF mice (Kamada et al., 2012).

We next tested for the ability of GOS to reduce adherence *in vivo* using CF and GF mice. In mice, *C. rodentium* is the causative agent of murine colonic hyperplasia. The severity of the disease varies according to mouse strains. In C57Bl/6 mice, the lesions in affected animals include a grossly thickened distal colon, commonly devoid of formed feces. In those cases in which the severity of

disease is increased, the cecum and ileum may also be affected. Microscopically, murine colonic hyperplasia is accompanied by colonic crypt elongation, goblet cell depletion, sub-mucosal edema, and neutrophil and mononuclear cell infiltration. The disease peaks around 7-10 days post-infection, clearing after about 6 weeks (Barthold, 1980; Luperchio and Schauer, 2001; Mundy et al., 2005).

Our results indicated that GOS supplementation significantly reduced *C. rodentium* adherence in the distal colon of CF mice. In addition, a reduction in the gross lesions of the colon, as assessed by colon shortening and lack of fecal pellets, was observed when mice were supplemented with GOS. However, only the highest doses were effective at reducing adherence and causing these pathological effects. Histological analysis of the distal colon of CF flora mice indicated typical signs of *C. rodentium* infection. Epithelial hyperplasia, goblet cell depletion, neutrophil and mononuclear cell infiltration, loss of epithelial cell integrity, and sub-mucosal edema were evident in groups infected with *C. rodentium*. Lesions were scored as previously described, and no protection from disease was evident in mice supplemented with GOS.

The role that the commensal microbiota plays in controlling pathogen colonization has been well documented (Buffie and Pamer, 2013; Kamada et al., 2013). The resident population can restrict pathogen access to host tissue either directly, by production of toxins and bacteriocins, or indirectly, by lowering colonic pH via secretion of short chain fatty acids (SCFA). Collectively, these agents can result in inhibition of other competitors of the same or different bacterial species

(Buffie and Pamer, 2013; Hammami et al., 2013; Tejero-Sariñena et al., 2012). Dietary modulation of the gut microbiota due to consumption of GOS and other prebiotics has been shown to enhance the production of SCFA in the gut (Cummings and Macfarlane, 2007; Cummings et al., 2001). Hence, one mechanism by which GOS can prevent or reduce pathogen colonization, indirectly, is by modulating the gut microbiota, enhancing the production of SCFA, and lowering the environmental pH. Nevertheless, no reduction in *C. rodentium* shedding in CF mice was observed among the infected groups (with and without GOS supplementation), suggesting the reduction in adherence of *C. rodentium* was likely not due to a bactericidal effect mediated by the microbiota.

A GF mouse model was used to assess the effect of GOS on adherence of *C. rodentium* to the mouse colon, in the absence of the gut microbiota. Surprisingly, in contrast to the CF model, GOS did not reduce adherence of *C. rodentium* in the GF mouse colon. Evidently, the commensal microbiota was necessary for GOS to be effective as an anti-adherence agent. One mechanism pathogens employ to overcome colonization resistance is to occupy niches and adhere intimately to regions devoid of commensal bacteria. Thus, for EPEC, EHEC, and *C. rodentium* (Kamada et al., 2012, 2013), intimin, as well as other specialized virulence factors promote colonization by tightly binding to the epithelial cell lining. In the absence of a microbiota, all the niches become available. Thus, expression of adherence factors becomes less critical to the success of the organism.

Moreover, it has been shown that *C. rodentium* colonizes at higher levels in GF mice as compared to CF mice (Kamada et al., 2012), suggesting that in the absence of the gut microbiota, more niches would be open in the GF mice, hence allowing *C. rodentium* to colonize elsewhere, in addition to tightly binding to the epithelial cell lining (Kamada et al., 2012). In addition, avirulent *C. rodentium* strains are known to colonize GF mice, but not CF mice (Kamada et al., 2012), suggesting that a specific niche may not be essential for colonization in the absence of the gut microbiota. Colonization of different tissues relies on the expression of different adhesins by the pathogen, that recognize specific receptors in the chosen binding site (Ofek et al., 2003); different binding sites require the expression of different adhesins. Therefore, in the absence of the microbiota, *C. rodentium* could be expressing other adherence factors targeted for colonization of other available niches, in addition to the expression of the virulence factors carried in the LEE island. The latter are indistinctly expressed in *C. rodentium* colonization of GF or CF mice (Kamada et al., 2012). Hence, to reduce adherence of *C. rodentium* in the GF mouse colon, GOS might not be effective due to the other possible adhesins being expressed for colonization.

Although the exact adherence and colonization mechanisms of *C. rodentium* have not been thoroughly characterized, some factors have been identified that are required for colonization of CF mice (Deng et al., 2003; Kamada et al., 2012; Mundy et al., 2003). Intimin and the translocated intimin receptor (Tir), two important LEE encoded proteins, have been shown to have crucial roles in pedestal formation in A/E lesions. The mechanisms for pedestal formation have

been well established for EPEC and EHEC (Campellone, 2003; DeVinney et al., 2001; Marches et al., 2000). Tir, in addition to its role in pedestal formation, has been shown to be essential for *C. rodentium* colonization of the CF mouse colon (Deng et al., 2003). The *Ler* promoter is responsible for the regulation of most LEE encoded genes, including Tir (Barba et al., 2005; Deng et al., 2001, 2004); Kamada et al., 2012 showed that *C. rodentium ler* mutants were unable to colonize CF mice. Although Tir has been shown to be essential for *C. rodentium* colonization, other LEE-encoded factors might also play a central role. In addition to the importance of LEE for colonization, a non-LEE encoded colonization factor has been characterized for *C. rodentium.* The colonization factor *Citrobacter* (CFC) is a gene cluster encoding a type IV pilus, and has been shown to be required for colonization (Mundy et al., 2003). Hence, it has been suggested that initial colonization of *C. rodentium* of locations other than colonic epithelial cells, could be mediated by a fimbrial adhesin (Wiles et al., 2004). The role of other factors, either LEE or non-LEE encoded, that might be involved in *C. rodentium* adherence and colonization have not been so far elucidated. Moreover, factors used for *C. rodentium* colonization and adherence in GF mice are not clear.

Overall, this study shows that GOS may provide prophylactic protection against *C. rodentium* by reducing adherence of bacteria to epithelial cells *in vitro* as well as *in vivo* in a CF mouse model. Further research is required to elucidate the adherence mechanisms and factors required for *C. rodentium* colonization in GF mice. It is highly likely that GOS targets a specific factor used for adherence by *C. rodentium*, specifically expressed when colonizing CF mice and tissue culture

HEp-2 cells. The difference in adherence mechanisms in GF and CF mice should be further explored. If these are well characterized, other types of non-digestible oligosaccharides could be used to target different adhesins or adherence factors responsible for adherence to and colonization of the colonic epithelial cell lining.

References

Barba, J., Bustamante, V.H., Flores-Valdez, M.A., Deng, W., Finlay, B.B., and Puente, J.L. (2005). A positive regulatory loop controls expression of the locus of enterocyte effacement-encoded regulators *Ler* and *GrlA.* J. Bacteriol. *187*, 7918–7930.

Barthold, S.W. (1980). The microbiology of transmissible murine colonic hyperplasia. Lab. Anim. Sci. *30*, 167–173.

Bishop, A.L., Wiles, S., Dougan, G., and Frankel, G. (2007). Cell attachment properties and infectivity of host-adapted and environmentally adapted *Citrobacter rodentium.* Microbes Infect. *9*, 1316–1324.

Buffie, C.G., and Pamer, E.G. (2013). Microbiota-mediated colonization resistance against intestinal pathogens. Nat. Rev. Immunol. *13*, 790–801.

Campellone, K. (2003). Tails of two Tirs: actin pedestal formation by enteropathogenic *E. coli* and enterohemorrhagic *E. coli* O157:H7. Curr. Opin. Microbiol. *6*, 82–90.

Coppa, G. V, Zampini, L., Galeazzi, T., Facinelli, B., Ferrante, L., Capretti, R., and Orazio, G. (2006). Human milk oligosaccharides inhibit the adhesion to Caco-2 cells of diarrheal pathogens: *Escherichia coli, Vibrio cholerae, and Salmonella fyris*. Pediatr. Res. *59*, 377–382.

Cummings, J.H., and Macfarlane, G.T. (2007). Gastrointestinal effects of prebiotics. Br. J. Nutr. *87*, S145.

Cummings, J.H., Macfarlane, G.T., and Englyst, H.N. (2001). Prebiotic digestion and fermentation. Am. J. Clin. Nutr. *73*, 415S–420S.

Defoirdt, T., Halet, D., Sorgeloos, P., Bossier, P., and Verstraete, W. (2006). Short-chain fatty acids protect gnotobiotic *Artemia franciscana* from pathogenic *Vibrio campbellii*. Aquaculture *261*, 804–808.

Deng, W., Li, Y., Vallance, B.A., and Finlay, B.B. (2001). Locus of enterocyte effacement from *Citrobacter rodentium*: sequence analysis and evidence for horizontal transfer among attaching and effacing pathogens. Infect. Immun. *69*, 6323–6335.

Deng, W., Vallance, B.A., Li, Y., Puente, J.L., and Finlay, B.B. (2003). *Citrobacter rodentium* translocated intimin receptor (Tir) is an essential virulence factor needed for actin condensation, intestinal colonization and colonic hyperplasia in mice. Mol. Microbiol. *48*, 95–115.

Deng, W., Puente, J.L., Gruenheid, S., Li, Y., Vallance, B.A., Vázquez, A., Barba, J., Ibarra, J.A., O'Donnell, P., Metalnikov, P., et al. (2004). Dissecting virulence: systematic and functional analyses of a pathogenicity island. Proc. Natl. Acad. Sci. U. S. A. *101*, 3597–3602.

DeVinney, R., Puente, J.L., Gauthier, A., Goosney, D., and Finlay, B.B. (2001). Enterohaemorrhagic and enteropathogenic *Escherichia coli* use a different Tir-based mechanism for pedestal formation. Mol. Microbiol. *41*, 1445–1458.

Firon, N. (1982). Interaction of mannose-containing oligosaccharides with the fimbrial lectin of *Escherichia coli*. Biochem. Biophys. Res. Commun. *105*, 1426–1432.

Ganan, M., Collins, M., Rastall, R., Hotchkiss, A.T., Chau, H.K., Carrascosa, A. V, and Martinez-Rodriguez, A.J. (2010). Inhibition by pectic oligosaccharides of the invasion of undifferentiated and differentiated Caco-2 cells by *Campylobacter jejuni*. Int. J. Food Microbiol. *137*, 181–185.

Gibson, G.R., and Roberfroid, M.B. (1995). Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J. Nutr. *125*, 1401–1412.

Gibson, D.L., Ma, C., Rosenberger, C.M., Bergstrom, K.S.B., Valdez, Y., Huang, J.T., Khan, M.A., and Vallance, B.A. (2008). Toll-like receptor 2 plays a critical role in maintaining mucosal integrity during *Citrobacter rodentium*-induced colitis. Cell. Microbiol. *10*, 388–403.

Hammami, R., Fernandez, B., Lacroix, C., and Fliss, I. (2013). Anti-infective properties of bacteriocins: an update. Cell. Mol. Life Sci. *70*, 2947–2967.

Van Immerseel, F. (2003). Invasion of *Salmonella enteritidis* in avian intestinal epithelial cells *in vitro* is influenced by short-chain fatty acids. Int. J. Food Microbiol. *85*, 237–248.

Kamada, N., Kim, Y.-G., Sham, H.P., Vallance, B.A., Puente, J.L., Martens, E.C., and Núñez, G. (2012). Regulated virulence controls the ability of a pathogen to compete with the gut microbiota. Science *336*, 1325–1329.

Kamada, N., Chen, G.Y., Inohara, N., and Núñez, G. (2013). Control of pathogens and pathobionts by the gut microbiota. Nat. Immunol. *14*, 685–690.

Kisiela, D., Laskowska, A., Sapeta, A., Kuczkowski, M., Wieliczko, A., and Ugorski, M. (2006). Functional characterization of the FimH adhesin from *Salmonella enterica* serovar Enteritidis. Microbiology *152*, 1337–1346.

Luperchio, S.A., and Schauer, D.B. (2001). Molecular pathogenesis of *Citrobacter rodentium* and transmissible murine colonic hyperplasia. Microbes Infect. *3*, 333–340.

Marches, O., Nougayrede, J.-P., Boullier, S., Mainil, J., Charlier, G., Raymond, I., Pohl, P., Boury, M., De Rycke, J., Milon, A., et al. (2000). Role of Tir and Intimin in the virulence of rabbit enteropathogenic *Escherichia coli* Serotype O103:H2. Infect. Immun. *68*, 2171–2182.

Mundy, R., Pickard, D., Wilson, R.K., Simmons, C.P., Dougan, G., and Frankel, G. (2003). Identification of a novel type IV pilus gene cluster required for

gastrointestinal colonization of *Citrobacter rodentium*. Mol. Microbiol. *48*, 795–809.

Mundy, R., MacDonald, T.T., Dougan, G., Frankel, G., and Wiles, S. (2005). *Citrobacter rodentium* of mice and man. Cell. Microbiol. *7*, 1697–1706.

Mundy, R., Girard, F., FitzGerald, A.J., and Frankel, G. (2006). Comparison of colonization dynamics and pathology of mice infected with enteropathogenic *Escherichia coli*, enterohaemorrhagic *E. coli* and *Citrobacter rodentium*. FEMS Microbiol. Lett. *265*, 126–132.

Neeser, J.R., Koellreutter, B., and Wuersch, P. (1986). Oligomannoside-type glycopeptides inhibiting adhesion of *Escherichia coli* strains mediated by type 1 pili: preparation of potent inhibitors from plant glycoproteins. Infect. Immun. *52*, 428–436.

Nicholson, J.K., Holmes, E., Kinross, J., Burcelin, R., Gibson, G., Jia, W., and Pettersson, S. (2012). Host-gut microbiota metabolic interactions. Science *336*, 1262–1267.

Ofek, I., Hasty, D.L., and Doyle, R.J. (2003). Bacterial adhesion to animal cells and tissues (ASM Press).

Olano-Martin, E., Williams, M.R., Gibson, G.R., and Rastall, R.A. (2003). Pectins and pectic-oligosaccharides inhibit *Escherichia coli* O157:H7 Shiga toxin as

directed towards the human colonic cell line HT29. FEMS Microbiol. Lett. *218*, 101–105.

Quintero, M., Maldonado, M., Perez-Munoz, M., Jimenez, R., Fangman, T., Rupnow, J., Wittke, A., Russell, M., and Hutkins, R. (2011). Adherence inhibition of *Cronobacter sakazakii* to intestinal epithelial cells by prebiotic oligosaccharides. Curr. Microbiol. *62,* 1448-1454.

Quintero-Villegas, M.I., Aam, B.B., Rupnow, J., Sorile, M., Eijsink, V.G.H., and Hutkins, R.W. (2013). Adherence inhibition of enteropathogenic *Escherichia coli* by chitooligosaccharides with specific degrees of acetylation and polymerization. J. Agric. Food Chem.

Roberfroid, M. (2007). Prebiotics: The Concept Revisited. J. Nutr. *137*, 830S–837.

Searle, L.E.J., Best, A., Nunez, A., Salguero, F.J., Johnson, L., Weyer, U., Dugdale, A.H., Cooley, W.A., Carter, B., Jones, G., et al. (2009). A mixture containing galactooligosaccharide, produced by the enzymic activity of *Bifidobacterium bifidum*, reduces *Salmonella enterica* serovar Typhimurium infection in mice. J. Med. Microbiol. *58*, 37–48.

Searle, L.E.J., Cooley, W.A., Jones, G., Nunez, A., Crudgington, B., Weyer, U., Dugdale, A.H., Tzortzis, G., Collins, J.W., Woodward, M.J., et al. (2010). Purified galactooligosaccharide, derived from a mixture produced by the enzymic activity

of *Bifidobacterium bifidum*, reduces *Salmonella enterica* serovar Typhimurium adhesion and invasion *in vitro* and *in vivo*. J. Med. Microbiol. *59*, 1428–1439.

Shoaf, K., Mulvey, G.L., Armstrong, G.D., and Hutkins, R.W. (2006). Prebiotic galactooligosaccharides reduce adherence of enteropathogenic *Escherichia coli* to tissue culture cells. Infect. Immun. *74*, 6920–6928.

Shoaf-Sweeney, K.D., and Hutkins, R.W. (2008). Adherence, anti-adherence, and oligosaccharides: preventing pathogens from sticking to the host. Adv. Food Nutr. Res. 101–161.

Tejero-Sariñena, S., Barlow, J., Costabile, A., Gibson, G.R., and Rowland, I. (2012). *In vitro* evaluation of the antimicrobial activity of a range of probiotics against pathogens: evidence for the effects of organic acids. Anaerobe *18*, 530–538.

Wiles, S., Clare, S., Harker, J., Huett, A., Young, D., Dougan, G., and Frankel, G. (2004). Organ specificity, colonization and clearance dynamics in vivo following oral challenges with the murine pathogen *Citrobacter rodentium*. Cell. Microbiol. *6*, 963–972.

Wong, J.M.W., de Souza, R., Kendall, C.W.C., Emam, A., and Jenkins, D.J.A. (2006). Colonic Health: fermentation and short chain fatty acids. J. Clin. Gastroenterol. *40*, 235–243.

Figure 1 Adherence of *C. rodentium* DBS100nal^{r} to HEp-2 in the presence and absence of GOS. Treatments marked with an (*) are significantly different from the control ($p<0.05$). Treatments with different letters are significantly different from each other ($p<0.05$).

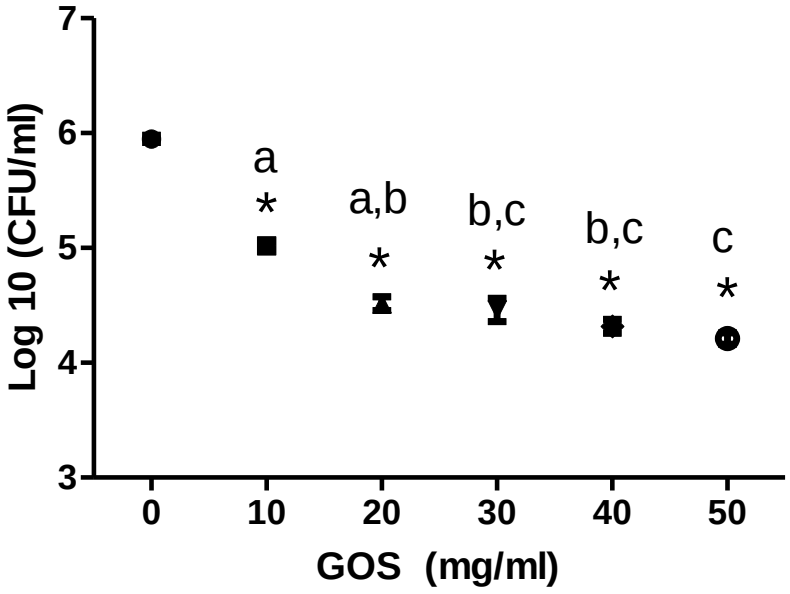
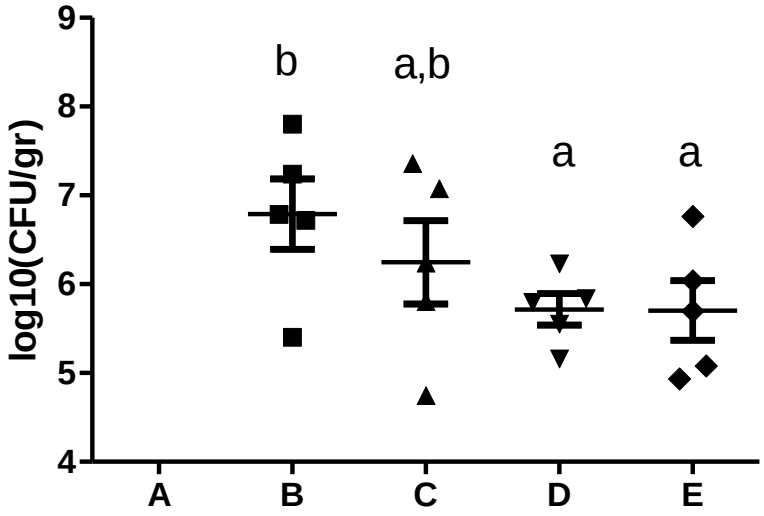
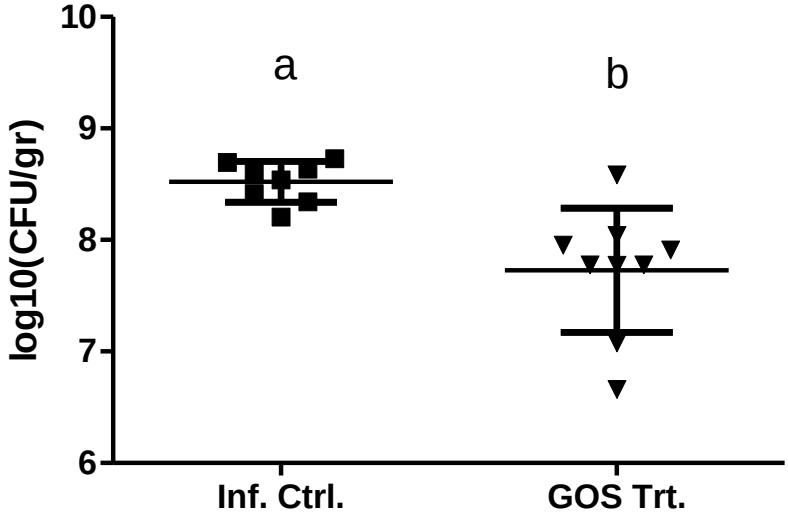

7
6
5
4
3
Log 10 (CFU/ml)
a
*
a,b
*
b,c
*
b,c
*
c
*
0
10
20
30
40
50
GOS (mg/ml)

Figure 2 (A) Adherence of *C. rodentium* DBS100 Nal^r to CF mice distal colon. Mice were supplemented with different doses of GOS and the groups are defined as follows (A) Non-treated group; (B) Infected control; (C) GOS 140 mg/kg body weight (infected); (D) GOS 2500 mg/kg body weight (infected); (E) GOS 5000 mg/kg body weight (infected). Treatments with different letters are significantly different from each other ($p<0.05$). **(B)** Adherence of *C. rodentium* to CF mice distal colon. (GOS treatment: 5000 mg GOS/kg body weight). Groups with different letters are significantly different from each other ($p<0.05$). **(C)** Adherence of *C. rodentium* to GF mice distal colon. (GOS treatment: 5000 mg GOS/kg body weight). Groups with different letters are significantly different from each other ($p<0.05$).

A


log10(CFU/gr)
9
8
7
6
5
4
b
a,b
a
a
A
B
C
D
E

B


log10(CFU/gr)
10
9
8
7
6
a
b
Inf. Ctrl.
GOS Trt.

C

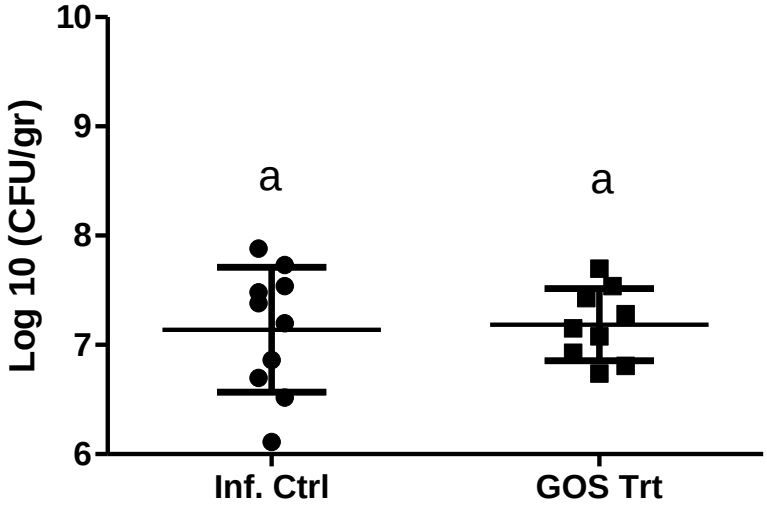
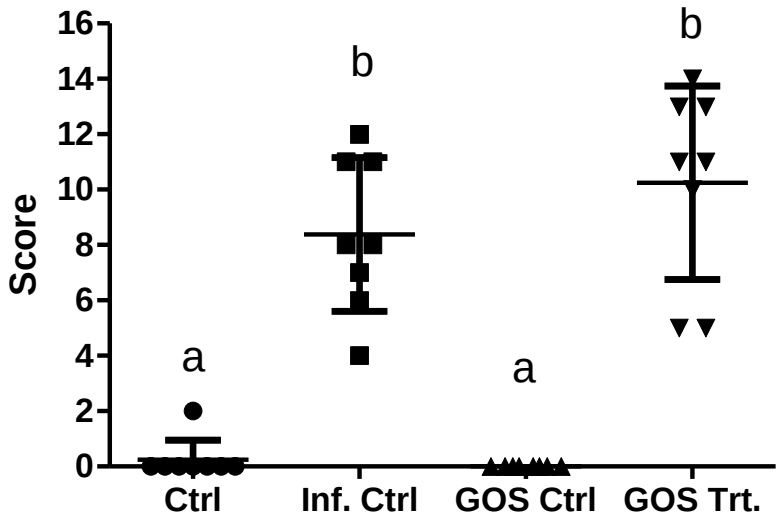


Figure 3 Total histopathological score of CF mice distal colon. Treatments with different letters are significantly different from each other ($p<0.05$).


16
14
12
10
8
6
4
2
0
Score
a
b
a
b
Ctrl
Inf. Ctrl
GOS Ctrl
GOS Trt.

Chapter 5

Biological Properties of Yeast-derived Mannan Oligosaccharides: Antiadherence and Prebiotic Activity

Abstract

Non-digestible oligosaccharides have been shown to have several biological properties that can contribute to host health. Some are classified as prebiotics and stimulate the growth of specific members of the gut microbiota and enhance production of short chain fatty acids (SCFA). In contrast, others are able to inhibit pathogen adherence to the surface of epithelial cells and thereby reduce infection and carriage. Yeast-derived mannan oligosaccharides (MOSy) are widely used in animal feed as health and growth promoters. However, their anti-adherence and prebiotic activities have not been established. In this study, the soluble fraction of MOSy was tested for its ability to reduce adherence of *Vibrio cholerae*, the causative agent of cholera. Anti-adherence experiments were done using HEp-2 cells at different concentrations of MOSy. In addition, MOSy was subjected to *in vitro* digestion and human fecal fermentations to assess their bifidogenic effect and the ability to stimulate the production of short chain fatty acids (SCFA). Results indicated that MOSy inhibited adherence of *V. cholerae* at a minimum concentration of 1 mg/ml in a dose-dependent manner. In addition, after 12 hours of fecal fermentation, growth of bifidobacteria was stimulated, and production of SCFAs (acetate, butyrate and propionate) was increased, compared to a control (no carbohydrate added) fermentation. Acetate was produced in the highest proportion, followed by propionate and then butyrate. This suggests that the soluble fraction of MOSy possesses anti-adherence activity against *V. cholerae* and also has prebiotic activity. Therefore, this material could be used as a prophylactic ingredient to prevent the onset of

cholera in areas where the disease remains endemic, as well as contribute to overall gut health.

Key Words: mannan oligosaccharides, anti-adherence, *Vibrio cholerae*, prebiotic

Introduction

Prebiotic oligosaccharides are defined as selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microflora that confer benefits upon host well-being and health (Gibson et al., 2010). A similar group of substances, referred to as non-digestible oligosaccharides (NDOs), have been shown to have biological properties that confer health benefits on the host, but that do not necessarily meet the definition of a prebiotic. Among the NDOs that have received much commercial and research interests are the mannan oligosaccharides (MOS). Most commercial MOS are derived from yeast cell walls, mainly *Saccharomyces cerevisiae* (Tassinari et al., 2007). They are manufactured and marketed as cell extracts, containing unmodified cell walls, or are partially purified containing primarily MOS and glucans (Spring et al., 2000). The latter are the most common MOS products among the animal feed industry. The process of purification involves yeast cell lysis, followed by centrifugation and isolation of the cell wall components. They are then washed and spray dried (Hill et al., 2008; Spring et al., 2000).

Most of the research on the biological and functional properties of MOS has been done with livestock, including broiler chickens and weanling pigs. Properties attributed to MOS include immune modulation, as well as inhibition of pathogen adherence to the surface of epithelial cells (Heinrichs et al., 2003; Muchmore et al., 1990; Newman et al., 1994; Savage et al., 1996). These activities may therefore provide protection of the host against colonization by

intestinal pathogens in the animals. In addition, MOS supplementation in animal feed has been shown to support overall animal health, including increased feed intake and enhanced growth performance (Davis et al., 2000; Pettigrew, 2000). Despite these biological properties, MOS are not generally fermented by the colonic microbiota of livestock (Fairchild et al., 2001; Flickinger et al., 2003). There is also very little information regarding the ability of the human colonic microbiota to ferment MOS.

Some bacterial adhesins, such as the Type 1 fimbriae produced by *Salmonella* spp. and *Escherichia coli,* are known to be mannose sensitive. Thus in the presence of free mannose, Type 1-mediated adherence to host cell tissues is inhibited (Firon, 1982; Kisiela et al., 2006; Neeser et al., 1986). For example, *in vitro* studies have shown that yeast-derived mannoproteins inhibit adherence of *Campylobacter jejuni* to a Caco-2 tissue culture cell line (Ganan et al., 2009). Moreover, the addition of mannose and its derivatives has been shown to inhibit adherence of *E. coli* to epithelial cells, as well as inhibit adherence and further colonization of the mouse urinary tract (Aronson et al., 1979; Ofek and Beachey, 1978).

Another group of NDO that has been well studied is the galactooligosaccharides (GOS). These are widely used in food applications due to their prebiotic activity. However, in addition to their ability to modulate the gastrointestinal microbiota, GOS have also been shown to reduce adherence of pathogens to epithelial cells *in vitro* and *in vivo* (Quintero et al., 2011; Searle et al., 2009, 2010; Shoaf et al., 2006). In addition, GOS have been shown to inhibit

binding of the *Vibrio cholerae* toxin to its cell surface receptor (Sinclair et al., 2009).

V. cholerae is the causative agent of cholera, an enteric infection caused by the ingestion of water contaminated with the organism (Reidl and Klose, 2002). Cholera is endemic in many parts of the world (Codeco, 2001). The most frequently affected areas generally consist of low income and other vulnerable populations, often following exposure to natural disasters when poor sanitation conditions exist. Although the mechanism by which *V. cholerae* adheres to the surface of cells has not been clearly established, it appears clear that intestinal adherence and colonization are essential steps in the infection process (Jones et al., 1976; Nelson et al., 1976) . Jones and Freter, 1976 proposed that flagella are required for adherence, not necessarily for the bacteria to come in contact with the cells, but due to an adhesin carried in the tip of the flagella. Thus, prophylactic approaches based on preventing or interfering with adhesins necessary for initial adherence may be effective at reducing infections caused by this organism.

In this study, the soluble fraction obtained from a commercial source of yeast cell wall-derived mannan oligosaccharides was tested for its ability to inhibit *V. cholerae* at the surface of tissue culture epithelial cells. In addition, the fraction was characterized for its ability to resist digestion *in vitro* and serve as a substrate during *in vitro* fermentations. Moreover, GOS were also assessed for their ability to reduce adherence of *V. cholerae* to the tissue culture epithelial cells.

Materials and Methods

Organisms and growth conditions *Vibrio cholerae* ATCC 14035 was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Prior to each experiment, frozen stock cultures were plated onto Tryptic Soy Agar (TSA; Difco) and grown overnight at 37°C. A single colony was inoculated into Tryptic Soy Broth (TSB; Difco) and incubated aerobically, at 37°C, without shaking for 18 hours. Cells were harvested by centrifugation (3,184 x g for 8 minutes), washed once with phosphate buffered saline (PBS) pH 7.0, and re-suspended in minimal essential medium (MEM; Hyclone, Logan, Utah), supplemented with 10% fetal bovine serum (FBS; Hyclone). MEM was pre-equilibrated at tissue culture conditions (5% CO_2, 95% relative humidity, 37°C).

Yeast cell wall-derived Mannan oligosaccharides (MOSy) MOSy were obtained from Lallemand (Ontario, Canada) as a powdered material. A stock solution was prepared by mixing 100 mg of powder per ml of distilled water. To separate the soluble fraction, the stock solution was centrifuged (201 x *g* for 10 minutes) and the supernatant was collected, filtered through a 0.4 µm filter, and subsequently freeze dried. Stock solutions from the freeze dried material were prepared as required for adherence experiments. This material contained about 60% protein and 30% carbohydrate.

Galactooligosaccharides (GOS) Purimune GOS were obtained from GTC Nutrition. The powder contained 92% GOS with background sugars including lactose (7%) and glucose and galactose (1%). GOS was prepared as a stock

solution (100 mg/ml) in distilled water and filter sterilized using 0.22 µm membrane filters.

Mannose D-(+)-mannose (Sigma) was prepared as a stock solution at a final concentration of 100 mg/ml in distilled water. The solution was filter sterilized using 0.22 µm membrane filters.

Tissue culture cells HEp-2 cells were obtained from ATCC. Cells were grown in 75 cm^2 tissue culture flasks containing 25 ml of MEM supplemented with 10% FBS in a CO_2 incubator at tissue culture conditions. Confluent HEp-2 cells were harvested by removing MEM and washing the cells once with PBS. Subsequently, 0.5 ml of a 0.25% Trypsin-EDTA solution was added followed by a 10 minute incubation at tissue culture conditions. After incubation, 0.5 ml of FBS were added to inactivate the trypsin. Cells were then seeded onto 12 mm diameter glass coverslips in 24-well tissue culture plates at approximately 3.6×10^5 cells per well, and 500 µl of MEM supplemented with 10% FBS was added to each well. Plates were incubated under tissue culture conditions for about 20 hours prior to the start of each experiment. Cells were checked before the experiment under an inverted microscope to make sure they had reached about 70% confluency.

Adherence Assays *V. cholerae* was grown and harvested as described above. MOSy was mixed with bacterial cultures at a concentration of 10^8 CFU/ml prior to addition to tissue culture cells. Distilled autoclaved water was added as a control instead of MOSy. Bacteria mixed with MOSy were added to the cells and plates

were incubated for 30 minutes at tissue culture conditions. Assays were done in duplicate and replicated 3 times (n=6). ANOVA was used to compare treatments to the control and Tukey's test to compare all treatments using GraphPad Prism5 software.

Quantitative PCR (qPCR). qPCR was used for enumeration of adhered *V. cholerae* cells. Bacterial cells from adherence assays were detached by the addition of 1 ml of 0.1% Triton X-100 for 30 minutes at room temperature. Bacterial cells were harvested and centrifuged for 5 min at 10,000 x *g*, supernatants were discarded, and pellets were resuspended 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.

Species-specific forward (5'-CACCAAGAAGGTGACTTTATTGTG-3') and reverse (5'-GAACTTATAACCACCCGCG-3') primers described by Nandi et al. (Nandi et al., 2000) were used. A standard curve was prepared with known cell counts of *V. cholerae*. DNA was extracted as previously described. The qPCR was performed using a Mastercycler Realplex2 (Eppendorf AG, Hamburg, Germany). Each PCR was conducted in a 25 µl volume. The reaction mixture comprised 11.25 µl of 20x SYBR solution and 2.5-µl Real-MasterMix (5Prime), 0.5 µM of each primer, and 1µl of DNA template. The program consisted of an initial denaturation step at 94°C for 5 min, and 30 cycles of denaturation at 94°C for 30 sec, 64°C for 30 sec for annealing, and 68°C for 30 sec for extension.

The percent (%) inhibition was calculated as the control (adhered *V. cholerae* without MOS) minus the treatment (adhered *V. cholerae* with MOS), divided by the control, times 100. Detection limit was determined and prepared by running DNA samples of known *V. cholerae* concentration. Standard curve was prepared from DNA extracted from *V. cholerae* pure culture at a known concentration.

***In vitro* digestion** Gastric and small intestine digestions were simulated *in vitro* for MOSy as described by (Mishra and Monro, 2009), with modifications. MOSy (1 g) was added to a 50 ml screw cap conical tube and 12 ml of water was added. Digestion was started by adding 400 µl of 10% pepsin (Sigma) dissolved in 50 mM HCl. The pH was adjusted to 2.5 with 1 M HCl. Tubes were placed in a water bath at 37°C, incubated for 30 min, shaking at 130 rpm. Subsequently, 800 µl of 1 M sodium bicarbonate, 2 ml of 0.1 M sodium maleate buffer (pH 6, containing 2.5 mM $CaCl_2$), and 2 ml of 2.5% pancreatin (Sigma) dissolved in sodium maleate buffer were added. The tubes were further mixed for 120 min at 130 rpm. Following digestion, samples were dialyzed (500 molecular weight cutoff) and freeze dried.

***In vitro* fermentation** *In vitro* human fecal fermentations were performed in an anaerobic chamber (Bactron X, Sheldin Manufacturing, Cornelius, OR). First, 150 mg of digested, freeze-dried MOSy (see above) were transferred into 50 ml screw cap Erlenmeyer flasks. Flasks containing inulin or no added carbohydrate were also included as positive and negative controls. Then, 8 ml of Nutrient Basal Media (NBM) (Hartzell et al., 2013) were added to each flask. Fecal samples were collected from volunteers in the Department of Food Science and

Technology at the University of Nebraska – Lincoln and immediately transferred to the anaerobic chamber to prepare the fecal slurry. The subjects had no reported symptoms of gastrointestinal distress or disease and had not used antibiotics for the previous three months. Slurries were prepared by diluting pooled portions from three subjects 1:10 in PBS using a hand blender. Then, 2 ml of fecal slurry were added to each flask. Flasks were incubated at 37°C shaking at 130 rpm. Samples (1 ml) were collected in 1.5 ml microcentrifuge tubes at 0 (baseline), 8, 12, and 24 hours, centrifuged (10,000 x g for 3 min), and frozen at -80°C for microbial analysis.

Bifidobacteria and lactobacilli quantification

DNA was extracted from the samples using the phenol-chloroform method described by Martinez et al. (2009). Incubation times were increased to 30 minutes. Populations of bifidobacteria and lactobacilli were quantified by qPCR as described above. *Bifidobacterium*-specific forward (5' TCGCGTCYGTGTGAAAG 3') and reverse (5' CCACATCCAGCRTCCAC 3') primers were used for detection of bifidobacteria. The amplification program used consisted of an initial denaturation at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 15 sec, annealing at 58°C, and extension at 68°C for 60 sec. Standard curves were prepared using overnight cultures of *Bifidobacterium adolescentis* IVS-1 at known concentrations. *Lactobacillus*-specific forward (5' AGCAGTAGGGAATCTTCCA 3') and reverse (5' ATTYCACCGCTACACATG 3') primers were used for detection of lactobacilli. The amplification program consisted of an initial denaturation at 95°C for 10 min, followed by 40 cycles of

denaturation at 95°C, annealing at 61°C, and extension at 68°C for 60 sec. Standard curves were prepared using overnight cultures of *Lactobacillus reuteri* DSM 17938 (BioGaia, Stockholm, Sweden) at known concentrations.

Short chain fatty acid (SCFA) analysis

Samples (900 µl) were collected after 12 hours of fermentation for SCFA analysis, 100 µl of phosphate buffer (0.8 M, pH 2.0) were added, and immediately frozen at -80°C. For SCFA analysis, samples were prepared, as described by Pollet et al., 2012 with modifications. Briefly, samples were thawed at room temperature, and 400 µl were transferred to a 2 ml microcentrifuge tube containing sulfuric acid (200 µl, 9 M), sodium chloride (~0.16 g), and internal standard (100 µl of 7 mM 2-ethyl butyrate in 2 M KOH). Immediately before injection into the GC, diethyl ether (500 µl) was added to the tubes, mixed well, and centrifuged at 10,000 x g for 3 min. The diethyl ether phase was collected and placed into glass vials. SCFAs were measured by gas chromatography (GC) as described by Hartzell et al., 2013. Standards were injected and response factors for each SCFA relative to 2-ethyl butyric acid were calculated for quantification.

Results

MOSy, but not the mannose monomer, inhibits adherence of *V. cholerae* to HEp-2 cells

Adherence assays were performed using a HEp-2 cell line as previously described. MOSy solutions were prepared from the initial stock in concentrations

ranging from 0.5 to 50 mg/ml. Mannose solutions were prepared from the initial stock in concentrations ranging from 10 to 50 mg/ml. Adherence in the presence and absence of MOSy and mannose was measured by qPCR. Significant adherence inhibition was observed at a minimum concentration of 1 mg/ml MOSy and increased with higher concentrations. No additional inhibition was observed when a concentration of 4 mg/ml was reached as compared with all the higher concentrations tested (Fig. 1). In contrast, the mannose monomer had no effect on adherence of *V. cholerae* to HEp-2 cells (Fig. 2).

GOS does not inhibit adherence of *V. cholerae* to HEp-2 cells

Adherence assays were performed using a HEp-2 cell line as previously described. GOS solutions were prepared from the initial stock in concentrations ranging from 10 to 50 mg/ml. Adherence in the presence and absence of GOS was measured by qPCR. No reduction in adherence of *V. cholerae* was observed in the presence of GOS (Fig. 3).

MOSy increases bifidobacteria levels but not lactobacilli in *in vitro* fermentations

The bifidogenic effect of MOSy was assessed *in vitro* with fecal samples obtained from three different donors. Inulin was used as a positive control. The levels of bifidobacteria were assessed after 8, 12, and 24 hours of fermentation compared to the baseline (0 hours) and the control (no carbohydrate added). Results indicated that 1.5% MOSy significantly stimulated the growth of bifidobacteria in human fecal samples after 12 hours of fermentation as

compared to the baseline and the control (Fig. 4). However, no significant differences were observed at 8 and 24 hours of fermentation (data not shown). *Lactobacillus* levels were also assessed after 8,12, and 24 hours of fermentation and compared to the baseline (time 0) and the control. No significant differences were observed for any of the time points (data not shown).

MOSy increases production of SCFA

SCFA were measured after 12 hours of fermentation. Concentrations of total SCFA (butyrate + acetate + propionate) in samples from the fermentations with added carbohydrate and with the control were determined. The total SCFA production in the presence of inulin (positive control) and MOSy was significantly different from the control. No significant difference in total SCFA production between inulin and MOSy was observed. Significant differences were observed among the different types of SCFAs between inulin and MOSy. Propionate was significantly higher in the MOSy treatment, whereas butyrate was significantly higher in the inulin treatment. No significant differences were observed in the production of acetate with the two carbohydrates (Fig. 5).

Discussion

There is now considerable public health interest in identifying prophylactic strategies to prevent or reduce gastrointestinal infections caused by enteric pathogens. One such approach, using molecular decoys to inhibit pathogen adherence, was proposed more than a decade ago (Cozens and Read, 2012; Sharon and Ofek, 2000, 2002). This is an attractive approach since several

food-grade ingredients, including prebiotics and NDOs, have been shown to inhibit pathogen binding to the surface of intestinal cells *in vitro* and *in vivo*. In these studies, adherence of enteropathogenic *E. coli* (EPEC), *Salmonella* Typhimurium, and *C. sakazakii* was reduced by GOS *in vitro* (Quintero et al., 2011; Searle et al., 2009; Shoaf et al., 2006). In addition, Searle et al., 2009 showed that GOS reduced adherence of *S.* Typhimurium in mice. Moreover, GOS was also shown to prevent binding of cholerae toxin to its receptor in host cells (Sinclair et al., 2009). Other studies have shown that yeast-derived mannoproteins inhibit adherence of *C. jejuni* to a Caco-2 cell line (Ganan et al., 2009) and mannose and its derivatives inhibit adherence of *E. coli* to epithelial cells (Ofek and Beachey, 1978). These studies led us to consider whether MOSy and GOS would be effective at reducing adherence of *V. cholerae*, an enteric pathogen responsible for 3–5 million cases and 100,000–120,000 deaths every year.

In this study, adherence of *V. cholerae* was assessed in the presence of MOSy and mannose, the monomer of MOSy, as well as GOS. Our results show that MOSy inhibits adherence *V. cholerae* to the surface of HEp-2 cells. Interestingly, mannose did not inhibit adherence of *V. cholerae,* suggesting that a special structural conformation of the mannose monomers might be required to inhibit *V. cholerae* adherence. Nevertheless, this is consistent with other reports showing that D-mannose does not reduce adherence of *V. cholerae* to rabbit brush borders (Jones and Freter, 1976). Moreover, our results indicated that GOS does not reduce adherence of *V. cholerae* to the surface of HEp-2 cells.

Interestingly, it has been shown that GOS prevents cholerae toxin from binding to its GMP1 receptor (Sinclair et al., 2009), suggesting that GOS might resemble the structure to which cholerae toxin binds, but not the receptor that *V. cholerae* recognizes for adherence and colonization. In addition to the ability to inhibit adherence, MOSy was also assessed for its prebiotic properties. MOSy was subjected to *in vitro* digestion and human fecal fermentation to determine if it confers a bifidogenic effect. It has been well established that prebiotics, such as inulin, increase levels of bifidobacteria in human fecal fermentations (Rao, 1999; Wang and Gibson, 1993) and *in vivo* in human studies (Gibson et al., 1995; Ramirez-Farias et al., 2009). Nevertheless, the ability of MOSy to stimulate the growth of bifidobacteria *in vitro* with human fecal samples had not yet been assessed. In this study, we demonstrated that 1.5% MOSy stimulates the growth of bifidobacteria, which is one of the bacterial species most commonly targeted in prebiotic research, since they are considered beneficial to gut health (Gibson et al., 2004).

While stimulating the growth of specific members of the gut microbiota, prebiotics or NDOs are fermented mainly to SCFAs, principally acetate, propionate, and butyrate. SCFAs have been shown to be beneficial to the host in multiple ways. They have been shown to enhance colonic metabolism and function by serving as an energy source for the colonic epithelium (Flint et al., 2012). Butyrate has also been suggested to have anti-cancer and anti-inflammatory properties (Flint et al., 2012; Hamer et al., 2008). Therefore, ingredients that stimulate the production of SCFAs could have a positive impact

on gut health and physiology. In this study, we demonstrated that MOSy stimulated the production of total SCFAs (butyrate, propionate, and acetate), suggesting that MOSy consumption as a prebiotic can positively impact gut health.

The amount of each type of SCFA was also analyzed. MOSy stimulated the production of acetate to a greater extent than propionate and butyrate. Acetate has been shown to be released and become available systemically, in contrast to butyrate, which is consumed mainly by the colonic epithelium (Flint et al., 2012), hence their effects are also different. Acetate promotes and increases colonic blood flow and enhances motility in the ileum, while butyrate has been shown to be a preferred energy source for colonocytes and play a role in preventing certain types of colitis (Scheppach, 1994). Almost invariably, acetate reaches the highest concentration among the different SCFAs, and it is known to act as an intermediate that is utilized by butyrate-producing bacterial species in the gut (Louis and Flint, 2009).

Yeast-derived mannan oligosaccharides are commonly used in the animal feed industry to promote health and growth of livestock animals. Besides being used as a health promoter for livestock, MOSy has been shown to have other biological properties, which can be targeted to promote not only animal, but also human health if used as a food ingredient/prebiotic. Prophylactic approaches to prevent or mitigate bacterial infections are warranted, since antibiotic resistance in bacterial pathogens is rapidly increasing and has become a topic of public concern. MOSy could be proposed as a prophylactic agent to prevent or mitigate

V. cholerae infections, although further research is needed to determine if it could also be used to target other bacterial pathogens. In addition, MOSy could be used as a prebiotic to promote gut health and stimulate the growth of beneficial members of the gut microbiota.

References

Aronson, M., Medalia, O., Schori, L., Mirelman, D., Sharon, N., and Ofek, I. (1979). Prevention of colonization of the urinary tract of mice with *Escherichia coli* by blocking of bacterial adherence with methyl-D-mannopyranoside. J. Infect. Dis. *139*, 329–332.

Codeco, C. (2001). Endemic and epidemic dynamics of cholera: the role of the aquatic reservoir. BMC Infect. Dis. *1*, 1.

Cozens, D., and Read, R.C. (2012). Anti-adhesion methods as novel therapeutics for bacterial infections. Expert Rev. Anti. Infect. Ther. *10*, 1457–1468.

Davis, E., Maxwell, C., Kegley, B., Rodas, B. de, Friesen, K., Hellwig, D., Johnson, Z.B., and Kellogg, D.W. (2000). Efficacy of mannan oligosaccharide (Bio-Mos®) addition at two levels of supplemental copper on performance and immunocompetence of early weaned pigs. In Arkansas Animal Science Department Report 1999., (Arkansas Agricultural Experiment Station, University of Arkansas), pp. 15–18.

Fairchild, A., Grimes, J., Jones, F., Wineland, M., Edens, F., and Sefton, A. (2001). Effects of hen age, Bio-Mos, and Flavomycin on poult susceptibility to oral *Escherichia coli* challenge. Poult. Sci. *80*, 562–571.

Firon, N. (1982). Interaction of mannose-containing oligosaccharides with the fimbrial lectin of *Escherichia coli*. Biochem. Biophys. Res. Commun. *105*, 1426–1432.

Flickinger, E.A., Grieshop, C.M., Bauer, L.L., Murphy, M.R., and Fahey, G.C. (2003). *In vitro* fermentation characteristics of selected oligosaccharides by swine fecal microflora. J. Anim. Sci. *81*, 2505–2514.

Flint, H.J., Scott, K.P., Louis, P., and Duncan, S.H. (2012). The role of the gut microbiota in nutrition and health. Nat. Rev. Gastroenterol. Hepatol. *9*, 577–589.

Ganan, M., Carrascosa, A.V., de Pascual-Teresa, S., and Martinez-Rodriguez, A.J. (2009). Inhibition by yeast-derived mannoproteins of adherence to and invasion of Caco-2 cells by *Campylobacter jejuni.* J. Food Prot. *72*, 55-59.

Gibson, G., Scott, K., and Rastall, R. (2010). Dietary prebiotics: Current status and new definition. J. Food Sci. *7*, 1-19.

Gibson, G.R., Beatty, E.R., Wang, X., and Cummings, J.H. (1995). Selective stimulation of bifidobacteria in the human colon by oligofructose and inulin. Gastroenterology *108*, 975–982.

Gibson, G.R., Probert, H.M., Loo, J. Van, Rastall, R.A., and Roberfroid, M.B. (2004). Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. Nutr. Res. Rev. *17*, 259–275.

Hamer, H.M., Jonkers, D., Venema, K., Vanhoutvin, S., Troost, F.J., and Brummer, R.-J. (2008). Review article: the role of butyrate on colonic function. Aliment. Pharmacol. Ther. *27*, 104–119.

Hartzell, A.L., Maldonado-Gómez, M.X., Hutkins, R.W., and Rose, D.J. (2013). Synthesis and *in vitro* digestion and fermentation of acylated inulin. Bioact. Carbohydrates Diet. Fibre *1*, 81–88.

Heinrichs, A.J., Jones, C.M., and Heinrichs, B.S. (2003). Effects of mannan oligosaccharide or antibiotics in neonatal diets on health and growth of dairy calves. J. Dairy Sci. *86*, 4064–4069.

Hill, T.M., Ii, H.G.B., Aldrich, J.M., and Schlotterbeck, R.L. (2008). Oligosaccharides for Dairy Calves. *24*, 460–464.

Jones, G.W., and Freter, R. (1976). Adhesive properties of *Vibrio cholerae*: nature of the interaction with isolated rabbit brush border membranes and human erythrocytes. Infect. Immun. *14*, 240–245.

Jones, G.W., Abrams, G.D., and Freter, R. (1976). Adhesive properties of *Vibrio cholerae*: adhesion to isolated rabbit brush border membranes and hemagglutinating activity. Infect. Immun. *14*, 232–239.

Kisiela, D., Laskowska, A., Sapeta, A., Kuczkowski, M., Wieliczko, A., and Ugorski, M. (2006). Functional characterization of the FimH adhesin from *Salmonella enterica* serovar Enteritidis. Microbiology *152*, 1337–1346.

Louis, P., and Flint, H.J. (2009). Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. FEMS Microbiol. Lett. *294*, 1–8.

Martínez, I., Wallace, G., Zhang, C., Legge, R., Benson, A.K., Carr, T.P., Moriyama, E.N., and Walter, J. (2009). Diet-induced metabolic improvements in a hamster model of hypercholesterolemia are strongly linked to alterations of the gut microbiota. Appl. Environ. Microbiol. *75*, 4175–4184.

Mishra, S., and Monro, J.A. (2009). Digestibility of starch fractions in wholegrain rolled oats. J. Cereal Sci. *50*, 61–66.

Muchmore, A., Sathyamoorthy, N., Decker, J., and Sherblom, A. (1990). Evidence that specific high-mannose oligosaccharides can directly inhibit antigen-driven T-cell responses. J. Leukoc. Biol. *48*, 457–464.

Nandi, B., Nandy, R.K., Mukhopadhyay, S., Nair, G.B., Shimada, T., and Ghose, A.C. (2000). Rapid method for species-specific identification of *Vibrio cholerae* using primers targeted to the gene of outer membrane protein OmpW. J. Clin. Microbiol. *38*, 4145–4151.

Neeser, J.R., Koellreutter, B., and Wuersch, P. (1986). Oligomannoside-type glycopeptides inhibiting adhesion of *Escherichia coli* strains mediated by type 1 pili: preparation of potent inhibitors from plant glycoproteins. Infect. Immun. *52*, 428–436.

Nelson, E.T., Clements, J.D., and Finkelstein, R.A. (1976). *Vibrio cholerae* adherence and colonization in experimental cholera: electron microscopic studies. Infect. Immun. *14*, 527–547.

Newman, K., Lyons, T.P., and Jacques, K.A. (1994). Mannan-oligosaccharides: natural polymers with significant impact on the gastrointestinal microflora and the immune system. 167–174.

Ofek, I., and Beachey, E.H. (1978). Mannose binding and epithelial cell adherence of Escherichia coli. Infect. Immun. *22*, 247–254.

Pettigrew, J.E. (2000). Bio-Mos effects on pig performance: a review. In Biotechnology in the Feed Industry: Proc. Alltech's 16th Symp, T.P. Lyons, and K.A. Jacques, eds. (Loughborough, UK: University Press),.

Pollet, A., Van Craeyveld, V., Van de Wiele, T., Verstraete, W., Delcour, J.A., and Courtin, C.M. (2012). *In vitro* fermentation of arabinoxylan oligosaccharides and low molecular mass arabinoxylans with different structural properties from wheat (*Triticum aestivum L.*) bran and psyllium (*Plantago ovata Forsk*) seed husk. J. Agric. Food Chem. *60*, 946–954.

Quintero, M., Maldonado, M., Perez-Munoz, M., Jimenez, R., Fangman, T., Rupnow, J., Wittke, A., Russell, M., and Hutkins, R. (2011). Adherence inhibition of *Cronobacter sakazakii* to intestinal epithelial cells by prebiotic oligosaccharides. Curr. Microbiol. *62,* 1448-1454.

Ramirez-Farias, C., Slezak, K., Fuller, Z., Duncan, A., Holtrop, G., and Louis, P. (2009). Effect of inulin on the human gut microbiota: stimulation of *Bifidobacterium adolescentis* and *Faecalibacterium prausnitzii.* Br. J. Nutr. *101*, 541–550.

Rao, A. V. (1999). Dose-response effects of inulin and oligofructose on intestinal bifidogenesis effects. J. Nutr. *129*, 1442S–1445S.

Reidl, J., and Klose, K.E. (2002). *Vibrio cholerae* and cholera: out of the water and into the host. FEMS Microbiol. Rev. *26*, 125–139.

Savage, T.F., Cotter, P.F., and Zakrzewska, E.I. (1996). The effect of feeding a mannanoligosaccharide on immunoglobulins, plasma IgA and bile IgA of Wrolstad MW male turkeys. Poult. Sci. Suppl. 1 *75,* 143.

Scheppach, W. (1994). Effects of short chain fatty acids on gut morphology and function. Gut *35*, S35–S38.

Searle, L.E.J., Best, A., Nunez, A., Salguero, F.J., Johnson, L., Weyer, U., Dugdale, A.H., Cooley, W.A., Carter, B., Jones, G., et al. (2009). A mixture containing galactooligosaccharide, produced by the enzymic activity of

Bifidobacterium bifidum, reduces *Salmonella enterica* serovar Typhimurium infection in mice. J. Med. Microbiol. *58*, 37–48.

Searle, L.E.J., Cooley, W.A., Jones, G., Nunez, A., Crudgington, B., Weyer, U., Dugdale, A.H., Tzortzis, G., Collins, J.W., Woodward, M.J., et al. (2010). Purified galactooligosaccharide, derived from a mixture produced by the enzymic activity of *Bifidobacterium bifidum*, reduces *Salmonella enterica* serovar Typhimurium adhesion and invasion *in vitro* and in *vivo*. J. Med. Microbiol. *59*, 1428–1439.

Sharon, N., and Ofek, I. (2000). Safe as mother's milk: Carbohydrates as future anti-adhesion drugs for bacterial diseases. Glycoconjugate J. *17*, 659-654.

Sharon, N., and Ofek, I. (2002). Fighting infectious diseases with inhibitors of microbial adhesion to host tissues. Crit. Rev. Food Sci. Nutr. *42*, 267–272.

Shoaf, K., Mulvey, G.L., Armstrong, G.D., and Hutkins, R.W. (2006). Prebiotic galactooligosaccharides reduce adherence of enteropathogenic *Escherichia coli* to tissue culture cells. Infect. Immun. *74*, 6920–6928.

Sinclair, H.R., de Slegte, J., Gibson, G.R., and Rastall, R.A. (2009). Galactooligosaccharides (GOS) inhibit *Vibrio cholerae* toxin binding to its GM1 receptor. J. Agric. Food Chem. *57*, 3113–3119.

Spring, P., Wenk, C., Dawson, K., and Newman, K. (2000). The effects of dietary mannaoligosaccharides on cecal parameters and the concentrations of enteric

bacteria in the ceca of *Salmonella*-challenged broiler chicks. Poult. Sci. *79*, 205–211.

Tassinari, M., Pasto, L.., Sardi, L., and Andrieu, S. (2007). Effects of mannan oligosaccharides in the diet of beer cattle in the transition period. ”In *Proceedings of the 13th International Congress in Animal Hygiene, Tartu,* Estonia" . *2,* 810–815.

Wang, X., and Gibson, G.R. (1993). Effects of the *in vitro* fermentation of oligofructose and inulin by bacteria growing in the human large intestine. J. Appl. Microbiol. *75*, 373–380.

Figure 1 % adherence inhibition of *V. cholerae* to HEp-2 cells by MOSy. ANOVA with Tukey's post-test was used for statistical analysis (n=6). Treatments marked with an (*) are significantly different from the control. Treatments with different letters are significantly different from each other.

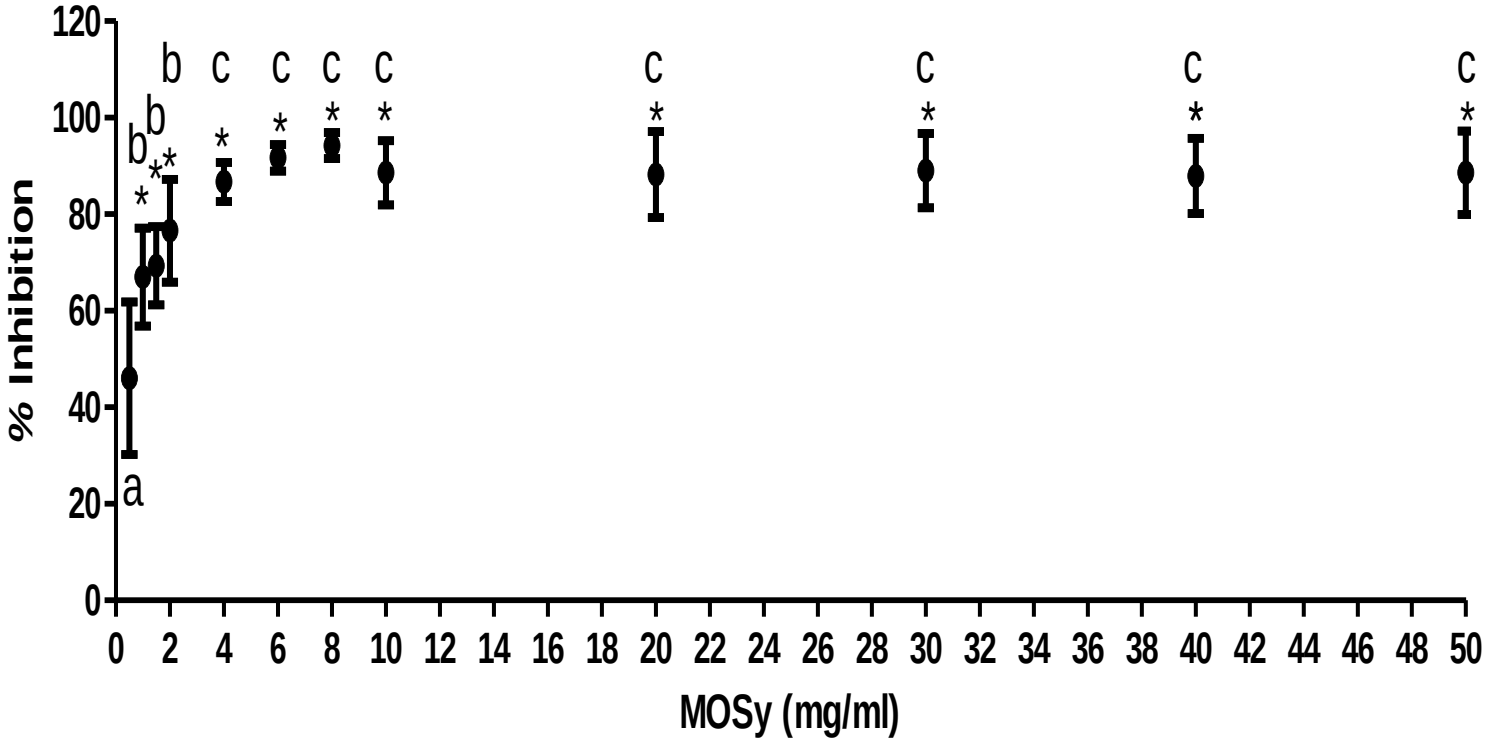

% Inhibition
MOSy (mg/ml)
0
20
40
60
80
100
120
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50
a
b
b
b
c
c
c
c
c
c
c
c
*
*
*
*
*
*
*
*
*
*
*

Figure 2 Adherence of *V. cholerae* to HEp-2 cells in the presence and absence of Mannose. ANOVA with Tukey's post-test was used for statistical analysis (n=6). No treatments differed significantly from the control (α=0.05).

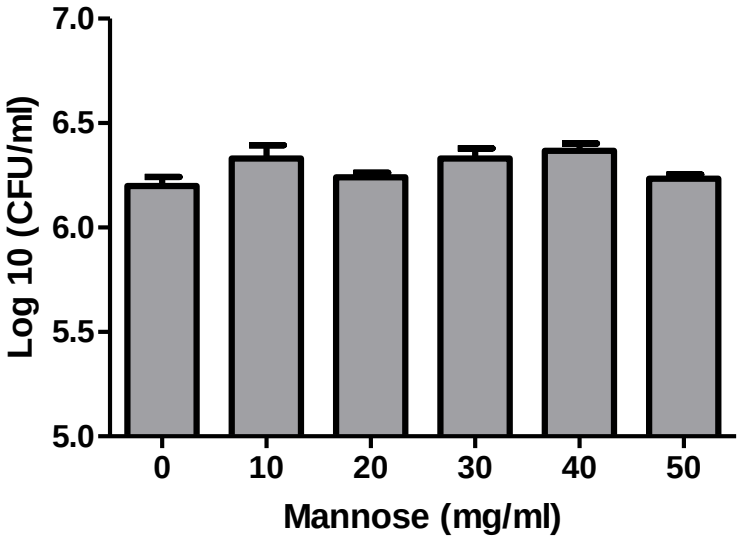

7.0
6.5
6.0
5.5
5.0
Log 10 (CFU/ml)
0
10
20
30
40
50
Mannose (mg/ml)

Figure 3 Adherence of *V. cholerae* to HEp-2 cells in the presence and absence of GOS. ANOVA with Tukey's post-test was used for statistical analysis (n=6). No treatments differed significantly from the control (α=0.05).

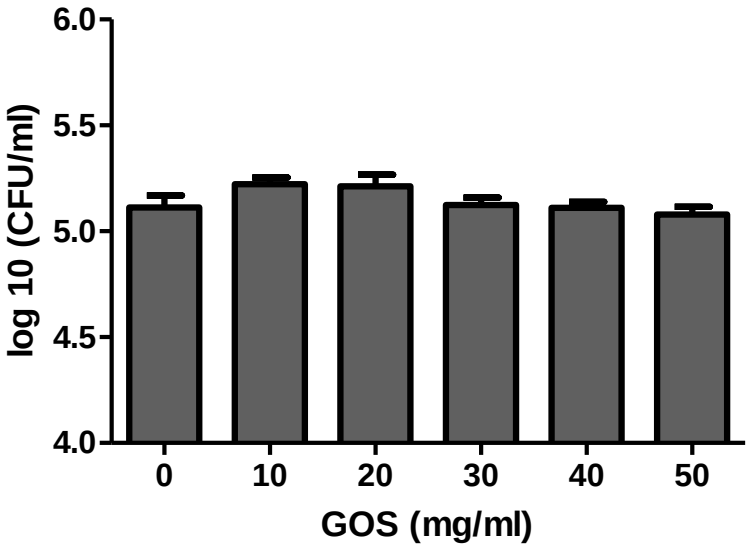

6.0
5.5
5.0
4.5
4.0
log 10 (CFU/ml)
0
10
20
30
40
50
GOS (mg/ml)

Figure 4. Enumeration of bifidobacteria in human fecal *in vitro* fermentation, in the presence of 1.5% inulin (positive control) or 1.5% MOSy or absence of carbohydrate (negative control) after 12 hours of fermentation. ANOVA with Tukey's post-test was used for statistical analysis (n=3). Significant differences from the control are indicated with an *. Treatments with different letter are significantly different from each other (α=0.05).

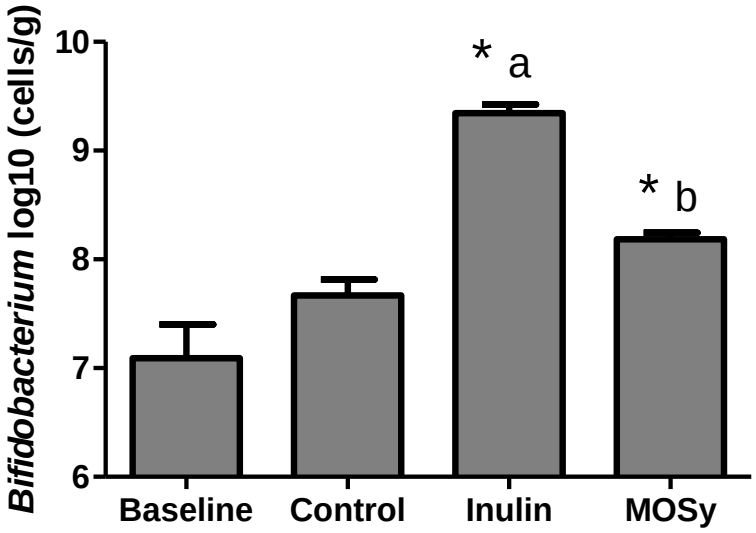
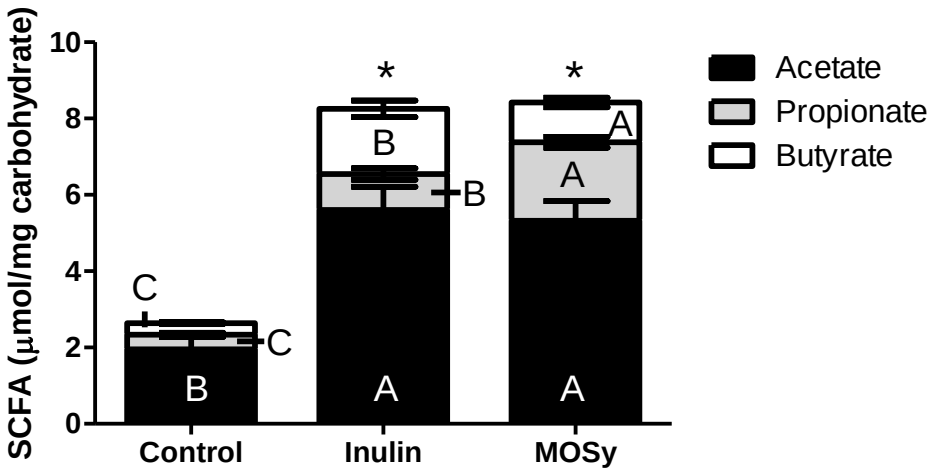

Bifidobacterium log10 (cells/g)
10
9
8
7
6
* a
* b
Baseline
Control
Inulin
MOSy

Figure 5. Analysis of SCFA concentration after 12 hours of *in vitro* human fecal fermentation. Significant differences are shown with different letters within SCFA type. For total SCFA production, samples marked with an (*) are significantly different from the control (n=3, α=0.05).


SCFA (µmol/mg carbohydrate)
10
8
6
4
2
0
Control
Inulin
MOSy
Acetate
Propionate
Butyrate
*
*
A
B
B
C
C
B
A
A

Chapter 6

Conclusions

In this research we established that NDOs reduce adherence of pathogens to epithelial cells. Specifically, CHOS reduced adherence of EPEC to a HEp-2 cell line, as well as lactoferrin reducing adherence of *C. sakazakii.* In addition, MOSy reduced adherence of *V. cholerae* in a dose-dependent manner. Moreover, GOS was shown to reduce adherence of *C. rodentium* to the distal colon of conventional flora mice but not to germ-free mice. The major findings of this research and proposed future experiments are described below.

- Different fractions of CHOS effectively reduced adherence of EPEC to HEp-2 tissue culture cells.
- F_A and DP were not determinant in the ability of CHOS to reduce adherence of EPEC in tissue culture cells.
- An oligomeric conformation is required for CHOS to inhibit EPEC adherence, since the monomers did not exhibit any antiadherence activity.
- Lactoferrin, alone and in combination with a blend of GOS-PDX, reduced adherence of *C. sakazakii* in tissue culture experiments.
- No synergistic effect was observed in reducing adherence of *C. sakazakii* when lactoferrin was mixed with a blend of GOS-PDX.
- GOS reduced adherence of *C. rodentium* to the surface of tissue culture HEp-2 cells at different concentrations.
- GOS reduced adherence of *C. rodentium* to the distal colon of conventional C57Bl/6 mice.

- Supplementation with GOS did not ameliorate lesions caused by *C. rodentium* in conventional C57Bl/6.
- GOS did not reduce adherence of *C. rodentium* to the distal colon of germ free mice.
- MOSy effectively reduced adherence of *V. cholerae* to HEp-2 cells in a dose dependent manner.
- MOSy increased bifidobacteria levels in human fecal *in vitro* fermentations as compared to the control and the baseline.
- MOSy increased the production of SCFA in human fecal *in vitro* fermentations, as compared to the control.
- GOS did not reduce adherence of *V. cholerae* to HEp-2 cells.
- Collectively, these results suggest that NDOs are suitable food ingredients to be used to prevent adherence of enteric pathogens, likely by acting as molecular decoys, hence preventing or mitigating the onset of an infection.

The results from the *in vivo* experiments open further questions on understanding how NDOs inhibit adherence in the presence and absence of the gut microbiota, as well as the mechanisms by which *C. rodentium* causes infection in the two models. The expression of *C. rodentium* adhesins during colonization of germ-free and conventional flora mice requires further study. The differential expression of adhesins allows pathogens to colonize different tissues and different niches that have diverse cellular receptors to which they bind. Determining which adhesins are expressed by *C. rodentium* in the absence of

the gut microbiota, and identifying its target sites, can help target adherence with different NDOs than the ones used in this experiment.