The influence of milk oligosaccharides on host-commensal interactions in the GI tract

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By

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not been submitted for any degree or examination at the National University of Ireland, Galway or elsewhere, and that all the sources I have used or quoted have been indicated and acknowledged by means of complete references.

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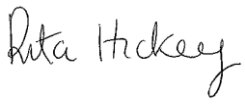
Supervisor 2 Signed: 

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THESIS ABSTRACT

This thesis sets out to explore the influence of milk oligosaccharides on the colonisation of the intestinal microflora and their potential downstream impact upon components of the gastrointestinal tract. Milk oligosaccharides are known to act as prebiotics for the early microflora, but it was not yet known if these molecules possessed alternative bioactivities relating to intestinal adhesion.

Various oligosaccharides present in human and bovine milk were screened for their ability to influence the adhesion of an infant-associated commensal, *Bifidobacterium longum* subsp. *infantis* ATCC 15697, to *in vitro* intestinal models (Chapter II). Screening of individual oligosaccharides identified that exposure to 6'sialyllactose, but not 3'sialyllactose, could promote adhesion to the HT-29 cell line. Interestingly, exposure to a combination of 3'- and 6'-sialyllactose resulted in a dramatic increase in adhesion to the HT-29 cell line. 3'- and 6'-sialyllactose, alone or in combination, increased adhesion to the Caco-2 model, though to a reduced extent. Parameters such as oligosaccharide concentration, duration of oligosaccharide exposure, enzymatic treatments of the bacteria, and a screen of other bacterial strains were used to explore the activity of 6'sialyllactose to promote adhesion to the HT-29 cell line in greater detail. The capability of 6'sialyllactose to induce an adhesive phenotype was unique to *Bifidobacterium longum* subsp. *infantis* ATCC 15697, required a threshold oligosaccharide concentration above 0.5mg/ml, and likely involves a surface protein or combination of proteins.

Following the discovery of the novel bioactivity of 6'sialyllactose, the genetic basis for the induced adhesive-phenotype was investigated. Oligosaccharide exposures were replicated as in the previous *in vitro* adhesion studies. Genetic expression was assessed through the use of whole genome DNA microarray analysis. Exposure to the combination of 3'- and 6'-sialyllactose resulted in both, the highest number of differentially transcribed genes and the greatest magnitude of transcription. Levels of gene transcription correlated with the trend of increased adhesion to the HT-29 cell line. The study identified several genes related to either stress-response or colonising factors (dnaK, groEL, sortase, dps-ferritin), which were further validated through the use of qPCR. Several of the genes have been confirmed as colonising factors in

alternative strains of bacteria. This study demonstrates a connection between exposure to a combination of predominant sialylated milk oligosaccharides and an adaptive colonising response of *B. longum* subsp. *infantis* ATCC 15697.

Milk oligosaccharides have demonstrated activity as both prebiotic and anti-infective molecules. Although human milk contains the highest concentration and greatest diversity of oligosaccharides, bovine milk and dairy whey streams contain several of the common acidic oligosaccharides and represent a scalable source of bioactive oligosaccharides. Bovine milk oligosaccharides (BMO) isolated from dairy whey streams and 6'sialyllactose were tested against an established commercial prebiotic, oligofructose, to assess their safety profile and impact on the microbial communities in the murine gut (Chapter III). Findings indicated that none of the treatments significantly affected markers of probiotic activity via short chain fatty acid production or altered IgA or cytokine profiles. Oligofructose demonstrated mixed activity to alter bacterial family proportions, while BMO and 6'sialyllactose were associated with significantly reduced proportions of bacterial families containing notable pathogens. The findings of chapter III demonstrate the overall safety of oligosaccharide supplementation and their ability to modulate the murine intestinal microbiome, with potential applications in infant formulations and functional foods.

With the ability of milk oligosaccharides to promote the growth and adhesion of bifidobacteria, which are in turn decorated with numerous glycans on their cell walls, it is likely that altering the numbers of bifidobacteria will influence many lectin-mediated interactions in the gastrointestinal tract. Accordingly, a panel of commensal bacteria were screened for their ability to interact with galectin-3, a galactose-binding glycoprotein, which is notably expressed in the epithelial cells of the respiratory and gastrointestinal tract and can influence both metastasis and pathogenic bacterial colonisation (Chapter IV). Surface plasmon resonance was employed for the screening assay, identifying two strains of HMO-consuming commensal bacteria, *B. longum* subsp. *infantis*, which interacted with galectin-3 to a greater extent than the pathogenic positive control. The interaction was further validated and characterized through the use of agglutination and solid-phase binding experiments. The galectin-3-bacteria interaction is mediated through a carbohydrate moiety; however, the entire

galectin-3 protein is required for optimal binding. The results demonstrate for the first time a novel interaction between galectin-3 and commensal bacteria. As the two strains can be influenced by HMOs via increased growth or colonisation, the findings present a potentially novel means to modulate the activity of galectin-3 in the GI tract.

Overall these studies build a strong case for the use of milk oligosaccharides to modulate the intestinal microflora, with bovine milk and dairy whey streams presenting an abundant and yet untapped resource. Future research will aid in further elucidating the intricacies of the oligosaccharide-microflora-GI tract interaction, thereby identifying novel therapeutic targets and means by which to maintain or restore host health.

PUBLICATIONS

1. **Kavanaugh, D.**, Kane, M., Joshi, L. and R. M. Hickey. 2013. Detection of galectin-3 interaction with commensal bacteria. Applied and Environmental Microbiology 79(11):3507-10

2. **Kavanaugh, D.**, O'Callaghan, J, Buttò, L. F., Slattery, H., Lane, J. A. Clyne, M., Kane M., Joshi, L. and R. M. Hickey. 2013. Exposure of *Bifidobacterium longum* subsp. *infantis* to milk oligosaccharides increases adhesion to epithelial cells and induces a substantial transcriptional response. PLoS ONE 8(6): e67224

3. **Kavanaugh, D**., Kane, M., Joshi, L. and R. M. Hickey. 2013. The intestinal glycome and its modulation by diet and nutrition: A review. In preparation

4. **Kavanaugh, D.,** Marques, T., O'Sullivan, O., Slattery, H., McCarthy, R., Kane, M., Mehra, R., Joshi L., Stanton C. and RM Hickey. 2013. Evaluation of bovine milk oligosaccharides versus a commercial prebiotic in mice. In preparation for submission to PlosOne

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6. Lane, J.A., Marino, K., Naughton, J., **Kavanaugh, D.**, Clyne, M., Carrington, S.D., and Hickey, R.M. 2012. Anti-infective bovine colostrum oligosaccharides: *Campylobacter jejuni* as a case study. International Journal of Food Microbiology 157(2):182-8

ABSTRACTS

1. **Kavanaugh, D.,** Slattery, H., Lane, J.A., Joshi, L., Kane, M., Clyne, M., and Hickey, R. M. 'Increased adherence of *Bifidobacterium longum* subsp. *infantis* to HT-29 cells following exposure to a predominant human milk oligosaccharide, 6'sialyllactose.' The 1st International Conference on Glycobiology of Human Milk Oligosaccharides, Copenhagen, Denmark, May 2011

2. **Kavanaugh, D.,** Slattery, H., Lane, J.A., Joshi, L., Kane, M., Clyne, M., and Hickey, R. M. 'Increased adherence of *Bifidobacterium longum* subsp. *infantis* to HT-29 cells following exposure to a predominant human milk oligosaccharide, 6'sialyllactose.' The 4th Annual GlycoScience Ireland Meeting, Teagasc Research Centre, Moorepark, Co. Cork, Ireland, October 2011

ORAL COMMUNICATIONS

1. **Kavanaugh, D.,** Slattery, H., Lane, J.A., Joshi, L., Kane, M., Clyne, M., and Hickey, R. M. 'Increased adherence of *Bifidobacterium longum* subsp. *infantis* to HT-29 cells following exposure to a predominant human milk oligosaccharide, 6'sialyllactose.' The 1st International Conference on Glycobiology of Human Milk Oligosaccharides, Copenhagen, Denmark, **May 16-17, 2011**.

2. **Kavanaugh, D.,** Slattery, H., Lane, J.A., Joshi, L., Kane, M., Clyne, M., and Hickey, R. M. 'Increased adherence of *Bifidobacterium longum* subsp. *infantis* to HT-29 cells following exposure to a predominant human milk oligosaccharide, 6'sialyllactose.' The 5th Annual GlycoScience Ireland Meeting, Galway, Ireland, **October 19, 2012**.

ABBREVIATIONS

ASF – Asialofetuin

B. - *Bifidobacterium*

BMO – Bovine milk oligosaccharide

BSA – Bovine serum albumin

cDNA – Complementary DNA

CFU/mL – Colony-forming units per millilitre

CRD – Carbohydrate recognition domain

DET – Differentially-expressed transcript

E. - *Escherichia*

EDC – 1-ethyl-3-(3-dimethylpropyl)-carbodiimide

EHEC – Enterohemorrhagic *E. coli*

ELISA – Enzyme-linked immunosorbent assay

EPEC – Enteropathogenic *E. coli*

EPS – Exopolysaccharide

FBS – Fetal bovine serum

FOS - Fructooligosaccharide

Fuc – Fucose

FUT2 - α-1,2-fucosyltransferase

Gal-3 – Galectin-3

Gal-3C – Carbohydrate-binding C-terminus of galectin-3

Gal – Galactose

GalNAc – N-acetyl-D-galactosamine

GOS – Galactooligosaccharide

GI tract – Gastrointestinal tract

GlcNAc – N-acetyl-D-glucosamine

HMO – Human milk oligosaccharide

HPAEC-PAD – High pH anion exchange chromatography with pulsed amperometic detection

HPLC – High performance liquid chromatography

IgA – Immunoglobulin A

LAC – Lactose

LacNAc – N-acetyllactosamine

LNB – Lacto-N-biose

LNT – Lacto-N-tetraose

LPS – Lipopolysaccharide

LTA – Lipoteichoic acid

Man – Mannose

MRS – deMan Rogosa Sharpe media

MWCO – Molecular weight cut-off

Neu5Ac – N-acetyl neuraminic acid

NHS – N-Hydroxysuccinimide

OD – Optical density

P95 – Beneo Orafti P95 oligofructose

PBS – Phosphate buffered saline

qPCR – real-time polymerase chain reaction

RU – Resonance unit

3'SL – 3'Sialyllactose

6'SL – 6'Sialyllactose

SCFA – Short chain fatty acid

SCS – Spent culture supernatant

SPR – Surface plasmon resonance

TAMRA – carboxytetramethylrhodamine

TLR – Toll-like receptor

TMB – 3,3',5,5'-tetramethylbenzidine

TNF-α - Tumor necrosis factor-α

VNTR – Variable number of tandem repeats

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For their unwavering source of motivation, support, love and encouragement, I dedicate this thesis to my family.

"Do not go where the path may lead.

Go instead where there is no path and leave a trail"

~ Ralph Waldo Emerson

THESIS INTRODUCTION

The human gut microbiota

The human gastrointestinal tract is a dynamic and complex environment, providing a variable landscape for commensal and pathogenic interaction. The microflora of the human gastrointestinal (GI) tract outnumber the cells of the human host by a factor of 10, with the bacterial population composed of greater than 1000 different species from 40-50 genera (Rajilić-Stojanović, M., Smidt, H., et al. 2007, Savage, D.C. 2005, Zocco, M.A., Ainora, M.E., et al. 2007). Altogether, the number of microbial genes present in the gastrointestinal tract are 100-fold greater than that present in the entire human genome (Bäckhed, F., Ley, R.E., et al. 2005).

The composition of the intestinal microbiota is variable, depending on the location within the GI tract and the stage of host development. The microbial composition changes not only along the length of the GI tract, but also by stratification of the intestinal layers, from the lumen to the mucosal barrier to the epithelial surface, each varying in microbial density. While the stomach and small intestine harbour similar bacterial communities (Hayashi, H., Takahashi, R., et al. 2005), a distinct shift from facultative anaerobes to obligate anaerobes is observed when moving from the stomach towards the distal intestine (Wang, X., Heazlewood, S., et al. 2003). With 10^{11}-10^{12} bacteria per gram of intestinal content, the large intestine is the most densely colonised region in the human body (Whitman, W.B., Coleman, D.C., et al. 1998). The vast majority of these bacteria (~90%) are predominated by two phyla, *Firmicutes* and *Bacteroides*, with *Actinobacteria* representing a sub-dominant group (Eckburg, P.B., Bik, E.M., et al. 2005, Turnbaugh, P.J., Hamady, M., et al. 2008).

The sterile neonatal intestinal tract is initially colonised by *Staphyloccocus*, *Streptococcus*, and *Enterobacteriaceae*, which influence and alter the early intestinal environment, rapidly followed by a transition to obligate anaerobes, including *Bacteroides*, *Clostridium*, and *Bifidobacterium* (Bezirtzoglou, E. 1997). Although there are relatively few different phyla present, the high number of both species and strains in the GI tract creates a high degree of bacterial diversity between individuals (Eckburg, P.B., Bik, E.M., et al. 2005, Qin, J., Li, R., et al. 2010). Despite the wide variability of bacteria observed, the fact that microbial function in healthy individuals is maintained

indicates the presence of a conserved group of necessary functional microbial genes (Qin, J., Li, R., et al. 2010, Tap, J., Mondot, S., et al. 2009).

The intestinal microflora, and accordingly the collective microbiome, differs markedly between neonates, infants and adults, undergoing a significant remodeling during development (Kurokawa, K., Itoh, T., et al. 2007). Factors affecting early intestinal colonisation include the duration of gestation (full-term or preterm), method of delivery (vaginal or caesarean), maternal microbiota, clinical intervention (antibiotics) and early nutrition (human breast milk or infant formula) (Favier, C.F., de Vos, W.M., et al. 2003, Morowitz, M.J., Denef, V.J., et al. 2011, Palmer, C., Bik, E.M., et al. 2007, Schwiertz, A., Gruhl, B., et al. 2003, Vaishampayan, P.A., Kuehl, J.V., et al. 2010).

Breastfeeding and intestinal colonisation

It has been recognized since the early 1900's that breastfeeding is associated with a bifidobacteria-dominant microflora in neonates. Following their discovery in the faeces of healthy breast-fed infants, bifidobacteria were thought to be accountable for lower incidences of infantile diarrhea due to their large numbers (Tissier, H. 1900). During the last thirty years, the concept of a bifidobacteria-dominant microflora in breast-fed infants has been debated and ultimately confirmed with the successive progression of novel techniques and technologies to quantify the gastrointestinal microbiome. The analytical methods include culture-based techniques, 16S rRNA sequencing, and, most recently, metagenomic analyses based on genetic regions outside that of the 16S rRNA gene (Sim, K., Cox, M.J., et al. 2012). Several studies have demonstrated that breastfeeding results in a microflora dominated by bifidobacteria (>60%) at one week of age (Balmer, S. and Wharton, B. 1989, Hudault, S. 1996, Kleessen, B., Bunke, H., et al. 1995, Roberts, A., Chierici, R., et al. 1992, Stark, P.L. and Lee, A. 1982, Yoshioka, H., Iseki, K., et al. 1983), as well as typically lower counts of bacteroides, eubacteria, peptococci, *Veillonella*, clostridia, and enterobacteria when compared to formula-fed neonates (Benno, Y., Sawada, K., et al. 1984, Mevissen-Verhage, E., Marcelis, J., et al. 1987). Contrary to breastfeeding which results in higher numbers of bifidobacteria and lactobacilli (Haarman, M. and Knol, J. 2005, Rinne, M.M., Gueimonde, M., et al. 2005), formula-feeding is associated with higher numbers of bacteroides and *Clostridium coccoides*

(Fallani, M., Young, D., et al. 2010, Rubaltelli, F.F., Biadaioli, R., et al. 1998). The use of culture-independent metagenomic approaches has confirmed that bifidobacteria constitute a large proportion of the faecal microbial population in unweaned infants (Fischer, A., Whiteson, K., et al. 2012, Kurokawa, K., Itoh, T., et al. 2007). Moreover, a recent study comparing the effects of breast milk, cow milk-, or goat milk-based formula on the microbial populations in the faeces of neonates reported a greater abundance of *Bifidobacteriaceae* in the faeces of breast-fed infants (Tannock, G.W., Lawley, B., et al. 2013). The species most commonly isolated from samples obtained from breast-fed or formula-fed infants is *Bifidobacterium breve*, followed by *B. longum* subsp. *infantis*, *B. longum*, and *B. bifidum* (Marteau, P., Pochart, P., et al. 2001). In addition, *B. catenulatum*, *B. adolescentis*, *B. pseudolongum*, and *B. dentium* have been detected though less frequently (Haarman, M. and Knol, J. 2005). In a recent infant study analyzing breast- and formula-fed faecal samples, *B. longum* subsp. *infantis*, *B. breve*, *B. bifidum*, and *B. longum* were detected in samples from all infants, with *B. longum* subsp. *infantis* being the major species found. *B. animalis* and *B. dentium* were not detectable and *B. angulatum* was present in very low numbers. *B. adolescentis*, which is most commonly found in adults, was detected at a low proportion in samples from the formula-fed infants but not at all in infants receiving breast milk (Klaassens, E.S., Boesten, R.J., et al. 2009).

Considering the overall association between breastfeeding and increased numbers and colonisation of bifidobacteria, one could infer a conserved mutualism between the human hosts and these commensal microbes mediated through the provision of breast milk. Breastfeeding results in increased numbers of bifidobacteria and healthier neonates, but the link between bifidobacterial colonisation, neonatal health and the potential mechanisms by which this is achieved require further research.

Probiotic properties associated with bifidobacteria

The individual species that make up the intestinal microflora are the result of rigorous niche-dependent selective pressure. From ingestion, they must survive gastric transit, overcome the physical characteristics of the GI tract, including temperature, pH, and bile salts, and avoid triggering an antimicrobial response by the innate and adaptive immune system of the host (Kailasapathy, K.

and Chin, J. 2000). Just as the host has selected for the microflora, the resident microbes provide necessary functions beneficial to the host, which would otherwise be absent. These beneficial bacteria can be defined as probiotic microorganisms, and satisfy the definition as "live organisms which when administered in adequate amounts confer a health benefit to the host" (FAO/WHO 2006).

From the time of their first discovery, bifidobacteria have been associated with increased benefits to infant health (Tissier, H. 1900). Bifidobacteria confer health benefits to human hosts through many diverse mechanisms. Recent studies have demonstrated a reduction in rotavirus infection, necrotizing enterocolitis, and antibiotic-induced GI tract distress (reviewed in Picard, C., Fioramonti, J., et al. 2005), as well as playing a modulatory role in the immune system of healthy neonates (Arunachalam, K., Gill, H.S., et al. 2000). Furthermore, consumption of bifidobacteria has been reported to exert antagonistic action towards intestinal pathogens, improve lactose utilization (thereby ameliorating lactose intolerance), provide anti-carcinogenic action and control serum cholesterol levels (Gilliland, S.E. 1990, Rahman, M.M., Kim, W.-S., et al. 2009). Bifidobacteria are capable of displacing pathogens in the intestinal environment either directly through niche occupation, the release of short chain fatty acids and the production of anti-bacterial materials (Collado, M.C., Gueimonde, M., et al. 2005, Fukuda, S., Toh, H., et al. 2011, Moroni, O., Kheadr, E., et al. 2006), or indirectly through their influence on the intestinal environment. For instance, higher numbers of bifidobacteria in breast-fed infant stools are associated with a more acidic faecal pH (<6.0), which is likely due to the production of lactic and acetic acids by bifidobacteria, while bottle-fed infants faecal pH is slightly alkaline (>7.0) (Bullen, C., Tearle, P., et al. 1976, Lee, J.-H. and O'Sullivan, D.J. 2010). The combination of low pH and the production of short chain fatty acids has been demonstrated to restrict the growth of pathogens (Van Limpt, C., Crienen, A., et al. 2004), thereby providing a potential link between breastfeeding and reduced pathogen colonisation.

It has been reported that continual microbial stimulation is required for the maturation of a balanced postnatal immune system (Hooper, L.V. 2004). From an immunomodulatory standpoint, colonisation by different commensal bacteria can exert unique effects on the host immune response. Exposure of

caecal epithelial cells to *B. longum* increases production of interferon gamma (IFN-γ), while *B. thetaiotaomicron* increases the production of tumor necrosis factor-α (TNF-α) (Sonnenburg, J.L., Chen, C.T., et al. 2006). Alternatively, a peptide derived from *B. lactis* Bb-12 (pCHWPR), is able to enhance gene expression of *c-myc* (a cellular transcription factor) and interleukin-6 *in vitro* (Mitsuma, T., Odajima, H., et al. 2008). Moreover, in a model of the human colonic microbiota, inoculation with *B. longum* subsp. *infantis* ATCC 15697 reduced colonic LPS concentrations, decreased the pro-inflammatory cytokine, TNF-α, and increased the anti-inflammatory cytokine, IL-4, which may have implications in reducing the overall intestinal inflammatory status (Rodes, L., Khan, A., et al. 2013). As such, surface proteins and carbohydrates of bifidobacteria may interact with both host structures and immune-mediators, potentially acting as mediators and effectors of intestinal immune recognition and activity.

The beneficial effects of bifidobacteria have been investigated in clinical trials examining diarrhea prevention, establishment of a healthy microflora in pre-term infants, colon regularity and lactose tolerance, cholesterol reduction, and immunostimulation (Lee, J.-H. and O'Sullivan, D.J. 2010), though translation of beneficial effects from *in vitro* studies to clinical studies do not always prove successful.

Bifidobacteria colonisation

In order for a host to consistently benefit from probiotic bacteria, they must either colonise and persist or be continuously ingested. The first step in bacterial colonisation of the gut is adherence to the intestinal surface; otherwise, colonization is transient, as the bulk of the inoculating bacteria are shed in the faeces (Simmering, R. and Blaut, M. 2001). The GI tract represents a gauntlet for bacteria, testing their ability to adapt to varying environments and available receptors, chemical and digestive hazards, and the host's innate immune system (Marteau, P., Minekus, M., et al. 1997). In return, the successful and adaptive microorganisms benefit from specific niches and nutritional resources. One of the beneficial attributes associated with probiotic bacteria, and specifically bifidobacteria, is the ability to antagonize pathogens through competitive

exclusion (Collado, M.C., Gueimonde, M., et al. 2005). Sites of potential competition include the intestinal lumen, mucosal layer, or even an exposed epithelial layer resulting from injury or disease state. Accordingly, the ability to compete for and interact with appropriate intestinal receptors is a very important characteristic of probiotic bacteria.

To date, the majority of studies focussing on bacterial adhesion have centred around pathogens (McSweegan, E. and Walker, R.I. 1986, Vallance, B. and Finlay, B. 2000, Weinstein, D.L., O'Neill, B.L., et al. 1998). To facilitate adhesion, microbes generally rely on adhesion to carbohydrates via lectins, protein-protein interactions, or hydrophobic interactions (Ofek, I., Hasty, D.L., et al. 2003). Early studies in this field have demonstrated bifidobacterial adhesion to intestinal models and their antagonism of pathogens, however, the mechanisms of adherence or responsible structures are typically unknown (Bernet, M.-F., Brassart, D., et al. 1993, Collado, M.C., Gueimonde, M., et al. 2005). Strains of *B. adolescentis* have been shown to bind extracellular matrix proteins in a manner which is inhibited by periodate treatment of the mammalian cell components or the presence of galactose, indicating the involvement of a bacterial surface lectin in adhesion (Mukai, T., Toba, T., et al. 1997). Recently, *B. longum* subsp. *infantis* ATCC 15697 was found to express Family 1 solute-binding proteins, which exhibit a preference for blood group and mucin oligosaccharides, and, in this manner, interact with intestinal epithelial cells *in vitro* (Garrido, D., Kim, J.H., et al. 2011).

The investigation of the role of protein-protein adhesion in intestinal colonisation is perhaps best explored with regard to bifidobacteria. Bernet *et al.* (1993) first demonstrated that the adhesion of *B. breve* to an *in vitro* model (Caco-2 cell line) was reliant on a surface-expressed and released proteinaceous component (Bernet, M.-F., Brassart, D., et al. 1993). Guglielmetti *et al.* were the first to identify the lipoprotein, BopA, expressed in *B. bifidum* MIMBb75 which is directly involved in the binding of bifidobacteria to Caco-2 cells (Guglielmetti, S., Tamagnini, I., et al. 2008). Furthermore, dnaK and enolase have been demonstrated to participate in adhesion (Candela, M., Centanni, M., et al. 2010), with enolase in particular binding to human plasminogen. Proteins believed to be cytoplasmic in origin have been found to be 'moonlighting' or performing additional functions at the bacterial cell surface and may also participate in host

adhesion. A recent study of the outer membrane of *B. animalis* subsp. *lactis* BB-12 identified the presence of 15 proteins capable of interacting with host epithelial cells or extracellular matrix proteins (Gilad, O., Svensson, B., et al. 2011).

Knowledge on the contribution of lipoteichoic acid to hydrophobic interactions with host cells is currently limited in bifidobacteria (Op den Camp, H.J., Oosterhof, A., et al. 1985), although emerging studies have identified the involvement of conserved cell surface pilus-like structures (tad type IVb pili) to be important in the ability of bifidobacteria to colonise host tissues (Foroni, E., Serafini, F., et al. 2011, O'Connell-Motherway, M., Zomer, A., et al. 2011, Turroni, F., Serafini, F., et al. 2013). It is clear from the results of numerous *in vitro* and *in vivo* studies that bifidobacteria possess the capacity to adapt to and colonise the human GI tract employing a plethora of adhesion mechanisms, often in a multifactorial manner.

Breast milk selectively promotes growth and colonisation of bifidobacteria in the GI tract.

The ingestion of maternal breast milk as a source of initial infant nutrition is conserved among mammals. The composition of milk has evolved to provide a survival advantage to newborns. In simplest terms, breast milk, through its various components, is a source of nutrition, immunity, developmental cues, and selective media guiding intestinal colonisation (Sela, D.A. and Mills, D.A. 2010). Human milk contains several components which negatively select against pathogens. For example, lysozyme and lactoferrin, separately, are bacteriostatic against a range of pathogens, but are bacteriocidal when used in combination against several Gram-negative strains, including *Clostridium butyricum*, *Vibrio cholera*, *Salmonella typhimurium*, and *Escherichia coli* (Ellison, R. and Giehl, T. 1991, Jenssen, H. and Hancock, R.E. 2009, Rockova, S., Rada, V., et al. 2011). Conversely, lactoferrin has been demonstrated to promote the growth of specific strains of bifidobacteria in culture. The growth promotion was dependent on the presence of an identified lactoferrin receptor in those strains (Kim, W.-S., Ohashi, M., et al. 2004, Rahman, M.M., Kim, W.S., et al. 2009). Alternatively, bifidogenic peptides have been produced through the peptic digestion of lactoferrin and soluble immunoglobulin. Two such peptides have been isolated

from lactoferrin. The peptides were active at low concentration, demonstrating an *in vitro* growth effect ~100 times stronger than that of *N*-acetyl-glucosamine (GlcNAc), a known bifidobacterial growth substrate found in human milk (Lönnerdal, B. 2003).

A characteristic common to the majority of bioactive molecules in milk is that they are glycosylated. Glycans in human milk can be encountered in their free form as human milk oligosaccharides (HMO) or when linked to proteins or lipids through glycosidic bonds. While glycans provide resistance against proteolytic digestion and may act as recognition molecules for interactions, one of their principal functions is the promotion of commensal colonisation by acting as prebiotics in the breast-fed infant (Scholtens, P.A., Oozeer, R., et al. 2012). Human milk contains between 5 and 23 g/L of oligosaccharides, with over 200 different HMO structures, which differ in their size, charge, and sequence (Zivkovic, A.M., German, J.B., et al. 2010). Due to their structural properties and complexity, HMOs are largely resistant to enzymatic digestion and arrive in the distal intestine relatively unaltered (Dallas, D.C., Sela, D., et al. 2012, Engfer, M.B., Stahl, B., et al. 2000, Gnoth, M.J., Kunz, C., et al. 2000). For this reason, it is believed that HMOs could potentially be enriched in the distal colon (Sela, D.A., Li, Y., et al. 2011). HMOs are structurally similar to the glycan moieties present on human intestinal epithelial surfaces used as attachment sites for pathogens. As a result, pathogens bind the soluble decoy receptor (HMO) rather than the host ligand, ultimately leading to expulsion in the faeces (Morrow, A.L., Ruiz-Palacios, G.M., et al. 2005, Newburg, D.S. 1999).

The high concentrations of HMO and conjugated oligosaccharides in milk are believed to be the main contributors to the predominance of *Bifidobacterium* species in the infant gut (Garrido, D., Dallas, D.C., et al. 2013, Scholtens, P.A., Oozeer, R., et al. 2012). In 1974, it was first determined that the 'bifidus factor' in human milk responsible for increased growth of bifidobacteria spp. is composed of oligosaccharides (Gyorgy, P., Norris, R.F., et al. 1954). Although HMO are typically referred to as 'bifidogenic', this term is technically incorrect due to the fact that not all bifidobacteria can effectively utilize HMO as a carbon source (Bode, L. 2012), as the ability to consume HMO is strain-dependent (Ward, R.E., Ninonuevo, M., et al. 2007). Examples of bifidobacteria with different oligosaccharide utilisation abilities are numerous; *B. longum*

subsp. *longum* (DJO10A and NCC2705) have retained the genes responsible for the digestion and usage of plant-based carbohydrate sources, while lacking the requisite genes for the efficient metabolism of and growth on complex HMOs (LoCascio, R.G., Desai, P., et al. 2010). *B. longum* and *B. breve* are typically dominant in infant faeces (Avershina, E., Storrø, O., et al. 2013), yet *B. longum* subsp. *longum* ATCC 15707 and *B. breve* ATCC 15700 show only modest growth on pooled HMOs (Asakuma, S., Hatakeyama, E., et al. 2011, LoCascio, R.G., Ninonuevo, M.R., et al. 2007), and were shown to only metabolise lacto-N-tetraose (LNT), of which, lacto-N-biose (LNB) is a building block. Interestingly, the Lacto-N-biose / Galacto-N-biose (LNB/GNB) gene cluster is conserved among all infant-gut associated bifidobacteria, with several strains of *B. breve* and *B. longum* demonstrating growth on LNB as the sole carbon source (Xiao, J.-z., Takahashi, S., et al. 2010).

Of those bacteria which can effectively digest HMOs, *B. longum* subsp. *infantis* ATCC 15697 is the gold standard. Ward *et al.* reported the ability of *B. longum* subsp. *infantis* ATCC 15697 to effectively use HMOs as a carbon source for growth (Ward, R.E., Ninonuevo, M., et al. 2006). As a result of whole genome sequencing, it was revealed that this particular strain of bacteria possessed a 43-kb gene cluster dedicated to the binding, import and metabolism of HMO (Sela, D.A., Chapman, J., et al. 2008). Putative HMO-utilization genetic loci are conserved across *B. longum* subsp. *infantis* strains with the HMO-consumer phenotype, while differing in *B. longum* subsp. *longum* strains with the HMO-non-consumer phenotype (LoCascio, R.G., Desai, P., et al. 2010).

Despite the fact that both *B. longum* subsp. *infantis* and *B. bifidum* display high levels of HMO consumption, they employ different catabolic pathways for HMO utilisation. *B. bifidum* expresses several extracellular glycosylhydrolases in order to digest HMO into smaller components prior to import, while *B. longum* subsp. *infantis* first imports the oligosaccharides, followed by the intracellular deglycosylation of the HMO components (Garrido, D., Barile, D., et al. 2012). Recent studies have further characterised the repertoire of glycan-cleaving enzymes associated with *B. longum* subsp. *infantis* ATCC 15697, identifying two genes encoding α-sialidases, five α-fucosidases, five β-galactosidases, and three β-N-acetylglucosaminidases (Garrido, D., Barile,

D., et al. 2012), several of which are up-regulated during growth on HMO (Sela, D.A., Garrido, D., et al. 2012). The provision of complex oligosaccharides through milk creates an ideal and unique nutrient niche for the establishment of, and colonisation by, *B. longum* subsp. *infantis* strains (LoCascio, R.G., Desai, P., et al. 2010).

The genome sequences of bifidobacteria confirm that these micro-organisms are highly adapted to the intestinal environment (Schell, M.A., Karmirantzou, M., et al. 2002), and the genomes of infant-associated bifidobacteria have been strongly influenced by complex carbohydrates (Sela, D.A. and Mills, D.A. 2010). Recent studies have demonstrated that growth of bifidobacteria in human milk or on pools of milk oligosaccharides results in increased expression of putative type II glycoprotein binding fimbriae and increased adhesion to *in vitro* intestinal cell models (Chichlowski, M., De Lartigue, G., et al. 2012, Gonzalez, R., Klaassens, E.S., et al. 2008).

Based upon these findings, the work described in this thesis assessed milk oligosaccharides of human and bovine origin for bioactivity with regards to the adhesive capabilities *B. longum* subsp. *infantis* ATCC 15697 using *in vitro* intestinal models, examined the safety profile and effects of supplemented milk oligosaccharides on the intestinal microbiome in mice, and investigated the potential of enriched bifidobacterial populations to interact with important structural components of the GI tract.

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CHAPTER I

LITERATURE REVIEW

The intestinal glycome and its modulation by nutritional and microbial factors: A review.

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CHAPTER I

ABSTRACT

The human gastrointestinal (GI) epithelium is responsible for adequate digestion and absorption of nutrients, as well providing an immunological interface and highly selective environment which facilitates colonisation by commensal bacteria and prohibits the entry, adhesion and invasion of pathogenic agents. The physical barrier that is maintained by the epithelial lining is reinforced by the presence of the intestinal glycome which is defined as the vast array of sugar structures and glycoconjugates expressed by cells of the GI tract. Aberrant epithelial glycosylation can be associated with altered responses to enteric infections, as well as immune dysregulation. Intestinal glycosylation is a dynamic process and is susceptible to alteration by genetic, physiological and pathological state, in addition to modification by nutritional and environmental stimuli. The involvement of nutritional influences upon glycan expression and topology is of particular importance in intestinal barrier reinforcement and homeostasis. For instance, milk contains factors which can alter intestinal glycosylation, which in turn contributes to the early immune development and maturation of the intestinal tract of the newborn. The present review will focus on the glycosylation status of intestinal cells and the means by which nutritional factors modulate the expression of intestinal glycans.

CHAPTER I

INTRODUCTION

The mucosal surface of the GI tract is the largest body surface (200-300 m^2) in contact with the external environment (Lievin-Le Moal, V. and Servin, A.L. 2006). The intestinal glycome comprises the total population of sugars and glycoconjugates associated with the intestinal epithelium and is associated with three distinct tiers of the intestinal mucosa; a layer of epithelial cells one cell thick overlaid with a layer of mucus. Firstly, the overlying mucus layer consists of water and extensive polymers of secretory mucins, as well as secreted proteins and immunoglobulins, such as trefoil factors and defensins and provides a barrier between the mucosal epithelium and the lumen of the GI tract and its contents. Additionally, toll-like receptors (TLRs), which exhibit specificity to carbohydrate ligands, sample the mucosal microbial population through the epithelium and modulate the host response to enteric pathogens and parasites (reviewed in Moncada, D.M., Kammanadiminti, S.J., et al. 2003). The intestinal mucus layer thus represents a highly-selective physico-chemical sensor layer that restricts the degree of contact between the external environment and the host epithelium. Secondly, covering the surface of the intestinal epithelium and partially integrated with the overlying mucus gel is the epithelial glycocalyx (Hattrup, C.L. and Gendler, S.J. 2008), of which membrane-anchored cell-surface mucin glycoproteins are a major constituent. Lastly, the surface of the intestinal epithelium is abundant with protein- and lipid-glycoconjugates which are integral components of the apical brush border membrane. As glycan moieties constitute an essential component of the intestinal mucosa, the intestinal glycome has a significant impact upon mucosal and intestinal barrier integrity and function, resistance to pathogen adhesion / colonisation, and the provision of site-specific niche environments to beneficial bacteria (Linden, S.K., Sutton, P., et al. 2008). The aim of the present review is to highlight the necessity of the intestinal glycome, to explore means by which dietary and microbial factors can modulate the intestinal glycome, and ultimately, facilitate improvements in host health through manipulation of the mucosal glycome.

CHAPTER I

GASTROINTESTINAL GLYCOME

The glycome is defined as the comprehensive array of complex sugar structures expressed by cells and tissues (van Kooyk, Y. and Rabinovich, G.A. 2008). Glycans decorate the surface of most living cells and organisms, creating a complex landscape of recognition sites and barriers generally representing the first point of contact at the interface of the biotic and abiotic environment of a cell. As such, they are integrally involved in immunological recognition, cell–cell adhesion, and defence against pathogen attack (Angeloni, S., Ridet, J.L., et al. 2005). The vital nature of the host glycome may be inferred from the observation that a substantial fraction (1-2 %) of animal genes encode glycosyltransferases, glycosidases and sugar transporters that function in glycan biosynthesis and modification (Bishop, J.R. and Gagneux, P. 2007). Unlike proteins that are coded by genes and generally ribosomally-synthesised, glycan moieties are synthesized by enzymes in an "assembly-line"-like system of glycan synthesis pathways (reviewed in Lowe, J.B. and Marth, J.D. 2003). These sequential synthesis pathways allow cells and organisms to make rapid phenotypic changes based on modification of their protein glycoconjugates.

As reviewed by Moran *et al.* (2011), the classification of mammalian glycoconjugates is based upon whether the glycan moiety is attached to a protein or lipid. In the case of glycoproteins, they can be designated as either N- or O-linked. N-glycosylation is characterized by the attachment of oligosaccharides to asparagine residues when the amino acid is present in the Asn-X-Ser / Thr consensus motif, (where X represents any amino acid, with the exception of proline). N-linked glycosylation involves several attachment steps in the endoplasmic reticulum and the Golgi complex, wherein the glycan additions primarily consist of N-acetyl-D-glucosamine (GlcNAc), L-fucose (Fuc), D-galactose (Gal), D-mannose (Man), and sialic acid (N-acetyl neuraminic acid – Neu5Ac). In contrast, O-glycosylation is limited to the Golgi complex and commences with the enzymatic addition of a single N-acetyl-D-galactosamine (GalNAc) residue on Ser/Thr sites of folded proteins. Typically, O-glycosylated chains are shorter than their N-linked counterparts but consist of the same monosaccharides. Both, secreted and cell-surface-bound mucins are examples of O-glycosylation (Perez-Vilar, J. and Hill, R.L. 1999). Proteoglycans are a

subclass of glycoproteins and are composed of a protein backbone with a number of repeating acidic disaccharide units attached. The disaccharide units (glycosaminoglycans) are made up of hexosamine and a hexuronic acid or hexose creating iduronic acid or glucuronic acid, respectively (Bartlett, A.H. and Park, P.W. 2010). Glycolipids contain glycan chains where the lipid ceramide acts as the attachment site. Chain commencement and elongation begins with either a glucose or Gal, creating either a glucosylceramide or a galactosylceramide (Hakomori, S. 2003).

Secreted glycoproteins present on the apical surface (e.g. mucus and digestive fluid constituents) and the basolateral surface (e.g. signalling molecules and extracellular matrix constituents), contribute to the array of intestinal glycoconjugates (Nanthakumar, N.N., Dai, D., et al. 2005). Moreover, the intestinal cell glycome or glycocalyx is highly variable from tissue to tissue. For instance, the glycocalyx of human intestinal microvilli tips is relatively thick (100 – 500 nm), while the glycocalyx of the lateral microvilli surface is thinner by comparison (30 – 60 nm), (Ito, S. 1969, Soler, M., Desplat-Jego, S., et al. 1998).

The common expression of species-specific glycans, distributed throughout host cells and secretions, provides a plethora of suitable receptor targets that may be used by microorganisms for host recognition, attachment, and invasion. The microbial ligands that interact with these glycans include adhesins, pili, fimbriae and hemagglutinins (Gilboa-Garber, N. and Garber, N. 1989, Wadstrom, T. and Ljungh, A. 1999). The presence of microbes in the intestine requires a finely balanced host environment that will favour the establishment of beneficial commensal bacteria without also facilitating colonisation by pathogens. It is of immense benefit to the host to possess a means by which to rapidly adapt to challenge by pathogens, while maintaining a hospitable environment for beneficial organisms. Accordingly, the mucosal barriers (both secreted and adherent) are continuously renewed and can therefore be modified rapidly in response to alterations in the environment (Linden, S.K., Sutton, P., et al. 2008). The oligosaccharide moieties of the glycocalyx are highly diverse, with a rapid average turnover time of 6-12 hours for the human jejunal glycocalyx (Madara, J.L., Trier, J. 1987). Recent murine studies have demonstrated a distinct separation of an inner, firmly-adherent mucus layer from

an overlying, loosely-adherent layer (Johansson, M.E., Larsson, J.M., et al. 2011). Following intraperitoneal injection of an O-glycan analogue in a murine model, mucosal incorporation and luminal presence of the tagged glycan has been observed as early as three hours from goblet cells in the luminal surface epithelium and after 6-8 hours in the goblet cells of the crypt epithelium. Even more interesting is the fact that the time between luminal emergence of a labelled mucin glycoprotein in the inner layer and its even distribution throughout the inner layer was just one hour, indicating an extremely rapid turnover timeframe for the tightly adherent inner mucus layer (Johansson, M.E.V. 2012).

2.1 *Mucins*

Mucins play a vital role in the interaction between microbes and epithelial surfaces. They act in microbial trophism, the presentation of ligands which may obstruct binding or aid in colonisation (Gonzalez-Rodriguez, I., Sanchez, B., et al. 2012), and the provision of nutritional and metabolic sources for commensal microorganisms (Ruas-Madiedo, P., Gueimonde, M., et al. 2008). There are currently 23 identified members of the mucin gene family (Table 1) which can be divided into three distinct subfamilies: (1) cell-surface mucins, which possess a membrane spanning domain, (2) secreted, gel-forming mucins and (3) secreted, non-gel-forming mucins (MUC7) (reviewed in Dharmani P, S.V., Kissoon-Singh V, Chadee K 2009, Linden, S.K., Sutton, P., et al. 2008). As reviewed by Moran *et al*, (2011), each mucin is composed of a filamentous protein with hundreds of variable glycan moieties which can be envisioned to have a ‘bottle-brush’ likeness. As a heterogeneous family of large intricate glycoproteins, the epithelial mucins express an abundant array of O-linked oligosaccharides, often contributing to over 70% of their mass. The expression profile of mucins varies between tissues within the gastrointestinal tract, which displays the highest and most diverse levels of mucin expression in the body. Cell-surface mucins are present on the apical membrane of all mucosal epithelial cells and possess large extracellular VNTR (variable number of tandem repeats) domains, which contain repeating amino-acid sequences containing serine and threonine residues responsible for the anchoring of glycans. These repeats vary in size and number between mucins, while substantial differences in individual

mucin variability exists due to VNTR polymorphisms, allowing for considerable inter-individual differences in mucin size (Moran, A.P., Gupta, A., et al. 2011).

Secreted mucins are the sole contributor to the formation of the mucus gel. These polymeric glycoproteins are synthesized and secreted in the intestine and colon (goblet cells), and the stomach (mucus cells). Mucins are characterized by one or more domains that are rich in the amino acids serine, threonine, and proline and are referred to as mucin or PTS domains. The mucin domain serves as a scaffold for O-linked glycans, which bind water and may interact with both, exogenous and endogenous lectins. The physical properties of mucins, including their rigidity, resistance to proteases and gel-forming capability, are reliant upon glycosylation patterns (reviewed in Moncada, D.M., Kammanadiminti, S.J., et al. 2003). The dense oligosaccharide clusters decorating the mucin protein are responsible for their proteolytic resistance and a stiff and extended conformation (Lang, T., Alexandersson, M., et al. 2004).

Table 1. Membrane-attached and secreted, gel-forming mucins.

MUC Gene	Species	Tissue Localization
Membrane-bound Mucins		
MUC1	H, R, M	Lung, cornea, salivary glands, esophagus, stomach, pancreas, large intestine, breast, prostate, ovary, kidney, uterus, cervix
MUC3A	H, R, M	Thymus, small intestine, colon, kidney
MUC3B	H, R, M	Small intestine, colon
MUC4	H, R, M	Lung, cornea, salivary glands, esophagus, small intestine, kidney
MUC10	R, M	Submandibular glands, testis
MUC11	H, R, M	Lung, middle ear, thymus, small intestine, pancreas, colon, liver, kidney, uterus, prostate
MUC12	H, R, M	Middle ear, pancreas, colon, uterus, prostate
MUC13	H, R, M	Lung, conjunctiva, stomach, small intestine, colon, kidney
MUC14	H, R, M	Ovary
MUC15	H, R, M	Conjunctiva, tonsils, thymus, lymph node, breast, small intestine, colon, liver, spleen, prostate, ovary, leukocytes, bone marrow
MUC16	H, R, M	Conjunctiva, ovary
MUC17	H, R, M	Intestinal cells, conjunctival epithelium
MUC18	H, R, M	Prostate
MUC20	H, R, M	Lung, liver, kidney, colon, placenta, prostate
MUC21	H, M	Lung, large intestine, thymus, testis
Secreted Mucins		
MUC2	H, R, M	Lung, conjunctiva, ear, stomach, small intestine, colon, nasopharynx, prostate
MUC5AC	H, R, M	Lung, conjunctiva, middle ear, stomach, gall bladder, nasopharynx
MUC5B	H, R, M	Lung, middle ear, sublingual gland, larynx, submucosal glands, esophageal glands, stomach, duodenum, gall bladder, nasopharynx
MUC6	H, R, M	Stomach, duodenum, gall bladder, pancreas, kidney
MUC7	H, R, M	Lung, lachrymal glands, salivary glands, nose
MUC8	H, R, M	Oviduct
MUC9	H, R, M	Submandibular glands
MUC19	H, R, M	Lung, salivary gland, kidney, liver colon, placenta, prostate
H = Human; M = Mouse; R = Rat		

2.2 *Glycolipids*

Beneath the luminal secreted mucus layer is a cell-surface layer rich in carbohydrates, the glycocalyx, which is characteristic of enterocytes (Madara, J.L., Trier, J.S., et al. 1994). While the mucus layer is composed of glycoproteins, electrolytes, enzymes, and water, the principal constituents of the glycocalyx are glycoproteins, proteoglycans, and glycolipids (Esko, J.D. 1999, Frey, A., Giannasca, K.T., et al. 1996, Kovbasnjuk, O.N. and Spring, K.R. 2000, Rambourg, A. and Leblond, C.P. 1967, Ugolev, A.M., Smirnova, L.F., et al. 1979). Glycolipids are prominent in the membrane of epithelial cells lining the small intestine, constituting up to 25% of their membrane mass (Forstner, G.G. and Wherrett, J.R. 1973). Furthermore, the glycolipids confer unique structural properties and offer specific recognition sites on the cell surface (Morita, A., Miura, S., et al. 1986). The attached glycan moieties of glycolipids act as receptors or binding sites for a number of endogenous ligands (including oncodevelopmental antigens and hormones), as well as exogenous ligands (viral hemagglutinins, bacterial toxins, and microbial adhesins) (reviewed in Dai, D., Nanthkumar, N.N., et al. 2000, Etizione, A. 1996, Falk, P.G., Hooper, L.V., et al. 1998, Hooper, L.V., Xu, J., et al. 1999, Morita, A., Miura, S., et al. 1986). Accordingly, it has been demonstrated that some glycolipids, and their attached glycan moieties, are often necessary in the process of infection or the development of responses to toxins (Mouricout, M. 1997), while others may provide a source of nutrients for the commensal microflora (reviewed in Dai, D., Nanthkumar, N.N., et al. 2000, Mukai, T., Kaneko, S., et al. 1998, Walker, W.A. 2000). Given their wide variety of biological functions, any deviations in the composition or distribution of membrane glycolipids may have adverse effects on cell growth and differentiation, pathogenic adhesion, and the colonising ability of commensal bacteria.

2.3 *Glycan moieties*

The common classes of oligosaccharides found on cell surface glycoproteins are defined by the nature of their linkage to the non-carbohydrate component, (Moncada, D.M., Kammanadiminti, S.J., et al. 2003) Many of the general properties of mucin glycoproteins are attributable to glycosylation,

including high-charge density from sialic acid and sulfate residues, protease resistance and water holding capacity (hydration). The addition of sulfate and O-acetyl-substituted sialic acid to terminal mucin oligosaccharides confers additional resistance to degradation by glycosidases (Corfield, A.P., Wagner, S.A., et al. 1992). By way of these properties, the oligosaccharides serve to protect the protein core from proteases, functioning to preserve the integrity of the mucin polymer.

Mucin glycosylation patterns and distribution have been examined along both the fetal and adult GI tract. The O-linked glycans of intestinal mucins isolated from blood type Le^b adult kidney donors during autopsy demonstrated that the adult intestinal tract exhibits an increasing gradient of sialic acid expression when going from the ileum to the colon, with an opposing gradient of fucose along the tract (Robbe, C., Capon, C., et al. 2003). The small intestine was found to possess highly fucosylated glycans, which were primarily based on core 4 structures, GlcNAc-(β1-3)[GlcNAc(β1-6)]GalNAc while the distal colon contained the sulfo-Le^x determinant containing core 2 glycans, Gal(β1-3)[GlcNAc(β1-6)]-GalNAc. Finally, blood group A and H determinants were found solely in the ileum and cecum while Sd^a/Cad related epitopes increased along the length of the colon (Robbe, C., Capon, C., et al. 2004). Later studies confirmed the presence of nearly 120 glycans along the fetal GI tract, primarily attached to core 2 structures. The majority of the structures were acidic, containing sialic acid residues (α-2,6 linked to N-acetylgalactosaminitol) as well as sulphate residues (3-linked to Gal or 6-linked to GlcNAc). Interestingly, no significant regional differences in glycosylation patterns were observed along the GI tract and the expression of the major oligosaccharides throughout was within the same order of magnitude (Robbe-Masselot, C., Maes, E., et al. 2009). These findings provide evidence that the region-specific expression and distribution of glycan moieties is obtained during the transitional period to adulthood. Interestingly, studies of colonic biopsies have demonstrated that MUC2 O-glycans in the sigmoid colon are uniform in all humans, with the exception of individuals diagnosed with colonic inflammation (Larsson, J.M.H., Karlsson, H., et al. 2009).

Terminal glycan moieties are responsible for the creation of histo-blood-group antigens, resulting in the A, B, H, Lewis-a (Le^a), Lewis-b (Le^b), Lewis-x

(Le^x), Lewis-y (Le^y), and sialyl-Le^a and sialyl-Le^x structures. GalNAc serves to initiate the attachment of the carbohydrate moieties via serine and threonine residues. Elongation is mediated through attachment of core-structures, followed by a backbone region. These chains typically terminate in Fuc, GalNAc, Gal, or sialic acid residues, with these terminal sugars creating the histo-blood-group antigens. Further sulfation of GalNAc and Gal residues results in increased diversification (Ho, J.J., Jaituni, R.S., et al. 2003, Kui Wong, N., Easton, R.L., et al. 2003, McCool, D.J., Okada, Y., et al. 1999). In addition, the host genome contributes to the glycosyltransferases present, which in turn impacts the resultant expression of mucin glycan moieties (Oriol, R., Le Pendu, J., et al. 1986). A contributor to the diversity of the blood group epitopes is an α-1,2-fucosyltransferase, the FUT2 enzyme, which determines 'secretor status'. The addition of terminal α-1,2-fucose residues produces the H type 1 epitope. Inactivating mutations of this gene affect ~20% of the population, in turn, impacting the expression of glycan epitopes on the cell surface and in mucosal secretions, and thus influencing interaction with the various lectins of luminal microorganisms and host immune cells (Magalhães, A., Gomes, J., et al. 2009). These variations allow the host to cope with adaptive pathogens, and interestingly account for the histo-blood-group specific susceptibility to particular pathogens (Marionneau, S., Cailleau-Thomas, A., et al. 2001).

CHAPTER I

FUNCTIONS OF THE GASTROINTESTINAL GLYCOME

Glycans exhibit a wide range of structural and functional diversity and play a critical role in a variety of biological processes. Glycosylation is an integral component in both mucin barrier activity and the development and moderation of an immune response (reviewed in Linden, S.K., Sutton, P., et al. 2008, van Kooyk, Y. and Rabinovich, G.A. 2008). The highly hydrated mucus components covering the mucosal surface serve to protect the underlying epithelial cells from environmental and endogenous factors (enzymatic, chemical, and mechanical) which may cause injury, while also functioning as a first point of contact and, if necessary, a defensive barrier against pathogenic microorganisms (Linden, S.K., Sutton, P., et al. 2008).

The diversity and magnitude of glycan decoration of the GI mucins play crucial roles in their ability to resist enzymatic degradation, the provision of region-specific bacterial niches, modulation of intestinal immune response, and their function as releasable pathogen decoy ligands. The importance of mucin-type O-glycans was highlighted in a murine model in which the O-glycosylation of core-1 structures was abolished leading to the development of colitis analogous to that of humans (Fu, J., Wei, B., et al. 2011). Furthermore, inflammatory disease states (Crohn's disease and ulcerative colitis) and cancer / tumorigenesis are associated which alterations in mucin glycosylation (Campbell, B.J., Finnie, I., et al. 1995, Corfield, A.P., Myerscough, N., et al. 1996). Tissue-specific expression of glycosyltransferases results in a site-specific display of glycans which can function as adhesion sites and nutritional sources for both commensal and pathogenic microorganisms. The ability of a microorganism to colonise a specific region will depend upon its repertoire of adhesins and glycosidases, thereby accounting for the creation of intestinal niches (Robbe, C., Capon, C., et al. 2004). As an example, *Entamoeba histolytica* relies upon a Gal-specific lectin for attachment to terminal Gal and N-acetylgalactosamine residues on colonic mucins (Belley, A., Keller, K., et al. 1999), while conversely, the presence of α-1,4-linked *N*-acetylglucosamine is an antimicrobial glycan epitope which inhibits the colonisation of *H. pylori* in deeper regions of the gastric mucosa (Kawakubo, M., Ito, Y., et al. 2004).

Another important factor affecting colonisation and susceptibility to pathogens, as mentioned, is the expression of blood-group type glycan epitopes. Expression of particular blood group antigens, as well as secretor status, is associated with differential susceptibility to a range of pathogens (Anstee, D.J. 2010). *Helicobacter pylori* expresses the BabA adhesin with specificity for Le^b or H glycotypes, meaning that blood group O secretors are particularly vulnerable to infection (Aspholm-Hurtig, M., Dailide, G., et al. 2004), while blood group A and B secretors are less vulnerable due to shielding of their Le^b and H epitopes by α-1,3-linked GalNAc or Gal, respectively (Kościelak, J. 2012). Additionally, secretor status contributes to pathogen susceptibility, whereby inactivation of the *FUT2* gene results in a 'non-secretor' phenotype and is associated with a lower susceptibility risk to pathogens including Norwalk virus (Lindesmith, L., Moe, C., et al. 2003), *Campylobacter jejuni* (Ruiz-Palacios, G.M., Cervantes, L.E., et al. 2003), and *Helicobacter pylori* (Ikehara, Y., Nishihara, S., et al. 2001).

Many immune factors depend on glycosylation for proper function (de Kivit, S., Kraneveld, A.D., et al. 2011). The migration of differentiated eosinophils across the intestinal epithelium is dependent upon the activity of L-selectin and PSGL-1 which recognize the sialyl Lewis x glycan epitope (Michail, S., Mezoff, E., et al. 2005). As such, *H. pylori* can affect the expression of sialyl Lewis x, and consequently influence the host immune response.

Lastly, pathogenic lectin binding sites (terminal glycans) of secreted mucins are similar to those on the underlying epithelium, acting as decoy binding sites, thereby preventing attachment of pathogens to the epithelial surface (Carlstedt-Duke, B. 1989). This method of competitive inhibition defends the host epithelium from pathogenic microorganisms, while permitting the colonisation of the commensal microbiota. The host is able to vary its glycan expression in response to pathogenic infection through the elimination or incorporation of a novel structure. If the glycan alteration does not result in the loss of an essential endogenous function, this adaptation may result in a survival benefit conferred to the host. In vertebrates, the modification of glycan moieties of glycosaminoglycan chains present on proteoglycans can create novel ligand

binding sites, while maintaining a distinction from bacterial attempts at mimicry (Esko, J.D. and Lindahl, U. 2001).

Taken together, these diverse functions of glycosylation demonstrate their necessity in the maintenance of a healthy and balanced gastrointestinal environment. The remainder of the review will explore the ability of dietary and microbial factors to influence the GI glycome (Fig. 1).

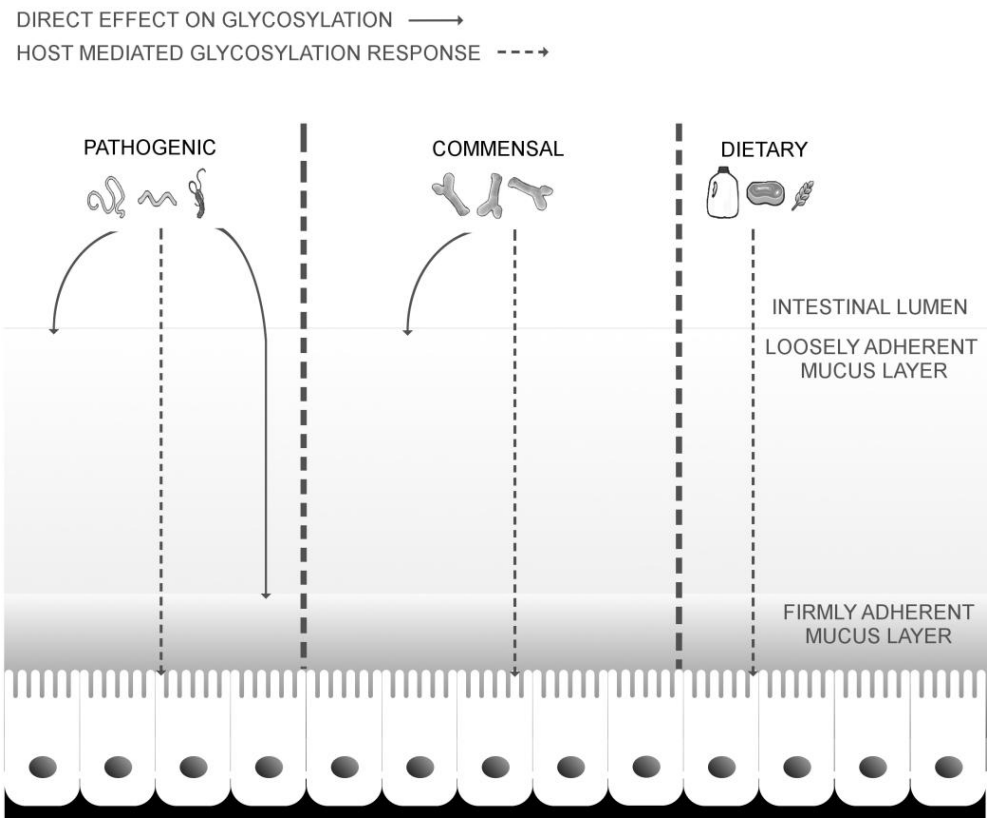

Fig. 1. The epithelial cells of GI tract are protected by an overlying mucosal layer which varies in thickness and composition along its length. Glycosylation of the mucosal constituents and the epithelial surface structures play an important role in several host processes, including infection, colonisation, and immunity. The abilities of dietary and microbial factors to influence the GI glycome are site-specific and are illustrated above.

CHAPTER I

DIETARY MODIFICATION OF THE GASTROINTESTINAL GLYCOME

Nutritional intake may influence most organs, but it is the epithelium of the gastrointestinal tract that has the most prolonged exposure to minimally digested components, and accordingly, the most significant response to variations in nutrient intake (Sanderson, I.R. 1998). Numerous factors influence the ontogeny of the intestinal glycome, with maternally-provided nutrition playing a large role, as well as other endogenous and extrinsic factors following weaning. While the extent of GI glycosylation is mainly dependent on the availability of glycosyltransferases, another rate-limiting factor can be the bioavailability of donor substrates for inclusion into glycan chains. Cellular levels of glycoprotein substrates (nucleotide-sugars) are controlled by their production and degradation, transport through cellular compartments, and the uptake and availability of dietary precursor sugars through the gut (Freeze, H. 1999). Consequently, nutritional studies have identified pathways in which diet and its individual components can regulate GI glycosylation through alterations in glycosyltransferase expression and substrate availability. In addition to the direct action of food components on the intestinal glycome, the presence of the intestinal microflora adds a further layer of complexity to the interaction between nutrients and the intestinal epithelial cells because changes to the microbiota induced by nutrition can in turn modulate the epithelial cells through microbial action. While there is a large body of research on the local or immediate effects of factors affecting glycosylation, there have been few studies relating the biological significance of GI glycan alterations with overall host health. The following section will address the influence of nutrition on the development and maintenance of the GI glycome.

4.1 *Parturition to weaning: development of the neonatal glycome*

Breast milk is considered to be the gold-standard of nutrition for neonates. Its composition varies over the course of lactation, beginning with colostrum for 3-5 days, transitional milk until the second or third week of life, and stabilising in diversity thereafter. Breast milk contains factors ranging in

function from immunological support and protection to nutrition and development (Hassiotou, F. and Geddes, D. 2012).

After lactose and lipids, oligosaccharides are the third largest solid component of human milk, with concentrations ranging from up to 50 g/L or more in colostrum to an average of 10-15 g/L in mature milk (reviewed in Kunz, C., Rodriguez-Palmero, M., et al. 1999, Kunz, C. and Rudloff, S. 2008). Interestingly, although human milk contains such a large amount of this molecule class, infants are unable to digest these milk components, (Engfer, M.B., Stahl, B., et al. 2000, Gnoth, M.J., Kunz, C., et al. 2000). They reach the colon intact where they stimulate the growth of bifidobacteria (Roger, L.C., Costabile, A., et al. 2010, Ward, R.E., Niñonuevo, M., et al. 2007). The remaining oligosaccharides which are not used in the colon are excreted in the faeces (Coppa, G., Pierani, P., et al. 2001) and to a lesser extent, in the urine (Rudloff, S., Pohlentz, G., et al. 2012). Given their abundance, it became clear that human milk oligosaccharides are likely to have specific biological functions, one of which may be the ability to modify the intestinal glycome (Bode, L. 2006).

Human studies addressing the subject of glycome modification are inherently invasive, which likely accounts for the absence of research in this particular area. Consequently, much of the research in this area has used animal models. While animal models may not always be a good representation of the human intestine, there is a general consensus among animal models that maturation of the GI glycome is associated with a shift in the predominant form of terminal glycosylation from sialylation to fucosylation (Bry, L., Falk, P.G., et al. 1996, Ohwada, S. and Suzuki, H. 1992, Shub, M., Pang, K., et al. 1983, Walker, W. 1999). If this conserved shift in GI glycosylation is representative of a mature glycan signature along the GI tract and a benchmark of intestinal health, then significant divergences in glycan expression and/or presentation would likely be detrimental to the organism as a whole.

In a porcine model, the effects of normal suckling were compared against the administration of formula for twenty-one days, at which point, the piglets were culled and colonic mucins were examined. The results indicate that sow-fed piglets had higher amounts of fucose (48%) and glucosamine (22%) than the artificially-fed piglets (Turck, D., Feste, A.S., et al. 1993). These findings

highlight the importance of maternal milk on GI maturation during the early developmental period. The findings of Jaswal *et al.* (1990) suggest that the importance of maternal milk may stem from its nutritional content as malnutrition imposed upon rat pups through restricted access to suckle subsequently resulted in an increase in sialic acid content and a reduced incorporation of ^{14}C-GlcNAc, ^{14}C-Man and ^{3}H-Fuc into membranes compared with controls (Jaswal, V.M., Babbar, H.S., et al. 1990a). Whether malnutrition results in reduced substrate availability or altered expression / activity of glycosyltransferases is unknown. However, glycan expression of GlcNAc and Man could be restored following administration of insulin or cortisone, indicating their altered expression did not result from reduced substrate availability, while the mechanistic effects on Fuc remain to be elucidated.

A recent study by Duncan *et al*., investigated the effects of milk oligosaccharides on the sialic acid utilisation and synthesis pathways in rat neonates. Notably, the level of colonic gene expression in the sialic acid synthesis pathway correlated strongly with the levels of sialic acid present in the milk. During early lactation the colonic gene expression profiles favour catabolism of sialic acid but as the levels of sialic acid decrease during lactation there is a switch to pathways involved in sialic acid biosynthesis (Duncan, P.I., Raymond, F., et al. 2009). This would seem to indicate that the supply of exogenous sialic acid in maternal milk directly influences intestinal gene expression and thus modulates the intestinal glycome. It was notable that in the study by Duncan *et al.* the effect of sialic acid was specific to the intestinal gene transcription with different patterns of gene expression observed in the liver and central nervous system. These findings raise the question as to whether the observed changes due to sialic acid levels are also inducible by altering the time from parturition to weaning.

The effect of premature and postponed weaning have been studied by Biol *et al*. The activities of β-1,3-galactosyltransferase and β-1,4-galactosyltransferase are low between birth and weaning in the rat small intestine and increase rapidly immediately after weaning to adult levels by the fourth week after birth. Early weaning resulted in a decrease in the activity of sialyltransferase and an increase in fucosyltransferase activity (Biol, M.C., Pintori, S., Mathian, B., Louisot, P. 1991), while delayed weaning of rat pups

(22-days) resulted in increased sialyltransferase activity and prevented the onset of increased galactosyl- and fucosyl-transferase activity. Following insulin treatment, the activities of galactosyltransferases and of α-1,2-fucosyltransferase reached levels nearing those observed in age-matched normally weaned rats (Biol, M.C., Lenoir, D., Greco, S., Galvain, D., Hugueny, I., Louisot, P. 1998). These results indicate that alterations in weaning induce changes in the typical sialyl-, galactosyl- and fucosyltransferase activities observed during normal postnatal development.

As mentioned earlier, due to the invasive nature of *in vivo* studies there has been a preponderance of animal studies and a paucity of information on the effects of milk oligosaccharides in humans. One alternative to *in vivo* study is to use *in vitro* models based on cultured epithelial cells. While *in vitro* models can never entirely replicate the physiological conditions occurring in the intestine it has been observed in recent studies that the effects of probiotic bacteria on epithelial cells *in vitro* are remarkably similar to those observed *in vivo*. A study performed by Angeloni *et al.* (2005), monitored changing glycosylation patterns of Caco-2 epithelial cells in response to treatment with 3'sialyllactose, the predominant sialylated human milk oligosaccharide which is common to a range of other mammals (Urashima, T., Saito, T., et al. 2001). Using glycans, glycol-conjugates and lectins arrayed on slides in micro-array formats, gene expression levels of a number of glycosyltransferases were observed to be reduced in seven day-cultured cells treated with 3'sialyllactose when compared with untreated cells. The significance of the resulting glycan changes was evaluated by challenging treated- and control-cells with enteropathogenic *Escherichia coli* (EPEC). Total bacterial adherence was reduced 50% in the treated cells (Angeloni, S., Ridet, J.L., et al. 2005). This reduction in adherence correlated with reduced sialic acid and lactosamine epitopes on the cell surface, which are key glycans for EPEC adhesion (Vanmaele, R.P., Finlayson, M.C., et al. 1995, Vanmaele, R.P., Heerze, L.D., et al. 1999). As a recent review points out (Bode, L. 2009), this appears to be the sole study on the influence of milk oligosaccharides on the GI glycome to the best of our knowledge and is an area that warrants further study.

The investigation and treatment of congenital glycosylation disorders provides some further insights into the role of glycosylation in human health and

some information on the effects of dietary saccharides on intestinal function. One notable glycosylation disorder is of particular relevance to this review as it describes treatment of carbohydrate-deficient glycoprotein syndrome (CDGS) by administration of dietary mannose. CDGS-type disorders typically result in severely diminished psychomotor and mental development together with blood disorders including thrombosis and severe life threatening bleeding episodes. The clinical symptoms in the study described were, however, very different. They did not include any psychomotor or mental impairment but rather presented as a severe gastrointestinal disorder with initial symptoms of diarrhoea and vomiting and abnormal small bowel biopsy that progressed to severe life-threatening intestinal bleeding. This particular disorder is due to phosphomannose isomerase (PMI) deficiency and has been named CDG-1b. The administration of mannose to PMI-deficient patients successfully reverses the clinical symptoms of this disorder (Niehues et al., 1994; Jaeken et al., 1998; Freeze and Sharma, 2010).

Human milk provides an immunomodulatory and protective function for developing neonates. One of the components of interest is tumor necrosis factor-alpha (TNF-α), which is present in human milk at concentrations of 620 pg/ml (Rudloff, H.E., Schmalstieg, F.C., et al. 1992). In the small intestine, TNF-α administration was associated with a loss of mucus-containing goblet cells in day-0 mice, while administration to day-21 mice resulted in increased mucin mRNA production, indicating a time-sensitive window in which TNF-α can influence GI glycosylation (McElroy, S.J., Prince, L.S., et al. 2011), though this study relied on intra-peritoneal injections of relatively high amounts of TNF-α, which are not representative of the intestinal concentrations of TNF-α encountered *in vivo*. However direct ingestion of TNF-α in human milk is likely to result in a more direct interaction with the intestinal epithelium.

While the components of human milk have varying effects on the GI glycome, it should be noted that the well-documented overall beneficial effects of breast feeding (reviewed in Eidelman, A.I., Schanler, R.J., et al. 2012) are likely to be due to a synergistic cooperation among several constituents of the milk.

4.2 *Post-weaning to adulthood: maintenance of optimal intestinal function*

Once the weaning process is successfully completed, an infant is transitioned to dietary sources which, when consumed in appropriate proportions, make up the fundamental components of the adult diet, namely – carbohydrates, protein, fat, and fibre, as well as various required or complementary substances, including polyamines, amino acids, and vitamins. The following section will explore the influences that these components may have upon the intestinal glycome.

4.2.1 *Direct dietary modification of the glycome: Proteins, fats and fibre*

The effects of dietary protein provision on the intestinal glycome have been studied predominantly in murine and rodent models and date from the early 1990's. Gupta *et al.* (1992b) investigated the effects of variations in dietary protein content on brush border glycosylation and enzyme activities in the murine small intestine. A high protein diet (30% vs. 18% control) administered for 21 days decreased total hexoses, sialic acid content and activities of several brush border enzymes, while hexosamine and fucose content remained unaltered (Gupta, R., Jaswal, V.M., et al. 1992b). Conversely, a neonatal low-protein diet produced an increase in sialic acid content together with a decrease in fucose expression in mice (Gupta, R., Jaswal, V.M., et al. 1992a).

Jaswal *et al.* (1990b) demonstrated the importance of both the fat and protein content of the maternal diet and their effects on the intestinal glycosylation of their pups in a rodent model. A high-fat or high-protein maternal diet significantly reduced levels of fucose in the brush border membrane with the sialic acid content remaining unaltered. Dams fed on a low protein (8%) diet versus those fed on a control diet containing 20% proteins, displayed reduced incorporation of [^{14}C]-GlcNAc into the microvillus membrane (Jaswal, V.M., Babbar, H.S., et al. 1990a). Additionally, Babbar *et al.* (1990) demonstrated that a maternal low-protein diet significantly increases the fucose level in the intestinal brush border membrane of neonate rat pups (Babbar, H.S., Jaswal, V.M., et al. 1990). Alterations in protein content appear to drive opposing changes in sialylation and fucosylation in neonates which are similar to

those evident during the weaning stage, exerting influences on expression of sialic acid and fucose,

For the most part, studies of dietary fat and their associated impact upon the GI tract have focussed upon the microvillus morphology (height and crypt depth) in rodent models and were conducted during a brief window of time nearly twenty years ago (1992-1996). Studies examining the effects of dietary fat upon the GI glycome are limited and often generalized to changes in sialic acid and fucose content. In a murine model, a high fat (26%) diet fed for 21 days compared against the control diet (10% fat) did not result in a significant change to fucose, sialic acid, hexoses, or hexosamines in the intestinal brush border membrane (Gupta, R., Jaswal, V., et al. 1993).

While the quantity of dietary fat does not affect intestinal glycosylation, Kaur *et al.* (1996), demonstrated that the quality of dietary fat does exert a minor influence on the glycosylation pattern in a rodent model. Diets were supplemented with fats from varying sources. In contrast to a previous study by Gupta *et al.* (1993), supplementation with coconut oil fat was found to increase membrane hexose and hexosamine content, while having a weaker ability to enhance sialic acid content in comparison to corn or fish oil (10% supplementation for 5 weeks). Dietary fat supplementation was found to have no effect upon levels of fucose (Kaur, M., Kaur, J., et al. 1996). Furthermore, an earlier study indicates that krill oil, a rich source of fatty acids, can induce a slight modification in the intestinal fucose and mannose proportions of rat pups at weaning compared to corn and fish oil (Ruggiero-Lopez, D., Servetto, C., et al. 1994). While it appears that dietary fats may have the potential to influence glycosylation and glycoprotein expression in the intestine, research on this topic is limited.

Soluble dietary fibre is often fermentable by intestinal bacteria, acting as a prebiotic, while insoluble fibre is metabolically inert, serving only to soften and add bulk to stool (Eastwood, M. and Kritchevsky, D. 2005). The physical characteristics of fibre and the quality of fibre ingested, either soluble (e.g. pectin) or insoluble (cellulose) have been shown to affect glycosylation in the GI tract. A rodent study by Tardy *et al.* (1994) demonstrated that feeding either pectin or cellulose (10% dietary intake in 23-day-old rat pups) resulted in

elevated levels of the neutral sugar content (Fuc, Man, and Gal) of intestinal glycoproteins.

The particle size and processing of fibre can influence the glycosylation of the GI tract. When ingestion of crude fibre of cereal origin (commercial feed) was compared against a finely powdered diet (with cellulose as source of fibre) in rats, the commercial diet resulted in a greater abundance of GlcNAc and sialic acid-linked D-galactose in small intestinal goblet cells, while a similar increase was observed for GalNAc in the large intestine (Sharma, R. and Schumacher, U. 1995). The effects of feed-processing were assessed in a porcine model. Ingestion of pelleted (versus non-pelleted) feed resulted in increased staining areas for neutral, acidic and sulfomucins in the distal small intestine, which was also associated with higher rate of *Salmonella enterica* serovar Typhimurium DT12 adhesion (Hedemann, M.S., Mikkelsen, L., et al. 2005). Interestingly, the above results indicate that the GI glycome can be influenced not only by the various components of the adult diet, but also by the manner in which they are processed.

4.2.2 *Indirect dietary modulation of the intestinal glycome: the role of the microflora*

A key feature of the maintenance of optimal intestinal function and health of an individual following weaning is the role played by the intestinal microbiota. The recent study by Claesson *et al*. (2012) demonstrated that the gut microbiota in elderly subjects correlated strongly with diet and health. The inference drawn from the study was that by adjusting diet it is possible to manipulate the intestinal microbiota and that this will lead to improved health in the elderly. One of the key points of interaction between commensal bacteria and the host is the intestinal mucosa, so it is timely to examine the effects of intestinal colonisation on the intestinal glycome.

During post-natal development there is a drastic modification in the surface glycosylation of the GI epithelium between birth and weaning (reviewed in Biol-N'garagba M, C. and Louisot, P. 2003, Dai, D., Nanthkumar, N.N., et al. 2000), and, as mentioned earlier, maturation is associated with a shift from terminal sialylation to fucosylation. This aspect of intestinal development is

associated with the neonatal transition to weaning (King, T.P., Begbie, R., et al. 1995, Lenoir, D., Ruggiero-Lopez, D., et al. 1995) and bacterial colonisation of the GI tract. The number of bacterial cells present in the gut greatly outnumbers that of human cells in the entire body and as such the microbiome has been referred to as a 'second genome'. The profound effects exerted by the microbiota on human health are well established, however, the precise molecular mechanisms by which those effects are achieved are yet to be elucidated fully. Unlike the human genome, the intestinal microbiota is effectively a dynamic system that can be altered by environmental influences, including diet, resulting in either beneficial or detrimental effects on the host. As an example, mice colonised with transplanted human microbiota demonstrate shifts in microbial communities as early as twenty-four hours following transition from a high-fat to a high-carbohydrate diet (Turnbaugh, P.J., Ridaura, V.K., et al. 2009). Though these shifts required several days to stabilize, the study results demonstrate the ability of the proportions of the intestinal microbiota to rapidly fluctuate with dietary changes.

In terms of the interaction of commensal bacteria and the intestinal mucosa, the symbiotic association between *Bacteroides thetaiotaomicron* and the host mucosa is perhaps the best understood. The genome of *B. thetaiotaomicron* contains nearly one hundred different polysaccharide utilization loci (Martens, E.C., Roth, R., et al. 2009), and is accordingly deemed a 'generalist', capable of digesting a broad range of glycan epitopes. However, despite being able to cleave sialic acid, it lacks the necessary pathway for sialic acid metabolism (Marcobal, A., Barboza, M., et al. 2011). There are several possible reasons for this, but it is likely these glycosidases are used to access underlying glycans (Lewis, A.L. and Lewis, W.G. 2012). As such, *B. thetaiotaomicron* has considerable potential to modify the host glycome. Despite its enzymatic capabilities, the levels of *B. thetaiotaomicron* are not stable in individuals and are highly prone to dietary-induced changes. This is most clearly demonstrated by Wu *et al.* (2011) who demonstrated that long term dietary patterns could lead to the development of characteristic gut microflora enterotypes (Wu, G.D., Chen, J., et al. 2011). Interestingly, diets high in protein and animal fat favoured the development of a *Bacteroides*-associated enterotype while a diet high in carbohydrates led to a *Prevotella*-dominated enterotype (De Filippo, C.,

Cavalieri, D., et al. 2010). Given the well established ability of *B. thetaiotaomicron* to utilise intestinal mucins, it is likely that dietary changes that modulate the intestinal *Bacteroides* population are also likely to alter mucus colonisation and utilisation by these bacteria. It is tempting to speculate that in a low carbohydrate diet the predominance of the *Bacteroides* enterotype is due, in part, to the ability of *B. thetaiotaomicron* to utilise intestinal mucins as a carbohydrate source, thus being independent of ingested dietary carbohydrate sources. Accordingly, a study implementing germ-free mice colonised with *Eubacterium rectale* and *B. thetaiotaomicron* demonstrated that switching to a high-fat, low-fibre diet resulted in the increased expression of host glycan-utilizing enzymes of *B. thetaiotaomicron* (Mahowald, M.A., Rey, F.E., et al. 2009). Additionally, *B. thetaiotaomicron* mutants lacking gene clusters related to host glycan foraging are greatly outcompeted by the wild-type bacteria in a germ-free mouse model (Martens, E.C., Chiang, H.C., et al. 2008).

CHAPTER I

BACTERIAL AND PARASITIC MODIFICATION OF THE GASTROINTESTINAL GLYCOME

Interactions of microorganisms with the intestinal glycome can be generalized to symbiotic or pathogenic, with commensal bacteria belonging to the former and pathogenic bacteria / parasites falling into the latter. Both groups have evolved multiple mechanisms to ensure their continued survival, specifically impacting the intestinal glycome while doing so. These alterations are generally mediated in one of two ways: 1) Host–response to bacterial colonisation and 2) Enzymatic cleavage of terminal glycan moieties. Although there is a marked difference in the ultimate activity of commensal and pathogenic microbes, adhesion is typically initiated by interaction with carbohydrates present in the lumen and on cell surfaces, potentially resulting in colonisation and / or infection (Bavington, C. and Page, C. 2005).The effects of colonisation / invasion on the GI glycome will be discussed below.

5.1 *Pathogens*

Adhesion and colonisation are prerequisites for the establishment of bacterial pathogenesis (Bavington, C. and Page, C. 2005). Pathogens can exploit multi-cellular host glycans in a variety of ways to aid their survival (van Kooyk, Y. and Rabinovich, G.A. 2008). Interestingly, pathogenic glycome modification can occur indirectly due to host response, and has been largely demonstrated in animal models (Mahdavi, J., Sondén, B., et al. 2002, Mantle, M., Atkins, E., et al. 1991). Studies of bacterial pathogen-induced host-mediated glycan changes are extremely limited, while those associated with parasites are more abundant (discussed in the following section). One of the only pathogens which have been thoroughly examined in relation to altering host glycosylation is *Helicobacter pylori*. Infection in multiple models (gastric cell lines, Rhesus monkeys, and humans) results in the induction of expression of the glycosyltransferase, β3GnT5. Though the exact mechanism for this response remains unknown, the ensuing over-expression of the enzyme leads to increased expression of sialyl-Lewis x in gastric tissue, and is associated with increased adherence of *H. pylori* (Mahdavi, J., Sondén, B., et al. 2002, Marcos, N.T., Magalhães, A., et al. 2008). A study involving Rhesus monkeys infected with *H. pylori* showed a similar

trend with increased gastric mucosal sialylation, but an associated loss in fucosylation (Lindén, S., Mahdavi, J., et al. 2008). *In vitro* studies using gastric cell lines attribute a loss of fucosylation with the induction of host α-L-fucosidase (FUCA2), which is induced only under co-culture conditions with *H. pylori*. FUCA2 expression was shown to be essential for bacterial adhesion and increased Lewis x antigen expression in *H. pylori* (Liu, T.W., Ho, C.W., et al. 2009). In a human study of mucin glycosylation in gastric juices, eradication of *H. pylori* was associated with an increase in lectin-staining for glycan structures containing sialyl-Lewis x, H type 1 structure, fucose, sialic acid, and GlcNAc-linkages (Radziejewska, I., Borzym-Kluczyk, M., et al. 2011). It should be noted, however, that the mucin glycan signatures were determined from released mucins, and may not have been entirely representative of the glycan status at the gastric mucosal surface. In a study of hospitalized patients, infection with *H. pylori* was associated with altered sialylation of gastric mucins obtained from biopsies, which subsided with subsequent bacterial treatment and clearance (Ota, H., Nakayama, J., et al. 1998). The lipopolysaccharide (LPS) of pathogens can contribute to changes in host glycosylation. When administered to germ-free rats, LPS results in altered mucin glycosylation, characterized by an increase in β-Gal, α-GalNAc, and mannose, which coincide with a reduction in α-fucose (Enss, M.L., Muller, H., et al. 1996). As *H. pylori* is a chronic infection afflicting more than half of the world's population (Kobayashi, M., Lee, H., et al. 2009), these resulting glycome changes may have a broad range of effects, impacting mucin barrier integrity and disease susceptibility to sialic acid-binding pathogens.

Beyond the host response to a pathogenic presence, the GI glycome can be altered by the direct enzymatic cleavage of mucin- and cell surface-associated glycans. Host glycans can be altered or destroyed by the expression of glycosidases in an attempt to expose more appropriate underlying epitopes for lectin interaction (Dwarakanath, A.D., Tsai, H.H., et al. 1995, Vimr, E.R., Kalivoda, K.A., et al. 2004). The removal of specific terminal glycans may favour pathogenic persistence through revealing binding sites for adhesins and toxins and eliminating decoy glycan epitopes responsible for their restriction to the outer mucus layer. *Vibrio cholerae* and *Clostridium perfringens* employ

sialidases to reveal binding sites and enhance potency of their toxins (Lewis, A.L. and Lewis, W.G. 2012, Li, J., Sayeed, S., et al. 2011). *Clostridium perfringens* also possesses an endo-α-N-acetylgalactosaminidase capable of releasing Galβ1-3GalNAc, Galβ1-3(GlcNAc1-6)GalNAc, GlcNAcβ1-3GalNAc, and GalNAc (Ashida, H., Maki, R., et al. 2008), while a screen of human *H. pylori* isolates has revealed the widespread presence of sialidase production in all isolates tested along with fucosidase activity in a smaller subset of isolates (9 of 49) (Dwarakanath, A.D., Tsai, H.H., et al. 1995).

In addition to the removal of host glycans to create or increase binding sites, the released glycans can be used as a carbohydrate source or structural precursors from which to create and express surface molecules to aid in immune evasion. Following the import of sialic acid, *Escherichia coli* cleaves the Neu5Ac structure to pyruvate and ManNAc, with the latter being further converted to fructose-6-phosphate and ammonia, and finally entering the organism's central metabolism (Vimr, E.R., Kalivoda, K.A., et al. 2004). Several pathogenic microorganisms have evolved the ability to enzymatically cleave glycans from their hosts or environment and incorporate these moieties into their own terminal glycans. Presentation of sialyl- and fucosyl-glycoconjugates in these pathogens appears to be critical for their colonisation and survival in a host by facilitating immune evasion (Liu, T.W., Ho, C.W., et al. 2009, van Kooyk, Y. and Rabinovich, G.A. 2008). Further examples of glycan-degrading pathogens are found in Table 2.

As a result of pathogens possessing this arsenal of entry and adhesion strategies to manipulate host glycans, there is an increased pressure on intestinal cells to maintain an intact mucosal barrier, whether through increased production of secretory decoy mucins, elimination of pathogen target glycans, or hosting commensal competitors.

Table 2. Pathogenic bacterial influence upon the GI glycome.

Pathogenic Bacteria	Host response	Reference
Helicobacter pylori	↑ Sialyl-Lewis x expression (due to β3GnT5); ↓ fucose (↑ α-L-fucosidase)	(Liu, T.W., Ho, C.W., et al. 2009) (Marcos, N.T., Magalhães, A., et al. 2008)
Yersinia enterocolitica	Rabbit - ↓ sialylation (small intestinal mucins), ↑ sulfation (small intestine, proximal colon); mucins contained fewer, but longer, oligosaccharide chains	(Mantle, M., Atkins, E., et al. 1991)

Pathogenic Bacteria	Enzymatic activity	Reference
Clostridium perfringens	β-N-acetylglucosaminidase, sialidase, endo-α-N-acetylgalactosaminidase	(Ficko-Blean, E., Gregg, K.J., et al. 2009) (Li, J., Sayeed, S., et al. 2011) (Yasui, H., Shida, K., et al. 1999)
Clostridium septicum	β-galactosidase, β-N-acetyl-D-glucosaminidase, neuraminidase	(Macfarlane, S., Hopkins, M.J., et al. 2001)
Escherichia coli (polluted water coliforms)	β-galactosidase	(Wutor, V., Togo, C., et al. 2007)
Helicobacter pylori	Neuraminidase, fucosidase, β-N-acetyl-D-glucosaminidase, β-N-acetyl-D-galactosaminidase, α-D-galactosidase,	(Dwarakanath, A.D., Tsai, H.H., et al. 1995) (Spice, W.M. and Ackers, J.P. 1998)
Salmonella typhimurium strain LT2	α-2,3-sialidase	(Lewis, A.L. and Lewis, W.G. 2012)
Vibrio cholerae	Neuraminidase, β-N-acetylhexosaminidase	(Almagro-Moreno, S. and Boyd, E.F. 2009) (Stewart-Tull, D., Ollar, R., et al. 1986)

5.2 *Parasites*

Parasitic infection of the GI tract is associated with negative consequences to the host tissues and physiology (Hoste, H. 2001). In contrast to the previous section, studies involving parasites and their ability to influence a host-related change in glycosylation are more abundant than those describing direct enzymatic modification of the GI glycome. Several studies depict the influence of parasites and the infection-related host-response in a diverse range of *in vivo* models with varying outcomes related to glycosylation (Table 3), whereas studies assessing the impact on the human GI tract appear to be absent (Hasnain, S., Thornton, D., et al. 2010, Hoang, V., Williams, M., et al. 2010, Redondo, M.J. and Alvarez-Pellitero, P. 2010, Rinaldi, M., Dreesen, L., et al. 2011, Tsubokawa, D., Nakamura, T., et al. 2009). A characteristic example of parasite infection affecting the host glycome is that of the nematode, *Nippostrongylus brasiliensis*. Seven days post-infection in a murine model, numbers of sialomucin-positive jejunal goblet cells significantly increased, likely mediated by thymus-derived T-cells, while sulfomucin-positive goblet cells increased 28-days post-infection. Changes in sulfomucin-positive goblet cells were found to correspond with increases in O-glycan sulfotransferases (Soga, K., Yamauchi, J., et al. 2008). In a separate study in the rodent small intestine, infection resulted in the expression of four sialylated oligosaccharides which were not found in uninfected animals. The novel sialyloligosaccharides contained the same trisaccharide epitope, NeuAc/NeuGc-α-2,3(GalNAcβ1-4)Galβ1, implicating the induction of a GalNAcβ1-4 glycosyltransferase (Karlsson, N.G., Olson, F.J., et al. 2000). Further research conducted by Holmen *et al.* identified a transient expression of two oligosaccharides carrying the blood group H epitope (Fucα1-2Gal-) in a murine model, which was associated with an increase in *Fut2* expression and likely responsible for the presence of the blood group H epitope. The results from this murine study and previous work in a rodent model (Olson, F.J., Johansson, M.E.V., et al. 2002) both resulted in a transient induced expression of blood group transferases (H versus A, respectively) midway through infection, with an induced transferase adding a terminal GalNAc towards the end of infection (Holmen, J.M., Olson, F.J., et al. 2002). The results demonstrate a species-specific glycosylation response to

infection with *N. brasiliensis*, and, accordingly, merits further consideration when attempting to extrapolate these findings in relation to a human model.

Table 3. Parasite influence upon the GI glycome.

Research on the direct glycan-degrading effects of parasites in the GI tract is comparatively sparse (Table 3). One of the best-characterized interactions between a parasite and colonic mucin is that of the enteric protozoan parasite *Entamoeba histolytica* (reviewed in Linden, S.K., Sutton, P., et al. 2008), which requires contact with and depletion of the mucosal barrier to elicit pathogenicity. In order to destroy the mucosal barrier, *E. histolytica* has several proteases and glycosidases at its disposal. Early studies show the parasite produces neuraminidase (Nok, A.J. and Rivera, W. 2003, Udezulu, I.A. and Leitch, G.J. 1987), β-N-acetylglucosaminidase and lower expression levels of β-mannosidase (Connaris, S. and Greenwell, P. 1997). A later study identified further glycosidases of *E. histolytica*, including α-D-glucosidase and modest levels of β-D-galactosidase, β-L-fucosidase, and α-N-acetylgalactosaminidase (Moncada, D., Keller, K., et al. 2005). The findings are in contrast to the early studies, where it was thought that *E. histolytica* did not produce the required glycosidases to degrade mucin. Current research suggests that the parasite is capable of independently subverting the protective mucus layer, thereby facilitating its own invasion, and possibly that of other microorganisms, while depleting or altering the GI glycome in the process.

Parasite	Host response	Reference
Echinostoma trivolvis	Murine – parasite expulsion associated with ↑fucose, GalNAc, GlcNAc and sialic acid	(Fujino, T. and Fried, B. 1993)
Haemonchus contortus	Ovine - ↓ fucosylation, ↓ sialylation, ↓ GalNAc, ↑glucosamine (fundus), ↑galactose (duodenum)	(Hoang, V., Williams, M., et al. 2010)
Nippostrongylus brasiliensis	Rat - ↑ GalNAcβ1-4 glycosyltransferase, ↓ N-glycolylneuraminic acid, 4 sialylated oligosaccharides not present in control	(Karlsson, N.G., Olson, F.J., et al. 2000)
	Rats and mice - ↑GalNAc, GlcNAc, sialic acid	(Ishikawa, N. 1994)
	Rat - ↑ $HCM31^{+}$ sialomucin in jejunal mucosa	(Tsubokawa, D., Goso, Y., et al. 2012)
Ostertagia ostertagi	Bovine - ↓ neutral, ↑ acidic mucins; ↓ fucosylation	(Rinaldi, M., Dreesen, L., et al. 2011)
Teladorsagia circumcincta	Ovine - ↓ fucosylation, ↓ sialylation, ↓ GalNAc	(Hoang, V., Williams, M., et al. 2010)
Trichinella spiralis	Rat - ↓sulfation, ↑ GalNAc and sialylation	(Theodoropoulos, G., Hicks, S.J., et al. 2001)
	Porcine – altered sulfation and sialylation in duodenum and jejunum	(Theodoropoulos, G., Hicks, S.J., et al. 2005)
Trichuris muris	Murine – altered glycosylation pattern, presence of highly charged mucins associated with expulsion	(Hasnain, S., Thornton, D., et al. 2010)

Parasite	Enzymatic activity	Reference
Entamoeba histolytica	β-N-acetylglucosaminidase, β-N-acetylgalactosaminidase, β-mannosidase,	(Connaris, S. and Greenwell, P. 1997)
	β-D-galactosidase, β-D-glucosidase, β-L-fucosidase	(Moncada, D., Keller, K., et al. 2005)
Giardia lamblia	β-N-acetylglucosaminidase, β-N-acetylgalactosaminidase	(Connaris, S. and Greenwell, P. 1997)
Trichinella spiralis	β-N-acetylglucosaminidase	(Nagano, I., Wu, Z., et al. 2009)

5.3 *Commensals*

Mammals have developed a delicately balanced relationship with commensal bacteria, essentially providing a favourable niche in return for a multitude of bacterial services, including that of maintaining the intestinal barrier and the provision of colonising resistance. In mammals, rapid colonisation by gut microflora begins at birth. The colonisation of the intestine is influenced by the mode of delivery (vaginal or caesarean section) (Guarner, F. and Malagelada, J.R. 2003), the surrounding environment and the ensuing nutritional intake (Lutgendorff, F., Akkermans, L.M., et al. 2008). Initial colonisation plays an essential role in the original shift in glycosylation of the intestinal glycome from sialylation to fucosylation (Bry, L., Falk, P.G., et al. 1996, Nanthakumar, N.N., Dai, D., et al. 2003), which may influence future susceptibility or resistance to intestinal infection (Freitas, M., Axelsson, L.G., et al. 2002, Mouricout, M. 1997). This transition is absent in mice raised in germ-free conditions. The glycosylation pattern can be partially rescued with administration of a soluble-factor present in spent culture supernatant (SCS) from *Bacteroides thetaiotaomicron,* and even more so with live bacteria, though a complete transition is observed when germ-free mice are colonised with the microflora of conventionally raised mice (Freitas, M., Axelsson, L., et al. 2005). In this manner, colonisation by a specific species of bacteria may be pre-requisite to the colonisation and stable establishment of other commensals. The spent culture supernatants of specific strains of bacteria are capable of influencing the glycosylation of the HT29-MTX intestinal model. SCS of *B. thetaiotaomicron* has been shown to increase cell surface Gal expression, while the SCS of *Lactobacillus casei* DN-114 001 increases the expression of Gal and sialic acid and decreases expression of GalNAc (Freitas, M., Tavan, E., Thoreux, K., Cayuela, C., Sapin, C., Trugnan, G. 2003). Among the conventional microflora, these appear to be the only species identified to date which directly influence the intestinal glycome through a host-mediated response. Further research is required to determine the contributions of other members of the complex GI microflora upon the GI glycome.

While the digestion and removal of mucin glycans is associated with a wide variety of pathogens, it must be emphasized that mucin degradation is a

recognized natural process for the turnover of mucus in the GI tract (Norin, K., Gustafsson, B., et al. 1985). The use of host-derived glycoconjugates has multiple advantages. Once cleaved, the glycans are rapidly fermented to carbon and energy. Furthermore, the source of glycans is abundant and competition is minimal since only certain bacterial species are capable of utilizing such glycans (Sonnenburg, J.L., Xu, J., et al. 2005). The establishment of mucin-degrading bacteria in the GI commences early in life and stabilizes nearing two years of age (Midtvedt, A.C., Carlstedt-Duke, B., et al. 1994). Reviews by Dallas *et al.* and Derrien *et al.* provide comprehensive information on the glycan-degrading enzymes produced by the GI microflora which act on the host (Dallas, D., Sela, D., et al. 2012, Derrien, M., van Passel, M.W.J., et al. 2010). Selectivity in glycan usage, paired with tissue-specific glycan expression is thought to contribute to the region-specific colonisation of GI bacteria (Robbe, C., Capon, C., et al. 2004). The availability of these enzymes within the GI tract highlights the capacity of these bacteria to degrade dietary oligosaccharides, mucin glycan moieties, and potentially host-associated blood group determinants. For example, secretory α-1,3-N-acetylgalactosaminidase is produced by *Ruminococcus torques* strains VIII-239 and is present in stool of term infants. This enzyme cleaves α-1,3-linked GalNAc, converting blood group A to blood group H through liberation of the terminal GalNAc-α-1,3-Gal (Larson, G., Falk, P., et al. 1988).

In addition to the ability of *B. thetaiotaomicron* to alter the immature intestinal glycome, it also produces a wide range of glycosidases (Xu, J., Bjursell, M.K., et al. 2003). Of particular interest is the ability of *B. thetaiotaomicron* to sense the presence of free fucose, and in its absence, induce the host to express fucosyltransferase, providing mucin-linked fucose which it can then cleave and catabolise (Hooper, L.V., Xu, J., et al. 1999). GI commensal bacteria may be termed ‘generalists’ able to cleave a broad range of glycans, or ‘specialists’ possessing enzymes for specific glycan structures (Table 4). As mentioned earlier, *B. thetaiotaomicron* is a generalist, reflected by its possession of 88 different polysaccharide utilization loci in its genome (Martens, E.C., Roth, R., et al. 2009). With its vast repertoire of glycan-cleaving enzymes likely used to reveal preferential binding sites, the broad activity of *B. thetaiotaomicron* may

serve to prune an array of intestinal glycans, creating favourable footholds for some, while potentially eliminating those of other sialic acid-binding bacteria, all the while shaping the host intestinal glycome.

Bifidobacterium longum subsp. *infantis* ATCC 15697, an infant isolate, has adapted to utilise human milk oligosaccharides (Sela, D., Chapman, J., et al. 2008). This species displays constitutive sialidase activity, while fucosidase activity is induced in the presence of human milk oligosaccharides (LoCascio, R.G., Ninonuevo, M.R., et al. 2007). These adaptations include the presence of genes encoding β-galactosidase, α-2,3- and α-2,6-sialidases, and α-1,2/4-fucosidases. Of these, only the fucosidases are known to be secreted, as the others lack identifiable export signals or cell wall anchor motifs (Sela, D.A., Li, Y., et al. 2011). In the presence of human milk oligosaccharides, the secreted fucosidases may act not only on the nutritional oligosaccharide source, but also on host-associated glycans as well. In this manner, cleavage of host α-1,2-linked fucose may result in the conversion of Le^b blood groups to Le^a, and degradation of blood group H (Larson, G., Falk, P., et al. 1988) and possibly the fucose-containing A and B blood group epitopes. Cleavage of α-1,4-linked fucose may result in the degradation of Le^a and Le^b blood group epitopes (Dallas, D., Sela, D., et al. 2012). Additionally, several strains of bifidobacteria have been demonstrated to possess endo-α-N-acetylgalactosaminidase activity capable of digesting and modifying the mucin glycoprotein disaccharide, O-linked galactosyl β-1,3-N-acetylgalactosamine (Katayama, T., Fujita, K., et al. 2005). These findings clearly demonstrate that the GI commensal microflora possess a diverse range of glycosidases which are likely to be continually ‘pruning’ and maintaining the GI glycome.

The vast contribution of commensal bacteria to the maintenance and homeostasis of the intestinal glycosylation status can be easily overlooked. Despite their direct involvement in the upkeep and maturation of the intestinal barrier and glycome, their most significant impact may be via ‘colonising resistance’ associated with their continued presence in the GI tract. Through denial of attachment sites and active defence against invading pathogens, commensal bacteria may impart their greatest benefit to the intestinal glycome.

Table 4. Commensal bacterial influence upon the GI glycome.

Commensal Bacteria	Host-mediated response	Reference
Conventional microflora	↑ GI fucosylation	(Freitas, M., Axelsson, L.G., et al. 2002)
Bacteroides thetaiotaomicron	↑ α-1,2-fucosyltransferase, ↑ terminal fucose expression	(Nanthakumar, N.N., Dai, D., et al. 2003)

Commensal Bacteria	Enzymatic activity	Reference
Human fecal bacteria	Sialidase, sialate O-acetylesterase, N-acetylneuraminate lyase, glycosulfatase	(Corfield, A.P., Wagner, S.A., et al. 1992)
Akkermansia muciniphila	α- and β-galactosidase, α-L-fucosidase, α- and β-N-acetylgalactosaminidase, β-N-acetylglucosaminidase, neuraminidase	(Derrien, M., van Passel, M.W.J., et al. 2010)
Bacteroides thetaiotaomicron	α-fucosidase, β-galactosidase, α-N-acetylgalactosaminidase, β-N-acetylglucosaminidase and neuraminidase; (88 different glycan utilization loci, in total)	(Tsai, H., Hart, C., et al. 1991) (Martens, E.C., Roth, R., et al. 2009)
Bacteroides fragilis	β-1,3-N-acetylglucosaminidase, sialidase	(Brigham, C., Caughlan, R., et al. 2009)
Bacteroides vulgatus	α- and β-galactosidase, α-fucosidase, β-N-acetyl-D-glucosaminidase, α- and β-N-acetylgalactosaminidase, neuraminidase	(Ruseler-van Embden, J., Van der Helm, R., et al. 1989)
B. longum subsp. *infantis* ATCC 15697	α-2,3/6-neuraminidase, α-2,6-sialidase, α-1,2-fucosidase, α-1,3/4-fucosidase, N-acetyl-β-hexosaminidases	(Garrido, D., Ruiz-Moyano, S., et al. 2012) (Sela, D.A., Garrido, D., et al. 2012) (Sela, D.A., Li, Y., et al. 2011)

Commensal Bacteria	Enzymatic activity	Reference
B. bifidum PRL2010	endo-α-N-acetylgalactosaminidase, putative 1,2-α-L-fucosidase, predicted 1,3/4-α-L-fucosidase, four putative N-acetyl-β-hexosaminidases, four predicted β-galactosidases, two putative exo-α-sialidases, and the GNB/LNB operon homolog, including lacto-N-biosidase	(Turroni, F., Bottacini, F., et al. 2010)
B. bifidum D119 and L22	endo-α-N-acetylgalactosaminidase, α-1,2-fucosidase	(Ruas-Madiedo, P., Gueimonde, M., et al. 2008)
B. bifidum JCM1254	α-1,2-fucosidase, α-1,3/4-fucosidase, α-2,3-neuraminidase, endo-α-N-acetylgalactosaminidase	(Ashida, H., Maki, R., et al. 2008) (Fujita, K., Oura, F., et al. 2005) (Kiyohara, M., Tanigawa, K., et al. 2011)
B. bifidum NCIMB 41171	Four β-galactosidases	(Tzortzis, G., Goulas, A.K., et al. 2005)
B. bifidum JCM 7004	endo-α-N-acetylgalactosaminidase	(Fujita, K., Oura, F., et al. 2005)
B. bifidum ATCC 29521	endo-α-N-acetylgalactosaminidase	(Fujita, K., Oura, F., et al. 2005)
B. longum strains C51, C61, C72, D12, H64, H92, L23	endo-α-N-acetylgalactosaminidase	(Ruas-Madiedo, P., Gueimonde, M., et al. 2008)
B. longum JCM 1217	endo-α-N-acetylgalactosaminidase	(Fujita, K., Oura, F., et al. 2005)
B. longum JCM 7054	endo-α-N-acetylgalactosaminidase	(Fujita, K., Oura, F., et al. 2005)
B. breve JCM 1192	endo-α-N-acetylgalactosaminidase	(Fujita, K., Oura, F., et al. 2005)
Clostridium cocleatum	β-galactosidase, β-N-acetylglucosaminidase, α-N-acetylgalactosaminidase, neuraminidase	(Boureau, H., Decre, D., et al. 1993)

Commensal Bacteria	Enzymatic activity	Reference
Escherichia coli K12	β-N-acetylglucosaminidase	(Yem, D.W. and Wu, H.C. 1976)
Ruminococcus AB strain VI-268	α-1,3-N-acetylgalactosaminidase, α-1,3-galactosidase, α-1,2/4-fucosidase, α-2,3-neuraminidase	(Larson, G., Falk, P., et al. 1988)
Ruminococcus gnavus	α-1,3-galactosidase	(Larson, G., Falk, P., et al. 1988)
Ruminococcus torques strain VIII-239	α-1,3-N-acetylgalactosaminidase, α-1,2-fucosidase, β-1,4-galactosidase, β-1,3-N-acetylglucosaminidase, α-2,3-neuraminidase	(Larson, G., Falk, P., et al. 1988)
Ruminococcus torques strain IX-70	α-1,3-N-acetylgalactosaminidase, α-1,2/4-fucosidase, β-1,3/4-galactosidase, β-1,3-N-acetylglucosaminidase, α-2,3-neuraminidase,	(Larson, G., Falk, P., et al. 1988)
Streptomyces sp.	α-L-fucosidase	(Derrien, M., van Passel, M.W.J., et al. 2010)

CHAPTER I

CONCLUDING REMARKS

Given the function and performance of the intestinal barrier as a selective legislator of the luminal interface, the integral role played by glycosylation has been well accepted. While the functional importance of the terminal glycans have been demonstrated in part through the study of glycosylation-related diseases, susceptibility/resistance to pathogens, and the provision of a nutritional niche for commensal bacteria, a comprehensive overview of the nutritional factors affecting the GI glycosylation status has been, until now, somewhat lacking.

Kreisman and Cobb (2012) have highlighted the failure to adequately bridge the gap between alterations in host glycosylation and the changes in overall host biology (Kreisman, L.S.C. and Cobb, B.A. 2012). Changes in glycosylation vary in severity, ranging from undetected to complete loss of function. Though macroscopic effects have proven difficult to study, they are even more difficult to predict (Varki, A. and Lowe, J.B. 2009). One of the greatest consequences of GI glycome alterations may manifest in immune recognition and functionality (van Kooyk, Y. and Rabinovich, G.A. 2008). For example, sialic acid acts as a recognition molecule for siglec-bearing immune cells and galectins. Alterations in terminal sialic acid, mediated through altered host expression or direct enzymatic cleavage, could translate to intestinal immune dysregulation resulting from reduced migration of eosinophils (Michail, S., Mezoff, E., et al. 2005) or aberrant galectin interactions with membrane glycoproteins (de Kivit, S., Kraneveld, A.D., et al. 2011). At the moment, there is a need for studies which effectively link these areas of research.

Breast milk plays an important intermediary role in bridging the effects of both nutrition and bacterial colonisation. Milk oligosaccharides can act as direct effectors upon intestinal glycosylation, while also acting as a modulator of the intestinal microflora, which in turn, influence the GI glycome. Unfortunately, large-scale production and distribution of human colostrum/milk is not a feasible means to proactively maintain the intestinal glycome and aid in the prevention of infection and disease. Methods to enrich and isolate the therapeutic components of non-human milks and whey streams are currently under investigation (Zivkovic, A.M. and Barile, D. 2011).

While there are several dietary factors capable of influencing the GI glycome, most are currently consumed as part of a healthy diet. Beyond the provision of dietary nutrients which function as glycan precursors or influence glycosyltransferase expression in the host GI tract, the area of prebiotic stimulation of the beneficial intestinal microflora is showing promise. This is mediated through the consumption of non-digestible carbohydrates which are preferentially consumed by commensal organisms which in turn confer benefits to the host (Gibson, G.R., Scott, K.P., et al. 2010). Although the current review has focussed on the direct effects of factors upon GI glycosylation, prebiotics can act to indirectly modify the GI glycome through the selection of specific commensal bacteria which possess varying glycosidases and glycan-specific lectins. The consumption of various prebiotic oligosaccharides, including galactooligosaccharides, fructooligosaccharides, and inulin, among others, aids in selecting beneficial bacterial populations, promoting the production of beneficial short chain fatty acids (Gaudier, E., Forestier, L., et al. 2004), and strengthens resistance against pathogen colonisation (Searle, L.E.J., Best, A., et al. 2009, Shoaf, K., Mulvey, G.L., et al. 2006).

In vivo trials aimed at elucidating the link between specific or general glycome alterations and the macroscopic effects upon host health are required. As a magic bullet or cure-all for GI glycome changes or deviations and their resulting disease conditions does not yet exist, an emphasis should be placed upon an ongoing, combinatorial approach to glycome maintenance through dietary provision of nutritional elements and promotion of beneficial bacteria, thereby strengthening the argument for the development of functional foods and pre-/probiotics.

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CHAPTER II

Exposure of *Bifidobacterium longum* subsp. *infantis* to milk oligosaccharides increases adhesion to epithelial cells and induces a substantial transcriptional response

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CHAPTER II

ABSTRACT

In this study, we tested the hypothesis that milk oligosaccharides may contribute not only to selective growth of bifidobacteria, but also to their specific adhesive ability. Human milk oligosaccharides (3'sialyllactose and 6'sialyllactose) and a commercial prebiotic (Beneo Orafti P95; oligofructose) were assayed for their ability to promote adhesion of *Bifidobacterium longum* subsp. *infantis* ATCC 15697 to HT-29 and Caco-2 human intestinal cells. Treatment with the commercial prebiotic or 3'sialyllactose did not enhance adhesion. However, treatment with 6'sialyllactose resulted in increased adhesion (4.7 fold), while treatment with a mixture of 3'- and 6'-sialyllactose substantially increased adhesion (9.8 fold) to HT-29 intestinal cells. Microarray analyses were subsequently employed to investigate the transcriptional response of *B. longum* subsp. *infantis* to the different oligosaccharide treatments. This data correlated strongly with the observed changes in adhesion to HT-29 cells. The combination of 3'- and 6'-sialyllactose resulted in the greatest response at the genetic level (both in diversity and magnitude) followed by 6'sialyllactose, and 3'sialyllactose alone. The microarray data was further validated by means of real-time PCR. The current findings suggest that the increased adherence phenotype of *Bifidobacterium longum* subsp. *infantis* resulting from exposure to milk oligosaccharides is multi-faceted, involving transcription factors, chaperone proteins, adhesion-related proteins, and a glycoside hydrolase. This study gives additional insight into the role of milk oligosaccharides within the human intestine and the molecular mechanisms underpinning host-microbe interactions.

CHAPTER II

INTRODUCTION

The microflora of the human gastrointestinal (GI) tract outnumber the cells of the human host by a factor of 10, with the bacterial population composed of up to 400 different species from 40-50 genera (Savage, D.C. 2005, Zocco, M.A., Ainora, M.E., et al. 2007). Intestinal colonisation by commensal bacteria is associated with several beneficial outcomes to the host, including resistance to colonisation by pathogens, the provision of nutrients through degradation of non-digestible food components, the production of metabolites and short-chain fatty acids, and modulation of mucosal immunity, among others (Zocco, M.A., Ainora, M.E., et al. 2007). Many benefits to the host are associated with colonisation by bifidobacteria in particular (Leahy, S.C., Higgins, D.G., et al. 2005) and therefore methods to increase the numbers of intestinal bifidobacteria have been actively pursued (Leahy, S.C., Higgins, D.G., et al. 2005). The two approaches most frequently employed are the delivery of live bacteria (probiotics) within a food source, or the use of specific carbohydrates or prebiotics (inulin, fructo- and galacto-oligosaccharides) which are known to survive gastric transit and are fermented in the colon by beneficial bacteria, thus promoting their growth (Boehm, G. and Stahl, B. 2007).

Following birth, the neonatal intestinal tract is colonised predominantly by *Staphyloccocus, Streptococcus,* and *Enterobacteriaceae,* rapidly followed by a transition to obligate anaerobes, including *Bacteroides, Clostridium,* and *Bifidobacterium* (Bezirtzoglou, E. 1997). The transition of the intestinal microflora during early life can be influenced by the source of nutrition. Human milk has evolved to become the optimal source of infant nutrition, containing various components that benefit the neonate including oligosaccharides, protein, and immune factors (Garrido, D., Ruiz-Moyano, S., et al. 2012). Breastfeeding is associated with higher counts of bifidobacteria in neonatal feces at one week of age, as well as typically lower counts of bacteroides, eubacteria, peptococci, *Veillonella*, clostridia, and enterobacteria than formula-fed neonates (Benno, Y., Sawada, K., et al. 1984, Mevissen-Verhage, E., Marcelis, J., et al. 1987). Human milk oligosaccharides (HMO) are believed to serve as a natural source of prebiotics for infants which stimulate the growth of bifidobacteria (Ward, R.E., Ninonuevo, M., et al. 2006, Ward, R.E., Niñonuevo, M., et al. 2007). Moreover,

HMOs have also been shown to possess anti-adhesive effects that reduce the binding of pathogenic bacteria to the host cells (Lane, J.A., Mehra, R.K., et al. 2010). Interestingly, human milk contains between 5 and 23 g/L of these oligosaccharides, with over 200 different HMO structures, which differ in their size, charge, and sequence (Zivkovic, A.M., German, J.B., et al. 2010). The genome of *Bifidobacterium longum* subsp. *infantis* ATCC 15697 has been fully sequenced, allowing investigations into the genetic basis and molecular mechanisms underlying the adaptation of this strain to the intestine, including metabolism of HMOs. Many of its genes encode enzymes that are active on HMOs and in particular, a novel 43-kbp region dedicated to oligosaccharide utilization is present in the genome (Sela, D.A., Chapman, J., et al. 2008). Research to date suggests that one of the primary roles of milk oligosaccharides is to act as a source of prebiotics, however, the first step in establishment of bacterial populations in the intestine is the adherence of the organisms in the gut environment.

Previously, Gonzalez *et al.*, (2008), have demonstrated that growth of *Bifidobacterium longum* in de-fatted human milk leads to the genetic up-regulation of putative type II glycoprotein binding fimbriae, which are implicated in attachment and colonisation (Gonzalez, R., Klaassens, E.S., et al. 2008). Recently, Chichlowski *et al*. (2012), demonstrated that growth of *Bifidobacterium longum* subsp. *infantis* ATCC 15697 to mid-exponential phase on human milk oligosaccharides as the sole carbon source increased adherence to the HT-29 cell line (Chichlowski, M., De Lartigue, G., et al. 2012). As the majority of oligosaccharides in breast milk are able to traverse the GI tract and reach the colon undigested (Engfer, M.B., Stahl, B., et al. 2000, Gnoth, M.J., Kunz, C., et al. 2000a), perhaps HMOs may contribute not only to selective growth of commensal bacteria, but also to their specific adhesive ability. The aim of the current study is to investigate the adhesion- and metabolic-related transcriptomic changes of *B. longum* subsp. *infantis* following transient exposure to milk oligosaccharides and to correlate the observed phenotypic changes with transcriptional changes as determined by microarray analysis.

CHAPTER II

MATERIALS AND METHODS

Bacterial strains and culture conditions

Bifidobacterium longum subsp. *infantis* ATCC 15697 was purchased from DSMZ (Germany), *Bifidobacterium longum* subsp. *infantis* ATCC 15702 was obtained from ATCC (Middlesex, UK), *Bifidobacterium longum* NCIMB 8809 and *Bifidobacterium angulatum* NCIMB 2236 from NCIMB (Aberdeen, Scotland), and *Lactobacillus reuteri* DPC 6100 from the Teagasc Food Research Centre culture collection, (Fermoy, Ireland). Strains were stored in deMan Rogosa Sharpe (MRS) [Difco, Sparks, MD, USA] broth containing 50% glycerol at -80°C and propagated twice in MRS media supplemented with L-cysteine (0.05% w/v) [Sigma, Steinheim, Germany] prior to use. Bacteria were routinely grown overnight at 37˚C under anaerobic conditions generated using the Anaerocult A system (Merck, Dannstadt, Germany). All cultures were grown to an optical density (OD_{600nm}) of 0.4-0.5 to ensure entry into mid-logarithmic growth prior to use. Following the initial carbohydrate screening and lactose inhibition assays using *Bifidobacterium longum* subsp. *infantis* ATCC 15697, subsequent adhesion studies were done using mid-exponential phase cultures. To prepare mid-exponential phase cells, bacterial growth time was measured to determine the time of entry into the mid-exponential growth phase at which time the optical density was adjusted to 0.3, and the cultures were allowed to grow for a further 2 hours and used at an optical density (OD_{600nm}) ~0.45-0.5 (corresponding to ~5 x 10^7 CFU/mL, ascertained by plate counts). The procedure was identical for the other bacterial strains tested.

Bacterial oligosaccharide exposure conditions

The time taken for *B. longum* subsp. *infantis* ATCC 15697 to enter the mid-exponential growth phase was determined by constructing growth curves by measuring the optical density at 600nm (OD_{600}) at intervals during growth. Bacteria were harvested in mid-exponential growth phase (18 hours) and the optical density was adjusted to an OD_{600} of 0.3, incubated for a further 2 hours and used at an optical density (OD_{600nm}) ~0.45-0.5 (corresponding to approximately 5 x 10^7 CFU/mL, ascertained by plate counts on MRS agar). The *B. longum* subsp. *infantis* cells were washed twice by centrifugation and

resuspension in PBS, before final resuspension in McCoy's 5A tissue culture media supplemented with 2% Fetal Bovine Serum (FBS). 1mg/mL of either 6'sialyllactose, 3'sialyllactose, or a combination of the two (1mg/ml each) was added and incubated for 3 hours at 37°C under anaerobic conditions. A control sample using tissue culture medium without oligosaccharide supplementation was also prepared. Bacteria were harvested by centrifugation (10,000 x g, 8 minutes), the supernatants removed and pellets resuspended in RNAprotect (Qiagen, Hilden, Germany) for 10 minutes at room temperature followed by storage at -80°C prior to RNA extraction. As a control, non-supplemented tissue culture media was used. Additionally, exposures were replicated in the presence of 1mg/ml lactose.

Epithelial cell line conditions

The HT-29 (human colonic adenocarcinoma) and Caco-2 cell lines were used as a model of the human intestinal epithelial layer. HT-29 and Caco-2 cells were routinely cultured in McCoy's 5A media (10% FBS; 1% penicillin/streptomycin) and DMEM (10% FBS; 1% penicillin/streptomycin; 1% non-essential amino acids) (Sigma, Steinheim, Germany), respectively, in 75 cm^2 tissue culture flasks at 37°C in a humidified 5% CO_2 atmosphere. The cultures were passaged by detaching with trypsin when the cell growth had reached approximately 80% confluency. Cultures between passage numbers 15 and 18 (HT-29) and 20 to 24 (Caco-2) were used for adhesion studies. For adhesion assays, cells were seeded at a density of 2 x 10^5 cells/mL into 12-well plates (Cellbind; Corning, New York, USA) and grown to late post-confluence as described by Coconnier *et al.* (1992). HT-29 and Caco-2 cells were used 21 days and 14 days post-confluence, respectively. Twenty-four hours prior to assay, media was substituted for McCoy's 5A or DMEM (2% FBS, no antibiotics) for the appropriate cell line. Both flask and plate cultures were fed by replacing the culture medium with fresh medium every other day.

Adhesion assays

Bacterial strains were grown overnight and harvested at an optical density (OD_{600}) between 0.4-0.5 (corresponding to ~5 x 10^7 CFU/mL, determined by plate counts) by centrifugation (4500g, 8min), washed twice in phosphate

buffered saline (PBS; Sigma, Steinheim, Germany) and resuspended in McCoy's 5A or DMEM tissue culture media (2% FBS; further referred to as non-supplemented media) supplemented with 3'sialyllactose, 6'sialyllactose (Carbosynth, Berkshire, UK), or Beneo Orafti P95 (oligofructose) (Beneo Orafti, Dublin, Ireland) at the concentrations indicated in the results section. Non-supplemented media was used as a control. The bacteria were exposed to the oligosaccharides at 37°C for the times indicated in the results section, washed once in PBS to remove the supplemented oligosaccharides, and resuspended in non-supplemented McCoy's 5A / DMEM media prior to use in the assays.

Eukaryotic cells were washed twice with PBS, and 500µl of the bacteria:media suspensions were added to the appropriate wells, corresponding to approximately 10-20 bacterial cells per cell. The number of viable cells per well was determined by detaching the cells with trypsin and counting in a Neubauer hemocytometer, and was about 3×10^6 cells per well. Bacterial exposure to eukaryotic cells was for 2 hours at 37°C under anaerobic conditions. The exposure conditions were optimized to ensure maximal viability of bacterial and mammalian cells, with the viability of the eukaryotic cells remaining at 96-98% throughout the assays. After two hours, the wells were washed three times with PBS to remove any non-adherent bacteria and lysed with 500µl of PBS containing 0.1% Triton X100; (Sigma, Steinheim, Germany) for 30 min at 37°C on a shaking platform at 110 agitations per minute to ensure maximal recovery of viable bacterial cells. The lysates were serially diluted and enumerated by spread-plating on MRS plates. Aliquots of the experimental inocula were retained, diluted and plated to determine original CFU/mL. The results were expressed as adherent bacteria as a percentage of the original inoculum, thereby accounting for variations in the original inocula between groups. Percentage adherent = [CFU/mL of recovered adherent bacteria ÷ CFU/mL of inoculum] X 100. Each adhesion assay was conducted independently in triplicate over 3 successive passages of intestinal cells.

During carbohydrate exposure, bacteria were harvested and plated for CFU/mL on MRS agar at the following timepoints: 0, 1.5, and 3 hrs exposure in order to determine if bacterial numbers increased over the duration of the assay. In addition, the effect of longer incubation times was examined by harvesting an overnight culture and adjusting the cell density to an $OD_{600} = 1.0$ (~$2x10^8$

CFU/mL). The cells were then washed in PBS and resuspended in McCoy's 5A tissue culture media supplemented with 2% FBS. One millilitre of the bacterial suspension was added to 3mL of either non-supplemented tissue culture media or media supplemented with 6'sialyllactose (final concentration of 1mg/mL). Aliquots were removed at 3, 6, 9, and 24 hours to determine CFU/mL by decimal dilutions and plating on MRS agar.

To determine if the oligosaccharide concentration had decreased during exposure to the bacteria, aliquots of the original and spent media following exposure were analysed by High pH Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) using a Dionex ICS-3000 system equipped with an ED40 electrochemical detector with a gold cell and LC30 chromatography oven. A CarboPac PA100 (250 X 4 mm) column protected with a CarboPac PA100 (50 X 4 mm) guard column was used. The mobile phase was 100 mM NaOH, 25 mM NaAc (0-40 min), 100 mM NaOH, 250 mM NaAc (40-40.01 min), and 100 mM NaOH, 25 mM NaAc (40.01-50 min). The limit of detection was 10 parts per million for this assay.

RNA isolation and microarray hybridisation

Bacterial RNA was isolated using the RNA isolate mini kit (Bioline, London, UK) with modifications. Bacterial aliquots were thawed on ice and pelleted at 5,000 x g for 10 minutes. The supernatant (RNAprotect) was removed and the bacterial pellets resuspended in 100 µL TE buffer (50 mg/mL lysozyme, 1000 units/mL mutanolysin; Sigma, Poole, Dorset, UK) at 37°C for 30 minutes. Bacteria were then added to 2mL screw-cap vials containing glass beads (Sigma) (filled to 0.25mL mark) and 800µL lysis buffer (Bioline kit). The cells were disrupted by bead-beating (Mini-Beadbeater-16; BioSpec Products Ltd, Bartlesville, USA) for 3 intervals of 20 seconds and placed on ice between each interval to cool the samples. The tubes were centrifuged at 10,000 x g for 10 minutes at 4°C to collect glass beads and cell debris. Supernatants were removed and processed according to the manufacturer's instructions commencing at the post-lysis step. RNA quantity and quality was assessed on a NanoDrop 1000 (Wilmington, DE, USA). Only samples with 260/280 nm readings >1.9, and 230/260 nm readings ≥ 1.00 were used. Sample quality was further assessed by agarose gel electrophoresis (NorthernMax buffer, Bioline,

London, UK). RNA samples were subsequently shipped to IMGM laboratories (Martinsried, Germany) for labelling and DNA microarray analysis. Prior to their use in the microarray experiments the quality of the RNA was assessed using an Agilent Bioanalyser (RNA600 Chip) to ensure that degradation of the samples had not occurred during transit. All samples used for microarray analysis had an RNA integrity number (RIN) of 10, indicating that the RNA was of excellent quality.

Microarray analysis

The RNA was labelled for analysis using an RT-IVT protocol. Prior to labelling, the RNA was spiked with synthetic polyadenylated transcripts (Agilent spike-in controls). For each RT-IVT reaction, 500 ng of RNA was used and labelled as follows. The spiked total RNA was reverse transcribed into cDNA using random priming (Full Spectrum™ MultiStart Primers for T7 IVT, System Biosciences (SBI)) and then converted into labelled cRNA by *in-vitro* transcription (Quick-Amp Labelling Kit One-Color, Agilent Technologies) incorporating Cyanine-3-CTP. The manufacturers protocols were followed for both the reverse transcription and labelling steps.

The efficiency of labelling was determined both by using a NanoDrop analyser and by analysis on an Agilent 2100 Bioanalyser with a 6000 Nano LabChip Kit (Agilent Technologies, Co. Cork, Ireland). Only samples with cRNA yields >825 ng and with a specific activity of 9.0 pmol cyanine3 per microgram of cRNA were used for array analysis.

Following cRNA clean-up and quantification (NanoDrop), 600 ng of each Cyanine-3-labeled cRNA sample was fragmented and prepared for One-Color based hybridization (Gene Expression Hybridization Kit, Agilent Technologies, Co. Cork, Ireland). cRNA samples were hybridized at 65°C for 17 hrs on separate custom *Bifidobacterium* GE Microarrays (8x15K format).

Afterwards, microarrays were washed with increasing stringency using Gene Expression Wash Buffers (Agilent Technologies) followed by drying with acetonitrile. Fluorescent signal intensities were detected with Scan Control A.8.4.1 Software (Agilent Technologies) on the Agilent DNA Microarray Scanner and extracted from the images using Feature Extraction 10.7.3.1

Software (Agilent Technologies) and the design file 033172_D_F_20110831.xml.

Analysis of microarray data

The raw microarray results were analysed using the Limma package of the R statistics suite. Background correction was performed by the normexp method with offset=12. The inter-array normalisation method chosen was the Cyclic Loess normalisation algorithm. The array data were clustered using the heatmap2 package in R to identify any experimental samples deemed to be outliers. The fold-changes in gene expression were calculated as actual fold-change relative to the control samples. The cut-off for identifying genes of interest was a fold change of >2 and an uncorrected p-value of <0.05. As the majority of the differentially expressed genes identified from the microarray data were confirmed by real-time quantitative RT-PCR, adjusted P-values to correct for multiple sample errors were not taken into consideration as false detections would be identified by qPCR.

qPCR analysis

Complementary DNA (cDNA) was synthesized from 1µg of RNA incubated with 3.2µg of random hexamers, 0.5µl of Transcript Reverse Transcriptase (Roche), 0.5µl of Protector RNAse inhibitor, 1mM dNTPs mix and 4µl of Transcriptor RT Reaction Buffer (Roche), in a final volume of 20µl. Template and random primers were incubated at 65°C for 10min, followed by addition of the remaining components. The mix was incubated at 55°C for 30min. Finally, Transcript Reverse Transcriptase was inactivated by heating to 85°C for 5min.

PCR primers and probes were designed using the Universal Probe Library Assay Design Centre (Roche, West Sussex, UK). Primer sequences and probe combinations are listed in Supplementary Table 1.

16S rRNA and cysteinyl-tRNA synthetase were used as endogenous controls to correct for variability in the starting total RNA and provide a stable expression marker, against which relative levels of expression could be determined. Amplification reactions contained 2.5µl of cDNA, 5µl of 2X

SensiMix II Probe Buffer (Bioline), 5 pmol/µl of each primer and probe mix and were brought up to a total of 10µl by the addition of RNase-free water.

All reactions were performed in duplicate in 384-well plates on the LightCycler®480 System (Roche). Positive and negative controls were included in each run. Thermal cycling conditions applied were 95°C for 10mins, [95°C for 10 seconds, 60°C for 45 seconds, and 72°C for 1 second to allow for fluorescence acquisition] (55 cycles), 40°C for 10 seconds as recommended by the manufacturer (Roche, West Sussex, UK). The $2^{-\Delta\Delta Ct}$ method (Livak, K.J. and Schmittgen, T.D. 2001) was employed to calculate relative changes in gene expression.

Statistical analysis

All adhesion studies were carried out on three separate occasions in triplicate. Results are presented as mean ± standard deviation of replicate experiments. Graphs were drawn using Microsoft Excel and the Student *t*-test and one-way ANOVA were used for pairwise or multiple comparisons, respectively, to determine statistically significant results, where $p<0.05$ was considered significant.

CHAPTER II

RESULTS

Promotion of bacterial adhesion by selected carbohydrates

B. longum subsp. *infantis* ATCC 15697 grown to mid-logarithmic phase were incubated separately with the two predominant milk oligosaccharides found in human and bovine milk (3'- and 6'-sialyllactose) or a commercial prebiotic (Beneo Orafti P95) for three hours at a concentration of 1mg/ml, after which oligosaccharides were removed and their ability to adhere to HT-29 and Caco-2 cells was determined. Addition of 6'sialyllactose to mid-exponential cells resulted in a marked increase in adhesive ability, as represented by a 4.7-fold increase in adhesion of *B. longum* subsp. *infantis* ATCC 15697 to the HT-29 cells versus the control ($p<0.0001$). No change in adhesion of *B. longum* subsp. *infantis* ATCC 15697 to HT-29 cells was observed following incubation alone with 3'sialyllactose, Beneo Orafti P95 (Fig. 1) or lactose (data not shown).

When combinations of the structurally similar milk sugars were tested, simultaneous exposure of *B. longum* subsp. *infantis* ATCC 15697 to 3'sialyllactose and 6'sialyllactose (1 mg/mL each) produced an enhanced effect with an increase in percent adhesion of 9.8-fold over control ($p<0.0001$; Fig. 1). In contrast, pairing lactose with 6'sialyllactose completely abolished the ability of 6'sialyllactose to enhance bacterial adhesion.

Adhesion of *B. longum* subsp. *infantis* ATCC 15697 to Caco-2 cells following pre-treatment with oligosaccharides displayed a different response than that of the HT-29 cell line. 3-Sialyllactose pre-treatment resulted in the greatest effect upon bacterial adhesion (1.85-fold increase), while pre-treatment with 6'sialyllactose or the combination of 3'- and 6'-sialyllactose increased adhesion by 1.53-fold and 1.58-fold, respectively ($p<0.01$) (Fig. S1). Pre-treatment with lactose or oligofructose (P95) did not significantly affect bacterial adhesion to the Caco-2 cell line.

Effect of 3'sialyllactose, 6'sialyllactose, lactose and P95 on the growth characteristics of B. longum subsp. infantis ATCC 15697

Growth studies were carried out to determine if the screened carbohydrates were capable of promoting the growth of *B. longum* subsp. *infantis* ATCC 15697 under the experimental conditions used in the adhesion study.

Lactose alone promoted the growth of the bacteria (Fig. 2) within the three hour incubation period. No change in growth rate was observed with 3'sialyllactose, 6'sialyllactose, oligofructose (P95), or the non-supplemented media. It was further confirmed that 6'sialyllactose did not affect growth of *B. longum* subsp. *infantis* following 3, 6, 9, and 24 hours of incubation in tissue culture media under identical conditions used in the adhesion assays (Fig. 3).

The effect of bacterial exposure on oligosaccharide concentrations

To assess whether the increased adhesion was associated with metabolism of the added oligosaccharides by the bacteria during the 3-hour exposure period, the oligosaccharide content of media before and after incubation with bacteria was determined by HPAEC-PAD (Fig. 4). No significant change was noted in the concentration of 6'sialyllactose following exposure at 1mg/mL to the bacteria. The concentration of 6'sialyllactose increased by 3.7%, which was non-significant. When lactose and 6'sialyllactose were incubated with bacteria together (1mg/mL each), a significant decrease of 29% in lactose concentration was detected ($p=0.0035$), while 6'sialyllactose showed an increase of 5.9% (non-significant). Interestingly, the media transitioned from red to yellow during the 3-hour bacterial incubation in the presence of lactose (alone or in combination with 6'sialyllactose; data not shown) indicating acidification. Bacterial exposure to the combination of 3'sialyllactose and 6'sialyllactose (1mg/mL each) resulted in non-significant increases in both 3'sialyllactose (1.9%) and 6'sialyllactose (2.6%). These results confirm that the two oligosaccharides, 3'sialyllactose and 6'sialyllactose, were not significantly catabolised during bacterial exposure, while lactose was preferentially consumed.

Influence of oligosaccharide concentration and exposure time on bacterial adhesion to HT-29 cells

To determine if the effect of 6'sialyllactose on the adhesion of *B. longum* subsp. *infantis* ATCC 15697 could be enhanced, the effects of increased exposure duration (1mg/ml, 6 hours) or exposure concentration (2mg/ml, 3 hours) on adhesion of bacterial cells were examined. Each treatment group resulted in a significant increase in percent adhesion in relation to the control

(4.7-fold, $p<0.0001$ [1mg/ml; 3 hours]; 3.3-fold, $p<0.0001$ [1mg/ml; 6 hours]; 5.1-fold, $p<0.0001$ [2mg/ml; 3 hours]). Neither increasing the concentration of 6'sialyllactose nor the length of exposure significantly increased adhesion to HT-29 cells in comparison to the original exposure conditions (1mg/mL; 3 hour incubation; Fig. 5A). Given the current findings, an incubation time of 3 hours appears to produce the maximal increase in adhesion of the bacteria to the HT-29 cells.

As increasing the concentration of 6'sialyllactose did not significantly increase bacterial adhesion beyond that previously achieved, lower concentrations were investigated. Subsequent assays were performed using lower concentrations of 6'sialyllactose (0.25 and 0.50mg/mL) with an exposure time of 3 hours and these concentrations did not significantly alter the adhesion of the bacteria in relation to the control, while exposure to 1mg/ml continued to demonstrate a significant increase in adhesion (4.2 fold; $p<0.0001$) (Fig. 5B). Additionally, no increase in adhesion was noted after 1-hour exposure of bacteria to 6'sialyllactose (1 mg/mL) (Fig. 5B).

Effect of trypsin-treatment on adhesion of B. longum subsp. infantis ATCC 15697 to HT-29 cells following oligosaccharide exposure

To explore whether the increased adhesion of the bacteria to HT-29 cells was mediated by a surface protein, *B. longum* subsp. *infantis* cells were treated with trypsin in PBS or PBS alone (1 hour) following 6'sialyllactose exposure (1mg/ml, 3 hours), and used subsequently in adhesion assays. The results indicate that trypsin-treatment following 6'sialyllactose exposure significantly reduces the adhesive percentage of the bacteria to HT-29 cells compared to the control ($p<0.05$), while 6'sialyllactose exposure followed by incubation in PBS for 1 hour still resulted in increased adhesion ($p<0.05$) (Fig. 6). Trypsin treatment was carried out on a control group following exposure to non-supplemented media for 3 hours, resulting in a non-significant reduction in the adhesive percentage versus the untreated control ($p=0.0561$). Furthermore, comparison among the 6'sialyllactose-treated groups indicates that enzymatic treatment resulted in a significant reduction in adhesion to the HT-29 cells ($p<0.0001$). The results provide support for the involvement of a surface protein or proteins in the

normal adhesion of *B. longum* subsp. *infantis* ATCC 15697 to HT-29 cells and the increased adhesion following exposure to 6'sialyllactose.

Increased adhesion response is unique to B. longum subsp. infantis ATCC 15697

To assess whether 6'sialyllactose could promote the adhesion of other commensal strains, adhesion studies were performed under identical conditions with three different strains of bifidobacteria and a *Lactobacillus* strain. No significant increases in adhesion were observed for any of the other strains tested (Table 1), suggesting that the observed effect was species/strain-specific. The percentage adhesion of the bifidobacteria strains was much lower than the *Lactobacillus reuteri* (DPC 6100) (0.09 – 2.29% of the inoculum versus 12.0%, respectively).

Gene expression analysis by DNA microarray

The overall numbers of genes differentially expressed following the experimental treatments are shown by Venn diagram (Fig. 7). It can be seen from this analysis that while 3'- and 6'-sialyllactose alone induced numerous differentially expressed transcripts (DETs) in comparison to control media, that there was a very substantial synergistic effect observed when the *B. longum* subsp. *infantis* ATCC 15697 cells were treated with a mixture of 3'- and 6'-sialyllactose. Following exposure to the mixed oligosaccharides, a total of 135 DETs were up-regulated in comparison to 66 following treatment with 6'sialyllactose and 52 following treatment with 3'sialyllactose (Fig. 7). A relatively low number of genes (22) were differentially regulated by all three treatments (Table 2). As well as inducing the highest number of DETs following upon oligosaccharide treatment, the oligosaccharide mixture induced the highest number of treatment specific genes (70) compared to 21 for 6'sialyllactose and 14 for 3'sialyllactose (Fig.7). The genes with altered levels of transcription are described in Tables S2-S4.

Given the effects of oligosaccharide treatment on adhesion, it is probable that only those genes that are up-regulated by more than one oligosaccharide treatment are central to the adhesive phenotype and may be considered the core component of the oligosaccharide response. The total number of core genes is

55, and of those, the 42 up-regulated genes shared between 6'sialyllactose and the 3'- and 6'-sialyllactose mixture (Fig. 7) are of particular interest as these treatments directly led to an increase in adhesion to HT-29 cells (Fig. 1). This group of 42 genes is listed in Table S5, where the function assigned to each (if known) is also stated.

Aside from the qualitative changes observed for gene expression, the quantitative aspect of the transcriptional response is also important. The synergistic effect of the oligosaccharide mixture on adhesion correlated strongly with a higher level of gene transcription induced by the mixed oligosaccharides. We have used boxplot type plots to represent the overall transcript levels for the 22 commonly up-regulated genes (Fig.8a) and from this plot it may be seen that the median value for gene transcription following each treatment correlated extremely well with the observed effects on adhesion.

The final set of genes of interest includes those genes whose up-regulation was unique to either 6'sialyllactose or 3' and 6'sialyllactose treatment. This group contains 21 genes specific to 6'sialyllactose treatment and 70 genes specific to the mixed 3'- and 6'-sialyllactose treatment (Fig.7). Finally, we examined the expression profile of all of the genes up-regulated by the 3'- and 6'-sialyllactose mixture based on their position in the Venn diagram. Of the 4 treatment groups, the genes common to all three treatment groups (22) had the highest transcript level followed by those genes commonly up-regulated by 6'sialyllactose and the mixture group ([6'SL] + [3'&6'SL]). The genes common to the 3'sialyllactose treatment and the 3'- and 6'-sialyllactose mixture ([3'SL] + [3'&6'SL]) and, lastly, those unique to the combination of 3'- and 6'-sialyllactose, had the lowest levels of transcription (Fig. S2). From this analysis it is clear that the genes most significant are those that are up-regulated by all treatments.

Our detailed analysis of the transcriptional response has identified two significant groups of genes that are likely responsible for the enhanced adhesion response which are those common to all 3 treatments (Table 2) and those common to the 6'sialyllactose and the 3'- and 6'-sialyllactose mixtures (Table S5).

The number of genes for which expression was reduced in response to oligosaccharide treatment was roughly similar to the number of up-regulated

genes. However, in general, the magnitude of the changes observed was lower than that for the up-regulated genes. The 13 genes down-regulated in common to all three treatments (Table 3) were down-regulated in inverse proportion to the observed adhesion effects, with the extent of down-regulation being highest for the cells treated with the oligosaccharide mixture (Fig 8b). Unlike the up-regulated transcripts, the majority of those genes that were down-regulated were associated with sugar transport and metabolism. Among the down-regulated genes that would be central to sugar metabolism are two transcriptional regulators (blon_0573 and blon_0879) from the ROK family of proteins. Both of these genes appear to be transcriptional repressors and their down-regulation is likely to result in the up-regulation of the genes that they regulate. Interestingly, one of the genes down-regulated by both 6'sialyllactose and the 3'- and 6'-sialyllactose mixture was an exo-alpha sialidase that is located in a large cluster of genes (blon_2331-blon_2361) that are involved in sugar metabolism. Following a closer examination of the expression patterns of milk oligosaccharide metabolism genes, it is clear that the majority of them are down-regulated slightly following treatment with 6'-sialyllactose and down-regulated more so by exposure to the mixture of 3'- and 6'-sialyllactose (Figs. S3 and S4).

Gene expression validation by qPCR

The genes of interest identified by DNA microarray were further validated through the use of real-time PCR. In addition to those selected from the array analysis, we included the sortase and tadE pilus in the qPCR screening, as these genes were previously reported to be significant contributors to the adhesive potential of different strains of bifidobacteria (Foroni, E., Serafini, F., et al. 2011, O'Connell-Motherway, M., Zomer, A., et al. 2011) (Fig. 9). The 16S rRNA gene target produced a stable level of expression against which the relative expression of the selected gene targets could be assessed.

Two genes, Blon_1687 and Blon_1688, were found to be significantly up-regulated in the DNA microarray analysis, but were not validated through qPCR, as their levels of expression were nearly identical to those of the 16S rRNA control. Interestingly, four genes associated with bacterial adhesion were up-regulated following bacterial exposure to the combination of 3'- and 6'-sialyllactose; i) DPS-ferritin (Blon_0029); ii) GroEL (Blon_0694); iii) DnaK

(Blon_0141); and iv) Sortase (Blon_0162) (Fig. 7). When bacterial oligosaccharide exposures were performed in the presence of lactose, expression of each of the selected genes of interest was similar to that of the control.

CHAPTER II

DISCUSSION

By one month of age, bifidobacteria are the predominant fecal bacteria in neonates (Benno, Y., Sawada, K., et al. 1984), with several strains demonstrating the ability to utilize the abundant supply of breast milk oligosaccharides as a carbon source (Kiyohara, M., Tachizawa, A., et al. 2009, Marcobal, A., Barboza, M., et al. 2010). *Bifidobacterium longum* subsp. *infantis* ATCC 15697 is the archetypical human milk oligosaccharide consumer in the infant GI tract (Sela, D., Chapman, J., et al. 2008) and has previously been demonstrated to display an adherent phenotype following growth on human milk oligosaccharides in *in vitro* models (Chichlowski, M., De Lartigue, G., et al. 2012). However, the current study is the first to examine the effect of milk oligosaccharide treatment on the adhesive capacity of *B. longum* subsp. *infantis* ATCC 15697 and to identify specific transcriptional responses associated with an oligosaccharide-induced adherent phenotype.

While our findings were similar to that of Chichlowski *et al.*, the focus of our study was upon brief exposure (3 hours) to specific HMOs, as opposed to mid-exponential growth (48 hours) on a pool of HMOs (Chichlowski, M., De Lartigue, G., et al. 2012). Additionally, the current results were obtained through enumeration of live colonies by plating, compared to the methodology of qPCR. Bacterial adhesion to intestinal epithelial cells is strain- and species-specific (Bernet, M.F., Brassart, D., et al. 1993, Candela, M., Seibold, G., et al. 2005, Crociani, J., Grill, J.P., et al. 1995). Typically, the adhesive proportion of bifidobacteria, particularly *B. infantis* and *B. longum*, to Caco-2 and HT-29 cell lines is low (0-5%), with the exceptions of *B. bifidum*, *B. adolescentis* and *B. animalis* subsp. *lactis* Bb12 which display a greater degree of adhesion (10-30%) (Ali, Q.S., Farid, A. J., Kabeir, B. M., Zamberi, S., Shuhaimi, M., Ghazali, H. M., Yazid, A. M. 2009, Bernet, M.F., Brassart, D., et al. 1993, Candela, M., Seibold, G., et al. 2005, Del Re, B., Sgorbati, B., et al. 2000, Gleinser, M., Grimm, V., Zhurina, D., Yuan, J., Riedel, C. U. 2012, Laparra, J.M. and Sanz, Y. 2009, Preising, J., Philippe, D., et al. 2010, Riedel, C.U., Foata, F., et al. 2006). The current findings are in agreement with the previous studies in terms of percentage adherence and adherent bacteria/100 mammalian cells.

In agreement with the results of Chichlowski *et al.*, our results demonstrate that exposure of *B. longum* subsp. *infantis* ATCC 15697 to 3'SL, 6'SL, or a combination of the two, resulted in approximately 1.5-1.8-fold increased adhesion to Caco-2 cells and an increase in adhesion to HT-29 cells (3-4-fold increase following growth on a pool of HMOs versus 4-10-fold increase observed in the current study), though independent of growth upon the supplemented oligosaccharides. Confluent and fully-differentiated Caco-2 cells are similar to small intestine-like enterocytes (Chopra, D.P., Dombkowski, A.A., et al. 2010), while HT-29 cells are colonic adenocarcinoma epithelial cells remaining undifferentiated (95%) in the post-confluent state (Moss, A.C., Anton, P., et al. 2007). Accordingly, the HT-29 model may better represent the *in vivo* immature intestinal environment of a neonate, as infant-type bifidobacteria encounter milk oligosaccharides in the colon(Sela, D.A. 2011), a destination in which sialylated milk oligosaccharides may be enriched (Sela, D.A., Li, Y., et al. 2011). Further *in vivo* colonisation studies are necessary to validate the model-specific effects observed.

In the current study, increased adhesion conferred by exposure to HMOs was not accompanied by a change in oligosaccharide content in the spent culture supernatant, indicating that under the given conditions, 3'- and 6'-sialyllactose were not catabolised. Additionally, it has previously been reported that semi-synthetic media supplemented with human milk oligosaccharides at less than 0.8% (8mg/mL) resulted in only minimal growth of *B. longum* subsp. *infantis* ATCC 15697 (LoCascio, R.G., Ninonuevo, M.R., et al. 2007), indicating that the concentrations used in the current study were not sufficient to stimulate growth. It is clear from analysis of both bacterial growth and the oligosaccharide content of the assay medium that the oligosaccharides did not confer a growth advantage to the bacteria under the conditions used in this study, thereby eliminating the possibility that the increased adhesion was due to an increase in the bacterial numbers during the assay.

While *in vivo* experiments are ideal, the implementation of *in vitro* cell lines, such as HT-29 and Caco-2, prove valuable. Though the two cell lines are not completely representative of *in vivo* conditions, they provide a tightly-controlled environment in which to assay the ability of bacterial strains to adhere to differentiated and non-differentiated enterocytes. Though the intestinal cell

lines used do not produce the mucus layer that is typically present in *in vivo* conditions, the mucin producing cell lines currently available do not produce a mucus layer specifically representative of the large intestine or colon, while primary cell cultures present difficulties in reproducibility (Langerholc, T., Maragkoudakis, P.A., et al. 2011). Furthermore, there exist situations wherein the mucus layer has not yet fully developed or becomes disrupted. Under these circumstances, adhesion of intestinal bacteria to colonocytes becomes an issue. In the given scenario, it is of advantage to the host to promote or accommodate colonisation by beneficial bacteria, as their colonisation will negate the effects of invasion by other potentially pro-inflammatory species.

It is important to remember that the *in vivo* exposure of intestinal bacteria to oligosaccharides present in the digesta will always take the form of exposure to a mixture of different oligosaccharides. The synergistic effect between the oligosaccharides reported here is evidence that *B. longum* subsp. *infantis* produces a more effective adhesion phenotype under conditions that better represent the intestinal milieu. While 3'sialyllactose and 6'sialyllactose are identical in molecular weight and contain the same functional groups, a marked difference in adhesion-promoting ability exists between the two, which infers a specific response to α-2,6-linked sialic acid. Furthermore, the presence of lactose completely abolished the increased adhesion phenotype of *B. longum* subsp. *infantis* ATCC 15697 to the HT-29 cell line. As reported by Garrido *et al.*, (2011), growth of *Bifidobacterium longum* subsp. *infantis* ATCC 15697 on HMO as a sole carbon source, resulted in the expression of specific proteins that bind and import HMO isomers, but were also able to bind mucin and blood group glycans. If this finding is considered along with the fact that bacterial treatment with trypsin is accompanied by a significant reduction in adhesion, bacterial exposure to the milk oligosaccharide, 6'sialyllactose, may contribute to increased bacterial adhesion to the HT-29 intestinal model through expression of a proteinaceous surface receptor (Mukai, T., Toba, T., et al. 1997).

We observed a strong correlation between the number of differentially expressed transcripts following each oligosaccharide treatment and the observed increase in the adhesive potential of *B. longum* subsp. *infantis*. The use of Venn diagrams allowed us to identify genes that were induced by all of the treatments along with those genes induced specifically by certain treatments, thereby

allowing for the separation of the transcriptional responses to oligosaccharide treatments into two categories. *B. longum* subsp. *infantis* mounts what appears to be a general response to oligosaccharide exposure, wherein, 22 genes are induced in common to all three oligosaccharide treatments. It is clear that the magnitude of change in the transcript level for these genes is important in developing the adhesive response, as there was a strong correlation between transcript expression level and adhesion to the HT-29 cell line. A similar trend was observed for the 20 genes co-induced by the 6'sialyllactose treatment and the oligosaccharide mixture and these genes may be considered as being more specific to the adhesion process as they were only up-regulated by the treatments that enhanced adhesion, with the extent of the increase in transcription correlating well with the changes in adherence.

It appears that the contribution of the individual genes to epithelial adherence is greater if they are differentially regulated by more than one treatment and these may be designated the core genes. The increased transcription of those genes that were unique to each treatment may reflect changes in cell processes occurring as a consequence of the up-regulation of the core genes rather than contributing to the adhesive process.

In terms of the enhanced HT-29 adhesion observed, the most significant gene identified was the DNA-binding protein-ferritin (dps-ferritin) locus. The dps-ferritin type family is a widely diverged family of proteins that has evolved to perform a range of functions other than DNA protection, including the formation of fine tangle pili in *Haemophilus ducreyii* (Brentjens, R.J., Ketterer, M., et al. 1996) and possible roles in adhesion and protection against oxidative stress in *Helicobacter pylori* (Cooksley, C., Jenks, P.J., et al. 2003). The function of this protein homolog (NapA) in *H. pylori* is of particular interest because *H. pylori* is effectively a commensal organism in the human gut and it is therefore quite probable that the dps-ferritin protein plays a similar role in *Bifidobacterium.* The close correlation observed between transcription of this dps-ferritin gene and the increased adhesion support the assumption that it is involved in virulence or adhesion. Members of the dps-ferritin family also have a role in stress response, particularly towards oxidative stress, and it has been demonstrated for *Salmonella enterocolitica* and *Listeria monocytogenes* that the

dps-ferritin stress resistance is associated with increased virulence (Dussurget, O., Dumas, E., et al. 2005, Halsey, T.A., Vazquez-Torres, A., et al. 2004).

GroEL is a well characterised heat shock protein similar both in structure and function to the eukaryotic Hsp60. GroEL is virtually ubiquitous in prokaryotes, being present in >90% of genomes examined (Charlebois, R.L. and Doolittle, W.F. 2004) and is also present in bifidobacteria (Ventura, M., Canchaya, C., et al. 2004). The most important function of groEL is in its role as a molecular chaperone wherein it acts to ensure that certain proteins are folded to the correct tertiary structure. In its protein-folding role, groEL requires the co-chaperonin groES in equimolar quantities and in *Bifidobacterium breve* UCC2003 groEL and groES are transcribed at similar levels in response to heat shock even though they are located in different regions of the genome. It is noticeable, however, that the levels of groES transcript induced in our studies did not correlate with those of groEL and this may indicate that the excess groEL may fulfil a role that is independent of groES. Furthermore, there was no evidence that groEL was co-transcribed with the contiguous cold-shock gene cspA (blon_0693) in this study unlike the case for other bifidobacteria (Ventura, M., Canchaya, C., et al. 2004), as transcript levels for cspA were lower following all treatments. These observations may indicate that regulation of groEL mRNA levels occurs by means of decreased RNA turnover rather than increased transcription levels and may suggest a role for groEL in adhesion that is independent of its chaperone function. Surface expression of the groEL protein has been previously shown in several pathogens and has been implicated in attachment and/or immunomodulatory activities (Bergonzelli, G.E., Granato, D., et al. 2006, Dunn, B.E., Vakil, N.B., et al. 1997, Frisk, A., Ison, C., et al. 1998, Gillis, T., Miller, R., et al. 1985, Hennequin, C., Porcheray, F., et al. 2001). Surface expression of groEL has also been reported for the probiotic *Lactobacillus johnsonii* La1 (NCC 533), and was demonstrated to bind to both mucins and epithelial cells, as well as possessing the ability to aggregate *Helicobacter pylori* (Bergonzelli, G.E., Granato, D., et al. 2006). In this study, increased levels of groEL transcript followed all three treatments, with the greatest response following exposure to the mixture of 3'- and 6'-sialyllactose which correlated well with the increased adhesion to HT-29 cells observed.

DnaK is another chaperone protein, the transcription of which is induced on exposure to environmental stresses (temperature, bile salts, etc.). We observed transcript levels for *dnaK* at levels that were discordant with those of other known chaperone proteins apart from groEL. It is interesting because as reported for *groEL*, dnaK is also regarded as having a potential for acting as a surface bound receptor in *Bifidobacterium animalis* and has been associated with increased adhesion to host tissues (Candela, M., Centanni, M., et al. 2010). The experimental observations in this study appear to support a role for these two proteins in adhesion as exposure of bifidobacteria to milk oligosaccharides should not elicit a stress response. However in terms of intestinal colonisation, the ability to mount a response likely to facilitate adherence is advantageous to the bacteria and sustaining the level of dnaK and groEL transcript will obviously be of benefit under these conditions.

Although the sortase gene was not detected as being up-regulated by the microarray experiment, we did detect up-regulation by qPCR. The function of sortase enzymes in the anchoring of surface proteins is vital to their correct presentation on the external surface of bacterial cells. It is thought that the anchoring of sortase-dependent proteins may in some circumstances be limited by the availability of the sortase enzyme and therefore transcription of the sortase at higher levels would increase the anchoring of sortase-dependent proteins. Sortases are not essential for cell growth, as sortase deletion mutants are viable, however, adhesion of *Lactobacillus salivarius* to epithelial cells is reduced in sortase-negative mutants (Remus, D.M., Bongers, R.S., et al. 2013, van Pijkeren, J.-P., Canchaya, C., et al. 2006)

The tight adherence locus (*tad*) is homologous in members of *Actinobacteria* (Tomich, M., Planet, P.J., et al. 2007) and is responsible for the formation and assembly of type IVB pili. These pili bind to carbohydrate moieties present in glycoprotein and glycolipid receptors and are thought to mediate the initial encounter with host structures (Telford, J.L., Barocchi, M.A., et al. 2006). TadE transcription, as measured using the microarrays, was not significantly increased in response to oligosaccharide treatment, however because this locus has been reported elsewhere to be an important mediator of colonisation in *B. breve* (O'Connell-Motherway, M., Zomer, A., et al. 2011) we decided to examine the expression of the tadE gene by qPCR, where it was found

to be transcribed at a higher level. This increased expression of tadE and its role in the assembly of proteinaceous surface appendages could potentially contribute to the enhanced adhesive response of *B. longum* subsp. *infantis* ATCC 15697.

It is not clear why this discrepancy between the array results and the qPCR results occurred for those genes, however, differences between transcriptional data obtained from microarray and qPCR experiments are not unknown and may arise in this instance from the fact that the microarrays used directly-labelled mRNA while the qPCR analysis was done on cDNA. In addition, the selection process for deciding if a particular locus is significant in the microarrays was a combination of fold-change and statistical significance and this process leads to genes that narrowly exceed the statistical cut-offs being disregarded.

A glycosyl hydrolase active against the GlcNAcβ1-3 linkages found in lacto-N-hexaose, an oligosaccharide abundant in human milk (Garrido, D., Ruiz-Moyano, S., et al. 2012), was the only example of an up-regulated gene that was specifically related to sugar metabolism. In this study, this gene was only substantially differentially expressed in response to the mixture of 3' and 6'sialyllactose. In addition to the glycosyl hydrolase, a sugar-binding ABC transporter component was also up-regulated (blon_2061). Of interest regarding this gene is that there are 6 similar binding proteins present in the genome of *B. longum* subsp. *infantis* 15697 yet blon_2061 is the only one up-regulated, indicating that the induction of this gene is treatment-specific and not a component of a broad-spectrum global transcriptional response to oligosaccharide exposure. The general tendency was for genes involved in carbohydrate metabolism to be down-regulated.

Interestingly, the presence of lactose in experimental media at equal concentration during bacterial exposure resulted in the elimination of both, the adhesive *in vitro* response, as well as the associated up-regulation of the selected genes of interest determined through qPCR. Lactose is largely digested and removed in the small intestine, while HMOs are able to resist digestion and reach the colon (Engfer, M.B., Stahl, B., et al. 2000, Gnoth, M.J., Kunz, C., et al. 2000b). Accordingly, the current data demonstrate the potential function of HMOs to act as a site-specific adhesion-promoting factor in the large intestine.

Examination of the down-regulated genes revealed the interesting result that the HMO-utilisation cluster described previously by Sela *et al.* (Sela, D.A., Chapman, J., et al. 2008) was generally down-regulated following all three oligosaccharide treatments. As this cluster of thirty contiguous genes encodes sialidases, fucosidases, β-galactosidases, solute-binding proteins, and ABC transport permeases (LoCascio, R.G., Desai, P., et al. 2010, Sela, D.A., Chapman, J., et al. 2008), it is likely most effective in the degradation of complex milk oligosaccharides and the presence of the relatively simple structures of 3'sialyllactose and 6'sialyllactose may well act to suppress the transcription of those genes involved in complex oligosaccharide hydrolysis. Furthermore, digestion and growth upon HMOs as a sole carbon source is typically observed between 20-48 hours following inoculation (Ward, R.E., Ninonuevo, M., et al. 2007). A second cluster of genes reported to be involved in metabolism of lacto-N-biose and galacto-N-biose (LNB/GNB metabolic pathway; blon_2171-blon_2177) (LoCascio, R.G., Desai, P., et al. 2010) was also slightly down-regulated and again this down-regulation may be a consequence of the availability of less complex oligosaccharides. These findings relating to suppressed carbohydrate metabolism are supported by the HPLC results indicating that significant oligosaccharide metabolism was absent during the oligosaccharide exposure period of three hours.

This study has confirmed a substantial increase in the adhesive potential of *B. longum* subsp. *infantis* ATCC 15697 to HT-29 cells following certain oligosaccharide treatments and this correlated extremely well with the transcriptional response. The transcriptional response itself was composed of two distinct components in which there was a significant up-regulation of a number of genes likely to enhance adhesion to epithelial cells with an additional down-regulatory effect on the transcription of a group of genes likely to be involved in the metabolism of more complex oligosaccharides. The magnitude of the transcriptional response (and the enhanced adhesion phenotype) was greater during co-incubation with two oligosaccharides rather than either alone. Exposure of *B. longum* subsp. *infantis* ATCC 15697 to 6'sialyllactose at a concentration of 1mg/mL for a duration of 3 hours results in a substantial increase in adhesion to the HT-29 cells compared to the non-oligosaccharide exposed control. While increasing exposure time or concentration did not

produce a concentration-dependent effect, decreasing the oligosaccharide concentration to 0.5mg/ml or below resulted in levels of adhesion to HT-29 cells that did not differ from the control samples. This would suggest that while relatively low concentrations of oligosaccharides are effective in promoting adhesion, there is a threshold value below which they have no effect. This would suggest that *B. longum* subsp. *infantis* ATCC 15697 possesses a sensitive detection mechanism for the presence of oligosaccharides that is active in the micromolar range and which responds to oligosaccharide concentrations between 0.5mg/ml and 1 mg/ml. The quite narrow response range suggests a sensor that is adapted to respond to a relatively low and stable level of oligosaccharides likely to be present in the intestinal environment, as levels of 3'sialyllactose and 6'sialyllactose in human colostrum are 0.30 and 0.37 mg/mL, respectively, following the first 3 days of lactation (Asakuma, S., Akahori, M., et al. 2007) and thought to be further concentrated and enriched in the large intestine and colon (Sela, D.A., Li, Y., et al. 2011).

The current findings suggest that *B. longum* subsp. *infantis* ATCC 15697 is capable of sensing subtle environmental signals such as oligosaccharides and mounting a proportional transcriptomic and physiological response. The ability to detect and respond to the presence of potentially beneficial adhesion-promoting molecules is of immense benefit to a commensal species exposed to the constantly changing gut environment. The results obtained from this study provide a new insight into the mechanisms by which commensal bacteria adapt to their residency in the intestinal tract and the role of ingested oligosaccharides in eliciting an adaptive response. Of particular interest is the identification of a number of genes for potential colonisation factors that are very similar to known virulence factors employed by pathogenic bacteria. The expression of these genes may give beneficial commensals such as *Bifidobacterium* the opportunity to compete with pathogenic bacteria for specific intestinal niches thereby reducing colonisation by pathogens. The induction of these responses by milk oligosaccharides provides a molecular-level explanation to aid in the identification and characterisation of the protective effects of milk oligosaccharides previously observed in other *in vivo* studies.

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Table 1. Screen of commensal strains for increased adherence to HT-29 cells following exposure to 6'sialyllactose.

	B. longum subsp. *infantis* ATCC 15697	*B. longum* subsp. *infantis* ATCC 15702	*B. longum* NCIMB 8809	*B. angulatum* NCIMB 2236	*L.reuteri* DPC 6100
Control (% Adherent)	0.40% ± 0.18	0.094% ± 0.019	2.29% ± 0.38	1.14% ± 0.066	12.00% ± 2.30
6'SL-exposed (% Adherent)	1.81% ± 0.71	0.14% ± 0.050	1.61% ± 0.30	0.90% ± 0.53	11.57% ± 1.20
Fold-change	4.53	1.47	0.70	0.79	0.96

Table 2. Up-regulated genes common to all three oligosaccharide treatments.

		3'SL		6'SL		3'+6'SL	
Locus_tag	Gene Description	FC	pval	FC	pval	FC	pval
Blon_0029	Ferritin, Dps family protein	1.67	0.0002	1.72	0.0002	2.79	0.0002
Blon_0036	FAD-dependent pyridine nucleotide-disulphide oxidoreductase	1.31	0.017	1.26	0.036	1.78	0.0001
Blon_0392	Cation efflux protein	1.66	0.006	1.84	0.0001	3.04	0.00001
Blon_0460	Binding-protein-dependent transport systems inner membrane component	1.32	0.037	1.38	0.02	1.81	0.001
Blon_0617	Glutamate-cysteine ligase, GCS2	1.32	0.022	1.63	0.0009	1.37	0.014
Blon_0758	Glutaredoxin-like protein	1.45	0.004	1.45	0.004	1.99	0.0007
Blon_0759	ABC transporter related	1.42	0.029	1.37	0.05	1.93	0.0007
Blon_0902	Initiation factor 3	1.65	0.005	1.59	0.008	1.43	0.012
Blon_0947	helix-turn-helix domain protein	1.32	0.01	1.35	0.007	1.54	0.0002
Blon_0948	hypothetical protein	1.3	0.02	1.3	0.02	1.5	0.003
Blon_0992	hypothetical protein	1.43	0.021	1.98	0.004	2.47	0.002
Blon_1687	TfoX, C-terminal domain protein	1.56	0.009	1.93	0.0006	2.26	0.001
Blon_1688	transcription activator, effector binding	1.78	0.0005	2.35	0.00002	2.88	0.00006
Blon_1950	hypothetical protein	1.26	0.01	1.2	0.03	1.35	0.001
Blon_1951	UMUC domain protein DNA-repair protein	1.28	0.012	1.22	0.03	1.41	0.001
Blon_2191	ribose 5-phosphate isomerase	1.23	0.025	1.25	0.016	1.58	0.001
Blon_2370	glycerophosphoryl diester phosphodiesterase	1.71	0.0002	1.83	0.00008	2.09	0.00001
Blon_2371	Glutamate--tRNA ligase	1.45	0.002	1.75	0.0001	2.29	0.000005
Blon_2372	ATPase AAA-2 domain protein	1.7	0.0002	1.88	0.00004	2.45	0.000004
dnaK	chaperone protein DnaK	1.44	0.002	1.57	0.0005	1.97	0.00006
groEL	chaperonin GroEL	1.33	0.02	1.37	0.015	2.11	0.0022
recA	recA protein	1.19	0.044	1.26	0.014	1.21	0.027

3'SL – 3'sialyllactose; 6'SL – 6'sialyllactose; 3'+6'SL – combined treatment of 3'- and 6'-sialyllactose; FC – fold change; pval – p-value

Table 3. Down-regulated genes common to all treatments.

		3'SL		6'SL		3'+6'SL	
Locus tag	Gene Description	FC	pval	FC	pval	FC	pval
Blon_0335	trc regulator merR	-1.20	0.0400	-1.28	0.0100	-1.29	0.0060
Blon_0505	Hypothetical protein	-1.26	0.0200	-1.26	0.0190	-1.27	0.0100
Blon_0644	ROK family protein	-1.29	0.0400	-1.36	0.0200	-1.65	0.0080
Blon_0645	N-acetylglucosamine-6-phosphate deacetylase	-1.38	0.0300	-1.36	0.0300	-1.69	0.0030
Blon_0790	Proteinase inhibitor	-1.25	0.0500	-1.28	0.0300	-1.46	0.0040
Blon_0884	Extracellular solute binding protein	-1.31	0.0200	-1.29	0.0300	-1.47	0.0500
Blon_0885	Binding protein dependent sugar transport inner membrane protein	-1.25	0.0200	-1.40	0.0020	-1.50	0.0000
Blon_2174	Hypothetical protein	-1.40	0.0200	-1.55	0.0060	-1.75	0.0020
Blon_2175	Solute transport	-1.35	0.0300	-1.56	0.0030	-1.43	0.0100
Blon_2176	Solute transport	-1.46	0.0060	-1.63	0.0010	-1.51	0.0020
Blon_2341	Hypothetical protein	-1.29	0.0190	-1.33	0.0100	-1.73	0.0020
Blon_2379	Transport system inner membrane protein	-1.55	0.0070	-1.41	0.0200	-1.85	0.0020
Blon_2380	Sugar transport system solute binding protein	-1.51	0.0030	-1.38	0.0100	-1.53	0.0007

3'SL – 3'sialyllactose; 6'SL – 6'sialyllactose; 3'+6'SL – combined treatment of 3'- and 6'-sialyllactose; FC – fold change; pval – p-value

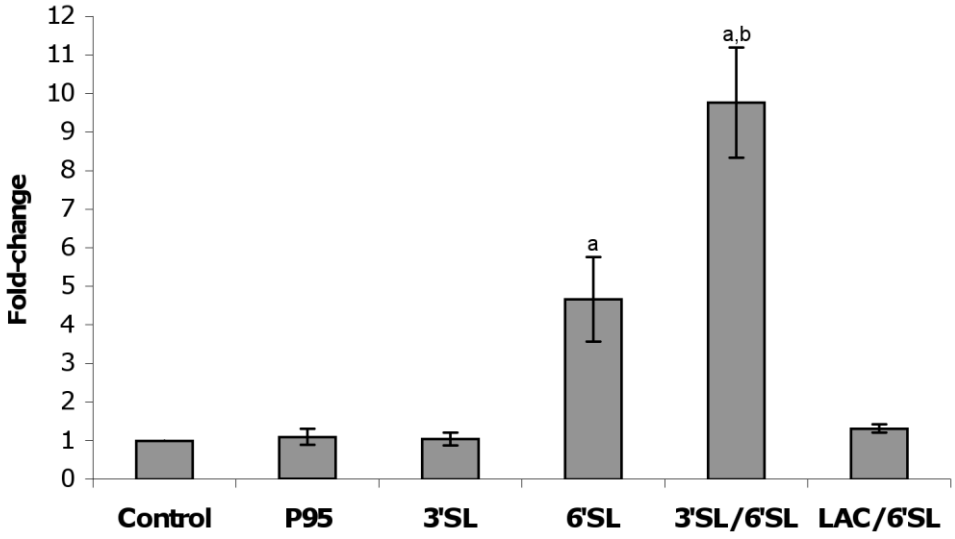

Fig. 1. Screening oligosaccharides for their ability to influence adhesion of *B. longum* subsp. *infantis* ATCC 15697 to HT-29 cells. Abbreviations: P95 - Beneo Orafti P95; 3'SL - 3'sialyllactose; 6'SL – 6'sialyllactose; Lac - lactose. Non-supplemented tissue culture media was used as control. Results are expressed as fold-change relative to control percent adhesion with error bars representing standard deviation. [a] denotes significant difference in relation to control; [b] denotes significance in relation to 6'SL group; $p<0.0001$.

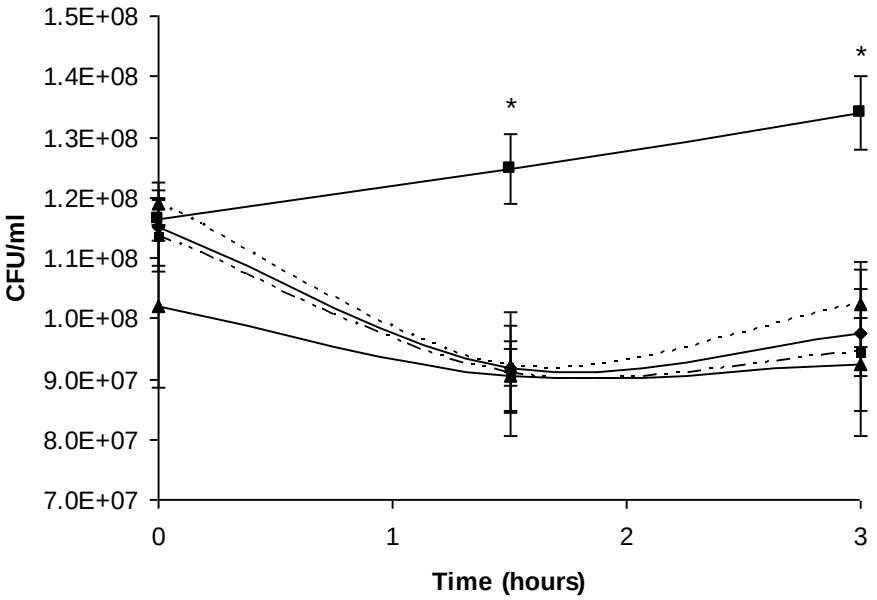

Fig. 2. Growth of *B. longum* subsp. *infantis* (ATCC 15697) under experimental oligosaccharide exposure conditions. 3'sialyllactose (-■-), 6'sialyllactose (-▲-), lactose (-■-), P95 (Beneo Orafti P95; -▲-) (1 mg/ml) and control tissue culture media (-◆-). Treatment groups were run in triplicate, with error bars representing the standard deviation. * denotes $p<0.05$.

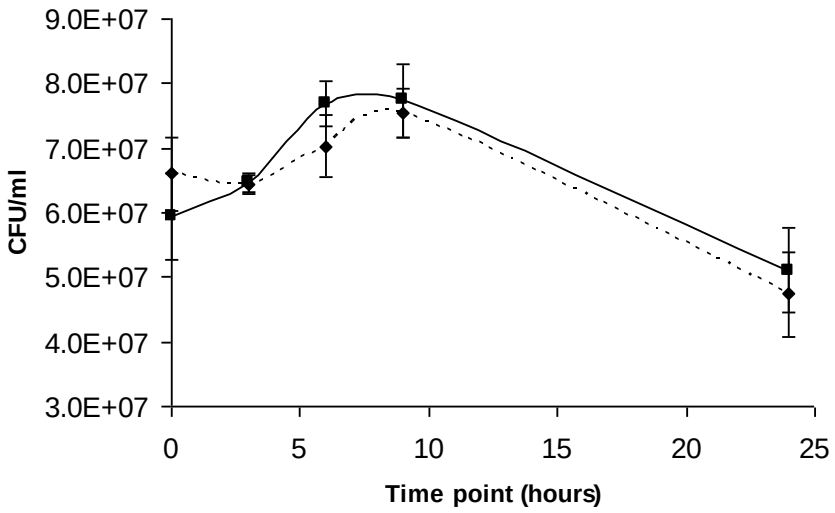

Fig. 3. Effects of 6'sialyllactose on growth and survival of *B. longum* subsp. *infantis* 15697. Growth curve of *Bifidobacterium longum* subsp. *infantis* ATCC 15697 in the presence of 6'sialyllactose (1mg/ml; square, solid line) or in control McCoy's 5A media (diamond, dashed line) after 24 hours of exposure. Treatment groups were run in triplicate, with error bars representing the standard deviation.

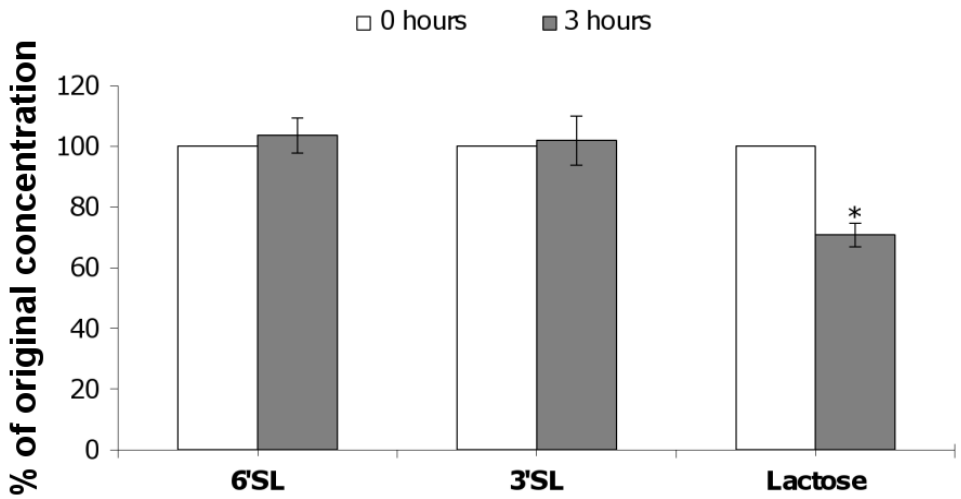

Fig. 4. Bacterial influence on oligosaccharide concentrations during exposure. Aliquots of media prior to and following exposure with *B. longum* subsp. *infantis* ATCC 15697 for 3 hours were assessed by HPLC. Abbreviations: 6'SL - 6'Sialyllactose; 3'SL - 3'sialyllactose. Results are expressed as percentage of initial oligosaccharide concentration in non-exposed media for each treatment group with error bars representing standard deviation. * denotes $p<0.005$.

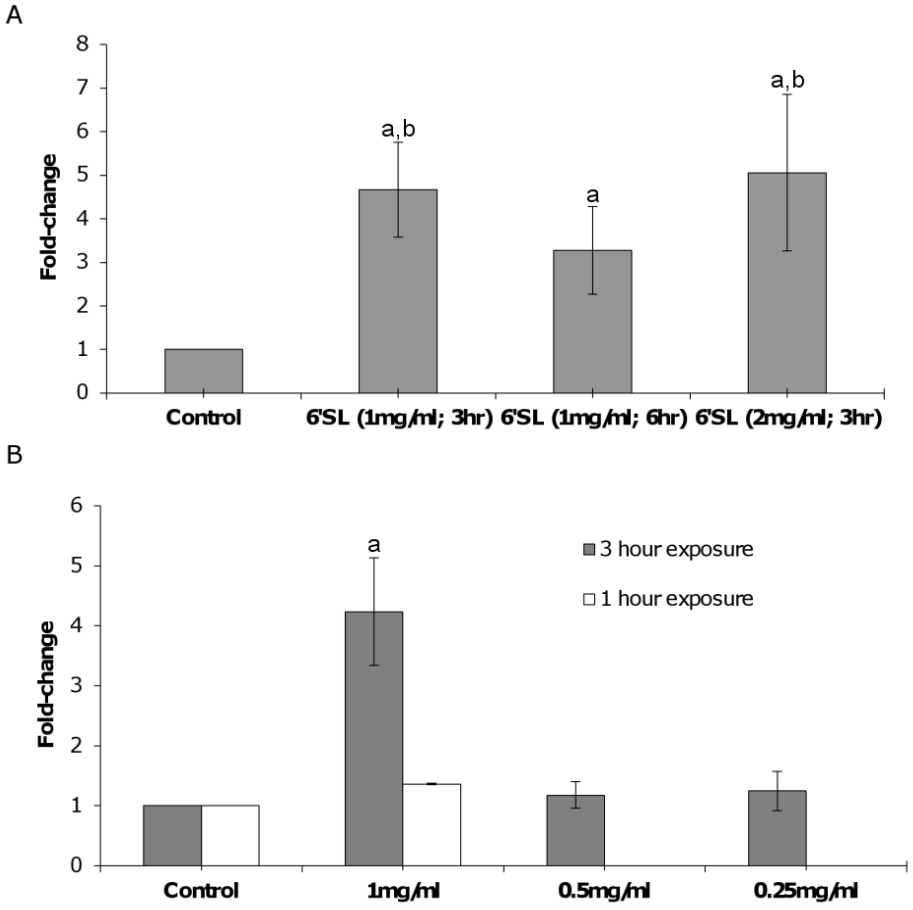

Fig. 5. Dose-response of *B. longum* subsp. *infantis* ATCC 15697 to 6'sialyllactose and subsequent adherence to HT-29 cells. (A) Bacterial exposure to original conditions (1mg/ml; 3 hours), increased duration (1mg/ml; 6 hours) or increased concentration (2mg/ml; 1 hour) in comparison to non-treated control bacteria. (B) Exposure to a gradient of 6'sialyllactose was assayed over 3 hours (gray shading) or control and 1mg/ml for 1 hour exposure (white shading). Results are expressed as fold-change in percent adhesion in relation to the respective control ± standard deviation. [a] denotes significance in relation to the control; [b] denotes significance in relation to 6'SL (1mg/ml; 6hr) treatment; $p<0.0001$.

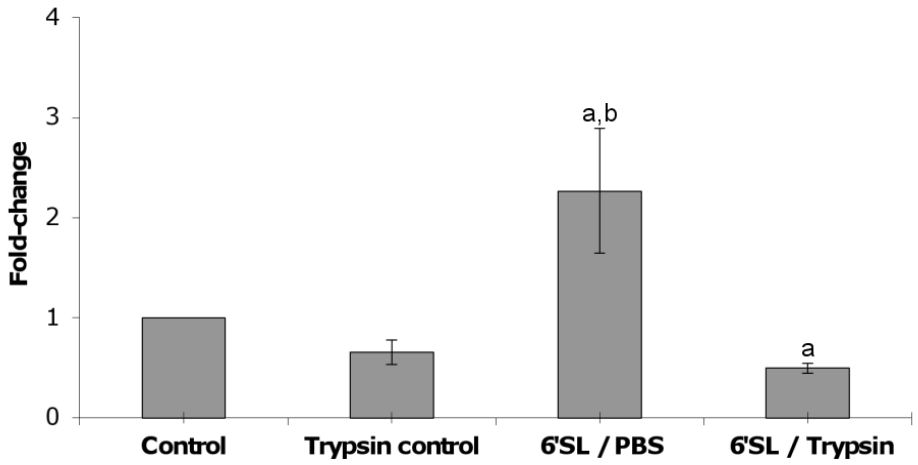

Fig. 6. Effects of trypsin treatment on adhesion of *B. longum* subsp. *infantis* ATCC 15697 to HT-29 cells following exposure to 6'sialyllactose (1mg/ml, 3 hours). Results are expressed as fold-change in percent adhesion in relation to the control with error bars representing standard deviation. [a] denotes significance in relation to the control, $p<0.05$; [b] denotes significance in relation to Trypsin control and 6'SL / Trypsin groups; $p=0.0001$.

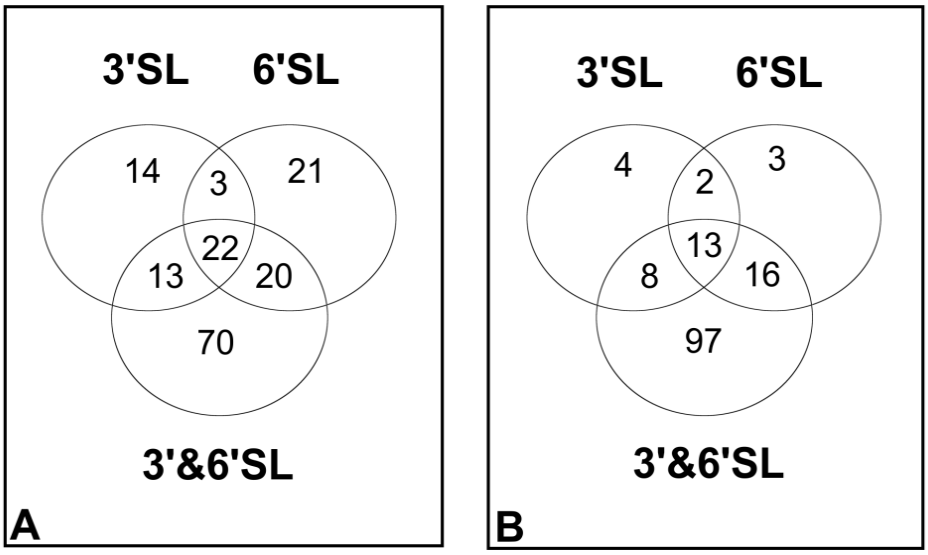

Fig. 7. Differentially expressed transcripts following exposure to the three oligosaccharide treatments. (A) up-regulated transcripts (B) down-regulated transcripts. The cut-off point for inclusion was a p-value <0.05.

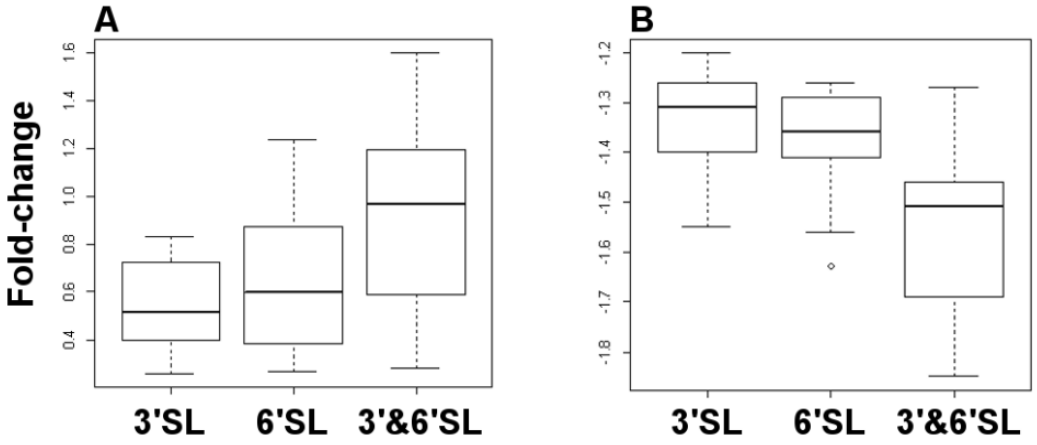

Fig. 8. Boxplot representation of the fold-change in expression for the genes up-regulated (A) and down-regulated (B) by all three treatments.

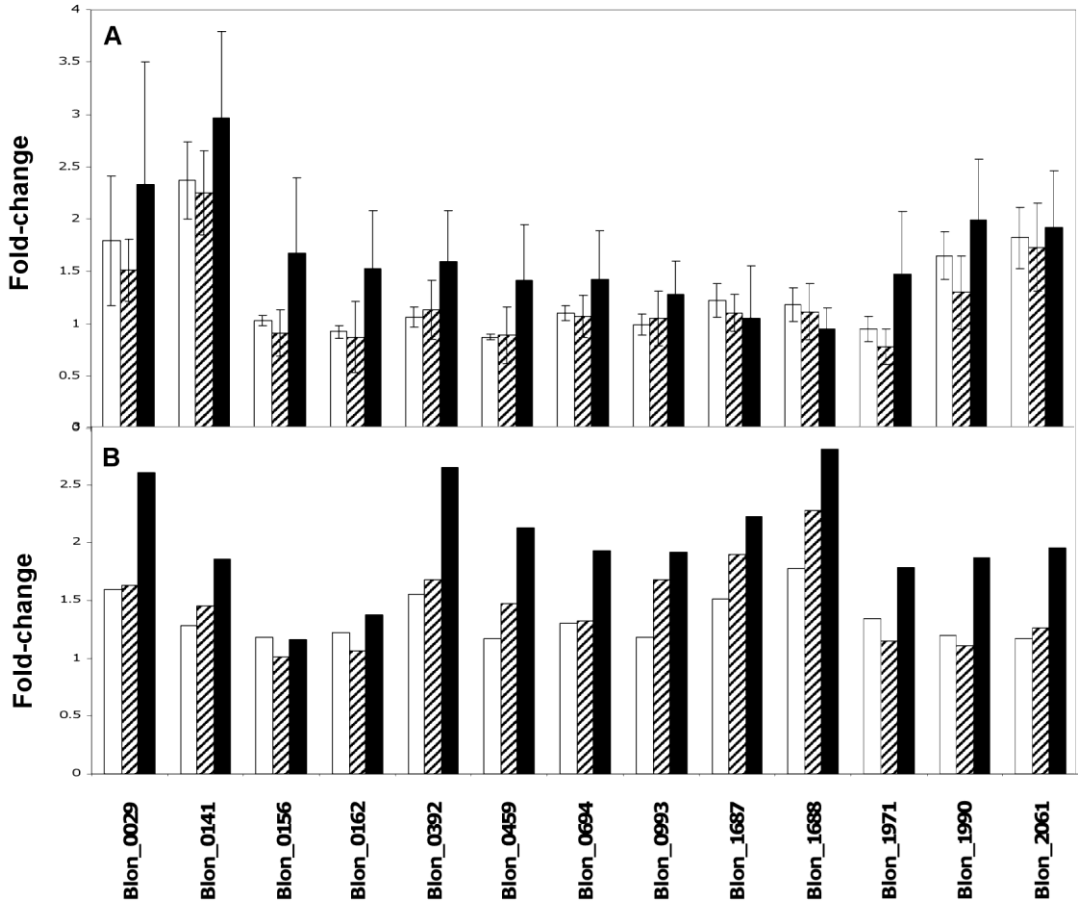

Fig. 9. Transcript levels for thirteen (13) selected genes as determined by qPCR (A) and microarray analysis (B). 3'sialyllactose (white shading); 6'sialyllactose (black and white diagonal shading); 3'- and 6'-sialyllactose mixture (black shading). Blon_0029 – Ferritin; Blon_0141 - Chaperonin protein DnaK; Blon_0156 - TadE family protein; Blon_0162 – Sortase; Blon_0392 - Cation efflux protein;Blon_0459 - Glycoside hydrolase, family 20; Blon_0694 – GroEL; Blon_0993 - Magnesium-translocating P-type ATPase; Blon_1687 - TfoX, C-terminal domain protein; Blon_1688 - Transcription activator, effector binding; Blon_1971 - High-affinity zinc ABC transporter; Blon_1990 - Glycine dehydrogenase; Blon_2061 – Extracellular solute binding protein.

SUPPLEMENTARY FIGURES AND TABLES

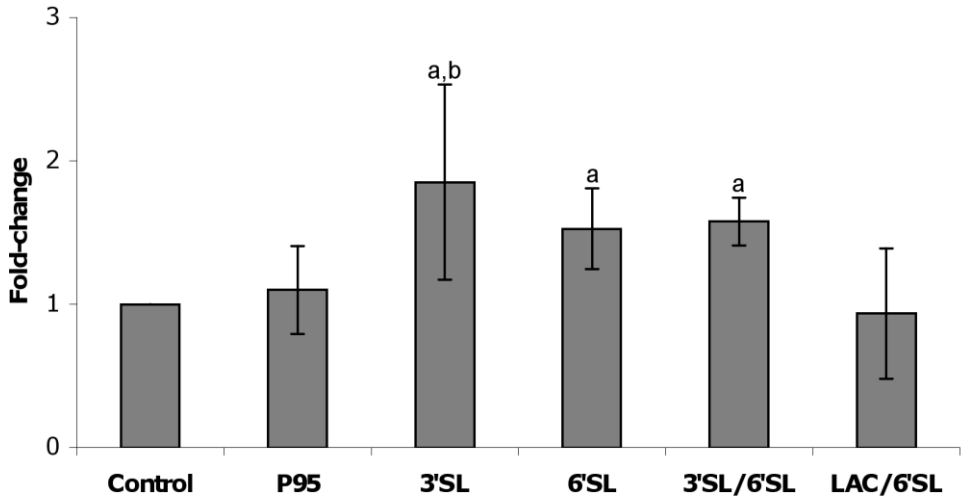

Fig. S1. Screening oligosaccharides for their ability to influence adhesion of *B. longum* subsp. *infantis* ATCC 15697 to Caco-2 monolayers. Abbreviations: P95 - Beneo Orafti P95; 3'SL - 3'sialyllactose; 6'SL – 6'sialyllactose; Lac - lactose. Non-supplemented tissue culture media was used as control. Results are expressed as fold-change relative to control percent adhesion with error bars representing standard deviation. [a] denotes significant difference in relation to control; [b] denotes significant difference in relation to P95 and LAC / 6'SL groups; p=0.0027.

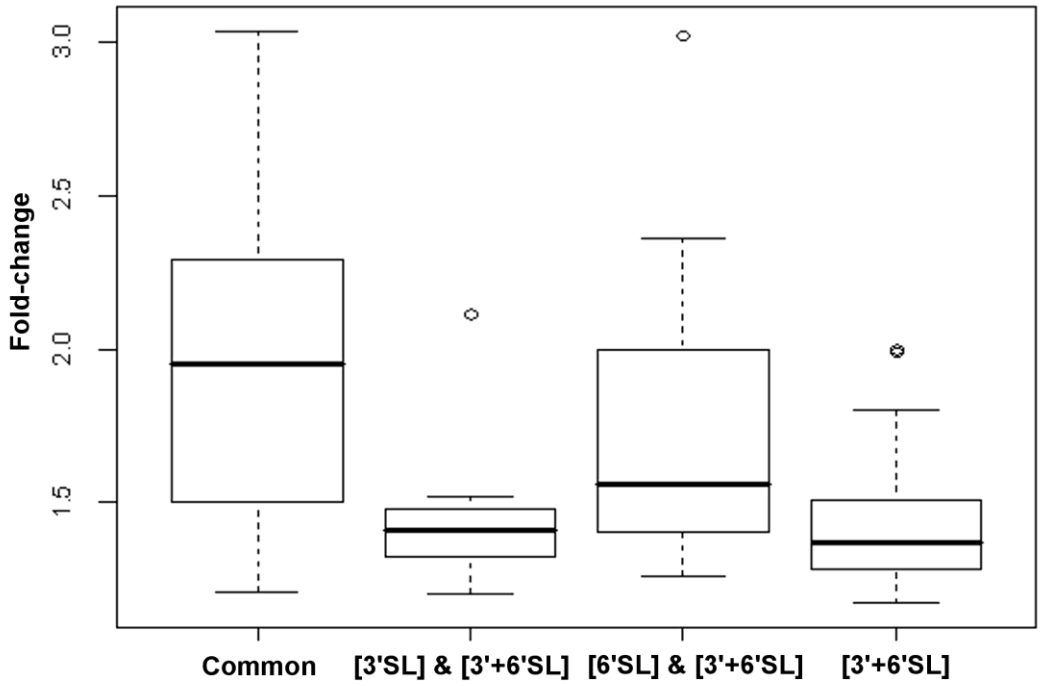

Fig. S2. Boxplot analysis of the pool of genes up-regulated by the mixture of 3'- and 6'-sialyllactose. Common = those genes upregulated by all treatments, [3'SL] & [3'+6'SL] = genes also upregulated by 3'SL; [6'SL] & [3'+6'SL] = genes also up-regulated by 6'SL alone; [3'+6'SL] = genes only up-regulated by the 3'- and 6'-sialyllactose mixture.

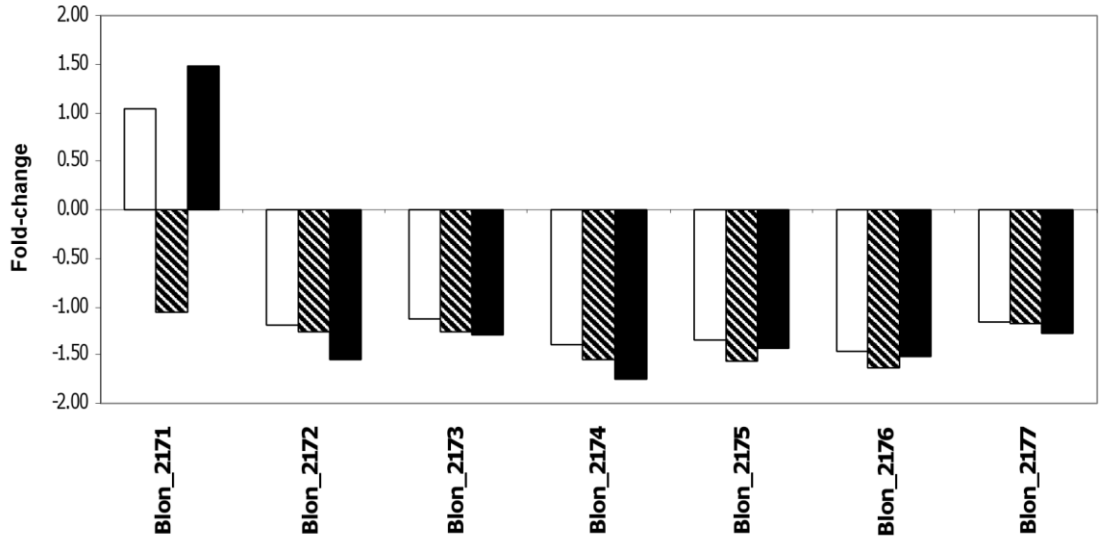

Fig. S3. Transcription levels (fold-change) for the cluster of genes for LNB / GNB metabolism. 3'sialyllactose (white shading); 6'sialyllactose (black and white diagonal shading); 3'- and 6'-sialyllactose mixture (black shading).

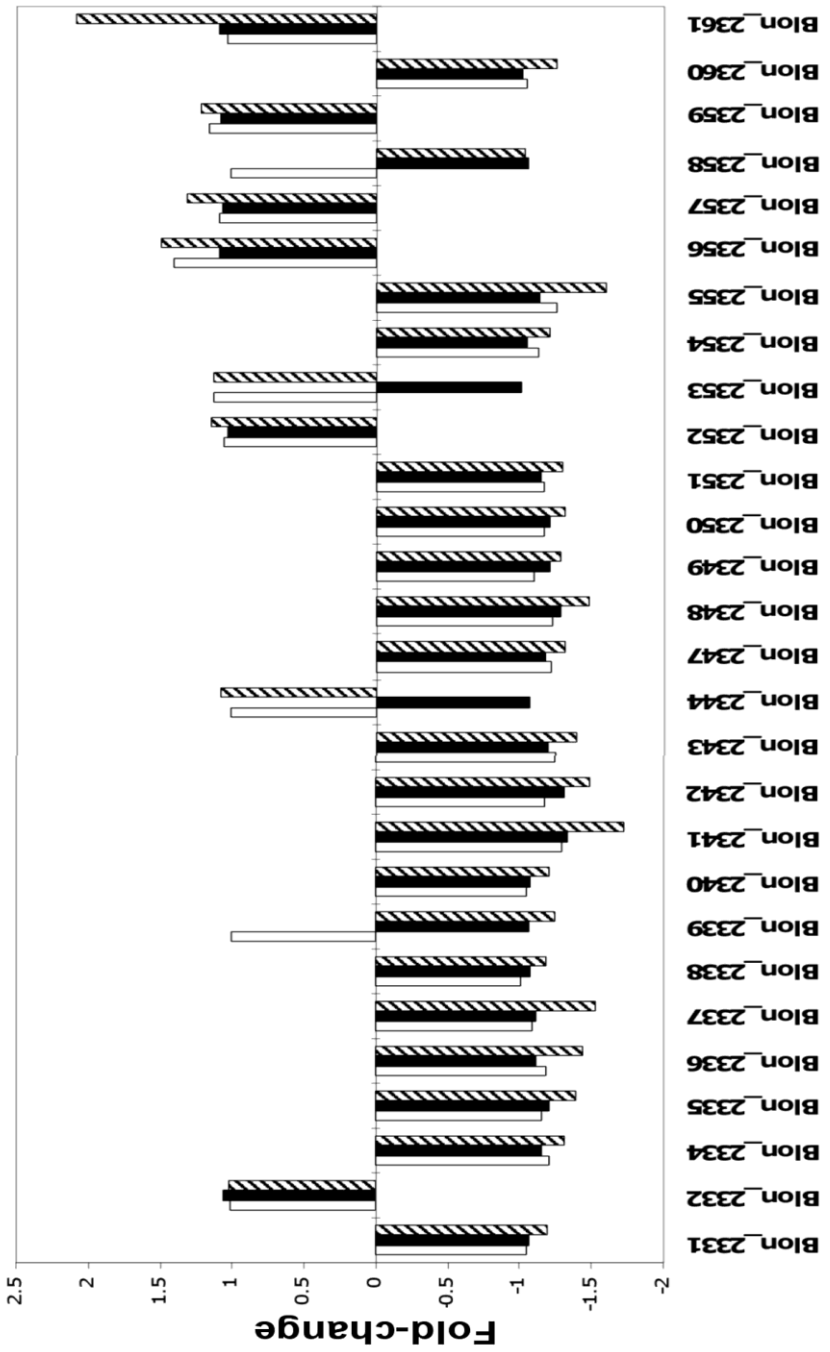

Fig. S4. Transcription (fold-change) of the genes in the HMO-utilisation cluster (blon_2331-blon_2361) following exposure to the three oligosaccharide treatments. 3'sialyllactose (white shading); 6'sialyllactose (black shading); 3' - and 6'-sialyllactose mixture (black and white diagonal shading).

Table S1. Selected genes, primers, and probes for qPCR.

Gene / Putative function	Primer (5' – 3')	Roche Probe #
Blon_1971 - High-affinity zinc ABC transporter	F: gtacgagcattcccacgac R: acaggtgcgggttcgtag	9
Blon_0029 - Ferritin	F: tcctgcagaaccgactgag R: gttccagtgtgcgtgcttc	15
Blon_0392 - Cation efflux protein	F: ggtcatctacgagatcctgacc R: tggaacgccggtaatacg	41
Blon_0993 - Magnesium-translocating P-type ATPase	F: gtgaaaccggatcgtgatg R: tccggtctggaagtagctgt	65
Blon_1687 - TfoX, C-terminal domain protein	F: ggtattttagatgaagacgatttgaa R: tttgctgtattttcaaccatgc	68
Blon_1688 - Transcription activator, effector binding	F: cgctggaactggcaaaaa R: caaccatgttccgaagatatactg	4
Blon_1990 - Glycine dehydrogenase	F: cgtatcatcgtccgcagtc R: ccggatacgccgtctgt	76
Blon_0459 - Glycoside hydrolase, family 20	F: ccgacaagctctgcctgta R: gcgaaggtgtgttcgatgt	78
Blon_2061 – Extracellular solute binding protein	F: aggtttccggcactcacat R: gcatctggagtcccagctt	20
Adhesion-related targets		
Blon_0141 - Chaperonin protein DnaK	F: gcaggcgaagaaggaactc R: gccaggtactgcatggaaat	10
Blon_0156 - TadE family protein	F: agactgccgccagacctat R: cgtcctattgccagtatcagc	61
Blon_0162 - Sortase	F: cagcccatcttgggtgaa R: ctggcttgggatcctcct	70
Blon_0694 - GroEL	F: gatgaggaagcccgtcag R: tgaccttgacggtgttgg	67
Endogenous controls		
Blon_0393 - Cysteinyl-tRNA synthetase	F: gcgatggcttcgacattc R: ccatctcgttttcgtggtg	70
Blon_R0085 – 16S rRNA	F: acgggtgagtaatgcgtga R: acccgtttccaggagctatt	43

Table S2: List of genes differentially regulated by 3'sialyllactose treatment.

Gene Name	Description	P-Value	Fold Change
Blon_0004	DNA replication and repair protein RecF	0.045	1.28
Blon_0029	Ferritin, Dps family protein	0.000	1.67
Blon_0036	FAD-dependent pyridine nucleotide-disulphide oxidoreductase	0.017	1.31
Blon_0121	hypothetical protein	0.019	0.75
Blon_0122	hypothetical protein	0.027	0.75
Blon_0125	Camphor resistance CrcB protein	0.020	0.79
Blon_0142	GrpE protein	0.045	1.24
Blon_0221	conserved hypothetical protein	0.033	0.80
Blon_0225	(Protein-PII) uridylyltransferase	0.015	0.76
Blon_0264	FAD dependent oxidoreductase	0.025	1.35
Blon_0335	putative transcriptional regulator, MerR family	0.041	0.84
Blon_0336	major facilitator superfamily MFS_1	0.050	0.75
Blon_0392	cation efflux protein	0.001	1.66
Blon_0460	binding-protein-dependent transport systems inner membrane component	0.037	1.32
Blon_0505	hypothetical protein	0.020	0.80
Blon_0612	anaerobic ribonucleoside-triphosphate reductase	0.022	1.27
Blon_0613	anaerobic ribonucleoside-triphosphate reductase activating protein	0.026	1.38
Blon_0617	glutamate--cysteine ligase, GCS2	0.022	1.32
Blon_0644	ROK family protein	0.044	0.77
Blon_0645	N-acylglucosamine-6-phosphate 2-epimerase	0.029	0.73
Blon_0646	glycosyl hydrolase, BNR repeat-containing protein	0.028	1.21
Blon_0697	two component transcriptional regulator, winged helix family	0.009	1.37
Blon_0702	ATPase AAA-2 domain protein	0.015	1.28
Blon_0758	Glutaredoxin-like protein	0.004	1.45
Blon_0759	ABC transporter related	0.029	1.42
Blon_0779	1-deoxy-D-xylulose 5-phosphate reductoisomerase	0.024	1.45
Blon_0790	proteinase inhibitor I4, serpin	0.047	0.80
Blon_0862	ABC transporter related	0.039	1.26
Blon_0884	binding-protein-dependent transport systems inner membrane component	0.024	0.76
Blon_0885	binding-protein-dependent transport systems inner membrane component	0.020	0.80
Blon_0892	conserved hypothetical protein	0.043	1.26
Blon_0902	initiation factor 3	0.005	1.65
Blon_0947	helix-turn-helix domain protein	0.010	1.32
Blon_0948	hypothetical protein Blon_0948	0.021	1.30
Blon_0992	hypothetical protein Blon_0992	0.021	1.43
Blon_1007	pyridoxamine 5'-phosphate oxidase-related, FMN-binding	0.018	1.24
Blon_1157	Holliday junction DNA helicase RuvA	0.048	1.26
Blon_1205	hypothetical protein	0.042	0.80
Blon_1337	hypothetical protein	0.024	0.71
Blon_1417	ABC transporter related	0.040	0.80
Blon_1574	branched-chain amino acid aminotransferase	0.040	0.83
Blon_1575	ribosomal 5S rRNA E-loop binding protein Ctc/L25/TL5	0.048	0.80
Blon_1640	DEAD/DEAH box helicase domain protein	0.004	1.57
Blon_1672	IstB domain protein ATP-binding protein	0.039	1.22
Blon_1687	TfoX, C-terminal domain protein	0.008	1.56

Gene Name	Description	P-Value	Fold Change
Blon_1688	transcription activator, effector binding	0.001	1.78
Blon_1776	GreA/GreB family elongation factor	0.023	1.27
Blon_1786	hypothetical protein	0.049	1.26
Blon_1850	regulatory protein GntR, HTH	0.048	1.30
Blon_1926	hypothetical protein	0.014	0.66
Blon_1950	hypothetical protein	0.010	1.26
Blon_1951	UMUC domain protein DNA-repair protein	0.012	1.28
Blon_1958	amidophosphoribosyltransferase	0.029	1.30
Blon_2174	conserved hypothetical protein	0.024	0.72
Blon_2175	binding-protein-dependent transport systems inner membrane component	0.028	0.74
Blon_2176	binding-protein-dependent transport systems inner membrane component	0.006	0.68
Blon_2191	ribose 5-phosphate isomerase	0.025	1.23
Blon_2341	protein of unknown function	0.019	0.77
Blon_2370	glycerophosphoryl diester phosphodiesterase	0.000	1.71
Blon_2371	Glutamate--tRNA ligase	0.002	1.45
Blon_2372	ATPase AAA-2 domain protein	0.000	1.70
Blon_2379	binding-protein-dependent transport systems inner membrane component	0.007	0.65
Blon_2380	extracellular solute-binding protein, family 1	0.003	0.66
Blon_2476	glycosyl transferase, family 2	0.046	0.83
dnaK	chaperone protein DnaK	0.002	1.44
groEL	chaperonin GroEL	0.024	1.33
hrcA	heat-inducible transcription repressor HrcA	0.043	1.25
pheT	phenylalanyl-tRNA synthetase, beta subunit	0.049	1.36
recA	recA protein	0.045	1.19
rnc	Ribonuclease III	0.041	1.39
rplD	ribosomal protein L4/L1e	0.047	1.29
rplT	ribosomal protein L20	0.048	1.23
rplW	Ribosomal protein L25/L23	0.042	1.29
rpmA	ribosomal protein L27	0.042	1.23
rpmF	ribosomal protein L32	0.027	1.28
rpmI	ribosomal protein L35	0.005	1.49
ruvB	Holliday junction DNA helicase RuvB	0.043	1.25

Table S3: List of genes differentially regulated by 6'sialyllactose treatment.

Gene Name	Description	P-Value	Fold Change
Blon_0028	conserved hypothetical protein	0.0491	1.35
Blon_0029	Ferritin, Dps family protein	0.0002	1.72
Blon_0036	FAD-dependent pyridine nucleotide-disulphide oxidoreductase	0.0365	1.26
Blon_0268	glycoside hydrolase family 2, sugar binding	0.0044	0.71
Blon_0286	lactoylglutathione lyase (LGUL) family protein, diverged	0.0441	1.91
Blon_0291	conserved hypothetical protein	0.0095	1.58
Blon_0307	ATP synthase F1, alpha subunit	0.0147	0.79
Blon_0332	hypothetical protein	0.0270	0.79
Blon_0335	putative transcriptional regulator, MerR family	0.0102	0.78
Blon_0392	cation efflux protein	0.0001	1.84
Blon_0394	glutamine amidotransferase class-I	0.0102	1.61
Blon_0450	hypothetical protein	0.0308	1.31
Blon_0459	glycoside hydrolase, family 20	0.0118	1.65
Blon_0460	binding-protein-dependent transport systems inner membrane component	0.0191	1.38
Blon_0505	hypothetical protein	0.0194	0.79
Blon_0518	hypothetical protein	0.0197	0.74
Blon_0536	hypothetical protein	0.0216	1.26
Blon_0573	ROK family protein	0.0341	0.75
Blon_0615	Resolvase, N-terminal domain protein	0.0260	1.24
Blon_0616	transposase, IS605 OrfB family	0.0446	1.25
Blon_0617	glutamate--cysteine ligase, GCS2	0.0009	1.63
Blon_0619	DNA polymerase, beta domain protein region	0.0014	1.59
Blon_0620	nucleotidyltransferase substrate binding protein, HI0074 family	0.0085	1.49
Blon_0621	Glucan 1,3-beta-glucosidase	0.0462	1.34
Blon_0643	conserved hypothetical protein	0.0308	0.78
Blon_0644	ROK family protein	0.0204	0.73
Blon_0645	N-acylglucosamine-6-phosphate 2-epimerase	0.0344	0.74
Blon_0748	Cystathionine gamma-synthase	0.0484	0.83
Blon_0758	Glutaredoxin-like protein	0.0044	1.45
Blon_0759	ABC transporter related	0.0463	1.37
Blon_0789	periplasmic binding protein/LacI transcriptional regulator	0.0456	0.83
Blon_0790	proteinase inhibitor I4, serpin	0.0330	0.78
Blon_0852	UDP-N-acetylmuramoylalanyl-D-glutamyl-2,6-diaminopimelate--D-alanyl-D-alanyl ligase	0.0453	0.82
Blon_0862	ABC transporter related	0.0141	1.34
Blon_0863	ABC-2 type transporter	0.0319	1.23
Blon_0864	ISXoo15 transposase	0.0343	1.24
Blon_0865	putative transcriptional regulator	0.0028	1.52
Blon_0884	binding-protein-dependent transport systems inner membrane component	0.0295	0.77
Blon_0885	binding-protein-dependent transport systems inner membrane component	0.0020	0.72
Blon_0902	initiation factor 3	0.0083	1.59
Blon_0947	helix-turn-helix domain protein	0.0067	1.35
Blon_0948	hypothetical protein	0.0199	1.30
Blon_0991	conserved hypothetical protein	0.0032	1.90
Blon_0992	hypothetical protein	0.0004	1.98
Blon_0993	hypothetical protein	0.0008	1.79
Blon_0994	transcriptional regulator, Fis family	0.0299	1.24

Gene Name	Description	P-Value	Fold Change
Blon_1037	conserved hypothetical protein	0.0485	1.41
Blon_1205	hypothetical protein Blon_1205	0.0234	0.78
Blon_1315	transposase, IS605 OrfB family	0.0072	1.29
Blon_1495	conserved hypothetical protein	0.0114	1.39
Blon_1496	helix-turn-helix domain protein	0.0095	1.54
Blon_1539	hypothetical protein	0.0392	1.26
Blon_1540	hypothetical protein	0.0293	1.25
Blon_1541	hypothetical protein	0.0186	1.41
Blon_1542	hypothetical protein	0.0218	1.39
Blon_1545	Cpl-7 lysozyme, C-terminal domain protein	0.0364	1.28
Blon_1664	GCN5-related N-acetyltransferase	0.0463	1.41
Blon_1687	TfoX, C-terminal domain protein	0.0006	1.93
Blon_1688	transcription activator, effector binding	0.0000	2.35
Blon_1689	GTP-binding protein YchF	0.0200	1.41
Blon_1691	proline iminopeptidase	0.0064	1.31
Blon_1692	integral membrane sensor signal transduction histidine kinase	0.0004	1.83
Blon_1693	two component transcriptional regulator, LuxR family	0.0007	1.72
Blon_1697	Phosphomethylpyrimidine kinase type-1	0.0020	1.74
Blon_1698	protein of unknown function UPF0102	0.0006	1.82
Blon_1700	SMF family protein	0.0422	1.26
Blon_1713	narrowly conserved hypothetical protein	0.0063	0.74
Blon_1714	pyruvate formate-lyase activating enzyme	0.0252	0.80
Blon_1761	1,4-alpha-glucan branching enzyme	0.0241	0.77
Blon_1902	conserved hypothetical protein	0.0035	0.73
Blon_1910	conserved hypothetical protein	0.0103	1.34
Blon_1950	hypothetical protein	0.0313	1.20
Blon_1951	UMUC domain protein DNA-repair protein	0.0332	1.22
Blon_2061	extracellular solute-binding protein, family 1	0.0254	1.29
Blon_2064	transcriptional regulator, DeoR family	0.0334	1.24
Blon_2081	conserved hypothetical protein	0.0199	1.29
Blon_2082	lipopolysaccharide biosynthesis	0.0112	1.50
Blon_2173	aminoglycoside phosphotransferase	0.0236	0.79
Blon_2174	conserved hypothetical protein	0.0060	0.64
Blon_2175	binding-protein-dependent transport systems inner membrane component	0.0033	0.64
Blon_2176	binding-protein-dependent transport systems inner membrane component	0.0013	0.62
Blon_2186	narrowly conserved hypothetical protein	0.0345	1.27
Blon_2191	ribose 5-phosphate isomerase	0.0158	1.25
Blon_2335	conserved hypothetical protein	0.0427	0.83
Blon_2341	protein of unknown function DUF624	0.0113	0.75
Blon_2342	binding-protein-dependent transport systems inner membrane component	0.0086	0.76
Blon_2348	Exo-alpha-sialidase	0.0355	0.78
Blon_2349	dihydrodipicolinate synthetase	0.0487	0.83
Blon_2370	glycerophosphoryl diester phosphodiesterase	0.0001	1.83
Blon_2371	Glutamate--tRNA ligase	0.0001	1.75
Blon_2372	ATPase AAA-2 domain protein	0.0000	1.88
Blon_2379	binding-protein-dependent transport systems inner membrane component	0.0221	0.70
Blon_2380	extracellular solute-binding protein, family 1	0.0115	0.73

Gene Name	Description	P-Value	Fold Change
dnaK	chaperone protein DnaK	0.0005	1.58
groEL	chaperonin GroEL	0.0147	1.37
recA	recA protein	0.0135	1.26
rpmI	ribosomal protein L35	0.0055	1.47
thiG	thiazole biosynthesis family protein	0.04100675	0.80

Table S4: List of genes differentially regulated by treatment with a mixture of 3'- and 6'-sialyllactose.

Gene Name	Description	P-Value	Fold Change
aspA	Fumarate Lyase	0.0025	1.52
Blon_0019	Metallophosphoesterase	0.0205	1.60
Blon_0022	phosphonate ABC transporter, ATPase subunit	0.0146	1.35
Blon_0029	Ferritin, Dps family protein	0.0002	2.79
Blon_0035	alkyl hydroperoxide reductase/ Thiol specific antioxidant/ Mal allergen	0.0010	1.49
Blon_0036	FAD-dependent pyridine nucleotide-disulphide oxidoreductase	0.0001	1.78
Blon_0096	ThiJ/PfpI domain protein	0.0140	1.54
Blon_0119	Uracil-DNA glycosylase superfamily	0.0106	0.76
Blon_0120	fructose-bisphosphate aldolase, class II	0.0058	0.73
Blon_0121	hypothetical protein	0.0016	0.68
Blon_0122	hypothetical protein	0.0041	0.66
Blon_0123	Adenylosuccinate synthase	0.0168	0.73
Blon_0124	Cl- channel, voltage-gated family protein	0.0015	0.71
Blon_0125	Camphor resistance CrcB protein	0.0001	0.59
Blon_0127	regulatory protein, LacI	0.0054	0.76
Blon_0139	4-alpha-glucanotransferase	0.0428	1.55
Blon_0142	GrpE protein	0.0095	1.34
Blon_0176	regulatory protein, LacI	0.0424	1.27
Blon_0182	conserved hypothetical protein	0.0046	1.29
Blon_0200	protein of unknown function DUF214	0.0050	0.77
Blon_0221	conserved hypothetical protein	0.0100	0.76
Blon_0222	signal recognition particle-docking protein FtsY	0.0031	0.74
Blon_0223	ammonium transporter	0.0284	0.80
Blon_0224	nitrogen regulatory protein P-II	0.0231	0.83
Blon_0225	(Protein-PII) uridylyltransferase	0.0006	0.64
Blon_0255	tRNA/rRNA methyltransferase (SpoU)	0.0304	0.85
Blon_0261	GCN5-related N-acetyltransferase	0.0490	0.83
Blon_0266	periplasmic binding protein/LacI transcriptional regulator	0.0463	0.80
Blon_0268	glycoside hydrolase family 2, sugar binding	0.0023	0.72
Blon_0286	lactoylglutathione lyase (LGUL) family protein, diverged	0.0019	3.03
Blon_0291	conserved hypothetical protein	0.0004	2.02
Blon_0292	helix-turn-helix domain protein	0.0295	1.75
Blon_0306	ATP synthase F1, delta subunit	0.0434	0.77
Blon_0308	ATP synthase F1, gamma subunitC	0.0265	0.74
Blon_0326	MscS Mechanosensitive ion channel	0.0069	1.46
Blon_0328	transcriptional regulator, TetR family	0.0143	0.77
Blon_0332	hypothetical protein Blon_0332	0.0255	0.78
Blon_0335	putative transcriptional regulator, MerR family	0.0056	0.77
Blon_0336	major facilitator superfamily MFS_1	0.0116	0.72
Blon_0339	DEAD/DEAH box helicase domain protein	0.0180	0.68
Blon_0341	Binding-protein-dependent transport systems inner membrane component	0.0263	0.65
Blon_0342	Binding-protein-dependent transport systems inner membrane component	0.0326	0.72
Blon_0349	Putative methyltransferase	0.0463	1.17
Blon_0392	Cation efflux protein	0.0000	3.04

Gene Name	Description	P-Value	Fold Change
Blon_0450	Hypothetical protein	0.0009	1.57
Blon_0459	Glycoside hydrolase, family 20	0.0029	2.36
Blon_0460	Binding-protein-dependent transport systems inner membrane component	0.0013	1.81
Blon_0488	Hypothetical protein	0.0435	1.55
Blon_0505	Hypothetical protein	0.0103	0.79
Blon_0517	ABC transporter related	0.0359	0.70
Blon_0527	Hypothetical protein	0.0037	0.59
Blon_0528	Hypothetical protein	0.0151	0.70
Blon_0529	Hypothetical protein	0.0042	0.68
Blon_0530	Conserved hypothetical protein	0.0045	0.68
Blon_0531	Transcription factor WhiB	0.0256	0.73
Blon_0536	Hypothetical protein	0.0456	1.46
Blon_0552	2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase	0.0229	0.75
Blon_0556	Hypothetical protein	0.0176	1.55
Blon_0567	2,5-didehydrogluconate reductase	0.0211	1.25
Blon_0570	Conserved hypothetical membrane protein possibly involved in transport	0.0133	1.26
Blon_0572	Carbohydrate kinase, FGGY	0.0195	1.30
Blon_0573	ROK family protein	0.0407	0.78
Blon_0577	Extracellular ligand-binding receptor	0.0010	0.54
Blon_0580	ABC transporter related	0.0006	0.60
Blon_0605	Endonuclease/exonuclease/phosphatase	0.0304	0.67
Blon_0612	Anaerobic ribonucleoside-triphosphate reductase	0.0494	1.20
Blon_0615	Resolvase, N-terminal domain protein	0.0205	1.26
Blon_0617	Glutamate--cysteine ligase, GCS2	0.0143	1.37
Blon_0638	Purine nucleosidase	0.0223	0.74
Blon_0642	GntR domain protein	0.0228	0.76
Blon_0643	Conserved hypothetical protein	0.0017	0.61
Blon_0644	ROK family protein	0.0082	0.61
Blon_0645	N-acylglucosamine-6-phosphate 2-epimerase	0.0028	0.59
Blon_0646	Glycosyl hydrolase, BNR repeat-containing protein	0.0001	1.48
Blon_0647	Extracellular solute-binding protein, family 5	0.0160	0.71
Blon_0648	Binding-protein-dependent transport systems inner membrane component	0.0461	0.55
Blon_0649	Oligopeptide/dipeptide ABC transporter, ATPase subunit	0.0047	0.61
Blon_0650	ABC transporter related	0.0066	0.59
Blon_0651	Dihydrodipicolinate synthetase	0.0229	0.62
Blon_0667	Mandelate racemase/muconate lactonizing enzyme, N-terminal domain protein	0.0043	0.69
Blon_0697	Two component transcriptional regulator, winged helix family	0.0174	1.34
Blon_0699	Putative cold-shock DNA-binding domain protein	0.0416	1.27
Blon_0701	UspA domain protein	0.0048	1.51
Blon_0702	ATPase AAA-2 domain protein	0.0058	1.28
Blon_0710	Extracellular solute-binding protein, family 3	0.0164	1.23
Blon_0732	Glycoside hydrolase, family 20	0.0306	1.21
Blon_0744	Polar amino acid ABC transporter, inner membrane subunit	0.0465	0.63
Blon_0748	Cystathionine gamma-synthase	0.0189	0.79

Gene Name	Description	P-Value	Fold Change
Blon_0758	Glutaredoxin-like protein	0.0007	1.99
Blon_0759	ABC transporter related	0.0067	1.93
Blon_0761	Polar amino acid ABC transporter, inner membrane subunit	0.0252	1.27
Blon_0762	Band 7 protein	0.0220	1.32
Blon_0772	Conserved hypothetical protein	0.0100	1.33
Blon_0785	Membrane lipoprotein lipid attachment site	0.0034	0.72
Blon_0789	Periplasmic binding protein/LacI transcriptional regulator	0.0016	0.72
Blon_0790	Proteinase inhibitor I4, serpin	0.0035	0.68
Blon_0799	Putative TIM-barrel protein, nifR3 family	0.0413	0.62
Blon_0838	Methyltransferase small	0.0351	0.75
Blon_0840	L-lactate dehydrogenase	0.0226	1.72
Blon_0841	Cation diffusion facilitator family transporter	0.0301	0.65
Blon_0850	Peptidoglycan glycosyltransferase	0.0075	0.72
Blon_0851	Conserved hypothetical protein	0.0007	0.68
Blon_0852	UDP-N-acetylmuramoylalanyl-D-glutamyl-2,6-diaminopimelate--D-alanyl-D-alanyl ligase	0.0066	0.77
Blon_0865	Putative transcriptional regulator	0.0012	1.89
Blon_0879	ROK family protein	0.0324	1.34
Blon_0884	Binding-protein-dependent transport systems inner membrane component	0.0484	0.68
Blon_0885	Binding-protein-dependent transport systems inner membrane component	0.0001	0.67
Blon_0902	Initiation factor 3	0.0121	1.43
Blon_0916	Hypothetical protein	0.0212	1.80
Blon_0917	hypothetical protein	0.0068	1.36
Blon_0918	Prephenate dehydratase	0.0106	1.55
Blon_0938	protein of unknown function UPF0005	0.0029	1.53
Blon_0947	helix-turn-helix domain protein	0.0002	1.54
Blon_0948	hypothetical protein	0.0025	1.50
Blon_0951	sigma 54 modulation protein/ribosomal protein S30EA	0.0166	1.41
Blon_0960	RNA polymerase, sigma 70 subunit, RpoD family	0.0213	0.76
Blon_0971	(p)ppGpp synthetase I, SpoT/RelA	0.0101	0.73
Blon_0991	conserved hypothetical protein	0.0039	2.17
Blon_0992	hypothetical protein	0.0021	2.47
Blon_0993	hypothetical protein	0.0047	2.05
Blon_0994	transcriptional regulator, Fis family	0.0098	1.48
Blon_0995	ABC transporter related	0.0377	1.49
Blon_0999	ABC transporter related	0.0198	1.28
Blon_1002	hypothetical protein	0.0418	1.45
Blon_1007	pyridoxamine 5'-phosphate oxidase-related, FMN-binding	0.0020	2.11
Blon_1023	hypothetical protein	0.0084	1.38
Blon_1028	hypothetical protein	0.0468	1.52
Blon_1037	conserved hypothetical protein	0.0034	1.85
Blon_1038	hypothetical protein	0.0108	0.63
Blon_1054	AMP-dependent synthetase and ligase	0.0177	1.20
Blon_1063	imidazoleglycerol phosphate synthase, cyclase subunit	0.0331	0.63
Blon_1092	aminotransferase, class I and II	0.0259	0.74
Blon_1150	fructosamine kinase	0.0305	1.27
Blon_1154	histidine triad (HIT) protein	0.0110	0.71
Blon_1157	Holliday junction DNA helicase RuvA	0.0195	1.32

Gene Name	Description	P-Value	Fold Change
Blon_1166	pseudouridine synthase	0.0289	0.82
Blon_1225	Resolvase, N-terminal domain protein	0.0424	1.24
Blon_1338	putative transcriptional regulator, XRE family	0.0385	1.22
Blon_1387	conserved hypothetical protein	0.0300	1.29
Blon_1426	hypothetical protein	0.0069	0.71
Blon_1455	5-methyltetrahydropteroyltriglutamate--homocysteine S-methyltransferase	0.0280	0.74
Blon_1492	hypothetical protein Blon_1492	0.0360	1.20
Blon_1494	hypothetical protein	0.0357	1.32
Blon_1495	conserved hypothetical protein	0.0153	1.40
Blon_1496	helix-turn-helix domain protein	0.0170	1.47
Blon_1541	hypothetical protein	0.0288	1.33
Blon_1574	branched-chain amino acid aminotransferase	0.0009	0.67
Blon_1613	ABC transporter related	0.0216	0.70
Blon_1615	conserved hypothetical protein	0.0144	0.68
Blon_1634	protein of unknown function DUF47	0.0290	1.44
Blon_1664	GCN5-related N-acetyltransferase	0.0080	1.60
Blon_1679	transcriptional modulator of MazE/toxin, MazF	0.0065	1.39
Blon_1687	TfoX, C-terminal domain protein	0.0014	2.26
Blon_1688	transcription activator, effector binding	0.0001	2.88
Blon_1693	two component transcriptional regulator, LuxR family	0.0349	1.32
Blon_1698	protein of unknown function UPF0102	0.0096	1.38
Blon_1708	Endopeptidase Clp	0.0166	0.73
Blon_1709	hypothetical protein	0.0033	0.68
Blon_1710	Cl- channel, voltage-gated family protein	0.0335	0.78
Blon_1712	3'-5' exonuclease	0.0281	0.65
Blon_1713	narrowly conserved hypothetical protein	0.0054	0.65
Blon_1745	Pyruvate kinase	0.0323	1.34
Blon_1756	periplasmic solute binding protein	0.0037	1.28
Blon_1761	1,4-alpha-glucan branching enzyme	0.0107	0.74
Blon_1771	cell envelope-related transcriptional attenuator	0.0317	0.67
Blon_1773	transcription factor WhiB	0.0260	1.38
Blon_1774	signal transduction histidine kinase	0.0329	0.81
Blon_1776	GreA/GreB family elongation factor	0.0003	1.52
Blon_1777	peptidylprolyl isomerase, FKBP-type	0.0022	1.40
Blon_1780	phage integrase family protein	0.0185	0.77
Blon_1786	hypothetical protein	0.0146	1.31
Blon_1839	Aminoacyl-tRNA hydrolase	0.0161	0.69
Blon_1850	regulatory protein GntR, HTH	0.0067	1.41
Blon_1855	regulatory protein GntR, HTH	0.0239	1.33
Blon_1856	two component transcriptional regulator, LuxR family	0.0469	1.32
Blon_1857	Signal transduction histidine kinase-like protein	0.0106	1.30
Blon_1873	thiamine biosynthesis protein ThiS	0.0488	0.84
Blon_1876	arginine repressor, ArgR	0.0103	0.75
Blon_1879	acetylglutamate kinase	0.0181	0.62
Blon_1901	ABC transporter related	0.0117	0.74
Blon_1902	conserved hypothetical protein	0.0089	0.73
Blon_1950	hypothetical protein	0.0017	1.35
Blon_1951	UMUC domain protein DNA-repair protein	0.0012	1.41
Blon_1968	ribosomal protein L31	0.0095	1.50

Gene Name	Description	P-Value	Fold Change
Blon_1969	ribosomal protein L36	0.0345	1.47
Blon_1971	putative high-affinity zinc ABC transporter	0.0139	2.01
Blon_1990	hypothetical protein	0.0152	1.99
Blon_2022	extracellular solute-binding protein, family 3	0.0115	1.35
Blon_2048	DNA-directed RNA polymerase, beta' subunit	0.0441	0.79
Blon_2061	extracellular solute-binding protein, family 1	0.0000	1.98
Blon_2062	galactokinase	0.0079	1.58
Blon_2063	galactose-1-phosphate uridylyltransferase	0.0078	1.52
Blon_2064	transcriptional regulator, DeoR family	0.0047	1.55
Blon_2082	lipopolysaccharide biosynthesis	0.0175	1.41
Blon_2086	transcriptional regulator, Fis family	0.0202	1.39
Blon_2098	IS3 family transposase	0.0358	0.75
Blon_2138	protein of unknown function	0.0072	1.36
Blon_2171	UDP-glucose 4-epimerase	0.0028	1.47
Blon_2172	UDP-glucose--hexose-1-phosphate uridylyltransferase	0.0313	0.64
Blon_2173	aminoglycoside phosphotransferase	0.0214	0.77
Blon_2174	conserved hypothetical protein	0.0028	0.57
Blon_2175	binding-protein-dependent transport systems inner membrane component	0.0146	0.70
Blon_2176	binding-protein-dependent transport systems inner membrane component	0.0019	0.66
Blon_2177	extracellular solute-binding protein, family 1	0.0307	0.78
Blon_2187	transcriptional regulator, BadM/Rrf2 family	0.0077	1.45
Blon_2188	pyridine nucleotide-disulphide oxidoreductase dimerisation region	0.0452	1.20
Blon_2191	ribose 5-phosphate isomerase	0.0014	1.58
Blon_2264	large hypothetical protein	0.0126	0.74
Blon_2273	ABC transporter related	0.0479	0.77
Blon_2287	hypothetical protein	0.0210	1.23
Blon_2326	regulatory protein, IclR	0.0382	1.27
Blon_2335	conserved hypothetical protein	0.0029	0.72
Blon_2336	alpha-1,3/4-fucosidase, putative	0.0151	0.69
Blon_2337	RbsD or FucU transport	0.0180	0.65
Blon_2341	protein of unknown function	0.0017	0.58
Blon_2342	binding-protein-dependent transport systems inner membrane component	0.0003	0.67
Blon_2343	binding-protein-dependent transport systems inner membrane component	0.0311	0.72
Blon_2348	Exo-alpha-sialidase	0.0247	0.68
Blon_2349	dihydrodipicolinate synthetase	0.0125	0.78
Blon_2355	glycoside hydrolase, family 20	0.0449	0.63
Blon_2370	glycerophosphoryl diester phosphodiesterase	0.0000	2.09
Blon_2371	Glutamate--tRNA ligase	0.0000	2.29
Blon_2372	ATPase AAA-2 domain protein	0.0000	2.45
Blon_2379	binding-protein-dependent transport systems inner membrane component	0.0022	0.54
Blon_2380	extracellular solute-binding protein, family 1	0.0007	0.65
Blon_2386	ABC transporter related	0.0237	0.80
Blon_2387	ABC-2 type transporter	0.0451	0.66
Blon_2397	K+ potassium transporter	0.0411	0.71

Gene Name	Description	P-Value	Fold Change
Blon_2444	extracellular solute-binding protein, family 1	0.0405	1.61
Blon_2474	narrowly conserved hypothetical protein	0.0044	0.69
Blon_2475	ABC transporter related	0.0359	0.79
Blon_2476	glycosyl transferase, family 2	0.0365	0.84
Blon_2478	ribonucleoside-diphosphate reductase, alpha subunit	0.0149	0.57
Blon_2492	parB-like partition protein	0.0169	0.78
Blon_2496	60 kDa inner membrane insertion protein	0.0192	0.59
Blon_2497	protein of unknown function DUF37	0.0388	0.56
Blon_2498	ribonuclease P protein component	0.0228	0.72
dnaK	chaperone protein DnaK	0.0001	1.97
groEL	chaperonin GroEL	0.0002	2.11
groES	chaperonin Cpn10	0.0029	1.34
hisS	Histidine--tRNA ligase	0.0282	0.70
hrcA	heat-inducible transcription repressor HrcA	0.0018	1.52
ileS	isoleucyl-tRNA synthetase	0.0135	0.75
nagB	glucosamine-6-phosphate isomerase	0.0006	1.45
nrdI	NrdI protein	0.0146	0.60
pgi	Glucose-6-phosphate isomerase	0.0083	1.36
prfA	peptide chain release factor 1	0.0069	0.71
recA	recA protein	0.0267	1.21
rplQ	ribosomal protein L17	0.0160	0.73
rpmA	ribosomal protein L27	0.0043	1.42
rpmD	ribosomal protein L30	0.0314	0.65
rpmF	ribosomal protein L32	0.0025	1.45
rpoB	DNA-directed RNA polymerase, beta subunit	0.0259	0.77
rpsH	ribosomal protein S8	0.0205	0.65
rpsM	ribosomal protein S13	0.0082	0.79
smpB	SsrA-binding protein	0.0027	1.51
thiG	thiazole biosynthesis family protein	0.0027	0.70
thrS	threonyl-tRNA synthetase	0.0328	0.63
tsf	translation elongation factor Ts	0.0162	0.81

CHAPTER III

Evaluation of bovine milk oligosaccharides versus a commercial prebiotic in mice

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ABSTRACT

Human milk oligosaccharides exhibit an array of beneficial biological functions, including selective promotion of commensal bacteria and anti-adhesive activity against various pathogens. Milk oligosaccharides from animal species are currently of potential commercial interest given that they may produce similar biological outcomes. In this study, the effect of bovine milk oligosaccharides or the commercial prebiotic, Beneo Orafti P95 oligofructose, was investigated in mice. Mouse diets were supplemented with bovine milk oligosaccharides, 6'sialyllactose or oligofructose (1 mg/g body weight) by oral gavage for 21 days. The effects of the orally administered oligosaccharides on caecal short-chain fatty acid levels, systemic cytokine expression, and faecal microbiota composition were examined. As markers of probiotic activity, short chain fatty acid, IgA, and cytokine profiles were generally unaffected by any of the carbohydrate interventions. Oligofructose supplementation was the only dietary intervention to result in increased proportions of multiple families of bacteria; *Enterobacteriaceae*, *Enterococcaceae*, and *Alcaligenaceae*. Bovine milk oligosaccharide supplementation was associated with a significant reduction in proportions of pathogen-associated bacterial families in faecal contents. This study highlights the potential of bovine milk and commercial whey streams as an untapped nutritional resource which could be used to modulate the intestinal microbiota.

CHAPTER III

INTRODUCTION

Oligosaccharides are the third largest solid component of human milk following lactose and lipids, with concentrations of 22-24 g/L in colostrum and 12-13 g/L in mature milk (Urashima, T., Taufik, E., et al. 2013). Human milk oligosaccharides have been shown to have specific biological functions in *in vitro* experiments, including prebiotic and anti-adhesive activity, anti-inflammatory properties, modification of cell surface glycans, a role in brain development and influencing growth-related characteristics of intestinal cells (for reviews see Bode, L. 2006, Hickey, R.M. 2009, Kunz, C. and Rudloff, S. 2006, Newburg, D.S., Ruiz-Palacios, G.M., et al. 2005). Few *in vivo* studies exist demonstrating the beneficial effects of human milk oligosaccharides (HMO) ingestion. Among these, Fuhrer *et al.* demonstrated that milk sialyllactose influences colitis in mice through selective intestinal bacterial colonization (Fuhrer, A., Sprenger, N., et al. 2010). Ingestion of milk containing 3'sialyllactose (unaltered or 3'sialyllactose alone) resulted in reduced resistance to DSS-induced colitis, which was associated with the presence of clostridial cluster IV bacteria. Mielcarek *et al.* also demonstrated modulation of bacterial translocation in mice through exposure to HMO (Mielcarek, C., Romond, P., et al. 2011). In a mouse model of schistosome infection, the HMO, lacto-N-fucopentaose III and lacto-N-neotetraose induced production of IL-10, an anti-inflammatory cytokine, in isolated CD45R+ cells (Velupillai, P. and Harn, D.A. 1994).

However, there are very few commercial products on the market which capitalize on these functions. This is mainly due to the fact that the large quantities of human milk oligosaccharides required for clinical trials are unavailable. In contrast, commercial oligosaccharides, including short-chain galactooligosaccharides and long-chain fructooligosaccharides (scGOS / lcFOS), are supplemented in certain products such as infant formula, which are currently marketed based on prebiotic health claims (Fanaro, S., Jelinek, J., et al. 2005). However, the structure and composition of commercial oligosaccharides differ from that of human milk oligosaccharides. Many efforts are therefore, being made to replicate the effects of human milk oligosaccharides by searching for alternative compounds which may produce similar biological outcomes

particularly in relation to infant formula composition. However, little information exists regarding the biological activity associated with alternative compounds, such as milk oligosaccharides from animal species. Indeed, infant formulas designed for nutrition during the first weeks after birth are currently based on bovine milk (Oozeer, R., van Limpt, K., et al. 2013). Therefore, isolation, fractionation or enrichment of bovine milk oligosaccharides may have potential applications in infant formulas. Moreover, a number of *in vitro* studies have already shown that bovine oligosaccharides possess anti-adhesive activity against certain pathogens (Hakkarainen, J., Toivanen, M., et al. 2005, Lane, J.A., Mariño, K., et al. 2012, Matrosovich, M., Gambaryan, A., et al. 1993, Wang, X., Hirmo, S., et al. 2001). Interestingly, these studies demonstrate that sialylated oligosaccharides, such as 3'-sialyllactose and 6'-sialyllactose, which are present in both human and bovine milk are potent inhibitors of pathogen adhesion in the gut (Gopal, P.K. and Gill, H.S. 2000, Lane, J.A., Mehra, R.K., et al. 2010).

Given that bovine milk contains only trace amounts of these valuable components, a concentration step would be necessary to generate a suitable preparation for evaluation. The use of dairy streams, whey permeate in particular, for large-scale extraction is an attractive option. The advantage of using specific whey fractions stems from their wide availability and low cost compared to other dairy streams. Whey permeate is a dairy processing flow-through by-product, containing smaller molecules such as lactose, oligosaccharides and salts. Using pre-existing processing technology, the production of an enriched oligosaccharide powder with reduced lactose and protein can be achieved, creating an abundant source of bioactive oligosaccharides from a waste process. Together, the potentially wide availability of bovine milk oligosaccharides from dairy streams and the accumulation of positive *in vitro* studies on their health-promoting effects described earlier, make a strong case for their inclusion in functional foods, such as infant formula.

The objective of the current study was to assess physiological parameters (safety and tolerance) and modulatory effects of an enriched bovine milk oligosaccharide powder or 6'sialyllactose on the fecal microbiota of healthy mice in comparison with the commercial prebiotic fibre, Beneo Orafti P95 oligofructose.

CHAPTER III

MATERIALS AND METHODS

Oligosaccharides

Oligofructose (P95) (composed of 95% FOS [Degree of polymerization 3 to 10]) was kindly provided by Beneo Orafti (Tienen, Belgium), 6'sialyllactose was purchased from Carbosynth (Berkshire, UK). Isolation of bovine milk oligosaccharides (BMO) from mother liquor (Glanbia plc; Co. Kilkenny, Ireland) was performed according to the method of Mehra *et al.* (manuscript in preparation). Specific details on the isolation and enrichment method for the oligosaccharide powder are currently restricted due to a pending patent application relating to the bioactivity of the powder. Briefly, mother liquor was clarified through a centrifugal clarifier, followed by a microfiltration step. The microfiltrate was further fractionated and enriched through ultrafiltration to remove lactose and mineral salts. The resulting retentate was concentrated by evaporation and spray dried to produce an enriched powder. This was further purified by means of gel filtration chromatography and freeze-dried. The resulting powder was injected on to a C18 reverse-phase column to further reduce the residual peptide content. The eluate was then freeze-dried to produce the final bovine milk oligosaccharide (BMO) powder.

Compositional analysis of BMO powder

Compositional analysis of BMO powder included carbohydrate, protein, fat and ash content. Carbohydrate concentrations were determined using High pH Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) using a Dionex ICS-3000 system equipped with an ED40 electrochemical detector with a gold cell and LC30 chromatography oven. A CarboPac PA100 (250 X 4 mm) column protected with a CarboPac PA100 (50 X 4 mm) guard column was used. Elution was carried out with the following gradient: 100 mM NaOH (Eluent A) and 100 mM NaOH, 500 mM NaAc (Eluent B) (t = 0–3 min 95% Eluent A; t = 3–13 min 88% Eluent A; t = 13–30 min 50% Eluent A; t = 30–45 min equilibrated at 95% Eluent A). The limit of detection was 10 parts per million for this assay.

Protein content was determined using the Bio-Rad protein assay kit according to manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA,

USA). Ash content was determined according to Association of Official Analytical Chemists method (AOAC 923.03) (AOAC 2005).

Animals and treatments

BALB/c mice were purchased from Harlan (Becester, Oxfordshire, UK) and housed within the biological services unit, University College Cork (UCC), under barrier-maintained conditions. Mice were delivered at 6 weeks of age and allowed to acclimatise for one week prior the beginning of the study. Animals were divided into four groups (n=10) and housed under standard conditions with access to standard chow and water *ad libitum*. Beneo Orafti P95, BMO or 6'sialyllactose were resuspended in saline solution and administered daily (1mg/g body weight) for 21 days by oral gavage. Body weight was assessed weekly. Faecal pellets were collected at day-0 and day-21 of the trial and immediately placed on ice, and frozen at -80°C until the extraction of bacterial DNA. At the conclusion of the study, animals were sacrificed by decapitation, blood serum collected, and the liver, small intestine, and caecum harvested, washed with PBS, blotted dry on filter paper and weighed. Caecal content was divided for analysis of secretory IgA and short-chain fatty acid composition. All animal experiments were approved by the University College Cork Animal Ethics Committee, and experimental procedures were conducted under the appropriate license from the Irish Government.

Caecal secretory IgA analysis

Caecal secretory IgA concentration was determined using the mouse IgA ELISA quantification kit (Bethyl Laboratories, Cambridge, UK). Essentially, anti-mouse IgA antibody is adsorbed on the surface of microtitre wells and blocked using BSA. Following addition and binding of caecal sIgA, wells were washed and bound sIgA was detected using a biotinylated detection antibody, followed by streptavidin-linked horse radish peroxidase. sIgA levels were colorimetrically detected using the TMB (3,3',5,5'-tetramethylbenzidine) substrate and read on a BioTek plate reader at 450nm. Concentrations were determined based on a standard curve using the mouse IgA standard provided.

SCFA analysis

SCFA analysis was carried out according to previously published methods (Wall, R., Marques, T.M., et al. 2012).

Serum cytokine analysis

Measurement of cytokines IL-1β, IL-6, IL-10, IL-12, IFN-γ, mKC, and TNF-α in serum was carried out using the Meso Scale Discovery 7-plex mouse pro-inflammatory kit (Meso Scale Discovery, Gaithersburg, MD, USA) following the manufacturer's instructions.

Microbial composition analysis

DNA extractions and pyrosequencing were done as in Murphy *et al.* (Murphy, E.F., Cotter, P.D., et al. 2012). Total metagenomic DNA was extracted from individual faecal samples using the QIAmp DNA Stool Mini Kit (Qiagen, Crawley, West Sussex, UK). Samples were initially bead-beaten and then processed according to the manufacturer's instructions. For compositional analysis, isolated microbial DNA was subjected to pyrosequencing of 16S rRNA tags (V4 region; 239 nt long) amplified using universal 16S primers. The forward primer, F1, (5'-AYTGGGYDTAAAGNG) and a mixture of four reverse primers, R1 (5'-TACCRGGGTHTCTAATCC), R2 (5'-TACCAGAGTATCTAATTC), R3 (5'-CTACDSRGGTMTCTAATC), and R4 (5'-TACNVGGGTATCTAATC) (RDP's Pyrosequencing Pipeline: http://pyro.cme.msu.edu/pyro/help.jsp) were used. To allow emulsion-based clonal amplification for the 454 pyrosequencing system, proprietary 19-mer sequences at the 5'end were incorporated into the primers. Unique molecular identifier (MID) tags were incorporated between the adaptamer and the target-specific primer sequence, allowing for identification of individual sequences from pooled amplicons. The resulting amplicons were cleaned using the AMPure purification system (Beckman Coulter, Takeley, UK) and sequenced within the Teagasc 454 Sequencing Platform. Raw sequences were quality trimmed using the Qiime Suite of programmes (Caporaso, J.G., Kuczynski, J., et al. 2010); any reads not meeting the quality criteria of a minimum quality score of 25 and sequence length shorter than 150bps for 16S amplicon reads. Trimmed FASTA sequences were then BLASTed (Altschul, S.F., Madden, T.L., et al.

1997) against a previously published 16S-specific database (Urich, T., Lanzen, A., et al. 2008) by using default parameters. The resulting BLAST output was parsed by using MEGAN (Huson, D.H., Auch, A.F., et al. 2007). MEGAN assigns reads to NCBI taxonomies by using the Lowest Common Ancestor algorithm. A bit-score of 86, as previously used for 16S ribosomal sequence data (Urich, T., Lanzen, A., et al. 2008), was used from within MEGAN for filtering the results before tree construction and summarisation. Phylum, family and genus counts for each subject were extracted from MEGAN. Sequences were clustered into operational taxonomical units (OTUs), chimera checked and aligned using the default pipeline within Qiime (Kuczynski, J., Stombaugh, J., et al. 2011); subsequently alpha and beta diversities were generated. A phylogenetic tree was calculated using the FastTree software (doi:10.1093/molbev/msp077). Resulting Principal Coordinate analysis was visualised using KING.

Detection of bifidobacteria

To quantify bifidobacteria in murine faecal pellets, a plasmid standard was first created. A PCR fragment from within the 16 rRNA gene of *B. longum* subsp. *infantis* ATCC 15697 was generated using species-specific primers. The primers used were as follows: forward 5'- CTCCTGGAAACGGGTGG- 3' and reverse 5'- GGTGTTCTTCCCGATATCTAC- 3', according to Matsuki *et al.* (Matsuki, T., Watanabe, K., et al. 2002). Purified amplicons were cloned into the pCR®2.1-TOPO vector using the TOPO-TA cloning system (Invitrogen, Life Technologies, Carlsbad, California) in accordance with manufacturer's instructions. The complete vector was transformed into chemically competent TOP-10 *E. coli* cells (Invitrogen, Life Technologies, Carlsbad, California) and harvested on LB media containing 50μgml^{-1} ampicillin. The cloned amplicon was confirmed by restriction analysis and DNA sequencing. Quantitative real-time PCR (qPCR) standards were prepared following the linearization of plasmid DNA with *Kpn*I restriction enzyme and quantification using a Nanodrop ND-1000 (Thermo Fisher Scientific Inc, USA). A standard curve was then generated via a series of dilutions from 10^9 to 10^2 copies/μl DNA. The LightCycler 480 SYBR Green I Master kit (Roche Diagnostics GmbH, Mannheim, Germany) was used for quantification according to the manufacturer's instructions. Each PCR

reaction contained 10µl Sybr green master mix, 1µl of both forward and reverse primer (10pmol), 1µl of DNA and was made up to a final volume of 20µl with nuclease free dsH_2O. The PCR conditions were as follows: Denaturation at 95°C for 10min, followed by 35 cycles of denaturation at 95°C for 10 sec, annealing at 55°C for 5 sec and elongation 72°C for 25 sec. To quantify by qPCR, it was necessary to correct copy number of 16S rRNA gene to cfu/ml. Based on the amplification of DNA, which was isolated from bifidobacteria cultures of known cfu/ml against a standard curve generated from the Lightcycler 480 (copy number/µl), a formula was used to correct copy number values for cfu/ml as outlined in McCarthy and Quigley, 2013 (submitted).

[(C/µl)*(TV)]/TCN x T cfu/ml = cfu/ml(S)

Where; C/µl = Copy number/µl, TV = Template volume, TCN = Total copy number of the standard used, T cfu/ml = Total cfu/ml of standard used and cfu/ml(S) = cfu/ml of test sample.

Statistical Analysis

Data are presented as mean values ± standard errors of the mean (SEM). Statistical analysis was performed by ANOVA and the Student t-test (Graph-Pad Software, San Diego, CA, USA). For microbiota analysis SPSS was utilised to establish non-parametric significance using the Mann-Whitney and Kruskal-Wallis tests. $p<0.05$ was considered as statistically significant.

CHAPTER III

RESULTS

Oligosaccharide powder composition and their effect on body and organ weight, caecal secretory IgA, SCFA composition and serum cytokine levels

The BMO powder composition is found in Table 1. The growth of the BALB/c mice throughout the study duration did not differ significantly as a result of oligosaccharide supplementation, nor did final overall body weight (Table 2). Following 3 weeks of dietary supplementation with oligosaccharides there was no effect on liver, caecal, or small intestinal weight (Table 2).

The caecal secretory IgA (sIga) content was assessed at day 21. sIgA levels were reduced following treatment with P95 (62%; $p<0.01$) and BMO (61%; $p<0.05$), 6'SL supplementation showed a similar trend but did not reach statistical significance (Fig. 1).

To examine the effects of oligosaccharide supplementation on SCFA content in the caecum, levels of butyrate, propionate, and acetate were determined (Fig. 2). P95 and BMO supplementation did not result in significant changes in SCFA content when compared with the saline control. 6'Sialyllactose supplementation resulted in reduced levels of butyrate 41% ($p<0.01$) in comparison with the saline-treated control, while levels of propionate and acetate were unaltered in caecal contents.

Serum was isolated from collected blood and analysed to determine whether oligosaccharide supplementation affected serum cytokine levels (Table 3). None of the supplemented oligosaccharides were found to be associated with changes in the cytokine levels of IL-1β, IL-10, IFN-γ, or TNF-α. P95 treatment was associated with a reduction in IL-12 (18.1%; $p<0.01$) and IL-6 (21.1%; $p<0.05$) versus the saline control. BMO reduced levels of IL-12 (11.9%; $p<0.05$) versus the control, and increased mKC levels in relation to 6'SL (42.7%; $p<0.01$).

Effects of oligosaccharide supplementation on murine intestinal microbiota

At the conclusion of the 21-day trial, the relative proportions of gut bacteria among the treatment groups were assessed by DNA sequencing (Roche-454 titanium, Roche Diagnostics Ltd, West Sussex, UK) of the V4-region of 16S rRNA amplicons derived from total faecal DNA. Sequencing resulted in a total

of 421,986 reads with an average of 5540.481 reads per mouse. Reads were quality trimmed and clustered into operational taxonomical units (OTUs) based on 98% sequence identity. Subsequently alpha diversity was estimated using 5 different metrics (Shannon, Simpson, Chao1, Phylogenetic diversity and observed species). There were some temporal differences in diversity, with a reduction in diversity over time (phylogenetic diversity p=0.019; observed species p=0.032; data not shown). However, no significant differences were observed in alpha diversity between the treatment groups at Day 21 only. Rarefaction curves for each group were at or approaching parallel, indicating that sufficient depth of sequencing was undertaken (Fig. 3). Principal Coordinate analysis, based on unweighted Unifrac distances illustrated that the control and oligofructose samples cluster closer to each other than to the 6'SL and milk oligosaccharide samples (Fig. 4).

Consistent with previous studies, Firmicutes and Bacteroides are the predominant gut microbiota in the BALB/c murine model at the phylum level (Pedron, T., Mulet, C., et al. 2012) (Fig. 5A). Proteobacteria, Deferribacteres, Actinobacteria, Viridiplantae, and Candidate division TM7 contribute to the remaining proportion (Fig. 5B). A comparison of intestinal microbiota composition between control and oligosaccharide-treated mice revealed multiple alterations. To establish if an alteration was due to time or treatment, the groups were compared at day 21 only and also day 21 vs. day 0 (Tables 3 and 4). At the phylum level, P95 treatment was associated with a significant reduction in *Cyanobacteria* (p=0.003). BMO supplementation was associated with an increase in the relative proportion of *Candidate division TM7* (p=0.002) (Fig. 5) and decreases in *Actinobacteria* (p=0.0255) and *Cyanobacteria* (p=0.003) compared to the control, while administration of 6'SL was associated with a decrease in the relative proportion of *Actinobacteria* (p=0.017).

In total, 13 significant differences (Table 3 and 4) were observed at the family level when comparing all treatments against one another. More specifically, two of these differences were temporal (Day 0 versus Day 21); *Alcaligenaceae* (p=0.031) and *Porphyromonadaceae* (p=0.023) and a further two, *Vibrionaceae* (p=0.024) and *Pseudoalteromonadaceae* (p=0.024) were significantly different at Day 21 only.

At the family level, P95 was associated with increases in *Enterobacteriaceae* (p<0.0001), *Enterococcaceae* (p=0.016), *Alcaligenaceae* (p=0.018), a decrease in *Porphyromonadacaea* (p=0.04), and the non-detection of *Moraxellaceae* and *Vibrionaceae* in pair-wise comparisons against the saline control (Fig. 6). Supplementation with BMO resulted in a significant reduction in numbers of 3 families; *Veillonellaceae* (p=0.003), *Succinivibrionaceae* (p=0.001), *Peptostreptococcaceae* (p=0.038), an increase in *Alcaligenaceae* (p=0.006), and non-detection of *Moraxellaceae* and *Vibrionaceae*. 6'SL was associated with a significant reduction in numbers or non-detection of 7 families: *Prevotellaceae* (p=0.035), *Moraxellaceae*, *Vibrionaceae*, *Veillonellaceae* (p=0.003), *Porphyromonadaceae* (p=0.016) and *Succinivibrionaceae* (p=0.001). Interestingly, *Lactobacillaceae* numbers were significantly reduced in the 6'SL-treated mice, compared with the unsupplemented control and P95 samples (p=0.001 and 0.005 respectively).

At the genus level, 19 significant differences are observed either at the temporal level (day 0 vs. day 21) or at the day 21 comparison only (Table 3 and 4). In pair-wise comparisons versus the control, P95 was associated with significantly increased numbers of *Enterobacteriaceae* spp. (p<0.0001), *Pasteurellales* (p<0.0001), *Sutterella* (p=0.018), *Parabacteroides* (p=0.004), *Ruminococcaceae Incertae Sedis* (p<0.0001), *Enterococcus* (p=0.030), and *Erysipelotrichales Incertae Sedis* (p=0.001), and decreases in *Lachnospiraceae Incertae Sedis* (p=0.017), uncultured *Lachnospiraceae* (p=0.003), *Marvinbryantia – Bryantella* (p<0.0001), and *Acetitomaculum* (p=0.008) (Fig. 7). BMO was associated with a significant increase in *Sutterella* (p=0.006) and decreases in *Anaerobiospirillum* (p=0.001), uncultured *Lachnospiraceae* (p=0.016), *Acetitomaculum* (p=0.008), *Anaerovibrio* (p=0.018), and *Ruminococcaceae Incertae Sedis* (p=0.028). 6'SL was associated with an increase in *Parabacteroides* (p=0.008) and *Ruminococcaceae Incertae Sedis* (p=0.002), and the largest number of genus decreases; *Anaerobiospirillum* (p=0.001), *Lachnospiraceae Incertae Sedis* (p=0.049), uncultured *Lachnospiraceae* (p=0.009), *Marvinbryantia – Bryantella* (p<0.0001), *Acetitomaculum* (p=0.008), *Anaerovibrio* (p=0.018), and *Lactobacillus* (p=0.001). Notably members of *Ruminococcaceae Incertae Sedis* were significantly increased in the P95 and 6'SL treatment groups versus the control

($p<0.0001$ and $p=0.002$, respectively), while decreasing in the BMO-treated mice ($p=0.028$). Interestingly, members of *Lactobacillus* were significantly reduced in 6'SL treatment, compared with oligofructose and control samples ($p=0.006$ and 0.001 respectively).

CHAPTER III

DISCUSSION

Dairy whey streams present an abundant resource from which to mine biologically active bovine oligosaccharides, offering an attractive alternative to human milk oligosaccharides as functional food ingredients (Zivkovic, A.M. and Barile, D. 2011). In the current study, we report the influence of carbohydrate supplementation (1mg/g body weight) of commercially available oligofructose, an enriched bovine milk oligosaccharide powder, or 6'sialyllactose on the composition of the murine microbiota. The findings are the first to describe the effects of a dairy-derived oligosaccharide powder on the GI microflora of mice.

Following the 3-week administration period, individual carbohydrate interventions resulted in clustering of bacterial species detected, with similar effects on bacterial composition among the control and P95, and BMO and 6'SL. In the current study, P95 demonstrated the ability to modulate the fecal bacterial populations of mice, promoting the growth of specific families, while reducing others. P95 significantly increased the relative proportions of *Enterococcaceae* and *Enterobacteriaceae,* which have been demonstrated to be associated with the intestinal microbiota of preterm infants (Arboleya, S., Binetti, A., et al. 2012, Hoy, C.M., Wood, C.M., et al. 2000, Magne, F., Abély, M., et al. 2006). The current study findings are in contrast to a prior study in which supplementation with oligosaccharides resulted in reduced concentrations of *Enterococcaceae* in the cecum (Pan, X.D., Chen, F.Q., et al. 2009), although this may be attributable to differences naturally present between the caecal and fecal microbiota (Marteau, P., Pochart, P., et al. 2001). While pathogens have demonstrated the ability to utilize oligofructose in pure culture, their growth is inhibited when co-cultured in the presence of select bifidobacteria (Rycroft, C., Jones, M., et al. 2001). The fact that bifidobacteria were absent in the current model may account for the promotion of the less favourable families of bacteria. Of interest, P95 supplementation was associated with non-detectable numbers of the *Moraxellaceae* and *Vibrionaceae* families of bacteria, both of which include notable human pathogens, *Moraxella catarrhalis* and *Vibrio cholerae*, respectively.

Overall, BMO and 6'SL supplementation were generally associated with reductions in less favourable bacteria. At the phylum level, BMO reduced the

proportion of *Candidate division TM7*, a currently uncultivable bacteria which has previously been isolated from periodontitis (Paster, B.J., Boches, S.K., et al. 2001) and is thought to rely on group interactions within biofilms for survival (Wade, W. 2002). BMO was also associated with an increase in *Actinobacteria*, a diverse phylum to which the family *Bifidobacteriaceae* belong (Ventura, M., Canchaya, C., et al. 2007). BMO treatment was associated with the absence of 7 families of bacteria, the majority of which are associated with a negative influence on health. In contrast to P95, BMO significantly reduced *Enterococcaceae*, as well as *Enterobacteriaceae*, which are both predominant in the flora of preterm human infants (Arboleya, S., Binetti, A., et al. 2012). Of the three treatments, BMO was the sole carbohydrate source to significantly reduce the *Peptostreptococcaceae* family.

Opposite to the effect of BMO, 6'SL significantly reduced the proportion of *Actinobacteria* at the phylum level. At the family level, 6'SL reduced *Moraxellaceae, Lactobacillaceae,* and *Porphyromonaceae,* while 4 families of bacteria were no longer detectable. The inability to detect *Prevotellaceae* was unique to treatment with 6'SL. *Prevotellaceae* are commonly linked to periodontal disease and abscesses along with bacteremia and upper respiratory tract infections (Tanaka, S., Yoshida, M., et al. 2008). Although investigation into the mechanisms of action were not carried out, the fact that 6'SL did not promote the growth of any bacterial families may suggest this molecule is acting in an anti-infective manner or as a decoy molecule, as reported for acidic oligosaccharides in previous studies (Coppa, G.V., Zampini, L., et al. 2006, Hester, S.N., Chen, X., et al. 2013, Thomas, R. and Brooks, T. 2004).

The fact that indigenous bifidobacteria were undetectable through both real-time PCR analysis and 454 pyrosequencing in baseline and 3-week faecal samples limited our ability to comprehensively assess the prebiotic nature of the supplemented oligosaccharides, as prebiotic supplementation is often associated with increases in bifidobacteria and lactobacilli (Gibson, G.R., Beatty, E.R., et al. 1995, Kaplan, H. and Hutkins, R.W. 2000, Roberfroid, M.B. 2000). Additionally, lactobacilli typically comprise 0.2-1% of the total microbiota in infant fecal samples (Mueller, S., Saunier, K., et al. 2006), and display adaptations to specific ecological niches. Isolates from milk exhibit an adaptation for growth on lactose, while those isolated from the intestinal niche

often encode pseudogenes associated with sugar internalization and metabolism (Makarova, K., Slesarev, A., et al. 2006, Ventura, M., Canchaya, C., et al. 2007). While fructo-oligosaccharides may act as fermentable substrates for lactobacilli (Gänzle, M.G. and Follador, R. 2012), FOS supplementation has been demonstrated to reduce levels of lactobacilli in conventionally raised C57Bl/6J mice (Pachikian, B.D., Neyrinck, A.M., et al. 2011), while other studies have reported limited or no promotion of growth (Campbell, J.M., Fahey, G.C., et al. 1997, Petersen, A., Bergström, A., et al. 2010). Furthermore, digestion of sialyllactose and mixtures of complex human milk oligosaccharides lie largely beyond the scope of their enzymatic activities (Idota, T., Kawakami, H., et al. 1994, Schwab, C. and Gänzle, M. 2011), possibly accounting for the lack of effect of the supplemented oligosaccharides on the lactobacilli numbers.

While the murine model provides an accessible and comparatively cheap *in vivo* intestinal model for the study of prebiotic substances, there are several inherent and important differences to that of the human environment. The commensal flora of mice is adapted to the murine intestinal environment and daily diet (utilizing different nutrients, binding sites, and adhesins), and may respond differently to the provision of human/bovine oligosaccharides when compared to the human microflora. For example, lactobacilli likely employ different adhesion mechanisms depending on host tissue, given that a widespread mucus-adhesion protein of lactobacilli will bind to human, rabbit, and guinea pig tissues, yet does not adhere to the mucus of murine intestinal biopsies (Coïc, Y.-M., Baleux, F., et al. 2012), indicating significant differences in the murine mucosal composition. Despite numerous studies demonstrating the presence and dominance of bifidobacteria in human neonates, the microflora in mice depends on the particular strain used (ie. C57BL/6 or BALB/c) and the environment in the housing facilities (Buddington, R.K., Williams, C.H., et al. 2000). This may allow for variable inter-study intestinal microflora, and in some cases, an absence or low levels of bifidobacteria (Hasegawa, M., Osaka, T., et al. 2010).

The predominant bacterial fermentation end-products in the large bowel are butyrate, acetate, and propionate, which result from the breakdown of oligosaccharides, polysaccharides, proteins, peptides, and glycoproteins (Macfarlane, G.T. and Macfarlane, S. 2002). The production of SCFAs relies on both the number of bacteria present, as well as their associated ability to

metabolize relevant precursors. *Clostridium*, *Eubacterium*, and *Ruminococcus* genera are associated with the production of butyrate, while propionate and acetate are attributed to lactic acid producing bacteria of the *Lactobacillus* and *Bifidobacterium* genera (Gourbeyre, P., Denery, S., et al. 2011). In the current study, levels of SCFAs were generally unaffected, with the exception of 6'sialyllactose treatment, which resulted in decreased levels of butyrate. However, fecal butyrate concentrations in breast-fed infants have been found to be lower than their formula-fed counterparts (Knol, J., Scholtens, P., et al. 2005), potentially highlighting a benefit associated with supplementation of 6'sialyllactose. This reduction in butyrate may be the result of an elimination of a family or combination of families of bacteria which are involved in the production of butyrate. Accordingly, 6'SL and P95 treatment were associated with increased proportions of *Ruminococcus*, with only the 6'SL treatment reducing butyrate concentrations. In addition, 6'SL significantly reduced the proportion of *Lactobacillus* but did not affect caecal acetate concentrations, leading to the inference that the lactobacilli populations did not significantly contribute to the observed levels of acetate in this particular study. It should be noted that in the current study caecal flow and volume were not assessed. An increased volume or rate of caecal transit could explain the lack of impact on or reduced concentrations of SCFAs detected (Cheng, B.Q., Trimble, R.P., et al. 1987, Peuranen, S., Tiihonen, K., et al. 2004).

Blood serum cytokine levels were assessed to determine whether oligosaccharide supplementation would modulate the murine systemic immune response. Supplementation with oligosaccharides did not result in significant changes in the serum concentrations of the three pro-inflammatory cytokines - IL-1β, IFN-γ, and TNF-α, or the anti-inflammatory cytokine, IL-10. As these cytokines have previously been demonstrated to be produced in response to probiotic bacteria (reviewed in Gill, H.S. 2003), their lack of significant change may be explained by the fact that bifidobacteria were found to be absent during the present study and lactobacilli were significantly reduced following 6'sialyllactose supplementation or otherwise unaffected. The stimulation of intestinal epithelial cells by pathogens is associated with the production of TNF-α and IL-8 (Kagnoff, M.F. and Eckmann, L. 1997), both of which were

unaffected by oligosaccharide supplementation. Therefore, despite the fact that it appears that probiotic-associated cytokines were unaffected, there was also a lack of production of pathogen-associated cytokines, possibly indicating that the oligosaccharide supplements did not result in an increased pathogenic burden.

P95 significantly decreased levels of IL-12 and IL-6, while BMO decreased IL-12 levels. IL-12 is a pro-inflammatory cytokine (Shida, K., Takahashi, R., et al. 2002), while IL-6 may act as a pro- or anti-inflammatory cytokine, which is necessary to fight infection of *Streptococcus pneumoniae* in a murine model (van der Poll, T., Keogh, C.V., et al. 1997), thereby demonstrating both positive and negative properties associated with the two interventions. mKC is the murine analogue of human IL-8, which is associated with inflammation and is released in response to pathogenic stimulation of intestinal epithelial cells *in vitro* (Gill, H.S. 2003). 6'SL significantly decreased levels of mKC in relation to the BMO group, demonstrating the potential to modulate inflammation. Alternatively, prebiotic galactooligosaccharides have previously been demonstrated to act in a direct antimicrobial manner through interaction with enterocyte surfaces, thereby decreasing pathogenic binding sites (Gibson, G., McCartney, A., et al. 2005, Shoaf, K., Mulvey, G.L., et al. 2006), thus offering an alternative mechanism through which the supplemented oligosaccharides may potentially be acting. As the exact mechanism of action of the oligosaccharides in this particular study is unknown, further studies would prove beneficial.

The primary role of sIgA is in the prevention of pathogenic attachment to host tissues (Kagnoff, M.F. 1993). Furthermore, the induction of IgA antibodies and $CD4^+$ regulatory T cells mediate oral tolerance to intestinal contents (Mowat, A.M. 2003). In the current study, both P95 and BMO resulted in significant decreases in caecal sIgA, while 6'SL did not differ significantly from the saline control. In contrast to our current findings, previous studies typically report increased levels following the administration of prebiotics (Seifert, S. and Watzl, B. 2007). Ingestion of RaftiloseR Synergy1 (a commercial blend of inulin and oligofructose) resulted in increased levels of caecal sIgA in a rodent model (Roller, M., Rechkemmer, G., et al. 2004). Hosono *et al.* found a transient increase in fecal IgA levels at week 2, returning to control levels at weeks three and four (Hosono, A., Ozawa, A., et al. 2003), while Nakamura *et al.* found

elevated levels throughout the small and large intestine, though feeding the mice nearly ten times as much oligofructose (50g/kg diet) as used in the current study (Nakamura, Y., Nosaka, S., et al. 2004). Although 6'sialyllactose supplementation did not induce a significant change, BMO and P95 reduced caecal levels of secretory IgA. Pathogens and probiotic bacteria are both able to stimulate the production of IgA, though typically differing on whether they are T-cell dependent or independent, respectively (Cerutti, A. and Rescigno, M. 2008). The fact that P95 and BMO supplementation both significantly reduced the levels of caecal sIgA leads several different hypotheses. Though many scenarios are possible, the most probable situation in agreement with the sequencing data is that the BMO and P95 treatments are reducing either a particular bacterial group responsible for the production of IgA, or the overall bacterial numbers in the caecum, resulting in a decreased concentration of sIgA. Given the current findings, it appears likely that 6'SL supplementation does not produce a significant effect on intestinal and oral tolerance due to its negligible effects on levels of sIgA.

The present study has demonstrated the gut-modulatory properties of oligosaccharides in a murine model. While the two oligosaccharide treatments have resulted in a significant reduction in proportions of less favourable bacterial families, next steps will include determining their effects upon probiotic bacterial strains, with specific emphasis on bifidobacteria which were absent in the current study. Future studies would benefit from the use of mice colonized with a human-derived microflora or a synbiotic treatment (provision of bifidobacteria and oligosaccharides) as implemented in previous studies (Bielecka, M., Biedrzycka, E., et al. 2002, Furrie, E., Macfarlane, S., et al. 2005), to allow for a better interpretation of the interplay between supplemented oligosaccharides, their effects on pathogens, probiotic commensals, and overall host health.

CHAPTER III

CONCLUSION

The results from this study demonstrate the ability of BMO and 6'SL supplementation to significantly reduce or eliminate families of bacteria which potentially harbour pathogenic organisms. Additionally, supplementation with 6'SL reduced caecal butyrate, which is primarily produced by *Clostridia*, *Eubacteria*, and *Ruminococcus*, possibly indicating a linked reduction in unfavourable bacteria. Finally, caecal sIgA was not significantly impacted by 6'SL, demonstrating that supplementation with this specific oligosaccharide, which is naturally found in human and bovine milk, likely would not impact oral tolerance of the host. Despite the differences and limitations of the murine model, this study has demonstrated that ingestion of bovine milk oligosaccharides is a safe and effective approach to modulate populations of the intestinal microbiota.

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Table 1. BMO powder composition.

BMO component	Percent (%)
3'- and 6'Sialyllactose	33.7
Lactose	0.03
Protein	0.94
Ash	13.4

Table 2. Murine body and organ weights by treatment group.

	Control	P95	BMO	6'sialyllactose
Final body weight (g)	19.3 ± 1.0	19.4 ± 1.2	19.1 ± 1.1	19.5 ±0.9
Liver weight (g)	0.771 ± 0.16	0.822 ± 0.15	0.879 ± 0.16	0.887 ± 0.09
Caecal tissue weight (g)	0.059 ± 0.02	0.058 ± 0.02	0.065 ± 0.02	0.061 ± 0.01
Small intestine tissue weight (g)	0.397 ± 0.09	0.393 ± 0.06	0.366 ± 0.09	0.315 ± 0.07

BMO- bovine milk oligosaccharide powder; P95 – Beneo Orafti P95 oligofructose.

Table 3. Serum cytokines detected following 21-day oligosaccharide supplementation.

	Control	P95 Beneo Orafti	BMO	6'sialyllactose
IFN-γ	4.4 ± 0.4	3.9 ± 0.2	3.9 ± 0.2	4.3 ± 0.3
IL-10	55.3 ± 3.0	54.7 ± 2.6	55.3 ± 1.6	56.5 ± 1.9
IL-12	110.6 ± 3.3	90.6 ± 2.3**	97.4 ± 2.7*	99.6 ± 4.8
IL-1β	15.6 ± 0.8	14.7 ± 1.9	15.6 ± 0.9	13.3 ± 1.1
IL-6	57.7 ± 3.4	45.5 ± 2.3*	54.2 ± 2.7	55.7 ± 2.8
mKC	103.2 ± 7.3	92.7 ± 6.8	121.3 ±10.0^	85.0 ± 4.5^
TNF-α	16.2 ± 1.2	12.9 ± 0.3	14.7 ± 0.7	14.1 ± 0.8

Concentration is presented as pg/ml. Statistical significance was determined using a one-way ANOVA; p value ≤ 0.05*; ≤ 0.01**; ≤ 0.001*** in relation to control; ^ denotes significance between two treatment groups.

Table 4. Significant differences in microbial composition. Day 0 vs. Day 21

Taxa	p-value
Cyanobacteria (p)	0.010
Actinobacteria (p)	0.026
Candidate division TM7(p)	0.005
Moraxellaceae (f)	0.001
Enterobacteriaceae (f)	<0.001
Succinivibrionaceae (f)	0.001
Alcaligenaceae (f)	0.031
Porphyromonaceae(f)	0.023
Prevotellaceae (f)	0.003
Peptostreptococcaceae (f)	0.017
Veillionallaceae (f)	0.001
Lactobacillaceae(f)	0.011
Enterococcaceae (f)	0.005
Leuconostocaceae (f)	0.011
Enterobacteriaceae species (g)	0.001
Psychrobacter (g)	0.048
Pasteurellales (g)	<0.001
Anaerobiospirillum (g)	0.001
Sutterella (g)	0.031
Parabacteroides (g)	0.017
Prevotella (g)	0.003
Lachnospiraceae Incertae Sedis (g)	0.016
Uncultured Lachnospiraceae (g)	0.021
Marvinbryantia-Bryantella (g)	0.000
Acetitomaculum (g)	0.027
Anaerovibrio (g)	0.011
Ruminococcaceae Incertae Sedis (g)	0.004
Lactobacillus (g)	0.011
Enterococcus (g)	0.009
Erysipelotrichales Incertae Sedis (g)	0.003

p=phylum level, f=family level, g=genus level

Table 5. Significant differences in microbial composition. Day 21 comparison only.

Taxa	p-value
Cyanobacteria (p)	0.029
Actinobacteria (p)	0.016
Candidate division TM7(p)	0.010
Moraxellaceae (f)	<0.001
Enterobacteriaceae (f)	0.000
Vibrionaceae (f)	0.024
Pseudoalteromonadaceae (f)	0.024
Succinivibrionaceae (f)	0.002
Prevotellaceae (f)	0.002
Peptostreptococcaceae (f)	0.008
Veillionallaceae (f)	0.001
Lactobacillaceae(f)	0.050
Enterococcaceae (f)	0.003
Leuconostocaceae (f)	0.024
Enterobacteriaceae species (g)	0.004
Pasteurellales (g)	0.001
Anaerobiospirillum (g)	0.002
Vibrio	0.024
Pseudoalteromonas	0.024
Prevotella (g)	0.002
Lachnospiraceae Incertae Sedis (g)	0.014
Marvinbryantia-Byrantella (g)	0.002
Anaerovibrio (g)	0.006
Lactococcus (g)	0.049
Lactobacillus (g)	0.050
Enterococcus (g)	0.004
Erysipelotrichales Incertae Sedis (g)	0.005
Psychrobacter (g)	0.018

p=phylum level, f=family level, g=genus level

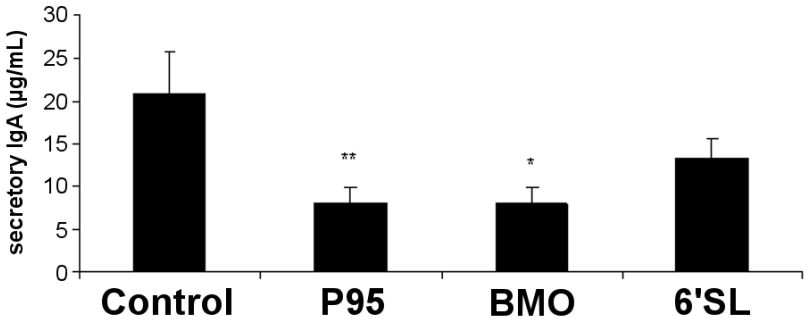

Fig. 1. Caecal secretory IgA measured following 21 days of oligosaccharide supplementation. * denotes $p<0.05$; ** denotes $p<0.01$.

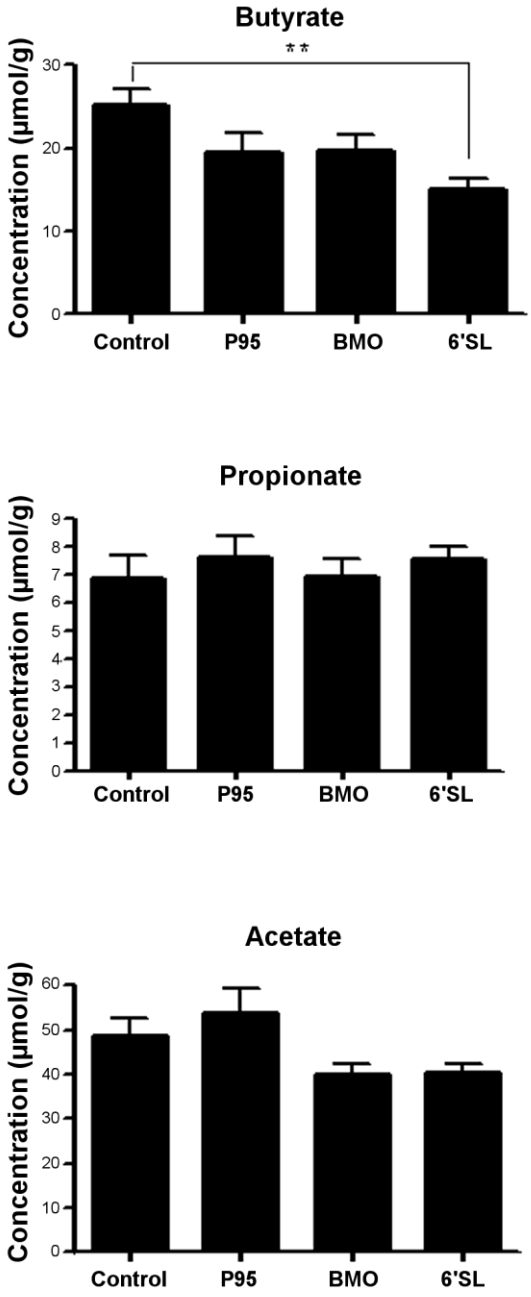

Fig. 2. Caecal short-chain fatty acids measured following 21 days of oligosaccharide supplementation. ** denotes $p<0.01$.

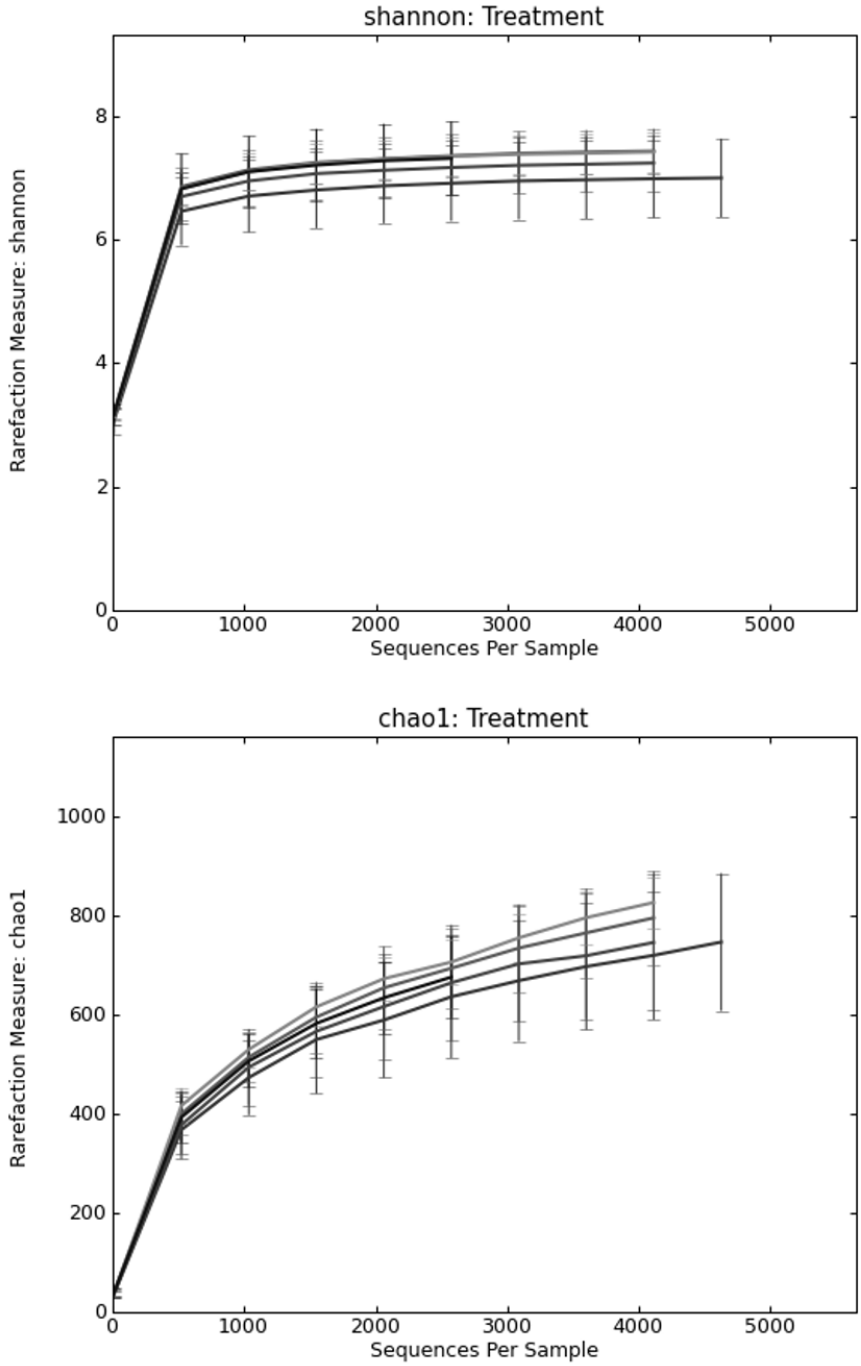

Fig. 3. 454 Pyrosequencing data rarefaction curves. A – Shannon treatment; B – Chao treatment.

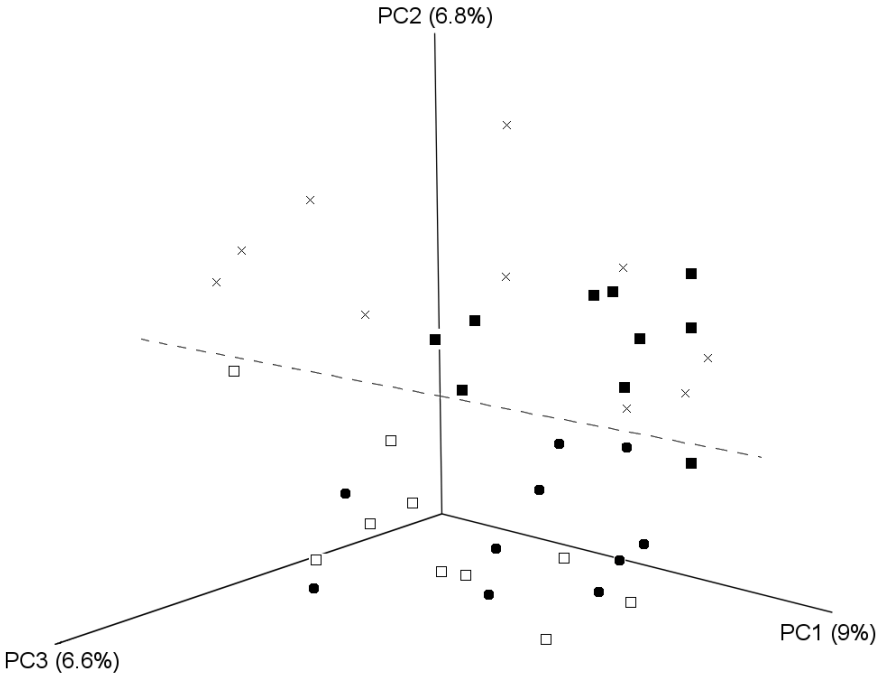

Fig. 4. Unweighted Unifrac analysis of oligosaccharide treatment clustering on microbial composition. Oligosaccharide treatments are denoted as follows: Control – black circle; P95 – white square; BMO – black square; and 6'SL – x symbol.

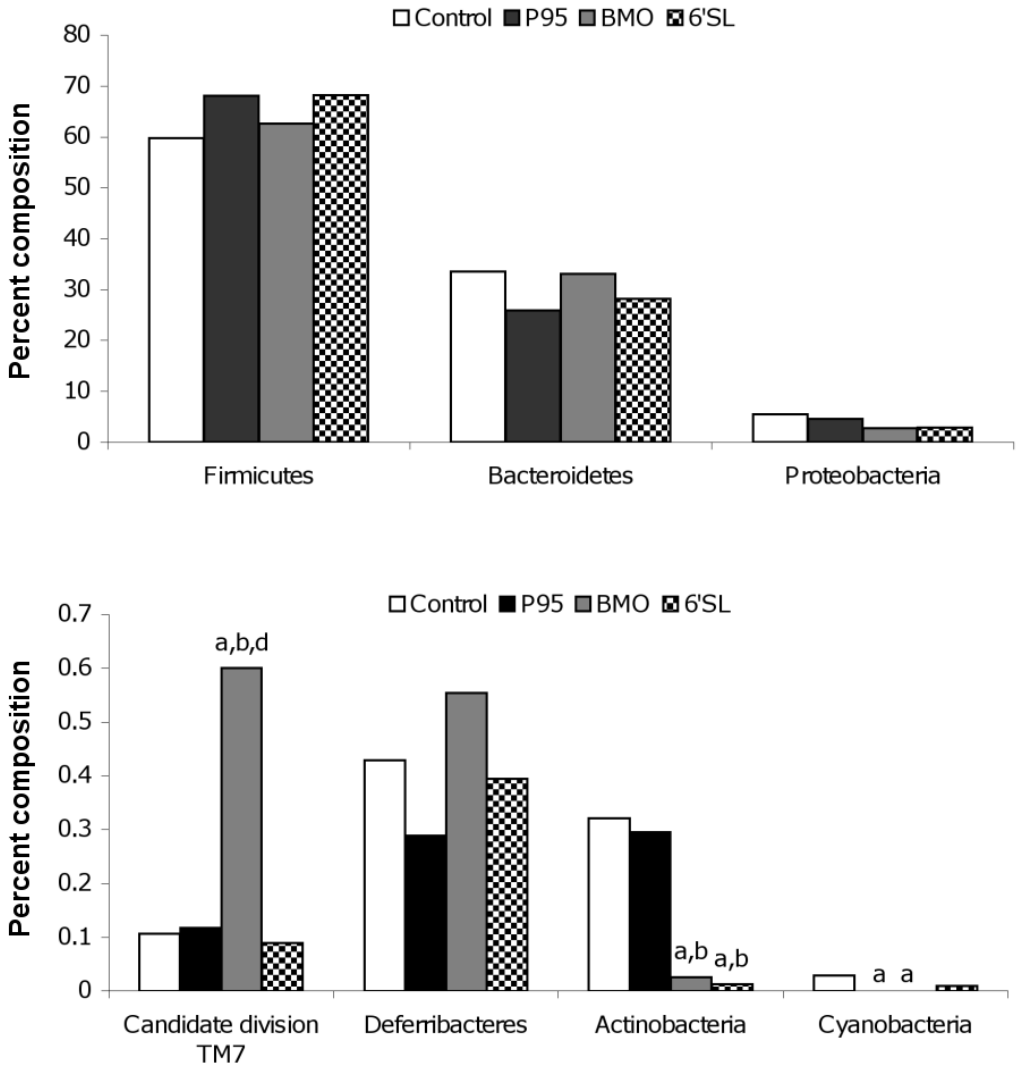

Fig. 5. Microbial composition at the phylum level. Top panel refers to microbial compositions > 2%, bottom panel refers to <1%. [a] denotes significance in comparison to control; [b] denotes significance in comparison to P95; [c] denotes significance in comparison to BMO; and [d] denotes significance in comparison to 6'SL. Significance is determined as $p<0.05$.

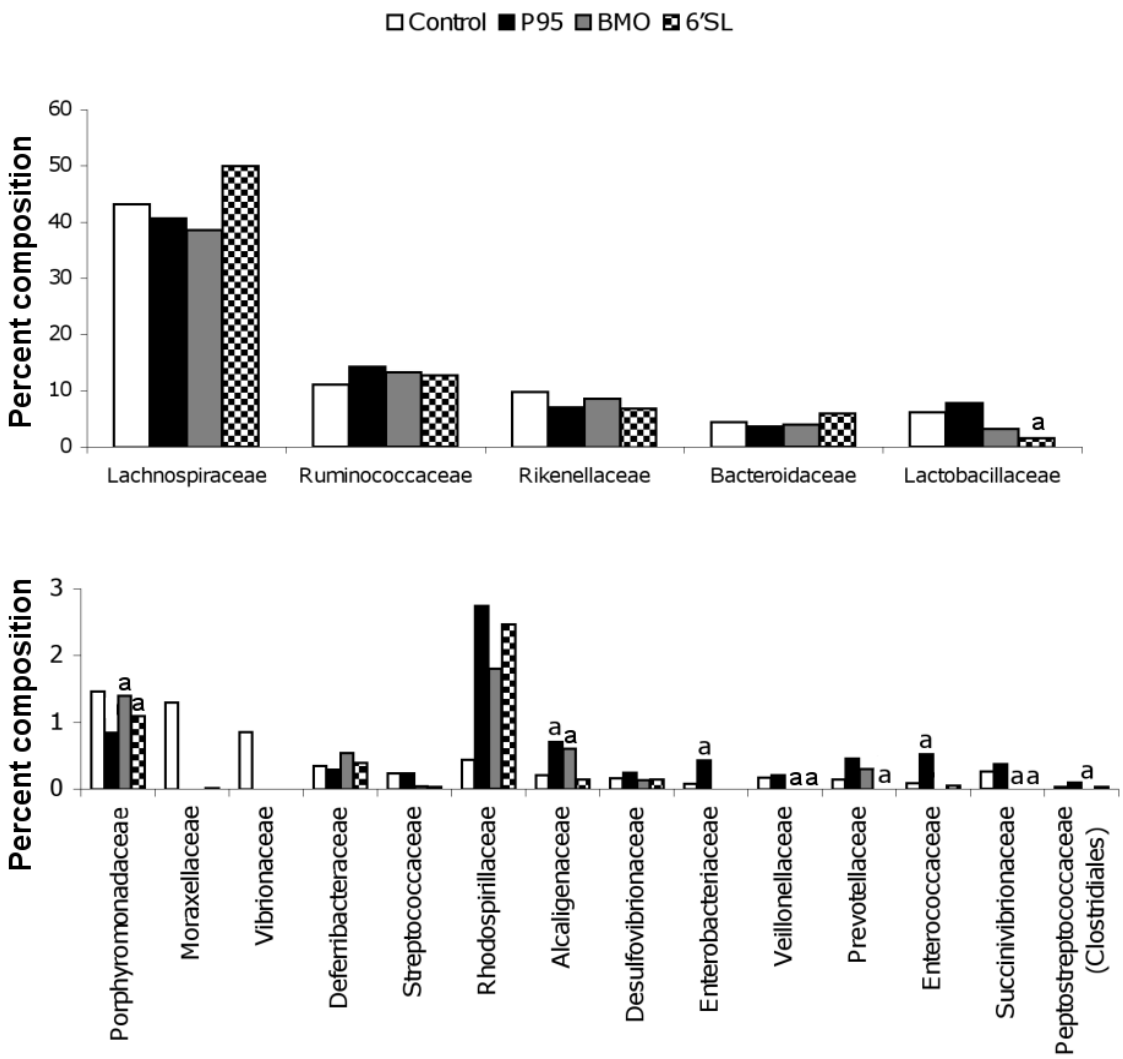

Fig. 6. Microbial composition at the family level. [a] denotes significance in comparison to control; [b] denotes significance in comparison to P95; [c] denotes significance in comparison to BMO; and [d] denotes significance in comparison to 6'SL. Significance is determined as $p<0.05$.

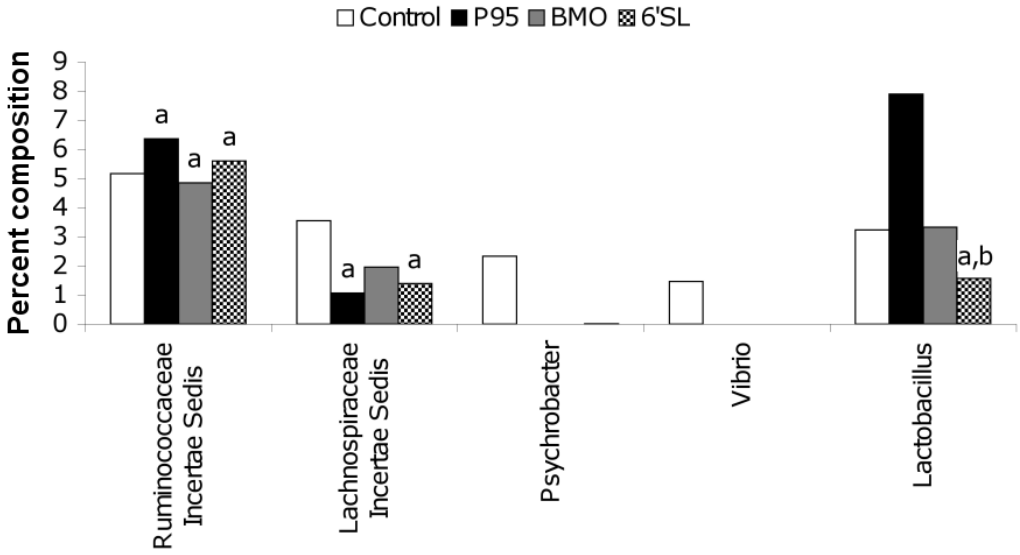

Fig. 7. Microbial composition at the genus level. [a] denotes significance in comparison to control; [b] denotes significance in comparison to P95; [c] denotes significance in comparison to BMO; and [d] denotes significance in comparison to 6'SL. Significance is determined as $p<0.05$.

CHAPTER IV

Detection of galectin-3 interaction with commensal bacteria

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ABSTRACT

Galectin-3 is a β-galactoside-binding lectin which functions in inflammation, cellular interactions, tumour progression and metastasis. The lectin has been shown to interact with pathogens via both its carbohydrate recognition domain and N-terminal region. Pathogen interaction with galectin-3 has been implicated in increased colonisation and survival of pathogens within the host. To date, interactions between galectin-3 and commensal bacteria have not been documented. In the current study, an indirect surface plasmon resonance (SPR) assay was used to screen a range of commensal bacteria for interactions with galectin-3. *Bifidobacterium longum* subsp. *infantis* ATCC 15702 and *Bifidobacterium longum* subsp. *infantis* ATCC 15697 exhibited a significantly higher ability to inhibit binding of galectin-3 (93% and 73%, respectively) to asialofetuin. This bacterial interaction was confirmed directly by agglutination and solid-phase binding assays using *B. longum* subsp. *infantis* ATCC 15702, with the latter study demonstrating the importance of the full-length protein for activity versus the carbohydrate recognition domain alone. The ability of bifidobacteria strains to significantly interact with galectin-3 may lead to a better understanding of galectin-3 and commensal function in the intestinal tract, as well as highlighting their potential as decoys in inhibiting inflammatory and disease states resulting from the interaction of pathogens with galectin-3.

CHAPTER IV

INTRODUCTION

Galectins comprise a family of evolutionarily-conserved proteins found across a variety of species ranging from lower invertebrates to mammals (Gunning, A.P., Bongaerts, R.J., et al. 2009). Presently, 15 galectin proteins have been identified in mammals with varying distribution throughout different types of cells and tissues. Galectins have been implicated in a wide range of biological processes, including tumour cell adhesion and progression, inflammation, wound-healing, development and immunity (Cao, Z., Said, N., et al. 2002, Dumic, J., Dabelic, S., et al. 2006, Leffler, H., Carlsson, S., et al. 2004). Galectins are secreted by a non-classical pathway without signal sequences and function through specific binding to their counterpart oligosaccharides present on glycoconjugates located on the cell surface and in the extracellular matrix (ECM) (Fowler, M., Thomas, R.J., et al. 2006, Iwaki, J., Minamisawa, T., et al. 2008). Previous studies have identified a number of natural ligands of galectins. These include galactose, lactose, polylactosamine, and N-acetyllactosamine (LacNAc) (Gunning, A.P., Bongaerts, R.J., et al. 2009). Recently, galectins have also been shown to bind to blood group antigens expressed on the surface of bacterial cells (Stowell, S.R., Arthur, C.M., et al. 2010).

Galectin-3 is a 31 kDa chimera-type lectin containing a C-terminal carbohydrate recognition domain (CRD) with an approximate molecular weight of 14 kDa (consisting of ~130 amino acids), and possessing an affinity for β-galactoside residues (Fowler, M., Thomas, R.J., et al. 2006, Gunning, A.P., Bongaerts, R.J., et al. 2009, Sato, S., St-Pierre, C., et al. 2009). The CRD is a globular structure which is attached to a long, thin tail composed of collagen-like material and resulting in a tadpole like appearance (Gunning, A.P., Bongaerts, R.J., et al. 2009). The N-terminal domain possesses a phosphorylation site and a collagenase-sensitive region (Frol'ova, L., Smetana, K., Jr., et al. 2009). Specific features of galectin-3 include its ability to associate into pentamers and the ability to bind proteins such as bcl-2 (involved in the regulation of cell apoptosis), and lipopolysaccharides (LPS) (Frol'ova, L., Smetana, K., Jr., et al. 2009). Upon binding to glycan ligands, galectin-3 undergoes a conformational change leading to oligomerization, a process that is mediated by the N-terminal domain (Sato, S., St-Pierre, C., et al. 2009). For example, the CRD of galectin-3

possesses the ability to bind bacterial LPS; however, the N-terminus is required for oligomerization and an enhanced response (Fermino, M.L., Polli, C.D., et al. 2011). Furthermore, galectin-3 can function in a multivalent fashion and is capable of cross-linking glycan ligands on cell surface receptors to form a lattice structure. For example, it has been shown that galectin-3 can interact with cell surface mucins on the ocular surface and contributes to the integrity of its mucosal barrier (Argueso, P., Guzman-Aranguez, A., et al. 2009).

Interactions of galectin-3 with pathogenic microorganisms are well documented. Galectin-3 was first demonstrated to associate with the LPS of *Klebsiella pneumoniae* through binding β-galactoside glycans in the outer core. The lectin also interacts with the lipid A/inner core region of LPS derived from *Salmonella minnesota* R7, in a manner that does not involve the CRD (Mey, A., Leffler, H., et al. 1996). In addition, interactions with *Pseudomonas aeruginosa* (outer core of LPS) (Gupta, S.K., Masinick, S., et al. 1997), *Neisseria gonorrhoeae* (lipooligosaccharides) (John, C.M., Jarvis, G.A., et al. 2002), and *Helicobacter pylori* (O-antigen of LPS) (Fowler, M., Thomas, R.J., et al. 2006), have been described. Many negative implications of the galectin-3 association with pathogens have been identified. For instance, the interaction of galectin-3 with the LPS of *Salmonella* results in the prevention of endotoxin shock, which attenuates the inflammatory response, ultimately favouring survival of the pathogen (Li, Y., Komai-Koma, M., et al. 2008), while galectin-3 pre-incubation with LPS of *Escherichia coli* resulted in enhanced neutrophil activation and ultimately increased mortality due to endotoxic shock (Fermino, M.L., Polli, C.D., et al. 2011). Furthermore, the presence of galectin-3 has been shown to increase the adhesion of *Trypanosoma cruzi* to host tissues (Kleshchenko, Y.Y., Moody, T.N., et al. 2004). These studies highlight the ability of pathogens to capitalize on the presence of galectin-3 to augment their capacity to colonize and survive within the host environment.

Despite numerous examples of pathogen interactions with galectin-3, there is very little evidence to suggest that commensal bacteria can interact with this protein. Recent research investigating the localization of galectin-3 in the murine intestinal tract has revealed that it is limited to the surface of the gastric mucosa. Moreover, galectin-3 was shown to interact with gastrointestinal microorganisms at this interface using electron microscopy (Nio-Kobayashi, J.,

Takahashi-Iwanaga, H., et al. 2009). The current study used an indirect SPR-based approach to screen commensal bacteria isolated from the human intestine for their ability to interact with galectin-3. The resulting interactions were further validated using agglutination and solid phase binding assays. Of six commensal strains examined, two specific strains of bifidobacteria were identified which interacted strongly with galectin-3.

CHAPTER IV

MATERIALS AND METHODS

Materials and reagents

All reagents and materials used for surface plasmon resonance studies were obtained from GE Healthcare UK Ltd (Buckinghamshire, United Kingdom). These include: 10-mM HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid) buffer (pH 7.5) containing 150 mM NaCl, 0.005% polyoxyethylenesorbitan (p20), and 3 mM ethylenediaminetetraacetic acid) (EDTA) (HBS–EP buffer); *N*-hydroxysuccinimide (NHS); *N*-ethyl-*N*′-(dimethylaminopropyl)carbodiimide hydrochloride (EDC); ethanolamine hydrochloride; NaOAc buffer, pH 4.5; and CM5 sensor chips (Carboxymethylated dextran surface). Lactose, phosphate buffered saline, L-cysteine hydrochloride, human-recombinanat galectin-3 and asialofetuin (ASF) were purchased from Sigma (Steinheim, Germany). Galectin-3C (the carbohydrate binding domain of galectin-3) and the Innocyte ECM adhesion galectin-1/galectin-3 kit were purchased from Calbiochem (Merck, Darmstadt, Germany). Anaerocult A gas packs were supplied by Ocon Chemicals (Cork, Ireland), deMan Rogosa Sharpe (MRS) and Brain Heart Infusion (BHI) bacterial growth media by Oxoid (London, United Kingdom), and TAMRA (carboxytetramethylrhodamine) from Invitrogen (Bio-Sciences, Dublin, Ireland).

Bacterial strains

Bacterial strains used in this study are listed in Table 1. All commensal strains used were human isolates obtained commercially from ATCC (Middlesex, UK) NCIMB (Aberdeen, UK), or DSMZ (Braunschweig, Germany). Commensal strains were routinely cultured on de Man, Rogosa, Sharpe medium (MRS) (Oxoid, London, United Kingdom) modified by the addition of 0.05% L-cysteine hydrochloride (Sigma, Steinheim, Germany). Strains were grown anaerobically at 37°C for 18-28 hours using Anaerocult A gas packs (Merck, Dannstadt, Germany) to maintain anaerobic conditions. The type strain *Escherichia coli* O157:H7 (NCTC 12900) (National Collection of Type Cultures, London, United Kingdom) was routinely cultured in Brain Heart Infusion (BHI) broth under aerobic conditions at 37°C for 16-18 hours. For the SPR inhibition assay, each bacterial strain was washed in PBS and adjusted to an optical density

A_{600} = 1.00 ± 0.069 in HBS-EP buffer. The colony forming units from each strain were determined at an optical density of 1.0 by decimal dilutions in maximum recovery diluent which were subsequently plated on their appropriate media and grown for 12-36 hours. Values were determined to be in the range of 1 x 10^8 and 4 x 10^8 CFU/ml (Table 1).

Interaction of bacterial strains with galectin-3 using SPR

Initially the CM5 chip was prepared for the capture of ASF, which is a ligand of galectin-3 due to its possession of terminal galactose residues on its structure. The carboxymethylated dextran layer in flow cell 1 was activated by injecting 70 µl of a 1:1 mixture of 0.05 M NHS and 0.2 M EDC at a flow rate of 10 µl/min, and ASF was immobilized (10 µl, 0.2 mg/ml in 10 mM NaOAc buffer, pH 4.5, flow rate: 5µl/min) to a level of ~11,000 resonance units (RU). The remaining NHS esters were blocked by the injection of a 1 M ethanolamine hydrochloride solution (70 µl, pH 8.5, flow rate 10µl/min). Flow cell 2 was also activated with NHS/EDC and then blocked with ethanolamine to create the reference flow cell to control for non-specific binding to the carboxymethylated dextran surface.

A stock concentration of 0.22mg/ml of human recombinant galectin-3 was used for all injections following preliminary optimisation experiments. 10µl of galectin-3 stock solution was incubated with 30µl (OD_{600}=1.0) of bacterial suspension in HBS-EP for 3 minutes prior to each injection, resulting in 55µg/ml of galectin-3 interacting with the bacteria. The mixture was injected over the sensor chip surface for 2 minutes. The results were calculated based upon the difference between the RUs immediately before injection and following the post-injection wash. Five measurements were taken, commencing with completion of the post-inject wash and every 20s thereafter for 80s. The 5 measurements were averaged to calculate the response value for each injection. The surfaces were then regenerated using 0.1 M lactose (a well characterised ligand of galectin-3) in HBS-EP (10µl) for 2 minutes. All measurements were performed at a flow rate of 5 µl/min. Each injection was repeated in triplicate for each bacterial strain tested and the average of the three results is reported. A representative diagram of the experimental protocol can be found in Fig. 1.

Agglutination assay to validate interaction of bacteria with galectin-3

The agglutination assay was performed as outlined in Hynes *et al.*, (1999) with modifications. Briefly, bacteria were harvested by centrifugation (8000 g, 10min), washed once with PBS, and resuspended to an optical density A_{550} = 0.8 in PBS. Aliquots of the bacterial cell suspension (40 µl) were mixed with 10 µl of galectin-3 (0.35mg/ml in PBS) solution in U-shaped microtiter wells (Corning, Sigma, Steinheim, Germany) for 5s and allowed to settle overnight, undisturbed, at 20°C. An identical assay using 10µl of PBS was used as a negative control. Results were read by visual inspection, with a positive reaction demonstrated as a carpet of aggregated cells on the bottom of the wells. Conversely, a negative reaction was evidenced by a pellet of cellular material on the bottom of a well. Negative results were confirmed by tilting the assay wells at an angle >45° and observing the movement of cellular material.

Solid-phase binding assay to investigate galectin-3 interaction with bacteria

Bacteria were fluorescently-labelled with carboxytetramethylrhodamine (TAMRA; Molecular Probes, Dublin, Ireland), by the method of Alemka *et al.*, (Alemka, A., Clyne, M., et al. 2010). Briefly, bacteria were grown overnight anaerobically at 37°C, adjusted to ~$2x10^8$ cfu/ml and washed in PBS. The bacterial suspensions were then incubated with 10 µg/ml of TAMRA in PBS for 40 minutes at 37°C in the dark followed by washing four times in PBS. The solid-phase binding assay was performed using the method of Fowler, *et al.*, (2006) with modifications. An InnoCyte ECM adhesion assay galectin-1/galectin-3 kit was used according to the manufacturer's instructions with modifications. TAMRA-labelled *Bifidobacterium infantis* ATCC 15702 cells (100µl) were added to the galectin-3-coated wells and incubated for 1 hour at 37°C under anaerobic conditions. The wells were then washed 3 times with 100µl of PBS and the bacterial adherence was quantified by fluorescence using an excitation wavelength of 530nm with detection at 590nm using a Synergy HT plate reader (BIO-TEK, Bedforshire, UK; sensitivity setting of 45). For inhibition studies, the TAMRA-labelled bacteria were exposed to the galectin-3-coated wells immediately after suspension in either 3.85µM galectin-3 or galectin-3C. The concentration of soluble galectin-3 and galectin-3C represents

a 20-fold molar excess in regards to that immobilized on the microplate well surface.

Statistical analysis

All experiments were performed in triplicate. Results are presented as the means ± standard deviations of replicate experiments. Statistical significance was determined using Student's t-test, where $p<0.05$ was considered significant.

CHAPTER IV

RESULTS

Surface plasmon resonance screening assay

The concentration of galectin-3 selected for use in the inhibition assay was 55µg/mL (based on prior optimisation experiments, data not shown) which gave an average RU value following the post-injection wash of 167 RU.

The degree of inhibition for all strains tested is shown in Fig. 2. Two strains were identified as significant inhibitors of the galectin-3:ASF interaction, namely, *Bifidobacterium longum* subsp. *infantis* ATCC 15702 and *Bifidobacterium longum* subsp. *infantis* ATCC 15697, which resulted in 93.1% and 73.1% inhibition, respectively, at similar densities. Interestingly, all strains tested showed some inhibition of binding of galectin-3 to ASF, ranging between 36-44%. Bacteria were injected over the ASF surface in the absence of galectin-3 as a negative control to ensure an absence of direct bacterial interference during injection. As the binding of galectin-3 to the LPS of *E. coli* has been reported, *E. coli* O157:H7 NCTC 12900 was screened as a positive control. At a density of 2×10^8 cfu/ml, this strain inhibited binding of galectin-3 by 54% in our assay, which suggested that the binding of at least two of the commensal species was stronger than that of the pathogenic positive control.

To eliminate the possibility of the bacteria either interfering with the surface directly (via binding to ASF) or indirectly (their presence passively obstructing access of galectin-3 to the ASF surface), bacteria were removed prior to injection of the solution over the chip surface. The assays were repeated as previously, except that following resuspension of the bacteria in the galectin-3 solution, samples were centrifuged to remove bacteria (and any potentially bound galectin-3) prior to injection over the chip. The results of the bacteria-free injections were identical to those where the bacteria was present in the injected solution (Fig. 3), in which only the unbound galectin-3 was free to interact with ASF. This demonstrates that the resulting inhibition was solely due to a bacterial interaction with galectin-3. Following SPR screening, the strain with the strongest interaction, *B. longum* subsp. *infantis* ATCC 15702, was selected for use in follow on experiments to determine the nature of the interaction with galectin-3.

Agglutination assay

The results of the SPR assays were confirmed by the agglutination assay (Fig. 3). The findings visually confirm the direct interaction between *B. longum* subsp. *infantis* ATCC 15702 (which displayed the strongest interaction) and galectin-3. Agglutination occurred in wells in which whole bacterial cells and galectin-3 were mixed and allowed to settle overnight (Fig. 4A), evidenced by the formation of a carpet of bacteria uniformly covering the bottom of the well. In the absence of galectin-3 (Fig. 4B), a compact pellet of cellular material could be observed in the bottom of the well, indicating that agglutination had not occurred.

Solid-phase binding and inhibition assay

TAMRA-labelled *B. longum* subsp. *infantis* ATCC 15702 cells were also found to adhere to galectin-3-coated microtitre wells at levels at least 5.8-fold greater than those of either *B. longum* subsp. *infantis* ATCC 15697 or *E. coli* O157:H7 (NCTC 12900) following a 1-hour incubation (Fig. 5A). Lactose was employed as a gal-3 inhibitor to determine whether the bacterial interaction with galectin-3 was carbohydrate-mediated, given the strong affinity of the lectin for the disaccharide. Addition of labelled bacteria, in the presence of lactose (50mM), to the galectin-3-coated wells resulted in a 90% reduction in adherent bacteria (Fig. 5B), indicating that galectin-3 can interact with this strain of commensal bacteria by a carbohydrate ligand. Furthermore, addition of the labelled *B. longum* subsp. *infantis* ATCC 15702 in the presence of soluble galectin-3 (3.85µM; 20-fold molar excess compared to that immobilised) resulted in a 44% reduction in adherent bacteria ($p = 0.0015$) (Fig. 5C). Addition of the bacteria in the presence of soluble galectin-3C (3.85µM) (containing only the carbohydrate-binding domain of galectin-3) did not affect adherence to the immobilized galectin-3 (Fig. 5C). These results demonstrate that the intact galectin-3 protein is able to interact with the labelled bacteria at a higher level than the galectin-3C domain alone.

CHAPTER IV

DISCUSSION

In the current study, the interaction of a panel of commensal bacteria with galectin-3 was investigated using an indirect surface plasmon resonance method that allows the detection of binding events in real time. This indirect method involved determining the ability of the various strains to inhibit the binding of galectin-3 to immobilised ASF. ASF immobilised on the CM5 chip provided a stable sensor surface that could be easily and consistently regenerated, which was not the case with immobilised galectin-3 as observed in a previous study (Maljaars, C.E., Andre, S., et al. 2008). The indirect method also avoided complications associated with the use of whole bacteria in SPR, wherein bacteria are limited to interact with the immobilized ligand at the uppermost surface of the carboxymethyldextran layer but not within it.

The results presented in this study have led to the identification of two commensal species which strongly interact with galectin-3 from a panel of 6 strains assessed. *B. longum* subsp. *infantis* ATCC 15702 and *B. longum* subsp. *infantis* ATCC 15697 have demonstrated an ability to interact with galectin-3 that is 2-fold and 1.59-fold greater than that of a known binding partner, the pathogen *Escherichia coli* NCTC 12900. The interaction was originally observed using SPR in an indirect inhibition assay and was subsequently confirmed directly by agglutination assay and solid-phase binding assays. To the best of our knowledge, this is the first report to date regarding the ability of commensal bacteria strains to interact with galectin-3.

The solid-phase binding assays demonstrate the ability of TAMRA-labelled *B. longum* subsp. *infantis* ATCC 15702 to adhere to immobilized recombinant human galectin-3. Inhibition studies were performed using soluble galectin-3, the CRD of galectin-3, or lactose, aiming to elucidate the nature of the interaction. The use of lactose as an inhibitor of the immobilised galectin-3 significantly reduced the amount of bacterial adherence to the galectin-3-coated wells, indicating that the galectin-3 interaction with this particular strain of bacteria is carbohydrate-mediated. However, the full-length galectin molecule is necessary for the strongest observable binding of the bacterial ligand, as the CRD alone was not sufficient to inhibit interaction between *B. longum* subsp. *infantis* ATCC 15702 and full-length galectin-3. These findings demonstrate the

mechanism of galectin-3 binding in a similar manner to previous research in which galectin-3C was able to bind to human neutrophils, and be significantly inhibited by the presence of lactose, while only full-length galectin-3 was able to effectively achieve an enhanced binding or response (Fermino, M.L., Polli, C.D., et al. 2011). This would suggest that the N-terminus of galectin-3, or additional amino acid residues beyond the 143 residues of the C-terminus CRD, contribute to a stronger overall interaction with the bacteria or ligand.

The data confirm that *B. longum* subsp. *infantis* ATCC 15702 binds galectin-3 by interacting with the CRD and possibly with alternative loci on the protein which indicates that the mechanism of binding is similar to that revealed for *Salmonella Minnesota* R7 (Mey, A., Leffler, H., et al. 1996). As galectin-3 is capable of interacting with two independent hydrophobic ligands on *Salmonella minnesota* R7 LPS (Mey, A., Leffler, H., et al. 1996), and mycolic acid from *Mycobacterium tuberculosis* (Barboni, E., Coade, S., et al. 2005) it seems galectin-3 may have a broader specificity than solely for LPS. As a result, it has been hypothesised that galectin-3 may interact with lipoteichoic acid (structurally similar to LPS), which is a predominant constituent in the cell wall of Gram-positive bacteria, especially bifidobacteria (Fermino, M.L., Polli, C.D., et al. 2011, Yi, Z.J., Fu, Y.R., et al. 2009). Additionally, structural analysis of the carbohydrate composition of the purified cell walls of *B. longum* subsp. *infantis* ATCC 15697 by Tone-Shimokawa *et al.* led to the isolation of two distinct neutral polysaccharides. One of the two isolated polysaccharides demonstrates a backbone structure which has been identified in *Klebsiella pneumoniae*, *Pasteurella haemolytica*, and *Serratia marcescens*, indicating that a bifidobacteria cell wall polysaccharide possesses the same structure as the O-specific antigen of some Gram-negative bacteria (Tone-Shimokawa, Y., Toida, T., et al. 1996). These current findings further emphasize the importance of the contribution of the N-terminus and intermediate residues which are involved in oligomerization, cross-linking of the protein, and a conformational change when bound to a ligand.

There is considerable evidence that the presence of galectin-3, either soluble or cell-surface-expressed, may facilitate increased cellular adhesion or survival of pathogens. The expression of galectin-3 by macrophages has been demonstrated to favour the survival of the pathogen *Salmonella minnesota* R7

(Li, Y., Komai-Koma, M., et al. 2008). Pathogens have evolved several methods to use galectin-3 to facilitate adhesion and pathogenesis. *Leishmania major* possesses the ability to bind galectin-3 through its surface lipophosphoglycans. Subsequently galectin-3 is cleaved, preventing its oligomerization, and reducing its ability to elicit an appropriate immune response (macrophage activation, neutrophil recruitment, and eosinophil-mediated cytotoxicity) (Pelletier, I. and Sato, S. 2002). Kleshchenko and colleagues (2004) have demonstrated that galectin-3 expression or exogenous administration is required for the attachment of *Trypanosoma cruzi* to coronary artery smooth muscle cells, wherein galectin-3 is able to adhere to both the pathogen and the cells (Kleshchenko, Y.Y., Moody, T.N., et al. 2004). Moreover, galectin-3 is thought to cross-link surface glycoconjugates in Madin Darby canine kidney (MDCK) cells, while allowing the N-terminal region of galectin-3 to interact with the non-agglutinating fimbriae of *Proteus mirabilis*, thus acting as a bridging molecule to promote pathogen adhesion (Altman, E., Harrison, B.A., et al. 2001).

Galectin-3 also plays a role in mucosal barrier function, acting to cross-link MUC1 proteins in the extracellular glycocalyx. Administration of the galectin-3 ligands, β-lactose or modified citrus pectin, results in decreased barrier function in intestinal Caco-2 epithelial cells. These findings were associated with decreased cell surface galectin-3, and a concomitant increase of galectin-3 in the media (Argueso, P., Guzman-Aranguez, A., et al. 2009). Further to this, lactose and pectin exposure in the intestinal lumen may serve to disrupt the mucosal barrier through the interaction with, and subsequent release of galectin-3 from the glycocalyx into the lumen. The presence of commensal bacteria capable of interacting with galectin-3 in this environment could serve to bind and reduce the amount of circulating galectin-3, preventing exploitation of the host immune lectin by pathogens.

The exact mechanism of interaction between galectin-3 and *B. longum* subsp. *infantis* ATCC 15702 is yet to be understood. Further research is required to assess the impact that these novel galectin-3 ligands may have on the presence, activity, and expression of this protein in the intestinal tract. Notably, both strains of bifidobacteria which were shown to interact strongly with galectin-3 by means of SPR inhibition assays are members of the subspecies of *Bifidobacterium longum*. Further investigation of the properties of these

commensal bacteria may allow the identification of a characteristic common to *B. longum* subsp. *infantis* strains to which galectin-3 binds. Such research may provide insights into a novel method by which commensal bacteria provide colonising resistance against their pathogenic counterparts through their interaction with immunity-related lectins, such as galectin-3.

Uncontrolled neutrophil activation is a predominant contributor to LPS-mediated endotoxic shock. Recently, Fermino *et al.* (2011) have demonstrated that pre-incubation of bacterial LPS with galectin-3 results in a sensitisation of neutrophils, which decreases the concentration of LPS required to initiate an immune response. Assessing mortality in mice due to endotoxic shock, LPS administration resulted in 60% mortality within 30 hours, while administration of the same concentration of LPS incubated with galectin-3 resulted in 100% mortality at 30 hours (Fermino, M.L., Polli, C.D., et al. 2011). Accordingly, soluble galectin-3 in the intestinal lumen could potentially harm the host by interacting with bacterial LPS, resulting in endotoxic shock. In light of the fact that nutritional intake of lactose and food pectins may release galectin-3 into the intestinal lumen through disruption of mucin cross-linkages (Argueso, P., Guzman-Aranguez, A., et al. 2009), the current study highlights the potential importance of commensal bacteria. Indeed, these microbes could interact with, and sequester, unbound galectin-3 in the intestinal lumen, potentially decreasing the likelihood of endotoxic shock to the host and pathogenic exploitation of galectin-3 to facilitate their colonisation and infection.

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Table 1. Bacterial strains screened as galectin-3 ligands.

Strain name	Accession No.	Colony forming unit/ml used for SPR screening
Bifidobacterium longum subsp. *infantis*	ATCC 15697	3.97×10^8
Bifidobacterium longum subsp. *infantis*	ATCC 15702	3.60×10^8
Bifidobacterium longum	NCIMB 8809	2.10×10^8
Bifidobacterium breve	ATCC 15700	4.00×10^8
Bifidobacterium breve	ATCC 15698	1.03×10^8
Bifidobacterium breve	NCIMB 8807	2.77×10^8
Escherichia coli O157:H7	NCTC 12900	2.00×10^8

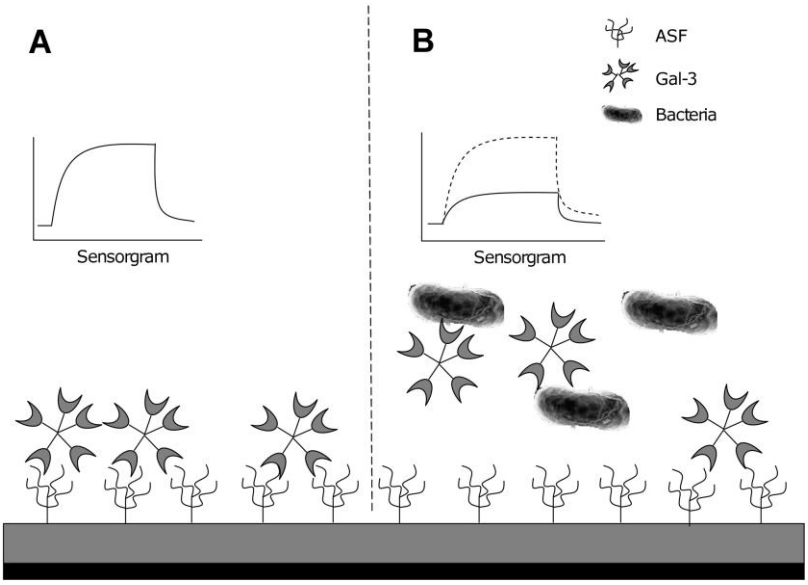

Fig.1. Experimental overview of SPR screening study. (A) Galectin-3 interacts with asialofetuin (ASF) on the chip surface. (B) Pre-incubation of galectin-3 with bacteria decreases galectin-3 interaction with ASF, indicated by a reduced resonance response.

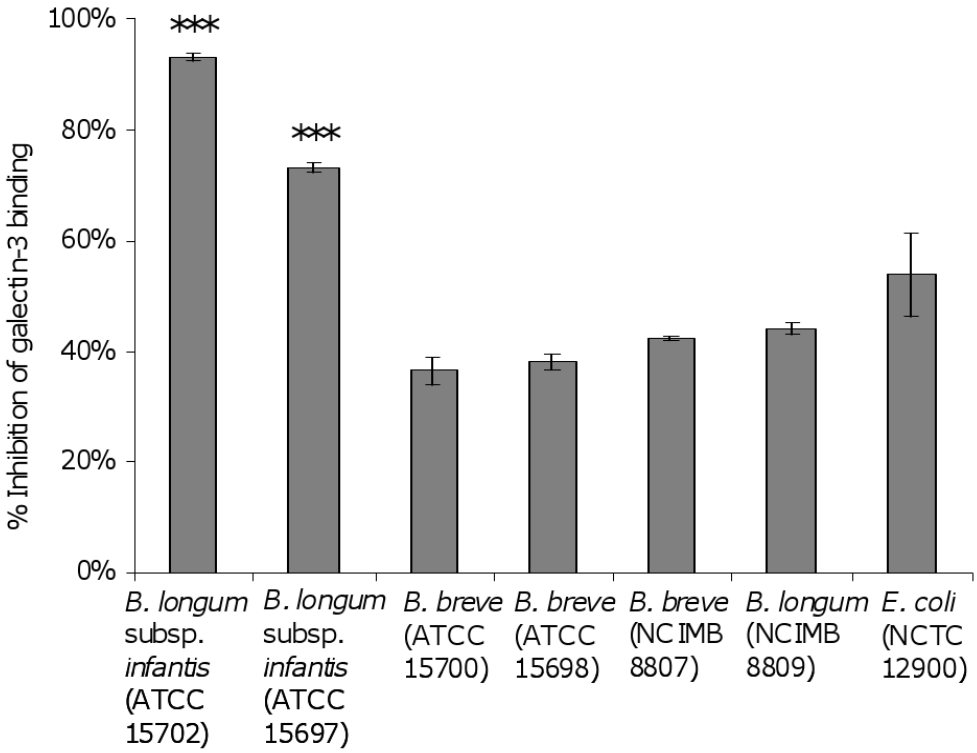

Fig 2. Inhibition of galectin-3 interaction with immobilized ASF by a panel of commensal bacteria using a surface plasmon resonance biosensor approach. *Escherichia coli* (NCTC 12900) was included as a positive control. Inhibition is defined as the ability of the bacteria to interact with gal-3, thereby reducing its ability to generate a surface plasmon response through interaction with the asialofetuin chip surface. Galectin-3 injected over the asialofetuin surface in the absence of bacteria was used to define the 0% inhibition SPR response. Experiments were carried out in triplicate (n=3) and the results are presented as mean inhibition ± standard deviation. *** denotes $p<0.0001$.

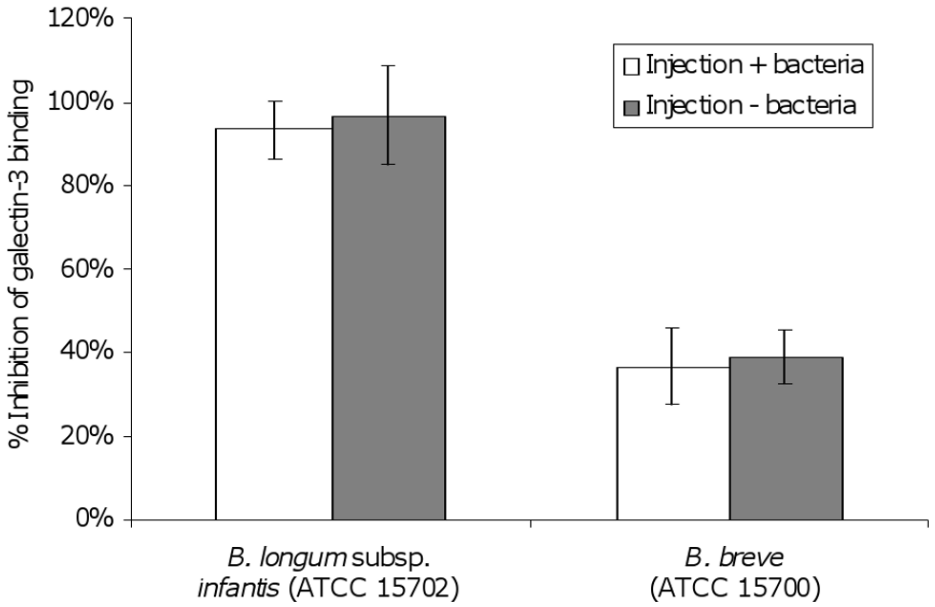

Fig 3. SPR analysis of bacteria-free injections of galectin-3 over an immobilised ASF surface. Inhibition is defined as the ability of the bacteria to interact with gal-3, thereby reducing its ability to generate a surface plasmon response through interaction with the asialofetuin chip surface. Galectin-3 injected over the asialofetuin surface in the absence of bacteria was used to define the 0% inhibition SPR response. The effects of injections with and without bacteria are compared and data are presented as the mean inhibition ± standard deviation. Experiments were carried out in triplicate (n=3). * denotes $p<0.05$.

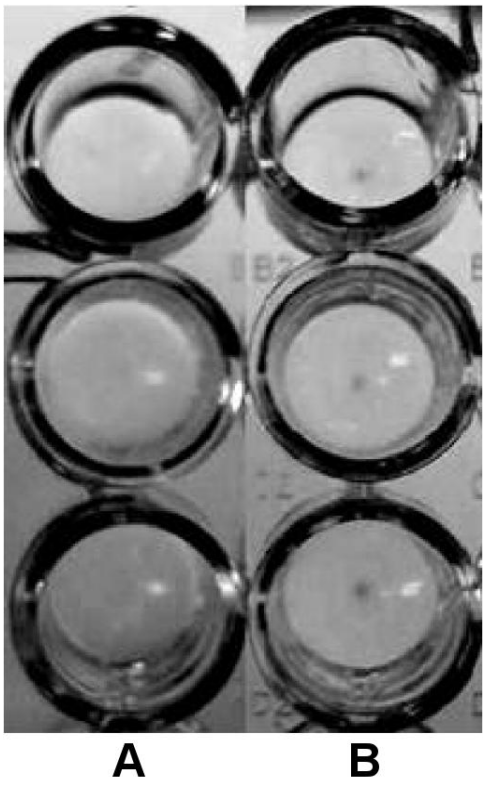

Fig 4. Agglutination assay for direct visualisation of bacteria:galectin-3 interaction. Galectin-3 or PBS alone (negative control) was incubated with *Bifidobacterium longum* subsp. *infantis* ATCC 15702. Galectin-3 exposure resulted in agglutination of the bacteria, evidence by the absence of a dense pellet of bacteria in the bottom of the well (A). PBS alone did not agglutinate the bacteria, resulting in pellet formation at the bottom of the well (B).

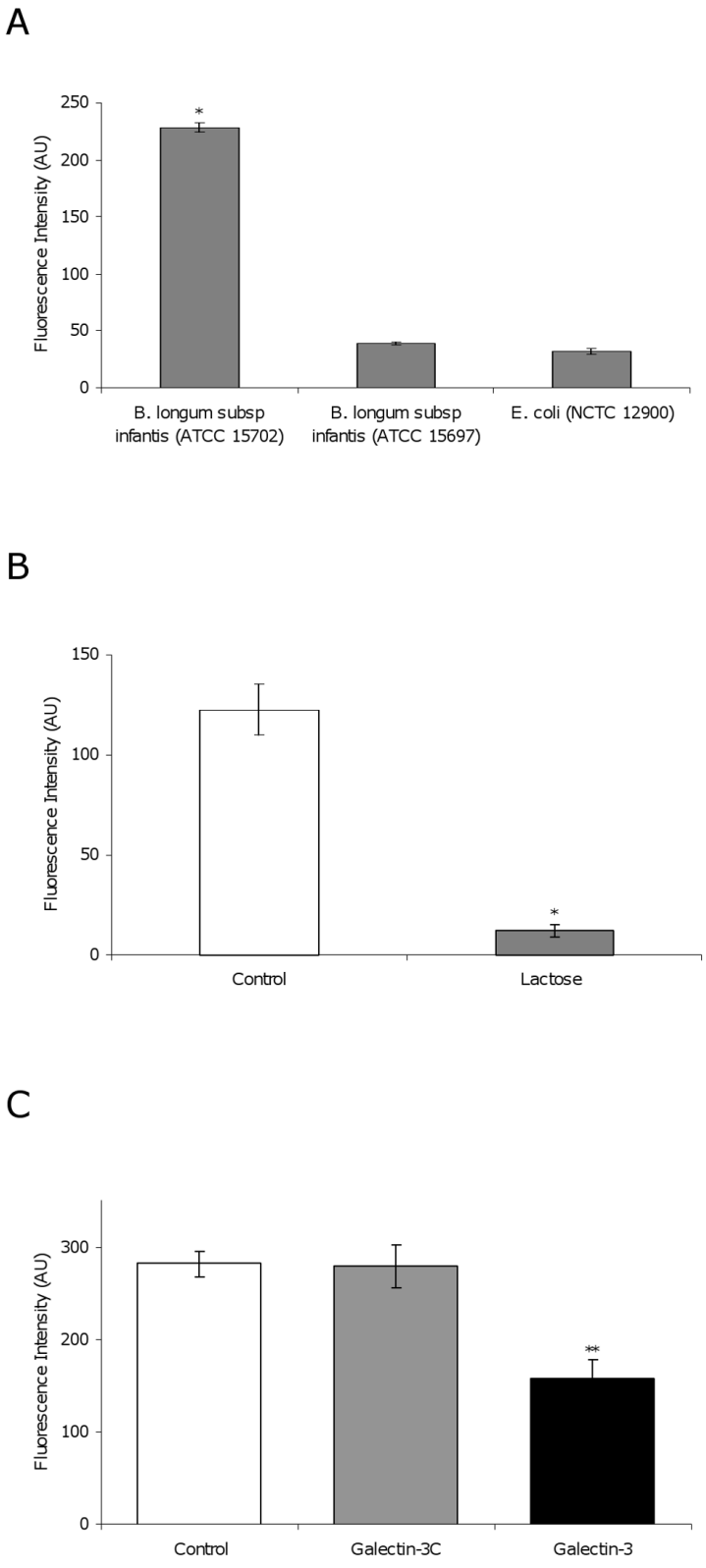

Fig 5. (A) Binding of TAMRA-labelled bacteria to recombinant galectin-3 in a solid-phase binding assay. *B. longum* subsp. *infantis* ATCC 15702, *B. longum* subsp. *infantis* ATCC 15697, and *E. coli* O157:H7 NCTC 12900 were incubated

with galectin-3-coated wells for 1 hour. (B) Binding of TAMRA-labelled *B. longum* subsp. *infantis* ATCC 15702 to recombinant galectin-3 in a solid-phase binding assay. TAMRA-labelled bacteria were incubated with galectin-3-coated wells (white bars) or galectin-3-coated wells in the presence of lactose (grey bars). (C) Binding of TAMRA-labelled *B. longum* subsp. *infantis* ATCC 15702 to recombinant galectin-3 in a solid-phase binding assay. Bacteria were incubated with galectin-3-coated wells (white), galectin-3-coated wells in the presence of soluble galectin-3C (grey) or full-length soluble galectin-3 (black). All experiments were carried out in triplicate (n=3). The data are presented as the means ± standard deviation. * denotes $p<0.05$, ** denotes $p<0.01$.

GENERAL SUMMARY AND DISCUSSION

Colonisation by bifidobacteria is associated with numerous health benefits, including stimulation of intestinal development, colonising resistance, and the digestion and metabolism of otherwise indigestible food components (reviewed in Lee, J.-H. and O'Sullivan, D.J. 2010). Beyond the ability to thrive on HMOs, *B. longum* subsp. *infantis* ATCC 15697 is known to favourably influence cellular tight junction proteins, cytokine production, and tumor regression in mice (Chichlowski, M., De Lartigue, G., et al. 2012, Sekine, K., Toida, T., et al. 1985). In scenarios which preclude breastfeeding, such as lifestyle choice or medical intervention, the ability to promote a bifidobacteria-rich intestine relies upon prebiotic or probiotic treatment. Commercial prebiotics, such as GOS, are widely accepted to selectively promote the growth of bifidobacteria, lactobacilli and other probiotic organisms, although recent studies indicate that in cases of infants possessing low numbers of bifidobacteria, they may, in fact, promote the growth of organisms, such as clostridia (Bunešová, V., Vlková, E., et al. 2012, Rada, V., Nevoral, J., et al. 2008). Probiotic bacteria, when administered, must survive gastric transit and gain a foothold in an environment that is already in a state of balance (prophylactic use), in a state of dysbalance following overgrowth by pathogenic species, or having a compromised structure, such as reduced mucus due to diarrheal diseases or IBD (interventional). These examples highlight the importance of their ability to compete for and adhere to receptors in the intestinal environment (Simmering, R. and Blaut, M. 2001). Thus, the ability to selectively aid the adhesion of probiotic bacteria, whether ingested or indigenous, would be of immense benefit.

Breast-feeding is associated with increased numbers of bifidobacteria in infant stools and reduced levels of infant morbidity. Human milk oligosaccharides have been identified as making a substantial contribution to bifidobacterial colonisation. HMOs and their interaction with commensal bacteria, specifically bifidobacteria, in the intestinal environment are the subject of increasing interest among researchers in the fields of glycobiology, nutritional science, and gastroenterology. This is due to the increasing appreciation of potential benefits associated with prebiotic oligosaccharide supplementation and concomitant establishment of bifidobacteria in the intestinal environment. Of particular interest are milk oligosaccharides and their ability to influence the intestinal colonisation of bifidobacteria. Commensal strains have demonstrated

genetic adaptations to utilize the abundant source of oligosaccharides delivered in human milk. The best example of which is *B. longum* subsp. *infantis* ATCC 15697, which possesses a vast array of genes responsible for the digestion and transport of milk and mucin oligosaccharides and freed glycans. While HMOs have proven to be a selectively fermentable prebiotic substrate, at the commencement of our research it was not known whether alternative bioactive properties existed relating to the promotion of adhesion of bifidobacteria, rather than acting solely through competitive exclusion of pathogens and supplying a nutritional resource for commensals (**Chapter II**). To investigate this hypothesis, various combinations of specific milk oligosaccharides and commensal bacteria were screened for evidence of oligosaccharide-induced enhancement of adhesion using *in vitro* intestinal models.

While several adhesion-related proteins and pathways have been identified in bifidobacteria, until recently, the ability of HMOs to stimulate bacterial adhesion had not been reported. At the same time as our study was being performed, Chichlowski *et al*. (2012) demonstrated that mid-exponential growth of *B. longum* subsp. *infantis* ATCC 15697 on pools of milk oligosaccharides resulted in increased adhesion to select intestinal cell models (Chichlowski, M., De Lartigue, G., German, J. B., Raybould, H. E., Mills, D. A. 2012). In **Chapter II**, we demonstrated that exposure of *B. longum* subsp. *infantis* ATCC 15697 to a specific milk oligosaccharide, 6'sialyllactose, resulted in an adhesive-phenotype with respect to HT-29 cell layers. This finding demonstrates a novel bioactivity of a specific milk oligosaccharide, as well as a novel means by which to increase the adhesion of an infant-associated bacteria to an intestinal cell line. Further phenotypic and transcriptomic observations outline the manner in which both the neonatal host environment and breast milk could potentially enhance the colonisation of this particular strain of bifidobacteria in the infant colon. Collectively, the findings of the study demonstrate that the adhesive-phenotype is influenced by factors such as oligosaccharide concentration or combination of oligosaccharides, exposure to lactose or trypsin, and the intestinal surface structural characteristics. As a result of these findings, a new question arises: If the presence of lactose abolishes the adhesion-promoting ability of 6'sialyllactose, where would *B. longum* subsp. *infantis* ATCC 15697 encounter 6'sialyllactose in the absence of lactose?

Looking at the natural digestion of breast milk, an answer may be evident. During gastric transit, lactose is digested in the small intestine, while 6'sialyllactose and other complex oligosaccharides arrive in the large intestine and colon unaltered (Engfer, M.B., Stahl, B., et al. 2000, Gnoth, M.J., Kunz, C., et al. 2000) and potentially enriched (Sela, D.A., Li, Y., et al. 2011). Furthermore, our findings indicate that the induced adhesive-phenotype displays a preference for the HT-29 colonocytes over differentiated Caco-2 monolayers, which resemble the epithelial cells of the small intestine. As such, this may represent an adaptation of the bacteria in response to the local environment of the GI tract, the evolution of HMO to direct colonisation of a commensal, or an example of a host-microbe co-evolution resulting in a mutually beneficial outcome. Overall, these findings shed light on the mechanism by which specific milk oligosaccharides can influence the degree and location of colonisation of an infant-associated strain of bifidobacteria.

Additional studies to further elucidate the phenotypic adhesive response of *B. longum* subsp. *infantis* ATCC 15697 to intestinal cells would prove beneficial. Assaying additional predominant acidic oligosaccharides, such as sialyl-LNT, and the plethora of neutral oligosaccharides, such as 2'fucosyllactose, independently, may allow a greater understanding of the bacterial response to HMOs. Pools of oligosaccharides isolated from varying sources would also serve to better define the bacterial response to a more representative mixture of oligosaccharides encountered while breastfeeding, though contamination with residual lactose may prove problematic and potentially complicate the interpretation of results. Additionally, the use of alternative commensal strains, including other highly active HMO-consumers, such as the *B. longum* subsp. *infantis* strains JCM 7011, JCM 7010, JCM 7009, JCM 7007 (outlined in Locascio, R.G., Ninonuevo, M.R., et al. 2009), the use or development of more representative *in vitro* models, such as those displaying mucus-secreting properties, and *in vivo* studies (murine and pig) would provide valuable insights into the ability of HMOs to promote the adhesion of this important family of bacteria. Investigation of pathogenic antagonism or displacement could demonstrate the nature of the bacterial interaction with the intestinal cells or mucus components, possibly indicating shared receptors/ligands. As the strain used here displays a vast adaptation to sense and

respond to HMOs in the infant gut, perhaps future attempts to promote the adhesion and colonisation of adult-derived strains of bifidobacteria, which demonstrate adaptations for the use of plant oligosaccharides, would benefit from a focus on plant-derived oligosaccharides.

At the transcriptional level, the DNA microarray analysis and qPCR experiments represent the first instance of identifying genetic elements potentially associated with an increased adherence to intestinal cells. Several genes were differentially expressed when exposed to a combination of 3'- and 6'-sialyllactose, the treatment which also resulted in the greatest proportion of bacteria adhering to HT-29 cells. Interestingly, two trends in gene expression emerged. The first trend was the up-regulation of stress- and colonisation-related genes. Among these genes were colonising factors that had previously been identified in other commensal strains, including dnaK, groEL, sortase, and the tight adherence locus common to bifidobacteria, the type IVb pili (Bergonzelli, G.E., Granato, D., et al. 2006, Candela, M., Centanni, M., et al. 2010, Lalioui, L., Pellegrini, E., et al. 2005, O'Connell-Motherway, M., Zomer, A., et al. 2011). The second trend to be observed was that the down-regulated genes are largely involved in the binding, transport, and catabolism of milk oligosaccharides. This is contrary to what one would expect to find when archetypical HMO-consuming bacteria are exposed to HMOs. However, growth on HMOs does not typically commence until nearly 20 hours of exposure (Ward, R.E., Niñonuevo, M., et al. 2007). Indeed, HMO may act initially as an environmental cue, stimulating adhesion and colonisation when exposed to sufficient quantities and combinations, followed by a colonisation- or possibly cell density-induced metabolic response which allows for further growth and proliferation. Our study is one of the first to identify a specific stimulus resulting in increased intestinal adhesion, supported by the transcriptomic analysis of the genome to identify colonisation genes responsible for the adhesive adaptation. Given the current findings, further research studying the bacterial response under *in vivo* conditions would serve to validate and expand the phenotypic- and transcriptional-response findings in a more representative environment. Although human trials would prove too invasive, pig trials may allow for luminal and mucosal sampling of bacteria in specific GI locations, as well as assessment of transcriptional activity. Alternatively, *ex vivo* intestinal biopsies or collection of intestinal mucus may

prove as suitable replacements for the current *in vitro* models currently in place. Additionally, future research would benefit from proteomic analysis of bacteria expressing the induced adhesive phenotype, targeted gene knock-out or mutant strains over-expressing specific genes identified here in adhesion studies.

Considering the properties of 3'- and 6'-sialyllactose, including resistance to intestinal enzymatic degradation and their positive influence on the adhesion of a predominant strain of infant-associated bifidobacteria (Klaassens, E.S., Boesten, R.J., et al. 2009), a case can be made for the inclusion of sialyllactose in infant or prebiotic formulations to reinforce the colonisation of this particular strain. Additionally, the concomitant administration of *B. longum* subsp. *infantis* ATCC 15697 and 6'sialyllactose as a synbiotic treatment may allow a more effective and selective establishment of the species in the infant colon.

It was previously presumed that the complexity of milk oligosaccharides could only be produced in nature. However, several methods to produce or enrich milk oligosaccharides have recently been developed, including large-scale chemical synthesis (Glycom, Elicityl, Carbosynth), the microbial biosynthesis of 2'fucosyllactose and 6'sialyllactose (Drouillard, S., Mine, T., et al. 2010), and the isolation of bioactive oligosaccharides from dairy whey streams (Barile, D. and Rastall, R.A. 2013). The findings in **Chapter III** demonstrate the safety, tolerability, and influence of a bovine milk oligosaccharide (BMO)-enriched powder and 6'sialyllactose on the gut microbiome in mice. To the best of our knowledge, this is the first study to assess the *in vivo* effects of either a dairy-derived oligosaccharide powder or 6'sialyllactose in regards to safety of administration and their ability to influence the gut microbiota when compared with a commercial prebiotic. While studies of this nature using commercial prebiotics typically focus on markers of immune and probiotic activity (Martin, F.-P.J., Wang, Y., et al. 2008, Vos, A., Van Esch, B., et al. 2007), the most significant observation in this study was the ability of both, the BMO powder and 6'sialyllactose to significantly reduce or eliminate proportions of bacterial families which are often associated with pathogens. Previous studies of commercial prebiotic supplementation associate reductions in pathogen numbers (clostridia and *E. coli*) with substrate fermentation and the production of carboxylic acids (Martin, F.-P.J., Wang, Y., et al. 2008). Although faecal pH was not determined in our study, there was very little effect on SCFAs (butyrate

and acetate) following oligosaccharide supplementation which indicates that the reduction in pathogen-associated bacterial families was likely not linked to acidification of the caecal and intestinal environment. The findings provide evidence to support the inclusion of milk oligosaccharides in infant formula and food additives, either alone or in combination with commercial prebiotics, to selectively modulate the gut microbiome. The fact that bifidobacteria were not detected in the faecal samples restricted our ability to assay the influence of BMO and 6'sialyllactose on this group of bacteria. Indeed, it has previously been shown that bifidobacteria only represent a minor constituent in the mouse GI tract (Weiss, G.A. and Hennet, T. 2012). As such, future studies using a synbiotic approach, by administering probiotic bacteria in combination with milk oligosaccharides, could shed light on the ability of HMO to influence the adhesion and colonisation of probiotic bacteria. Conversely, the use of mice inoculated with human microbiota and / or varying concentrations of supplemented oligosaccharides might demonstrate the ability of HMOs to influence the pre-established gut microbiota. Bacterial sampling from organ homogenates could give a more accurate portrayal of the firmly-adherent resident bacteria in each niche, as faecal sampling is a general representation of the entire GI tract. Indeed, with the emerging ability to produce milk oligosaccharides on a larger scale, a transition to pig trials may be more representative of the human intestinal landscape and community.

The outer membranes of Gram-positive and Gram-negative bacteria contain glycan epitopes (LPS, EPS, lipoteichoic acid, proteoglycans) capable of interacting with several lectins from various sources in the GI tract, including immune cells such as macrophages and neutrophils (Carlin, A.F., Uchiyama, S., et al. 2009, Comstock, L.E. and Kasper, D.L. 2006). Therefore, the ability to influence the resident microbiota through the ingestion of selective prebiotics, such as milk oligosaccharides, may present a feasible means to modulate the activity of specific GI immune cells and overall inflammatory tone. There is an accumulating body of reseach that confirms the ability of milk oligosaccharides to act as selective prebiotic substrates for probiotic bacteria, with a particular focus upon *B. longum* subsp. *infantis* (LoCascio, R.G., Desai, P., et al. 2010, Sela, D., Chapman, J., et al. 2008). Recent studies have demonstrated the beneficial effects of supplementation with this particular strain, including its

ability to significantly reduce the intestinal inflammatory status. In a human colonic microbiota model, *B. longum* subsp. *infantis* ATCC 15697 demonstrated the capacity to reduce colonic LPS concentrations, decrease the production of TNF-α, and increase IL-4 anti-inflammatory cytokines (Rodes, L., Khan, A., et al. 2013). As milk oligosaccharides can influence the colonisation of *B. longum* subsp. *infantis* ATCC 15697, this provides a means by which HMO could be implemented to modulate intestinal inflammation. In **chapter IV**, a panel of commensal intestinal isolates were screened for their ability to interact with the β-galactoside binding glycoprotein, galectin-3. Previously, only the interaction between galectin-3 and pathogens had been examined (Li, Y., Komai-Koma, M., et al. 2008, Mey, A., Leffler, H., et al. 1996). Our findings were the first to demonstrate an interaction between commensal bacteria and galectin-3 (Kavanaugh, D., Kane, M., et al. 2013). Interestingly, the two strains identified were *B. longum* subsp. *infantis* ATCC 15702 and ATCC 15697, which are known to consume HMOs (LoCascio, R.G., Desai, P., et al. 2010, Sela, D.A., Chapman, J., et al. 2008). As such, this may allow HMOs the possibility to modulate the function of intestinal galectin-3, both, directly and indirectly. Indirectly, HMOs influence the growth and colonisation of bifidobacteria, which can express lipoteichoic acid and a proteoglycan similar to LPS, while also reducing the presence of Gram-negative bacteria (Fermino, M.L., Polli, C.D., et al. 2011, Rodes, L., Khan, A., et al. 2013, Sekine, K., Toida, T., et al. 1985). There also exists the possibility that HMO may directly modulate the endothelial immune-related effects of galectin-3 (Chen, C., Duckworth, C.A., et al. 2013). Following feeding, HMO have been found in urine and are believed to enter systemic circulation, potentially reaching concentrations between 100-200 mg/L (Bode, L. 2006), allowing for the possibility to modulate the activity of galectin-3 in areas other than the intestinal lumen. Future studies would benefit from screening a wider range of commensal bacteria and the isolation of the bacterial component(s) responsible for the strong interaction with galectin-3. Additionally, competition assays between the two identified strains of bifidobacteria and those pathogens which are known to interact with galectin-3 to aid in their colonisation or survival would investigate the possibility that binding

of probiotic bacteria to galectin-3 may prevent pathogen colonisation through competitive exclusion.

For over one hundred years it has been known that breast feeding is the gold standard for infant nutrition and is associated with increased numbers of bifidobacteria in the infant intestine. Accordingly, the beneficial health outcomes from breast feeding have partially been attributed to bifidobacteria. The body of research presented here demonstrates the ability of milk oligosaccharides to influence the host-commensal interaction, with a particular emphasis upon bifidobacteria. As technology advances, the capability to isolate, or even synthesize *de novo*, complex milk oligosaccharides may enable the full deconstruction of the complete repertoire of HMO structures. Although oligosaccharides are currently broadly categorized as either neutral or acidic, further in-depth analysis and studies, should make it possible to eventually assign a specific function to each molecule, leading to a comprehensive understanding of the role of milk oligosaccharides in the modulation of the intestinal microbiota.

Specific oligosaccharide structures or combinations act as anti-infectives against specific pathogens or potentially aberrant commensals, such as those targeting sialic acid, therefore indicating the potential for targeted treatment. In tandem, the ability to rapidly and cheaply acquire personal metagenomic data on the gut microbiome through inexpensive sequencing is fast approaching. Eventually, based on bacterial sequencing and quantification data, it may be possible to identify bacterial overgrowths or shortages and prescribe a tailored regimen of oligosaccharides to treat intestinal dysbiosis and maintain intestinal homeostasis. The use of specific oligosaccharides or particular combinations of oligosaccharides may become commonplace as a prophylactic treatment for consumers who are most susceptible to infection, potentially leading to a reduction in the administration of antibiotics. The advantage of oligosaccharides is two-fold; 1) oligosaccharides act as decoys rather than lethal agents, making the development of resistance unlikely, and 2) oligosaccharides act as a nutritional resource for commensal bacteria, stimulating commensal proliferation rather than their elimination along with the pathogenic target, unlike treatment with antibiotics that has severe effects on the commensal bacteria as well as the pathogens. Within the last thirty years, the mystery surrounding the functionality

of milk oligosaccharides and their associated health benefits has dissipated and the role of individual oligosaccharides is better understood. Although the age of personalized-treatment remains just beyond the horizon, the continually growing body of research surrounding milk oligosaccharides and their influence on the host-microbiota relationship further supports their development and inclusion in infant formula, synbiotic treatments, and functional foods.

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