Mining Milk for Factors which Modulate Host-Microbial Interactions *In Vitro*

A Thesis Presented to National University of Ireland, Galway for the Degree of Doctor of Philosophy (Ph.D.) in the National Centre for Biomedical Engineering Science (NCBES)

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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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Thesis Abstract

Human milk oligosaccharides (HMO) have been reported to increase the adhesion of *Bifidobacterium longum* subsp. *infantis* ATCC 15697 (*B. infantis* ATCC 15697) to human colonic adenocarcinoma, HT-29 cells. In this thesis various commercial ingredients were selected as alternatives to HMO, including lactose (Chapter VI), and fractions isolated from bovine, caprine (listed in Table 1, Chapter II), and camel milk (Chapter V). *B. infantis* ATCC 15697 was chosen for screening assays based on previous *in vitro* adhesion studies and its evolutionary adaptation to the consumption of milk glycans.

As adhesion assays are slow-through-put, miniaturised high-throughput assay requiring substantially lower sample quantities were developed for screening purposes. Miniaturised and conventional adhesion assays were shown to be comparable. Increased *B. infantis* ATCC 15697 adhesion was observed in the presence of an immunoglobulin enriched powder (IGEP) and Goat Milk Oligosaccharides (GMO). These components were then studied in detail in Chapter III and Chapter IV respectively. In Chapter V, a camel milk fraction (CMF) was shown to increase the adhesion of three of the seven *Bifidobacterium* strains. In Chapter VI, lactose was found to increase the adhesion *B. breve* UCC2003 to HT-29 cells.

Campylobacter jejuni, a common bacterial cause of human gastroenteritis, was used to investigate if increased bifidobacterial adhesion to HT-29 cells could provide protection against pathogenic colonisation. GMO, IGEP, CMF and Lactose treated some *Bifidobacterium* strains decreased the adherence of *C. jejuni* to HT-29 cells by varying amounts as discussed in detail in Chapters, III, IV, V and VI.

Overall, these studies build a strong case for the use of milk components from a variety of domestic animal milks to modulate of intestinal bifidobacteria, with oligosaccharides showing multiple bio-functionalities, while lactose, CMF and IGEP offer more potential in terms of commercial viability. Future research should aim to elucidate the intricacies of how these milk components interact with bifidobacteria, thereby identifying novel therapeutic targets and a means by which to maintain or restore host gut health.

Publications

Chapter I. Quinn E. M.; Hickey R. M.; Lokesh J. Symposium Review: Dairy-Derived Oligosaccharides-Their Influence on Host-Microbe Interactions in the Gastrointestinal Tract of Infants. *J. Dairy Sci*. **2020**, *103* (4), 3816-3827. doi: 10.3168/jds.2019-17645.

Chapter II. Quinn, E. M.; Slattery, H.; Thompson, A.; Kilcoyne, M.; Joshi, L.; Hickey, R. M. Mining Milk for Factors Which Increase the Adherence of *Bifidobacterium longum* subsp. *infantis* to Intestinal Cells. *Foods.* **2018**, 7 (12), 196. https://doi.org/10.3390/FOODS7120196.

Chapter III. Quinn, E. M.; Slattery, H.; Walsh, D.; Joshi, L.; Hickey, R. M. *Bifidobacterium longum* subsp. *infantis* ATCC 15697 and Goat Milk Oligosaccharides Show Synergism *In Vitro* as Anti-Infectives against *Campylobacter jejuni*. *Foods* **2020**, 9 (3), 348. doi: 10.3390/foods9030348

Chapter IV. Quinn, E. M.; Slattery, H.; Walsh, D.; Joshi, L.; Hickey, R. M. A Whey Fraction Rich in Immunoglobulin G combined with *Bifidobacterium longum* subsp. infantis ATCC 15697 exhibits synergistic effects against *Campylobacter jejuni. International Journal of Molecular Sciences.* **2020**, *In press.*

Abbreviations

Asn – Asparagine

BF - Buttermilk Fraction

BMO - Bovine Milk Oligosaccharides

CF MRS media - Carbohydrate Free-DeMan Rogosa Sharpe media

CFU - Colony-Forming Units

CMF - Camel Milk Fraction

CMO – Caprine Milk Oligosaccharide

CMP – Caseinomacropeptide

DSL - Disialyl-Lactose

DSLNT - Disialyllactose-*N*-Tetraose

EPS – Exopolysaccharide

FBS – Fetal Bovine Serum

FOS – Fructooligosaccharide

Fuc – Fucose

FUT2 - α-1,2-Fucosyltransferase

Gal – Galactose

Gal-3 – Galectin-3

Gal-3C – Carbohydrate-Binding C-Terminus of Galectin-3

GalNAc – *N*-Acetyl-*D*-Glactosamine

GH25 - Glycoside Hydrolase (Family 25)

GI tract – Gastrointestinal Tract

GMO - Goat Milk Oligosaccharides

GMP – Glycomacropeptide

GMP-P - Sodium Metaperiodate Treated GMP

GOS – Galactooligosaccharides

HMO - Human Milk Oligosaccharides

HPAEC-PAD - High-Performance Anion Exchange Chromatography with Pulsed Amperometric Detection

HPLC – High-Performance Liquid Chromatography

Ig – Immunoglobulin

IgA – Immunoglobulin A

IGEP - Immunoglobulin G Enriched Powder

IgG - Immunoglobulin G

LAC – Lactose

LacNAc – *N*-Acetyllactosamine

LF – Lactoferrin

LNB – Lacto-*N*-Biose

LNDFH-I - Lacto-*N*-Difucohexaose I

LNFPI - Lacto-*N*-Fucopentaose I

LNFPII - Lacto-*N*-Fucopentaose II

LNFPIII - Lacto-*N*-Fucopentaose III

LNH - Lacto-*N*-Hexaose

LNnH - Lacto-*N*-Neohexaose

LNnT - Lacto-*N*-Neotetraose

LNT - Lacto-*N*-tetraose

LNT – Lacto-*N*-Tetraose

LPS – Lipopolysaccharide

LSTa - Sialyllacto-*N*-Tetraose (a)

LSTb - Sialyllacto-*N*-Tetraose (b)

LSTc - Sialyllacto-*N*-Tetraose (c)

Lys – Lysine

Man – Mannose

MFGM - Milk Fat Globule Membrane

MFGM-10 - Lacprodan® MFGM-10

MH – Mueller Hinton

MRS – DeMan Rogosa Sharpe media

MUC - Mucin

MWCO – Molecular Weight Cut-Off

NaCl - Sodium Chloride

NaIO4 - Sodium Metaperiodate

NAL - 6′-*N*-Acetyl-Glucosaminyl-Lactose

Neu5Ac – *N*-Acetyl Neuraminic Acid

Neu5GC - *N*-Glycolylneuraminic Acid

P95 - Fucutooligosacchride

PBS- Phosphate Buffered Saline

PL-20 -Lacprodan® PL-20

RP-HPLC - Reverse Phase HPLC

RT-qPCR – Real-Time Quantitative Polymerase Chain Reaction

SCFA – Short-Chain Fatty Acid

SCS – Spent Culture Supernatant

SDS-PAGE - Sodium Dodecyl Sulfate Polyacrylamide Gel

α-3'-GalNAcL - *α*-3'-*N*-Acetylgalactosaminyllactose

*α*3'-GL - *α*-3'-Galactosyllactose

*β*3'-GL - *β*-3'-Galactosyllactose

β-C - *β*-Casein

β-L - *β*-Lactoglobulin

κ-cn - Kappa Casein

2'-FL - 2'-Fucosyllactose

3-FL - 3-Fucosyllactose

3'-SL - 3′-Sialyllactose

4'-GL - *β*-4'-Galactosyllactose

6'-GL - *β*-6'-Galactosyllactose

6'-SLN - 6′-Sialyl-Lactosamine

6'-SL - 6′-Sialyllactose

Acknowledgements

First and foremost, I am incredibly grateful to my research supervisor in Teagasc, Dr Rita Hickey for her constant support and encouragement over the past few years, you have been a wonderful mentor and role model. Alongside Dr Rita Hickey, I would also like to thank Prof. Lokesh Joshi, for their invaluable knowledge, expert opinions and guidance. I would also like to give a special mention to Dr Jared Gerlach and Michelle Kilcoyne in NUIG, for the advice and support throughout my PhD, especially in the difficult times. Thank you for believing in me.

I would like to acknowledge the opportunity presented by Teagasc to pursue post-graduate research through the Walsh Fellowship programme. I am grateful to the Teagasc Food Research Centre for the provision of resources and a state-of-the-art research facility.

I would like to thank all the staff and students at Teagasc Moorepark. A special thank you to all the glyco-team, Sinead, Aoife, Sarah, Mariarosaria and Helen and the members of the team I have worked with throughout the years. A massive thank you to the friends and amazing researchers I met along the way; Fodhla, Aoife, Alan, Roberta and Drinalda, my journey would not have been the same without you all. A special thank you to Joshua Whorriskey for all the encouragement and support, and for being the only non-scientist I know to have read all my publications.

I have to give a huge thank you to my family. Thanks to my mother Margaret, for listening to my PhD woes throughout the years and Liam for all the dinners. Thank you to my sister Hazel Quinn, for all your help and support, my brothers David and Michael. Lastly, thank you to my wonderful grandparents Bridie and Michael Conway, for all that you have done for me. I dedicate this thesis to all of you.

'If you love life, don't waste time, for time is what life is made up of'

Bruce Lee

Thesis Introduction

Breastmilk components and Bifidobacteria

Breastmilk protects the developing neonate against infectious diseases while also meeting the nutritional requirements for neonatal growth and selectively enriching a beneficial gut microbiota. Breastmilk is considered the gold standard for infant health and is associated with lower incidences of diarrheal diseases and infant mortality [1, 2]. Many of the bioactivities associated with human breastmilk have been attributed to both free glycans, also known as oligosaccharides and the many glycoconjugates present [3, 4]. Oligosaccharides are carbohydrates composed of between three and ten monosaccharide residues, covalently linked through glycosidic bonds [5]. Oligosaccharides can be free or occur as structural motifs attached to proteins (glycoproteins) and lipids (glycolipids) [6]. Human milk oligosaccharides (HMO) are resistant to enzymatic digestion and arrive in the distal intestine relatively unaltered, where they have numerous beneficial effects [7, 8, 9]. These glycosylated compounds have been linked to anti-infective, prebiotic and anti-inflammatory capabilities, along with enhancing cognitive function [10, 11, 12, 13, 14, 15]. Structural similarities between HMO and the glycan moieties present on human intestinal epithelial surfaces exist [16]. For this reason, HMO can aid in preventing pathogen colonisation, as the pathogens bind to HMO which act as decoy receptors instead of to the host, ultimately leading to lower levels of infection [16, 17]. Additionally, many benefits associated with HMO are linked to the high levels of beneficial bacteria such as bifidobacteria which are characteristically present in the GI tract of the breast-fed infant with their growth being induced by glycan rich milk [18]. High concentrations of free and conjugated oligosaccharides in breastmilk correlate with the predominance of *Bifidobacterium* species in the infant gut [4, 19, 20]. Indeed, many bifidobacteria have specifically adapted to utilise milk glycans. *Bifidobacterium longum* subsp. *infantis* ATCC 15697 (*B. infantis* ATCC 15697) is known as a ‘champion coloniser’ because of its effectiveness in using milk glycans as a carbon source for growth [21]. Moreover, whole-genome sequencing has revealed that this strain possesses a 43-kb gene cluster dedicated to the binding, import and metabolism of milk glycans [22]. *B. infantis* ATCC 15697 contains genes which encode two *α*-sialidases, five *α*-fucosidases, several *β*-galactosidases, and three *β*-*N*-acetylglucosaminidases [23] and many of these are up-regulated during growth on HMO [24]. Notably, putative HMO-utilization genetic loci are conserved across *B. longum* subsp. *infantis* strains, in contrast to *B. longum* subsp. *longum* strains, which varies in its ability to consume HMO [25].

Pathogen protection

Enrichment of bifidobacterial strains in the gastrointestinal tract is associated with a variety of secondary beneficial effects. For example, the stools of breastfed infants tend to be rich in lactic and acetic acid which results in a more acidic faecal pH (<6.0), while bottle-fed infant stools are slightly alkaline (>7.0) [26]. The low pH and the production of short-chain fatty acids by bacteria such as bifidobacteria have been demonstrated to restrict the growth of pathogens, further promoting infant health [27]. Added to this, probiotic and pathogenic strains can share surface adhesins and as a result, compete for host receptor sites [28, 29]. Some probiotic strains are known to inhibit pathogens through competitive exclusion [30, 31]. Probiotic-mediated competition, exclusion and displacement of *Listeria monocytogenes* biofilm formation has been shown [32]. A combination of *L. casei* culture and maltodextrins has been shown to reduce adherence of *E. coli* O8:K88 in the gut of gnotobiotic pigs and conventional pigs [33]. Similarly, *L. plantarum* containing maltodextrin and/or fructooligosaccharides (FOS) has also been shown to reduce levels of *E. coli* O8:K88 in piglets [34]. Indeed, probiotic products currently on the market containing *Lactobacillus acidophilus*, *Saccharomyces cerevisiae*, *Enterococcus faecium*, *L. acidophilus* claim to reduce pathogen (e.g. *E. coli*, *Salmonella* and clostridia) colonisation in livestock, through competitive exclusion [30]. *Lactobacillus* spp., i.e., *acidophilus*, *casei*, *crispatus*, *gasseri*, *helveticus*, *pentosus*, *plantarum*, *rhamnosus* and *salivarius* have been shown to exclude *Campylobacter in vitro* and *in vivo* [35]. Thus, by increasing the colonisation of beneficial strains, it may be possible to competitively exclude a range of pathogens.

In order for the beneficial effects of bifidobacteria to be achieved, transient colonisation of such strains in sufficient numbers must occur. It appears such beneficial strains have evolved to interact and attach to the host's epithelial cell surface, facilitating transient colonisation of bifidobacteria in the gut. Solute binding proteins synthesised by *B. infantis* ATCC 15697 are known to have a binding affinity for mammalian carbohydrate structures such as Lewis antigens, polylactosamines and globotriose (Gb3) in addition to structures present in colonic mucins [36]. Additionally, *B. infantis* ATCC 15697 and *Bifidobacterium longum* subsp. *infantis* (Reuter) ATCC 15702 (*B. infantis* ATCC 15702) have been shown to interact with Galectin-3, a member of the beta-galactoside-binding protein family that plays an important role in cell-cell adhesion [37]. Consequently, there is much interest in developing methods to enhance such interactions in an effort to increase levels of transient attachment of these beneficial bacteria in the gut.

Recent research has shown that free and conjugated milk glycans may facilitate increased adhesion of *B. infantis* strains to intestinal cells. Increased expression of putative type II glycoprotein binding fimbriae in the presence of HMO has been shown, and in part, facilitates attachment and colonisation of the bacteria to mammalian cells [38]. A 9.8-fold increase in adhesion of *B. infantis* ATCC 15697 to HT-29 cells has been observed following exposure of the bacteria to 3' and 6'-sialyllactose [39]. To date, HMO have been associated with this ability to increase colonisation of bifidobacteria. However, HMO have not been available in the large amounts required for clinical trials until recently. Also, the commercial structures available such as 2'-fucosyllactose (2'-FL) and Lacto-*N*-tetraose (LNT) [40,41] may not fully mimic the beneficial effects observed with the entire pool of HMO which is known to consist of over 200 structures [42]. Domestic animal milk is also known to contain oligosaccharides which share some structural similarities with HMO (A list of domestic animal milk oligosaccharides and concentrations common in bovine, goat and human milk is reviewed in Chapter 1 Table 1). Furthermore, like human milk, animal milk contains a variety of other glycosylated components (Thesis introduction Figure 1). Until now, these milks have not been investigated for their ability to increase the overall colonisation potential of bifidobacteria in the gut.

The work described in this thesis identifies potential glycosylated fractions of domestic animal milks that may promote colonisation of bifidobacteria (Figure 1). The benefits of improving the colonisation potential of such strains can also have secondary effects such as protecting the host against pathogens e.g. a highly invasive strain of *Campylobacter jejuni (C. jejuni*). Other characteristics of these milk-derived components are also explored such as anti-infective and prebiotic capabilities. This work highlights the potential benefits of using animal-derived bioactivities as an alternative to human milk-derived components and indicates that there may be commercial applications in using such components as potential functional ingredients to promote health and protect the gut against pathogenic disease.

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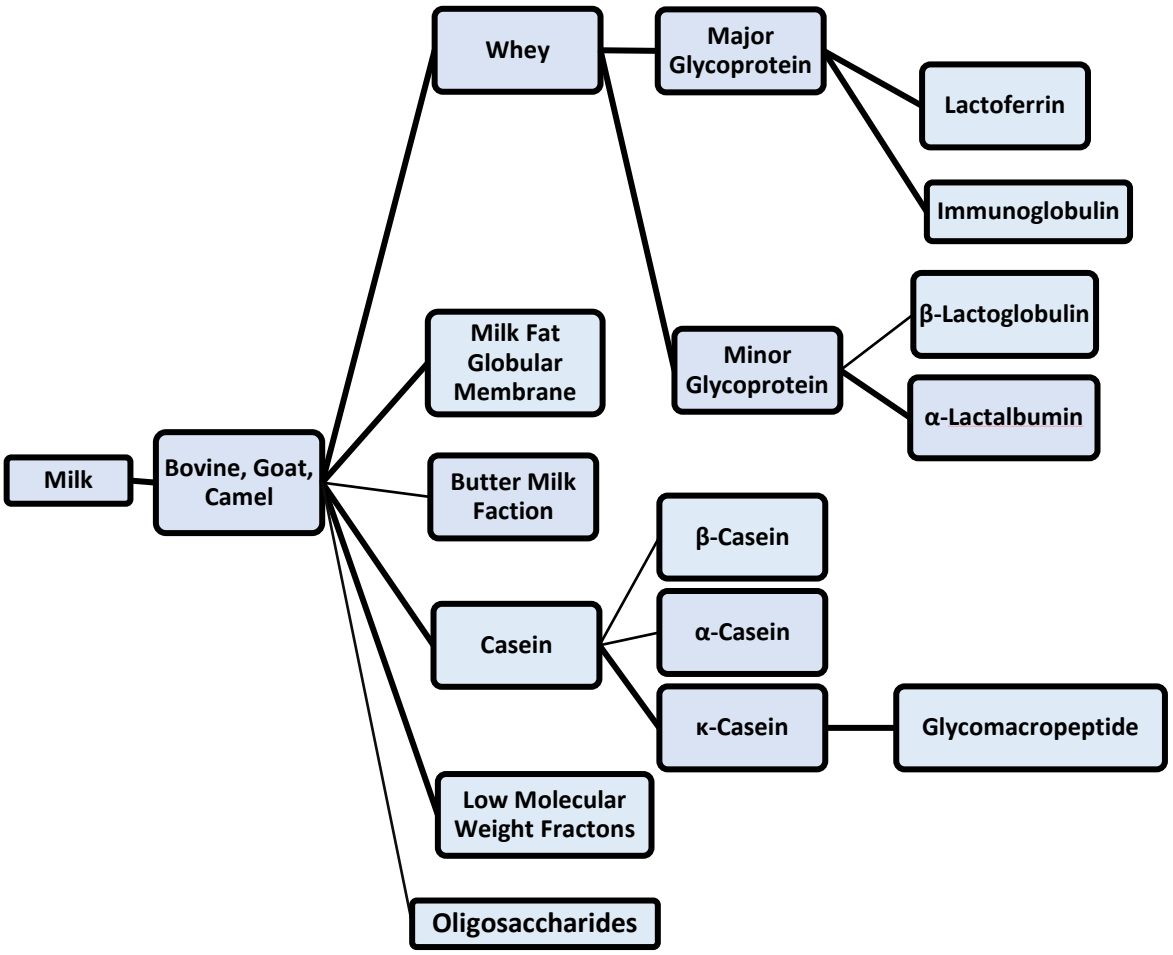

Figure 1. Overview of fractions present in domestic animal milks that may offer different biological activities.

Chapter I

Literature Review

Dairy-Derived Oligosaccharides: Their Influence on Host-Microbe Interactions in the Gastrointestinal Tract of Infants

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Published in The Journal of Dairy Science (2020), *103* (4), *3816-3827*.

Abstract

Oligosaccharides are the third most abundant component in human milk. It is widely accepted that they play several important protective, physiological and biological roles including selective growth stimulation for beneficial gut microbiota, inhibition of pathogen adhesion and immune-modulation. However, until recently, there were very few commercial products on the market that capitalised on these functions. This is mainly because the quantities of human milk oligosaccharides required for clinical trials have been unavailable. Recently, clinical studies have tested the potential beneficial effects of feeding infants formula containing 2'-fucosylactose which is the most abundant HMO in human milk. These studies have opened this field for further well-designed studies which are required to fully understand the role of HMO. However, one of the most striking features of human milk is its diversity of oligosaccharides with over 200 identified to date. It may be that a mixture of oligosaccharides is even more beneficial to the infant than a single structure. For this reason, the milk of domestic animals has become a focal point in recent years as an alternative source of complex oligosaccharides with associated biological activity. This review will focus specifically on free oligosaccharides found in bovine and caprine milk and the biological roles associated with such structures. These dairy streams are ideal sources of oligosaccharides, given their wide availability and use in so many regularly consumed dairy products. The aim of this review is to provide an overview of the state of the art in research regarding the functional role of bovine and caprine milk oligosaccharides in host-microbial interactions in the gut and provide current knowledge on the isolation of oligosaccharides as ingredients for incorporation in functional or medical foods.

1. Introduction

In recent times, there has been a growing awareness that breast milk beneficially supports the infant immune system [1] and modulates microbiota composition during the first months of life. Among the various bioactive components in breast milk, human milk oligosaccharides (HMO) are suggested to play a leading role [2, 3]. HMO are made of linear or branched monosaccharides, such as galactose, glucose, *N*-acetylglucosamine, fucose, and sialic acid varying in size from 3 to 22 monosaccharide units [4, 5]. Contrary to other mammals, human breast milk contains a very high amount and a structurally diverse set of oligosaccharides that even exceeds the protein content of breast milk [6, 5, 7]. Indeed, HMO constitutes the third-largest solid component of human milk after lactose and lipids [8, 9]. Table 1 outlines the overall concentrations of oligosaccharides found in human and dairy animal milks and highlights the major oligosaccharide structures common between these milks.

Surprisingly, HMO offer no direct nutritional value to the infant, and there is only minor absorption across the intestinal wall with approximately 0.1% and 4% detectable in plasma and urine respectively [10, 11, 12]. HMO are now recognized to have various additional benefits for the developing infant. They are the favoured substrates for several species of gut bacteria and act as prebiotics, promoting the growth of a beneficial microflora and shaping the gut microbiome, in turn beneficially influencing immune responses [13]. Fermentation products such as short-chain fatty acids (SCFAs) generated from gut bacteria breaking down HMO, are immuno-regulating and serve as nutrients for beneficial gut commensals and epithelial cells [14]. HMO also directly modulate host-epithelial responses, resulting in reduced binding of pathogens to the gut epithelium. In addition, HMO act as decoy receptors, inhibiting the binding of enteric pathogens to prevent infection and subsequent illness [15]. Furthermore, HMO provide a selective advantage for colonization by favourable bacteria, in turn inhibiting the colonization of pathogenic species. HMO and their metabolic products, such as sialic acid, also play a role in brain development, neural transmission, and synaptogenesis [16, 17].

However, the protective effects ascribed to HMO are in the most part, unavailable to formula-fed infants with the exception of 2'-fucosylactose and lacto-*N*-neotetraose which have been recently added to some formulas [18, 19]. Despite this advancement, the complexity of HMO makes it almost impossible for their associated functions to be duplicated in formulas. Infant milk formulas are based on bovine milk and to a lesser extent caprine milk, which contain lower concentrations of oligosaccharides (~0.03 and 0.30 g/L, respectively; [8, 20]. At least 10 bovine milk oligosaccharides (BMO) and 9 caprine milk oligosaccharides (CMO) do

however, share the same structure as certain HMO (outlined in Table 1), which could imply common functionalities [21, 22, 23, 24]. Therefore, value may lie in extracting and concentrating oligosaccharides from domestic animal milks with a view to their addition as an active ingredient to infant formulas. This review highlights recent studies from our group and others that demonstrate novel bioactivities associated with BMO and CMO, with a particular focus on their influence on host-microbial interactions in the gut. The review also provides an overview of the current developments in industrial-scale processes for milk oligosaccharide production.

2. Milk Oligosaccharide Structure

Milk oligosaccharides have a lactose core and are enzymatically elongated by β1-3 and β1-6 linkages to units of lacto-*N*-biose and *N*-acetyllactosamine, respectively. Milk oligosaccharides are further elongated by the addition of fucose and sialic acid at the terminal positions [25]. A large number (from 100 to 200) of different HMO can be found in individual milk samples [26, 27]. Concentrations in mature human milk ranges from 12 to 15 g/L, but relative proportions and amounts vary depending on the lactating stage, Lewis blood group, secretor status, feed practices and environmental factors [5, 28, 29, 30]. Among these elements, the individual maternal genetic disposition has a large impact on the HMO profile of human milk. In particular, the individual expression pattern of Lewis and secretor gene alleles which encode different fucosyl-transferases.

The significant difference between human and animal milk oligosaccharide pools is that human milk contains high levels of fucosylated oligosaccharides, which account for approximately 70% of oligosaccharides in human milk, with high levels of 2'-fucosyllactose being detected 4.65g/L [31, 32, 33]. Another difference between the milks is the predominance of type 1 oligosaccharides in human milk [34, 35, 36]. In contrast, animal milk counterparts contain higher levels of sialylated oligosaccharides containing *N*-acetylneuraminic acid or *N*-glycolylneuraminic acid [37, 35]. Added to this, only approximately 40 BMO and CMO structures have been identified to date [38, 39]. Despite these differences however, structurally identical oligosaccharides are found in human and animal milk oligosaccharide pools (Table 1). Indeed, many of these individual structures have been identified as having specific biological activities, so it is likely these structures may offer the same physiological benefits as oligosaccharides derived from human milk [40].

3. The Role of Milk Oligosaccharides in Host-Microbe Interactions

The diverse functions of milk oligosaccharides on the gut microbiome are as follows: (i) they act as prebiotics and stimulate the growth of beneficial microbes, (ii) they promote the colonization of beneficial microbes (iii) they exert direct and indirect defence mechanisms against pathogens and protect infants from infections and (iv) they act as signalling molecules and interact directly with the host cells (Figure 1). For insights into how milk oligosaccharides impact immune system development which is outside the scope of this review, we refer the reader to some excellent recent reviews [41, 7].

3.1 Role of Milk Oligosaccharides Prebiotics

The human gut lacks glycoside hydrolases and intestinal membrane transporters which can degrade milk oligosaccharides and therefore HMO are not digested in the upper part of the gastrointestinal tract of infants [42, 4]. As a result, the majority of HMO reach the colon, where they act as a substrate for specific bacteria, influencing the composition of the gastrointestinal microbiota [43]. HMO are specifically known to influence populations of beneficial bacteria, such as *Bifidobacterium* [43, 44], a dominant species in the intestine of breast-fed infants. These bacteria have the ability to utilize HMO with dedicated enzymes (glycoside hydrolases), transporters and other molecules which contribute to degradation [45]. Genomic analysis of a prototypical infant derived bifidobacteria, *Bifidobacterium longum* subsp. *infantis* (*B. infantis*) which grows well on HMO, revealed a single cluster of genes coding for enzymes dedicated to the degradation of HMO suggesting co-evolution of this strain with human milk [46, 47]. Analysis of other infant strains of *B. longum* subsp. *longum*, *B. bifidum* and *B. breve* which also grow on HMO [48, 49, 50] were shown to also possess specific milk glycan transporters and glycosyl hydrolases linked to milk glycan usage [51, 50, 52, 53, 54]. The role of HMO as prebiotics is well characterized and we refer the reader to many excellent reviews on the topic [55, 56, 57, 29].

The use of bovine milk oligosaccharides (BMO) as prebiotics is less well investigated with only a limited number of *in vitro* studies documented. A recent example is a study by Jakobsen et al. [58] where proton nuclear magnetic resonance (1H NMR) metabolomics and molecular biology methods were combined for quantification of bacteria to compare the effect of BMO and synthetic galacto-oligosaccharides on mono- and co-cultures of 8 major bacteria related to a healthy infant microbiome. The results revealed that BMO treatments supported the growth of *Bifidobacterium longum* subsp. *longum* and *Parabacteroides distasonis*, while at the same time inhibiting the growth of *Clostridium perfringens* and *Escherichia coli*. Perdijk

et al. [59] investigated the effect of sialylactose (isolated from bovine milk) on microbiota composition and SCFA production using *in vitro* fecal batch cultures. Sialylactose resulted in a distinct modulation of microbiota composition, promoting the outgrowth of *Bacteroides* and bifidobacteria which resulted in distinct changes in SCFA production profiles.

Caprine milk oligosaccharides have also been shown to have prebiotic characteristics *in vitro* [60]. Increased numbers of *bifidobacteria* spp. in the presence of CMO have been demonstrated using *in-vitro* fermentation models [61, 62]. A recent study by Leong et al. [63] examined the presence of naturally occurring oligosaccharides in commercial caprine milk-based stage 1 and stage 2 infant formulas and their prebiotic properties. Fourteen quantifiable oligosaccharides in caprine milk-based infant formula were detectable by liquid chromatography-mass spectrometry (LC/MS). These oligosaccharides were shown to significantly enhance the growth of bifidobacteria and lactobacilli *in vitro*.

The ability of BMO to modulate the gut microbiota *in vivo* has been the subject of a number of recent studies. Meli et al. [64] revealed positive trends in stool bacterial counts in infants fed BMO-supplemented formulae. The BMO in this case were generated from whey permeate. In a controlled, randomized double-blinded clinical trial, Simeoni et al. [65] tested the effect of feeding a formula supplemented with a mixture of BMO generated from whey permeate, containing galactooligosaccharides (GOS) and 3'- and 6'-sialyllactose, and the probiotic *Bifidobacterium animalis* subsp. *lactis* (*B. lactis*) strain CNCM I-3446. The same formula without addition of the prebiotic–probiotic blend was used in the control group. Breastfed infants served as the reference group. The test formula with the pre- and probiotic was well tolerated, and a strong bifidogenic effect was observed. The control, but not the test group, differed from the breastfed reference group by a higher faecal pH and a significantly higher diversity of the faecal microbiota. In the test group, the probiotic *B. lactis* increased by 100-fold in the stool and was detected in all supplemented infants. BMO stimulated a marked shift to a *Bifidobacterium*-dominated faecal microbiota via increases in endogenous bifidobacteria e.g. *B. longum, B. breve, B. bifidum, B. pseudocatenulatum* [65].

In another study, germ-free mice and newborn piglets were colonized with a consortium of cultured bacterial strains isolated from the fecal microbiota of a severely stunted Malawian infant and fed a representative Malawian diet with or without the addition of sialylated bovine milk oligosaccharides [66]. The results demonstrate that this BMO preparation produced a microbiota dependent promotion of growth and metabolic changes indicative of improved nutrient utilization in both host species. A study by Boudry et al. [67] investigated if a

combination of dietary BMO and *B. infantis* can reverse the gut microbial dysbiosis and altered gut permeability induced by ingestion of the Western diet. Male C57BL/6 mice were fed a Western diet (40% fat/kcal) or normal chow (14% fat/kcal) for 7 weeks. During the final 2 weeks of the study, the diet of a subgroup of Western diet-fed mice was supplemented with BMO (7% wt/wt). Supplementation with BMO normalized the cecal and colonic microbiota and the authors observed an increased abundance of *Lactobacillus* compared with both the Western Diet alone and control (chow-fed) mice. In addition, restoration of *Allobaculum* and *Ruminococcus* levels in the BMO group was observed which was comparable to that of the control group. A similar study by Hamilton et al. [68] investigated C57BL/6 mice that were fed a control diet, high fat (40% fat/kcal), or high fat and 6%/kg BMO for 1, 3, or 6 weeks. Gut microbiota and intestinal permeability were assessed in the ileum, cecum, and colon. BMO were found to completely abolish the high fat diet-induced increase in paracellular and transcellular permeability in the small and large intestine and increased abundance of *Bifidobacterium* and *Lactobacillus* in the ileum.

Obelitz-Ryom et al. [69] using preterm piglets, investigated effects of bovine milk supplements enriched with oligosaccharides to improve gut development and colonization. Supplements with BMO were well-tolerated, but did not improve gut maturation or clinical outcomes in artificially reared preterm piglets. The authors concluded that immaturity at birth, coupled with artificial rearing, may render the neonate unresponsive to the gut-protective effects of milk oligosaccharides. A study by Jena et al. [70], examined the effects of BMO and *B. infantis* in preventing non-alcoholic steatohepatitis (NASH) in Western diet-fed bile acid receptor FXR (farnesoid × receptor) knockout mice. The authors found that BMO increased the abundance of butyrate-generating bacteria which has a beneficial effect in NASH treatment. In a recent study, Cowardin et al. [71] colonized germ-free mice with cultured bacterial strains from a 6 month old stunted infant and fed the mice a diet supplemented with bovine sialylated milk oligosaccharides. Although this study was focused on bone biology, this diet was associated with BMO-dependent and microbiota-dependent increases in cecal levels of succinate, increased numbers of small intestinal tuft cells, and evidence for activation of a succinate-induced tuft cell signalling pathway linked to Th2 immune responses. In contrast, similar studies on CMO are limited. Thum et al. [72] found that consumption of CMO by mice during gestation and lactation improved the development of their pups, and the relative abundance of bifidobacteria and butyric acid in the colon, at weaning. Overall, these studies

highlight the potential of domestic animal milks as a source of oligosaccharides which can aid in shaping the enrichment of a protective intestinal microbiota.

3.2 Effect of Milk Oligosaccharides on the Colonization of Bifidobacteria

Bifidobacteria are associated with a number of health-related benefits for the host including inhibiting the growth of pathogenic organisms, modulating mucosal barrier function and promoting appropriate immunological and inflammatory responses [73, 74]. In order to exert a beneficial effect, a sufficient population of bifidobacteria must colonize the host, and therefore, initially adhere to intestinal cells [75]. Our group has recently found that oligosaccharides from domestic animal milks may contribute not only to the selective growth of bifidobacteria but also to their specific adhesive ability. Kavanaugh et al. [76] showed that treatment of *B. longum* subsp. *infantis* ATCC 15697 with a mixture of 3'- and 6'-sialyllactose found in human and domestic animal milks substantially increased bacterial adhesion (up to 9.8-fold) to human HT-29 intestinal cells *in vitro*. Moreover, transcriptomic analysis revealed that the increased adherence phenotype of the strain resulting from exposure to the oligosaccharide is likely multi-faceted, involving transcription factors, chaperone proteins, adhesion-related proteins, and a glycoside hydrolase [76]. More recently our group found that CMO also resulted in increased adhesion (8.3-fold) of *B. infantis* to HT-29 intestinal cells when used at physiological concentrations [77].

There is some evidence that bovine milk components may also modulate intestinal epithelial cell surface glycans thereby reducing or increasing the prevalence of attachment sites for bacteria [78]. In a recent study by our group, HT-29 intestinal cells were exposed to a bovine colostrum fraction rich in free oligosaccharides [79]. The adherence of several commensal bacteria, comprising mainly bifidobacteria, to the intestinal cells was significantly enhanced (up to 52-fold) for all strains tested which spanned species that are found across the human lifespan. Importantly, the changes to the HT-29 cell surface did not support enhanced adhesion of the enteric pathogens tested. The gene expression profile of the HT-29 cells following treatment with the colostrum fraction was evaluated by microarray analysis [79]. Many so called 'glyco-genes' (glycosyltransferases and genes involved in the complex biosynthetic pathways of glycans) were found to be differentially regulated suggesting modulation of the enzymatic addition of sugars to glycoconjugate proteins. Changes in the glycosylation pattern of the intestinal cells upon exposure to the colostrum fraction were also demonstrated through the use of lectin arrays [80]. These studies highlight the potential of bovine milk components

as functional ingredients that can potentially increase the attachment of health-promoting bacteria in the gut which may be important to individuals with lower counts of such bacteria.

3.3 Protective Role of Milk Oligosaccharides Against Pathogens and Infection

Milk oligosaccharides are considered to be soluble receptor analogs of epithelial cell surface carbohydrates as they are generated from similar enzymes [8, 15]. These structures display structural homology to host cell receptors and thus function as receptor decoys which pathogens can bind to instead of the host. Oligosaccharides can also inhibit pathogens by competitive binding with the host cell-surface receptor [81]. An expansive literature exists describing the action of HMO against a variety of pathogens [82, 83, 84, 85] while information on the role of oligosaccharides from domestic animal milks in preventing infection is more limited. There are examples of 3' and 6'-sialylactose being used as anti-infectives. Mysore et al. [86] investigated the effect of 3'-sialyllactose on colonization of *H. pylori* in rhesus monkeys. Of the 6 monkeys given the milk oligosaccharide, 2 were cured permanently, and 1 was transiently cleared and 3 animals remained persistently colonized suggesting that these oligosaccharides can cure or decrease *H. pylori* colonization in some cases. Martin et al. [87] investigated whether bovine milk oligosaccharides had potential to protect against 7 enterotoxigenic *Escherichia coli* strains, isolated from diarrheic calves. Inhibition of hemagglutination in the presence of oligosaccharides was used as an indicator of interaction between the oligosaccharides and bacterial adhesins. Mid-lactation milk oligosaccharides, in particular, proved to be the most efficient at inhibiting hemagglutination.

Coppa et al. [88] included a *Salmonella fyris* strain in their study on inhibition of pathogen adhesion to Caco-2 cells. The study showed that 6'-sialyllactose had an anti-adhesive effect on *S. fyris*. In a study by our group, we examined oligosaccharides isolated and purified from the colostrum of Holstein Friesian cows for anti-infective activity against a highly invasive strain of *Campylobacter jejuni* [89]. During our initial studies we structurally defined 37 bovine colostrum oligosaccharides (BCO) in our sample by hydrophilic interaction liquid chromatography (HILIC)-HPLC coupled with exoglycosidase digests and off-line mass spectroscopy [23]. We then examined the effect of the BCO on *C. jejuni* adhesion to, invasion of and translocation of intestinal HT-29 cells. We found that the BCO dramatically reduced the cellular invasion and translocation of *C. jejuni*, in a concentration dependent manner. Periodate treatment of the BCO prior to inhibition studies resulted in a loss of the anti-infective activity of the oligosaccharides suggesting a direct oligosaccharide-bacterial interaction. We found that 5 mg/mL BCO which is similar to physiological concentrations of HMO in breastmilk,

dramatically decreased the level of internalized bacteria by up to 50% when compared to the control [89]. Maldonado-Gomez et al. [90] also demonstrated that bovine colostrum oligosaccharides could prevent the adhesion of enteropathogenic *Escherichia coli*, *Cronobacter sakazakii* and *Salmonella enterica* serovar Typhimurium to HEp-2 cell monolayers cultured *in vitro* [90]. Recently, neutral and acidic oligosaccharides isolated from caprine milk and bovine colostrum were compared for their potency to inhibit the adhesion of *Salmonella enterica* IID604 to Caco-2 cells using HMO as a positive control [91]. Both the CMO and BCO inhibited the adhesion of *Salmonella* enterica to Caco-2 cells at concentrations ranging 2.5 and 5.0 mg/mL. The ability of CMO to prevent the adhesion of *Escherichia coli* NCTC 10418 and a *Salmonella typhimurium* isolate to Caco-2 cells was also recently demonstrated [63]. These studies build a strong case for exploring the possibility of using oligosaccharides sourced from dairy streams as potential anti-infectives.

3.4 Effect of Milk Oligosaccharides on Intestinal Epithelial Cells

Intestinal health and barrier function are considered to be the first line of defence in the gastrointestinal tract [92]. In this respect, HMO can modify host-microbe interactions by affecting epithelial cell turnover [93] and intestinal glycocalyx formation [78, 94, 95]. HMO are able to directly affect the intestinal cell response by reducing the cell growth and by inducing differentiation and apoptosis [93]. HMO have also been reported to increase the intestinal cell maturation [96]. Considering the consequences of pathogen invasion and the advantages of commensal colonization, it favours the gastrointestinal environment, and ultimately the host, to possess a means by which to rapidly adapt to possible threats, while maintaining a hospitable environment for beneficial organisms. There is some evidence that 3'-sialylactose, one of the main oligosaccharides of both human and domestic milks may modulate intestinal epithelial cell surface glycans thereby reducing or increasing the prevalence of attachment sites for bacteria [78]. Exposure of 3'-sialylactose to Caco-2 cells altered the glycan profile of the cell surface. The expression of the sialyltransferases ST3Gal1, 2 and 4 were reduced, but ST3Gal3, 5 and 6 were unaffected by 3′-SL. The subsequent reduction in α-2,3- and α- 2,6-linked sialic acid coincided with reduced adhesion of enteropathogenic *Escherichia coli* (EPEC) to the cells [78]. These results suggest that 3'-sialylactose modulates the intestinal cell surface glycome via differential regulation of genes associated with glycosylation in much the same way as described by our group [79, 80]. Interestingly, recent studies examining the impact of a CMO on barrier function of epithelial cell co-cultures found that the CMO at the maximum concentration tested (4.0 mg/mL) enhanced trans-epithelial

electrical resistance, mucin gene expression and mucin protein abundance in epithelial co-cultures, all of which are essential components of intestinal barrier function [62, 97]. Collectively, these findings suggest that milk components can directly modulate the intestinal cell surface and potentially alter the glycosylation state of the cells which in turn may facilitate the adherence of distinct communities of bacteria.

4. Industrial Scale Strategies to Produce Dairy Oligosaccharides

Considering the wide availability of dairy side streams from which oligosaccharides can be isolated, BMO and CMO show promise as future therapeutics that could be used to provide human milk oligosaccharide-associated health benefits to infants and adults at a large scale. Whey is a particularly attractive source of oligosaccharides and is the liquid part of milk that separates from the curd during cheese production. This stream is rich in lactose and protein, but has a high biochemical and chemical oxygen demand making it costly to dispose of within environmental regulations [98]. Martinez-Ferez et al. [99] were among the first to describe the use of membrane technology for the isolation of oligosaccharides from animal milk, in this case, pasteurized skimmed caprine milk. A two-stage tangential filtration process was used. At the end of the process, 80% of the oligosaccharides were obtained in the final retentate. Oliveria et al. [100] also used ultrafiltration to remove proteins and fat globules from caprine whey, and then the ultrafiltered permeate was further processed using a 1 kDa ‘tight’ ultrafiltration membrane. The final retentate was fractionated by preparative scale molecular size exclusion chromatography to yield 28 oligosaccharide rich fractions.

Ultrafiltered whey permeate is either disposed of at a cost to the whey processor or used to produce food-grade lactose by crystallization [101]. Milk oligosaccharides pass through the ultrafiltration membranes ending up in the whey permeate [21, 101]. The liquid that is separated from lactose crystals is known as mother liquor and is usually disposed of to sewage plants or sold as animal feed. A study by Mehra et al. [101] resulted in BMO concentration from mother liquor using membrane filtration. A combination of HPLC and accurate mass spectrometry allowed the identification of optimal processing conditions which resulted in the production of Kg amounts of BMO enriched powders. Among the BMO identified in the powder, 18 had high-molecular weights and corresponded in size to the most abundant oligosaccharides present in human milk. Interestingly, 6 oligosaccharides contained fucose which is rarely detected in bovine milk [101].

More commonly, a combination of lactose hydrolysis and membrane filtration is used to isolate oligosaccharides from milk in order to increase the purity. De Moura Bell et al. [102]

recently developed a novel pilot-scale approach for the recovery of highly pure oligosaccharides, from colostral bovine whey permeate. As the concentration of BMO in colostrum is much higher than in mature milk [103, 104], it does represent an available source from which to separate BMO at large scale. The method described by De Maura Bell et al. relies on the integration of optimized processing conditions that favour maximum lactose hydrolysis and monosaccharide fermentation prior to oligosaccharide concentration by selective membrane filtration. Upon complete lactose hydrolysis and fermentation of the monosaccharides by yeast, nanofiltration of fermented whey permeate from colostrum enabled the recovery of 95% of the oligosaccharides at high purity [102]. This processing strategy has also been applied to the recovery of CMO at pilot scale with a 75% recovery of oligosaccharides [105] and recently further optimised in terms of enzymatic hydrolysis [106]. Overall, processing conditions using temperatures ≤40 °C and an enzyme concentration of ≤0.25% resulted in a higher preservation/formation of caprine whey oligosaccharides. Martín-Ortiz et al. [106] were also successful in the selective removal of lactose, and the resulting glucose and galactose, from pooled caprine colostrum using a biotechnological procedure based on the combined use of *β*-galactosidase from *K. lactis* (optimal conditions: 0.68 U mL1 of enzyme, 37 °C, 15 min and pH 7) and yeast from *S. cerevisiae* (optimal conditions: 37 °C, 24 h).

Another recent study investigated the characterization and concentration of oligosaccharides naturally present in caprine cheese whey obtained from 2 types of caprine milk [108]. The caprine cheese whey was processed by a 2-step cross-flow filtration process and a hydrophilic interaction chromatography-Ultra-Performance Liquid Chromatography coupled to a High Definition Mass Spectrometry. A Quadrupole Time-of-Flight (HILIC UPLC-HDMS-Q-TOF) method was used to identify and measure oligosaccharides in the samples. A final product with recovery of 63–96% of oligosaccharides was obtained when compared with the original whey. Although membrane filtration is the most commonly investigated technique for producing dairy-derived oligosaccharides at large-scale, there has been some recent success using scalable chromatography approaches to produce bovine oligosaccharides from whey streams by our group (European Patent Application No. EP18214230.7), an area which we continue to explore.

5. Conclusion

As HMO are supplied through breastfeeding, these valuable effects are largely lost to formula-fed infants. Substitution of infant formula with dairy-derived oligosaccharides to impart HMO functions is a potential solution in addition to the benefits already observed by supplementation of formulas with 2'-fucosylactose. Oligosaccharides generated from bovine or caprine milk may have many applications in foods e.g. in infant milk formula, infant foods, follow-on formula, beverages and fermented milks. It may be that a mixture of oligosaccharides such as those generated from dairy streams may be even more beneficial to the infant rather than individual oligosaccharides. A mixture could better represent the great variety of chemical structures found in human milk and as such allow more than one function, however, the development of high yielding methods for oligosaccharide isolation and purification is required to achieve this. More studies are required to investigate the different *in vivo* actions of such ingredients on intestinal modulation and its technological implications when added to formula. Future clinical research should be directed at addressing the following questions: which specific milk oligosaccharides in the BMO and CMO pools confer beneficial activity, in what quantity are they required, for how long should they be administered and can production at large-scale be cost-effective?

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Figures and Tables

Table 1. Milk oligosaccharides found in human milk and dairy milks

Oligosaccharide (Abbreviation)	**g/L**		
	Human	**Bovine**	**Goat**
Total in Colostrum Milk	20-23	1.0	0.4-1.35
Total in Mature Milk	12–15	0.03-0.06	0.25-0.3
2'-Fucosyllactose (2'-FL)	1.88-4.9	Trace	Trace
3-Fucosyllactose (3-FL)	0.25-0.86	Trace	Trace
Lacto-*N*-tetraose (LNT)	0.5-1.5	Trace	Trace
Lacto-*N*-neotetraose (LNnT)	0.04-0.2	Trace	-
Lacto-*N*-fucopentaose I (LNFPI)	1.2-1.7	-	-
Lacto-*N*-fucopentaose II (LNFPII)	0.3-1.0	-	-
Lacto-N-fucopentaose III (LNFPIII)	0.01-0.2	-	Trace
3'-Sialyllactose (3'-SL)	0.1-0.3	0.035-0.119	0.03-0.05
6'-Sialyllactose (6'-SL)	0.3-0.5	0.014-0.088	0.05-0.07
Sialyllacto-N-tetraose (a) (LSTa)	0.03-0.2	Trace	-
Sialyllacto-N-tetraose (b) (LSTb)	0.01-0.16	-	-
Sialyllacto-N-tetraose (c) (LSTc)	0.1-0.6	Trace	–
6′-Sialyl-lactosamine (6'SLN)	-	0.009-0.176	Trace
Disialyl-lactose (DSL)	-	0.002-0.07	0.001-0.005
Disialyllactose-N-tetraose (DSLNT)	0.2-0.6	Trace	-
α-3'-Galactosyllactose (α3'-GL)	-	Trace	0.03-0.05
β-3'-Galactosyllactose (β3'-GL)	Trace	Trace	0.03
β-4'-Galactosyllactose (4'-GL)	Trace	-	-
β-6'-Galactosyllactose (6'-GL)	0.002	Trace	Trace
α-3'-*N*-acetylgalactosaminyllactose (α-3'-GalNAcL)	-	0.003-0.065	Trace
Lacto-N-difucohexaose I (LNDFH-I)	0.58	-	-
Lacto-N-neohexaose (LNnH)	Trace	–	–
Lacto-*N*-hexaose (LNH)	0.13	-	0.001-0.005
6′-*N*-Acetyl-glucosaminyl-lactose (NAL)	-	Trace	0.02–0.04

The ranges shown reflect differences due to variations in the analytical methods used in the different studies and reflect changes in abundance over lactation i.e. from colostrum to mature milk. Compiled date from: [8, 109, 110, 103, 111, 112, 113, 105, 60, 114, 115, 39, 116, 117, 118, 117, 119, 120].

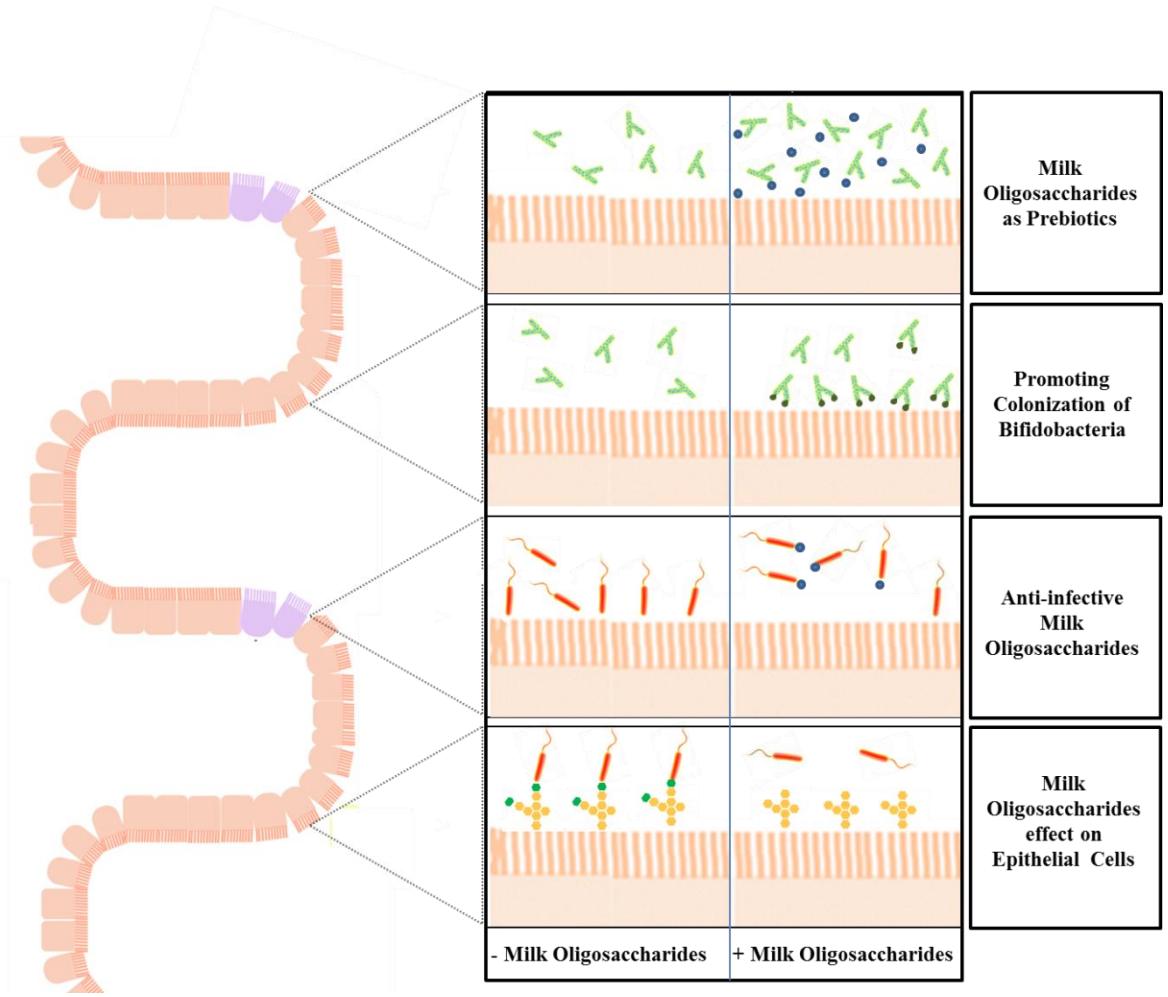

Figure 1. Schematic overview of the suggested mechanisms of action of milk oligosaccharides in the human intestine. Here, oligosaccharides may serve as prebiotics, promote the colonization of bifidobacteria, act as decoys for pathogens and modify epithelial glycan receptor expression.

Chapter II

Mining Milk for Factors which Increase the Adherence of *Bifidobacterium longum* subsp. *infantis* to Intestinal Cells

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Published in Foods (2018), *7* (12), 196.

Acknowledgements: Erinn Quinn performed all experimental work for this chapter with the exception of Dionex ICS-3000 Series HPLC which was conducted by Helen Slattery. Aoife Thompson aided with experimental assays. Sarah Ross provided the buttermilk powder. Rita Hickey and Michelle Kilcoyne assisted with manuscript preparation.

Abstract

Bifidobacteria play a vital role in human nutrition and health by shaping and maintaining the gut ecosystem. In order to exert a beneficial effect, a sufficient population of bifidobacteria must colonise the host. In this study, we developed a miniaturised high-throughput *in vitro* assay for assessing the colonising ability of bacterial strains in human cells. We also investigated a variety of components isolated from different milk sources for their ability to increase the adherence of *Bifidobacterium longum* subsp. *infantis* ATCC 15697, a common member of the gastrointestinal microbiota of breastfed infants, to HT-29 cells. Both conventional and miniaturised colonisation assays were employed to examine the effect of 13 different milk-derived powders on bacterial adherence, including positive controls which had previously resulted in increased bifidobacterial adherence (human milk oligosaccharides and a combination of 3′- and 6′-sialylactose) to intestinal cells. Immunoglobulin G enriched from bovine whey and goat milk oligosaccharides resulted in increased adhesion (3.3- and 8.3-fold, respectively) of *B. infantis* to the intestinal cells and the miniaturised and conventional assays were found to yield comparable and reproducible results. This study highlights the potential of certain milk components to favourably modulate adhesion of bifidobacteria to human intestinal cells.

1. Introduction

Milk is a complex and complete source of bioactive molecules that help to protect the newborn against infectious diseases and promote development, while selectively enriching a beneficial gut microbiota. Therefore, milk influences infant nutrition and microbial nutrition, which in combination promote infant health. Analyses of the infant microbiome indicate that breast milk selects for a highly adapted intestinal microbiota, dominated by bifidobacteria [1]. Indeed, the most commonly detected species in breastfed infants include *Bifidobacterium longum, B. infantis, B. breve,* and *B. bifidum* [2]. Bifidobacteria are associated with a number of health-related benefits for the host including inhibiting the growth of pathogenic organisms, modulating mucosal barrier function and promoting appropriate immunological and inflammatory responses [3, 4]. Based on these therapeutic effects, bifidobacteria are a popular choice as probiotics. In order to exert a beneficial effect, a sufficient population of bifidobacteria must colonise the host, and therefore, initially adhere to host cell components [5]. There are two approaches most traditionally employed to modulate the gut microbiota; delivery of live bacteria (probiotics) within a food source, or the use of specific prebiotics (inulin, fructo- and galactooligosaccharides), which are known to survive gastric transit and are fermented in the colon by beneficial bacteria, thus promoting their growth [6].

More recently, studies have suggested that components in human milk, such as oligosaccharides, may contribute not only to the selective growth of commensal bacteria but also to their specific adhesive ability. Gonzalez et al. [7] demonstrated that growth of *B. longum* in defatted human milk leads to the genetic up-regulation of putative type II glycoprotein binding fimbriae, which have been implicated in attachment and colonisation. Chichlowski et al. [8] demonstrated that the growth of *B. longum* subsp. *infantis* ATCC 15697 on human milk oligosaccharides (HMO) as the sole carbon source increased bacterial adherence to HT-29 intestinal cells. Kavanaugh et al. [9] showed that treatment of *B. longum* subsp. *infantis* ATCC 15697 with a mixture of the HMO, 3′- and 6′-sialyllactose (3′-SL and 6′-SL, respectively) substantially increased bacterial adhesion (up to 9.8-fold) to HT-29 cells. Moreover, transcriptomic analysis revealed that the increased adherence phenotype of the strain resulting from exposure to HMO is likely multi-faceted, involving transcription factors, chaperone proteins, adhesion-related proteins, and a glycoside hydrolase [9]. Garrido et al. [10] found that solute binding proteins produced by *B. longum* subsp. *infantis* ATCC 15697 had a binding affinity for mammalian carbohydrate structures including Lewis antigens, polylactosamines and globotriose (Gb3) and structures found in colonic mucins, suggesting that *B. infantis* may also interact with such structures on the epithelial cells.

Examining the effect of HMO structures on bifidobacterial colonisation *in vivo* may prove difficult as only certain structures are currently available and not the entire range of HMO found in human milk. Therefore, investigation of other milk sources for components with adhesion promoting capabilities may be an attractive alternative. For instance, domestic animal milk components including oligosaccharides may have biological functions similar to those of human milk. In addition, current methods for investigation of bacterial colonisation are time-consuming and require a high quantity of sample for testing. As a result, there is a need to develop high-throughput (HTP) techniques which can quickly define the colonising ability of bacterial strains to human cell lines and which require substantially lower sample quantities. This is particularly important as isolation yields of compounds from natural sources are often too low for investigation in such bioassays.

The aim of the current study was to investigate the changes in adhesion of *B. longum* subsp. *infantis* ATCC 15697, a model consumer of HMO in the infant gastrointestinal (GI) tract, to HT-29 cells following exposure to a panel of milk-derived components. We have also developed a miniaturised HTP method for screening bacterial interactions with cells and compared it with a conventional assay. In total, 13 different milk powders derived from a variety of sources were investigated for their ability to increase bacterial adherence to HT-29 cells, using both conventional and miniaturised colonisation assays. Free oligosaccharides and some of the major milk glycoproteins comprised the main components of these powders and their effects were compared with commercially available 3′- and 6′–sialyllactose and HMO, which were previously shown to increase bifidobacterial adherence to HT-29 cells.

2. Materials and Methods

2.1 Materials

The oligosaccharides 3′-sialylactose (3′-SL) and 6′-sialylactose (6′-SL) were purchased from Carbosynth Ltd. (Berkshire, UK). Beneo Orafti P95 (oligofructose) was kindly provided by Beneo Orafti (Oreye, Belgium)**.** Bovine *β*-casein (*β*-C) and *β*-lactoglobulin (*β*-L) were purchased from Merck (Darmstadt, Germany). Glycomacropeptide (GMP), with a maximum lactose content of 1% and containing approximately 8.5% sialic acid was kindly provided by Agropur Ingredients (Eden Prairie, MN, USA). Lyophilised Bovine Lactoferrin (LF) and lyophilised Immunoglobulin G enriched powder (IGEP) isolated from bovine whey were kindly provided by Upfront Chromatography (Copenhagen Denmark). Lacprodan® MFGM-10 from whey protein concentrate with a maximum lactose content of 3%, containing 2% sialic acid and 5% IgG was kindly provided by Arla Food Ingredients (Viby J, Denmark). Lacprodan® PL-20 with a maximum lactose content of 10%, containing a minimum of 16% phospholipids was also kindly provided by Arla Food Ingredients. The human colonic adenocarcinoma HT-29 cell line was used as a model of the human intestinal epithelial layer and was purchased from the American Type Culture Collection (ATCC, Middlesex, UK).

2.2 Generation of Milk-Derived Powders

All powders used in the study and their sources are listed in Table 1. For human milk oligosaccharides (HMO), human milk was kindly donated by the Irvinestown Human Milk Bank (Co. Fermanagh, Ireland). For Goats Milk Oligosaccharides (GMO), mature milk from goats was kindly donated by Ardsallagh Goat Farm (Carrigtwohill, County Cork). All samples were stored at −80 °C on arrival. To generate low molecular weight fractions, the milks were initially defatted by centrifugation at 4 °C, for 20 min at 3850× *g*. Caseins were then precipitated by adjusting the pH to 4.6, followed by centrifugation at 3850× *g* at 25 °C for 20 min. After neutralisation (by adjusting the pH to 6.7), large peptides and whey proteins were removed by ultrafiltration using a 5 kg/mol molecular weight cut off (MWCO) membrane (Millipore Helicon S10 Spiral Cartridge; Millipore). The permeates were freeze-dried and, to separate the lactose from the oligosaccharides, 100 mL of a 10% solution of each powder were applied to a BioGel P2 size exclusion column (92 × 5 cm; Bio-Rad Laboratories, Inc., Hercules, CA, USA) and eluted with deionised water at 3 mL/min. The 14 mL fractions collected were analysed for lactose, 3-SL and 6-SL using high pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) as detailed below, and peptide concentration was determined by the Bradford assay [11]. Peptide-free and low-trace lactose (<80 mg/L)

fractions were pooled and freeze-dried to give an oligosaccharide-enriched fraction (referred to as HMO and GMO for human and goat milk, respectively).

For bovine milk oligosaccharides (BMO), mother liquor (Glanbia plc, Co., Kilkenny, Ireland) was treated as described above. Following this, fractions low in lactose (<30 mg/L) from a number of separate runs were pooled and freeze-dried to give a bovine oligosaccharide enriched powder which still contained residual peptides. A 5% filtered solution (100 mL) of this powder was applied to a Nucleosil C18 reverse phase column (250 × 8 mm) on a 2695 Waters Alliance HPLC system (Waters Corporation, 34 Maple St, Milford, MA, USA) using deionised water as the eluent at a flow rate of 2 mL/min, which resulted in the binding of peptides to the column. After the sample was applied, the column was washed with one column volume of water. The sample flow-through and column wash were eluted, collected and freeze-dried to give an oligosaccharide-enriched and peptide-free fraction referred to as BMO.

For buttermilk fractions (BF), milk from Holstein-Friesian cattle was obtained from the bulk tank located at the milking parlour at the Teagasc Food Research Centre, Moorepark (Fermoy, Co., Cork, Ireland). Isolation of the buttermilk fraction was performed as previously described [12]. In brief, cream and milk fat globules were separated from the milk using a FT15 disc bowl centrifuge (Armfield Ltd., Ringwood, UK) and the cream was stored at 4 °C for 12–24 h. After chilling, the cream was churned using a mixer to produce butter and buttermilk. The buttermilk was then passed through glass wool (Merck) to remove minute butter granules. Washed cream was produced as described previously [13, 14]. Briefly, warm deionised water (37 °C) was added to the cream at a ratio of 1:10 and the mixture was added to the FT15 disc bowl centrifuge. This process was repeated twice, and the washed cream was then used to produce buttermilk as described above. A 5 kg/mol Viva-Flow 200 (Sartorius®, Göttingen, Germany) crossflow filtration system was used to separate lactose from the high molecular weight components leaving a sample enriched in glycoproteins and glycolipids. The sample was then lyophilised and the dry powder stored in a desiccator at room temperature. The final sample contained 0.223 mg/g of lactose which was quantified as previously described [15].

2.3 Milk Oligosaccharides Analysis

Oligosaccharide-enriched fractions were diluted in water and analysed in order to quantify levels of lactose, 3′-SL and 6′-SL using a Dionex ICS-3000 Series system (Dionex Corporation, Sunnyvale, CA, USA) equipped with an electrochemical detector. Samples were separated on a CarboPac PA100 column (250 × 4 mm) equipped with a guard column using the following

gradient; 95% 100 mM NaOH (Eluent A) and 5% 100 mM NaOH with 500 mM NaAc (Eluent B) for 3 min, 88% eluent A and 12% eluent B for 10 min, and 50% eluent A and 50% eluent B for 17 min for a 30 min separation. The column was re-equilibrated for 15 min with 95% eluent A and 5% eluent B after each separation.

2.4 Bacterial Strains and Culture

Bacterial culture conditions were maintained as previously described [9]. *Bifidobacterium longum* subsp. *infantis* ATCC® 15697™ (*B. infantis*) was obtained from the American Type Culture Collection (ATCC, Middlesex, UK). and the strain was stored in deMan Rogosa Sharpe (MRS) (Difco, Sparks, MD, USA) broth containing 50% glycerol at −80 °C. The strain was cultured twice in MRS media supplemented with L-cysteine (0.05% *w*/*v*) prior to use and was routinely grown overnight at 37 °C under anaerobic conditions generated using the Anaerocult A system (Merck, Dannstadt, Germany). For assays, *B. infantis* was incubated alone or in combination with each powder for 1 h and the bacteria were serially diluted and enumerated by spot plating on MRS agar to determine bacterial numbers. To determine the mid-exponential growth phase, the culture was monitored by measuring the absorbance at 600nm ($OD_{600\ nm}$) at intervals during growth.

2.5 Mammalian Cell Culture

Typically, HT-29 cells were grown in McCoy′s 5A modified medium (Merck) supplemented with 10% fetal bovine serum (FBS), which were maintained in 75 cm^2 tissue culture flasks and incubated at 37 °C in 5% CO_2 in a humidified atmosphere. For conventional 12-well plate preparations, once the cells were confluent (approximately 80–90%), cells were passaged into 12 well plates. For the assays, cells in 75 cm^2 flasks were trypsinised and seeded into a 12-well tissue culture plate (Sarstedt Ltd., Wexford, Ireland) at a density of 1×10^5 cells/mL between passages 15–21 and cells were used once fully confluent (approximately 4×10^6 cells/well at day 5–7). The media was changed every other day and supplemented with 2% FBS 24 h prior to use. For miniaturised 48-well plate preparations, the cells were grown and passaged as above. Cells were trypsinised and seeded into a 48-well tissue culture plate (Sarstedt Ltd., Wexford, Ireland) at a density of 1×10^5 cells/mL between passages 15–21 and cells were used once fully confluent (approximately 2×10^6 cells/well).

2.6 Exposure of Bacteria to Milk-Derived Powders

Exposure of the bacteria to milk-derived powders (Figure 1) was performed as previously described with minor modifications [9]. Bacteria were used at mid-exponential growth phase

(18 h) and the OD_{600nm} was adjusted to 0.3 at the start of the assay, after which the cells were cultured for 1.5–2 h and used once an OD_{600nm} of 0.5 was reached (corresponding to approximately 1.6×10^8 CFU/mL). Bacterial cells were washed twice with PBS by centrifugation. Cell pellets were re-suspended to a final OD_{600nm} of 0.5 in McCoy's 5A tissue culture media supplemented with 2% FBS and either 5 mg/mL of HMO or a total of 5 mg/mL of a mixture of 3′ and 6′-SL at a ratio of 1:1 (after filtration using a 0.45 um filter, Sarstedt Ltd.). A non-supplemented negative control was also included, i.e., bacteria resuspended in McCoy's 5A tissue culture media alone. Bacterial suspensions were then incubated for 1 h at 37 °C under anaerobic conditions. Following this, bacteria were harvested by centrifugation (3850× *g*, 5 min), the supernatants removed, and pellets were washed three times in PBS and then re-suspended in non-supplemented McCoy's media prior to use in the adhesion assays. For the miniaturised bacterial assay exposures, *B. infantis* was cultured and prepared as above except cell pellets were re-suspended to a final OD_{600nm} of 0.25 in non-supplemented or supplemented McCoy's 5A tissue culture media. A final concentration of 5 mg/mL of GMO, BMO, P95 and HMO was used plus a 5 mg/mL mixture of 3′ and 6′-SL at a ratio of 1:1. The results obtained for HMO and 3′ and 6′-SL were assessed and compared to ensure equivalence against the conventional assay (Figure 2). The same results were also included with the results obtained for the other oligosaccharides, GMO, BMO and P95 (Figure 3). A final concentration of 5 mg/mL of GMP, LF, *β*-C, MFGM-10, *β*-L, PL-20, BF or IGEP was also assessed in the miniaturised assay but was graphed separately (Figure 4).

2.7 Adhesion Assays

HT-29 cells were washed twice with PBS, and 500 µL of the bacteria and media suspensions were added to the wells, corresponding to approximately 40 bacterial cells per human cell in a 12-well plate. For the miniaturised assays, 250 µL of the bacteria and media suspensions were added to the wells, corresponding to approximately 40 bacterial cells per human cell, such that the ratio of bacteria to HT-29 cell was the same in the 12-well and 48-well assays. Bacterial cells were incubated with the HT-29 cells for 2 h at 37 °C under anaerobic conditions using an Anaerocult A system (Merck). The HT-29 cells were then washed five times with PBS to remove non-adherent bacteria. HT-29 cells were then lysed with 250 µL (for 48-well assay) or 500 µL (for 12-well assay) of 1% Triton™ X-100 (Merck) for 5 min at 37 °C. The lysates were serially diluted and enumerated by spot-plating on MRS plates. The adhesion of the bacteria was determined as the percentage of original inoculum which attached, thus accounting for variations in the starting inoculum. Percentage adhesion = (CFU/mL of recovered adherent

bacteria/CFU/mL of inoculum) × 100. Overall, in this study the percentage of the original inoculum which adhered to the HT-29 cells was 0.20% ± 0.13%; this is similar to adhesion rates observed for this strain previously (0.40% ± 0.18%) by Kavanaugh et al. [9]. Adhesion assays screens were performed in triplicate on one occasion, while assays conducted for the development of miniaturised assays were performed in triplicate on three separate occasions. Graphs were drawn using Microsoft Excel. The results are presented as the mean ± standard deviations of replicate experiments, and the student *t*-tests were used to determine statistically significant results in comparison to the control where *p*-value <0.05 was considered significant.

3 Results and Discussion

3.1 Comparison of Conventional and Miniaturised Assays

A miniaturised 48-well adhesion assay was compared with the conventional 12-well adhesion assay in an effort to reduce the volume of test sample consumption and increase the number of samples assayed together. Both the 48-well and 12-well assays demonstrated that pre-exposure of the strain to 5 mg/mL HMO, or a combination of 5 mg/mL 3′- and 6′-SL at a ratio of 1:1, resulted in increased adhesion to the HT-29 cells (Figure 2) consistent with previous work [8, 9]. The 12-well assay resulted in a 3.4-fold increase in *B. infantis* adhesion after incubation with HMO, while the 48-well assay resulted in a 5.7-fold increase in adhesion. Similarly, incubation of *B. infantis* with 3′- and 6′-SL in the 12-well assay resulted in a 4.4-fold increase in adhesion, while the 48-well assay resulted in a 3.5-fold increase in adhesion which demonstrated the scalability of the method. Due to the inherent variability between adhesion assays, differences in the significance of adhesion rates between the conventional assay and the miniaturised assay are not unexpected. Thus, the miniaturised assay was comparable to, and as reproducible as, the conventional colonisation assay.

3.2 Effect of Milk Components on Growth of B. longum subsp. infantis ATCC 15697

B. longum subsp. *infantis* ATCC 15697 (*B. infantis*) was chosen for this study as it is a prototypical HMO consumer in the GI tract of the developing infant [16]. Genomic studies on the strain have demonstrated evolutionary adaptations for the utilisation of milk glycoconjugates [17] galacto-oligosaccharides (GOS) [18] and fructo-oligosaccharides (FOS) [19], along with HMO [10, 16, 18, 20, 21]. Consumption of such carbohydrates is facilitated by a number of glycosyl hydrolases and ABC transporters within the strain [16, 17], making it a suitable model strain for this screening study. In this study, no major change in bacterial growth under adhesion assay conditions (1 h) was observed with the exception of supplementation with *β*-C, which resulted in a 65% increase in cell numbers. In whole bovine milk, there are four major forms of casein, *α*s1-, *α*s2-, *β*- and κ-caseins which make up 38%, 10%, 35% and 12%, respectively [22]. Some of these forms and fractions of these forms have been demonstrated to have growth-promoting activities [23, 24]. *β*-L, which resulted in a slight decrease (7 %) in bacterial cell numbers has been shown to have anti-microbial effects against mastitis-causing bacteria [25]. This anti-microbial effect could help explain the decrease in *B. infantis* numbers observed during the 1 h incubation period in the presence of *β*-L. However, further experiments are required to confirm these results.

3.3 Screening Milk Components for Increased Adhesion of B. longum subsp. infantis ATCC 15697 to HT-29 Cells

In order to identify milk components capable of modulating the adhesion of commensal strains using miniaturised adhesion assays, *B. infantis* was incubated separately with oligosaccharides isolated from different sources including human (HMO), goat (GMO), bovine (BMO), and a commercial prebiotic (Beneo Orafti P95) which acted as a negative control [9], for 1 h at 5 mg/mL, after which the strain's ability to adhere to HT-29 was determined. This concentration was selected based on physiological concentrations of oligosaccharides present in human milk. Bacterial adhesion to HT-29 cells following pre-treatment of the bacteria with GMO resulted in a marked increase in adhesive ability of the strain (Figure 3), as represented by an 8.3-fold increase in adhesion versus the control.

Previously, *B. infantis* was shown to have improved adherence following incubation with HMO using *in vitro* colonisation models [9, 8]. Kavanaugh et al. [9] reported a transcriptional response in the presence of three different oligosaccharide treatments (3′-SL, 6′-SL and 3′- and 6′-SL combined) and an overall up-regulation of genes involved in adhesion with a down-regulation of genes involved in complex oligosaccharide metabolism. However, while that study focused on only HMO and combinations of 3′- and 6′-SL, the current study is the first to examine the effect of oligosaccharides and other milk-derived components isolated from domestic animal milks on the adhesive properties of *B. infantis*, thereby potentially overcoming the limitation in the availability of many HMO. Each mammal derived oligosaccharide powder used in this study contained 3′ and 6′-SL (Table 2). In this study, bacterial cells were incubated with the HT-29 cells for 2 h at 37 °C under anaerobic conditions using an Anaerocult A system (Merck, Dannstadt, Germany). While anaerobic conditions are not optimal for HT-29 cells, previous studies have demonstrated that minimal negative effects occur within this time period while allowing for optimal conditions for the bacteria [9].

Previous studies have shown that goat milk and human milk share many common oligosaccharide structures [26] such as *β*3′-galactosyllactose, *β*6′-galactosyllactose, 2′-fucosyllactose, lactose-*N*-hexaose, 6′-*N*-acetylneuraminyllactose and 3′-*N*-acetylneuraminyllactose. In fact, the overall oligosaccharide profile of goat milk is more similar to human milk than either bovine or ovine milk [27]. Added to that, although goat milk has a substantially lower concentration of oligosaccharides (0.25–0.30 g/L) when compared to human milk (3–20 g/L), it contains 5 to 8 times more oligosaccharides than bovine milk (0.03–0.06 g/L) and 10 times more than ovine milk (0.02–0.04 g/L), [27, 28]. Interestingly, GMOs

have previously been shown to have other positive biological effects. For instance, GMOs can modulate the immune response [29, 30] and have also been shown to promote the growth of probiotic bacteria [31]. The current study further highlights their potential for inclusion in formulations where breastfeeding is not possible. Currently, many formulations based on goat milk are on the market, and methods for the large-scale isolation of GMOs have been investigated, which may allow future clinical trials [32].

In contrast to the other oligosaccharide powders under investigation, pre-treatment with P95 (0.12-fold increase) or BMO (0.1-fold decrease) did not result in a notable increase in adhesion of *B. infantis* to the intestinal cells (Figure 4). P95 had no effect on *B. infantis* adhesion in the study performed by Kavanaugh et al. [9], therefore the result obtained in this study is not surprising. The BMO powder, however, contains high quantities of 3′- and 6′-SL (Table 2) with minimal lactose and therefore may be expected to positively influence the adhesion of the strain. This result suggests that in contrast to pure 3′- and 6′-SL, which seem to have a synergistic effect on adhesion [9], the combination of multiple oligosaccharides within the BMO pool had no effect on the adhesion capacity of the strain and may actually inhibit the activity of 3′- and 6′-SL in promoting adhesion.

In this study, apart from free oligosaccharides, IGEP, which is an IgG enriched powder from bovine whey, also enhanced adhesion of *B. infantis* to intestinal cells by 3.3-fold (Figure 4). Numerous studies have highlighted IgG's bioactivity, specifically its anti-infective capabilities [33, 34]. IgG binds to many gastrointestinal pathogens including *Shigella flexneri*, *Escherichia coli*, *Clostridium difficile*, *Streptococcus mutants*, *Cryptosporidium parvum*, *Helicobacter pylori*, and rotavirus [35]. Indeed, the main role of milk IgG is to agglutinate bacteria, neutralise toxins, inactivate viruses and provide an environment favourable for the growth of beneficial bacteria [34, 36]. Considering the immunomodulatory and bacterial binding properties of IgG, IGEP, which is particularly rich in IgG, may promote adhesion of *B. infantis* by acting as a bridging molecule through interacting with both the mammalian and bacterial cells simultaneously. Alternatively, *B. infantis* is known to encode an endoglycosidase, EndoBI-1 (glycosyl hydrolase family 18), which cleaves many major types of *N*-linked oligosaccharides found on glycoproteins and could thus release intact oligosaccharides from immunoglobulin [37], thereby providing free oligosaccharides which may function similarly to HMO. However, further studies are required to investigate whether this is a possibility.

Reductions in bifidobacterial adhesion were observed with exposure to LF, which resulted in a 40% reduction in adhesion, *β*-lactoglobulin, which resulted in a 43% reduction, the butter

milk fraction, which resulted in a 34% reduction, and PL-20, which resulted in a 37% decrease (Figure 4). As mentioned, Kavanaugh et al. [9] reported an up-regulation in genes associated with adhesion, and a down-regulation in genes associated with oligosaccharide consumption following a 3 h pre-incubation period with 3′- and 6′-SL. The components resulting in reduced adhesion of the strain following the 1 h pre-incubation period could potentially cause *B. infantis* to preferentially up-regulate genes involved in carbohydrate metabolism, rather than use energy on adhesion activities. However, no major increase in growth was observed within the 1 h incubation period in this work with the exception of *β*-C (Table S1). LF is known to have prebiotic abilities, promoting the growth of various lactobacilli [38], and antimicrobial activity [39, 40], and also displays anti-adhesive effects against pathogens [41, 42]. Further investigations are required to explain the decrease in adhesion observed in the current study.

The buttermilk fraction and PL-20 are rich in glycoproteins and are associated with many bioactive properties. Previously, milk fat globule membrane glycoproteins were shown to have anti-adhesive properties against a range of pathogens including rotavirus and various enteric bacteria [12, 36, 43, 44]. Again, given the carbohydrate-rich nature of constituent glycoproteins, the bacteria may favour carbohydrate consumption rather than adhesion when incubated with the buttermilk fraction and PL-20. This however has yet to be confirmed, as no major increase in growth was observed within in the 1 h pre-treatment period. Another consideration is that commensal strains often adhere to cells using the same host ligands as employed by pathogens [9, 45]. As many of the milk components used in this study which resulted in reduced adhesion of the bifidobacterial strain are also anti-adhesive against pathogens, it may be that they are competing with the colonisation factors expressed by *B. infantis* to colonise the host.

In this study, high molecular weight compounds, such as lactoferrin, *β*-lactoglobulin and the buttermilk fraction resulted in a decrease in bifidobacterial adhesion (41%, 43% and 34% respectively). However, it is important to note that under *in vivo* conditions, these would likely be digested by the host prior to reaching the colon and therefore could potentially confer different biological effects to those observed *in vitro*. In contrast, the oligosaccharides tested in this study resulted in an increase in adhesion. Milk oligosaccharides are known to resist digestion, and thus can reach the colon where their presence can influence the development of a healthy gut microbiota. While IGEP is a high molecular weight protein that can be partially digested by the host, the resulting glycopeptides may still have bioactive effects on adhesion.

4. Conclusions

Considering the implications of human-derived products such as HMO and 3′- and 6′-SL, inclusion of GMO as a food ingredient in infant formulations to boost numbers of bifidobacteria could be a viable alternative, particularly considering the growing popularity of formulas based on goat milk. Bovine IGEP is relatively easy to isolate and is currently being produced by dairy companies in Europe for use as a food ingredient. In addition, while this research indicates the potential effects that milk-derived components may have on the gut microbiota, more supporting data are required. Future studies to investigate the effects that these powders may have on the colonisation of other gut-associated commensal and pathogenic bacteria are necessary. This study provides insight into the role milk components may have modulating commensal transient adhesion, as well as highlighting alternatives to HMO and 3′- and 6′-SL which are more commercially attractive to produce on a large scale. In addition, these results demonstrated that miniaturised assays are a feasible alternative to conventional assays where the yield of bioactive compounds is too low to support a conventional study. As the plate counting method is time-consuming and can be variable, fluorescent-labeling of the bacteria to measure the bacteria adhering using a fluorimeter and/or qPCR may improve the efficiency and yield of the miniaturised assay.

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Figures and Tables

Table 1. List of milk-derived components used in the study.

Powder	Abbreviation	Source
Goat milk oligosaccharides	GMO	Isolated in-house
Bovine milk oligosaccharides	BMO	Isolated in-house
Human milk oligosaccharides	HMO	Isolated in-house
3′-sialyllactose and 6′-sialyllactose	3′ and 6′ SL	Merck
Glycomacropeptide	GMP	Agropur foods international
Lactoferrin	LF	Upfront Chromatography A/S
β-casein	*β*-C	Merck
Lacprodan® MFGM-10	MFGM-10	Arla food ingredients
β-lactoglobulin	*β*-L	Merck
Lacprodan® PL-20	PL-20	Arla food ingredients
Butter milk fraction	BF	Isolated in-house
P95	P95	Beneo Orafti
Immunoglobulin G Enriched Powder	IGEP	Upfront Chromatography A/S

Table 2. Level of 3′ and 6′ sialyllactose present in the isolated oligosaccharide pools.

Powder	3′ Sialyllactose (μg/mL)	6′ Sialyllactose (μg/mL)
GMO	37.14	35.28
HMO	46.90	59.00
BMO	48.22	26.79

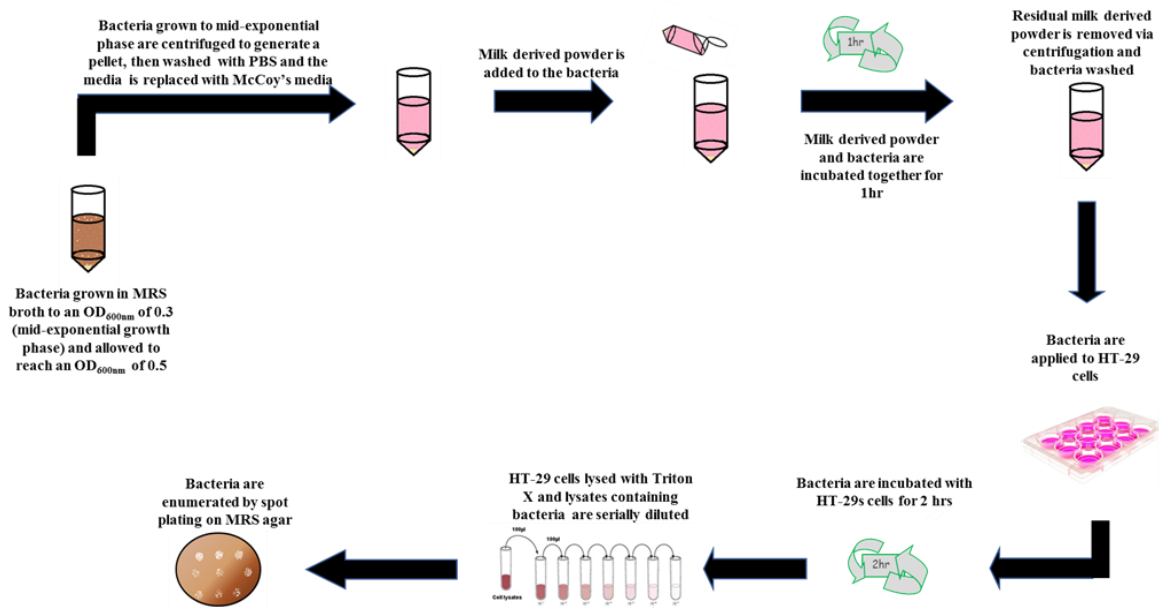

Figure 1. Schematic representation of *Bifidobacterium longum* subsp. *infantis* ATCC 15697 exposure to milk-derived components and subsequent testing for adherence to HT-29 cells.

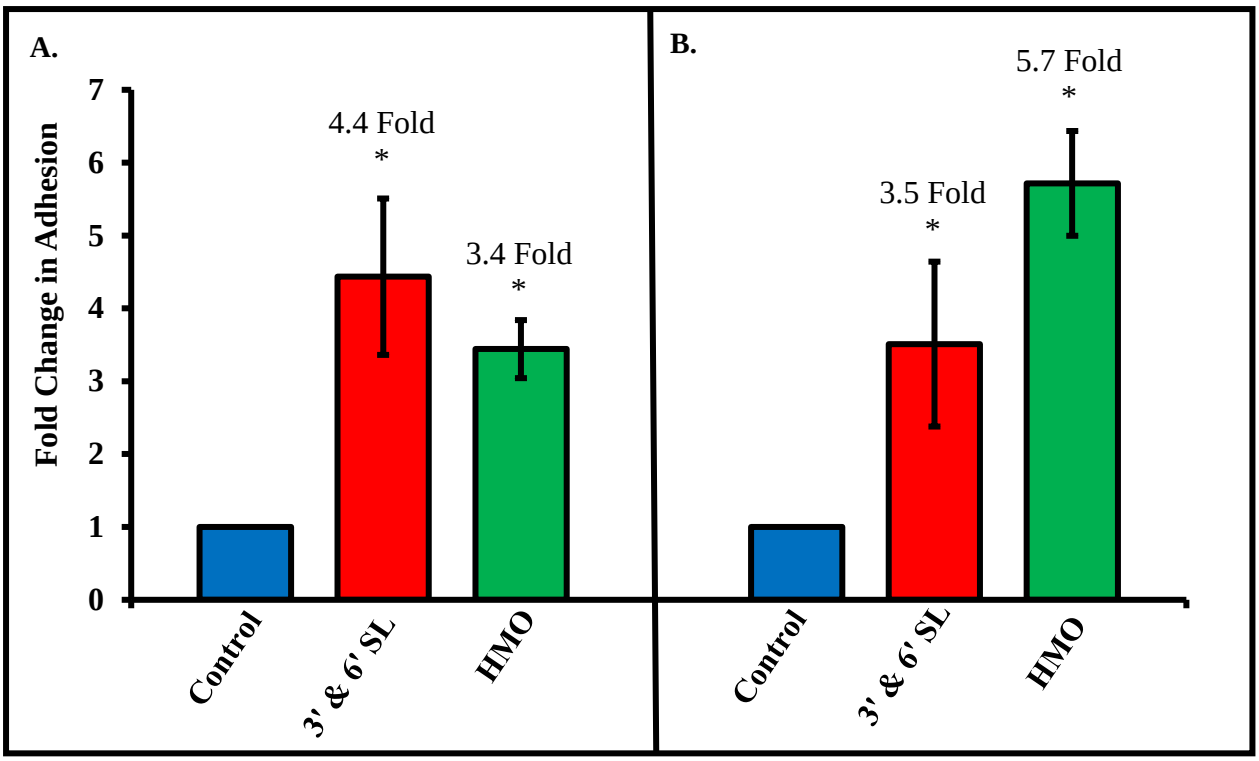

Figure 2. Comparison of adhesion of *B. longum* subsp. *infantis* ATCC 15697 to HT-29 cells following 1 h incubation with 5 mg/mL human milk oligosaccharides (HMO) and a mixture of 5 mg/mL 3′- and 6′-sialyllactose in a conventional adhesion assay (A) and miniaturised assay (B). Results are represented as percentage of adherent cells = [CFU/mL of recovered adherent bacteria ÷ CFU/mL of inoculum] × 100 and graphed as fold-change relative to percent adhesion of control, with error bars representing the standard deviation. (A) The student *t*-test was used to determine statistically significant results for biological triplicate experiments. For all experiments, $p<0.05$ was considered significant.

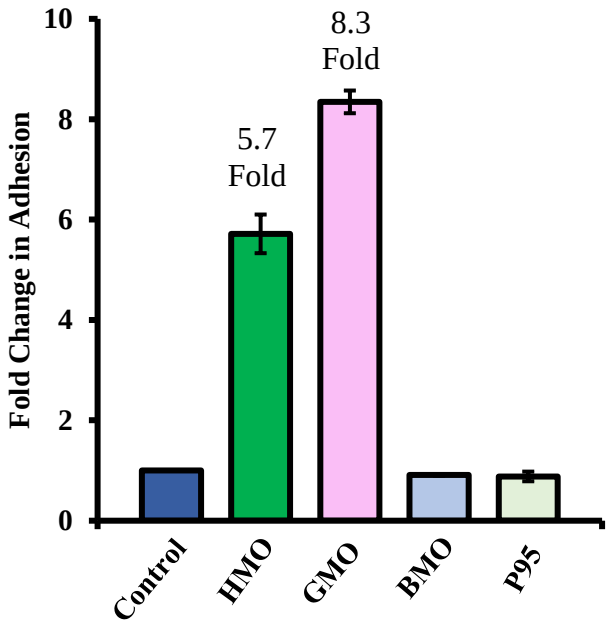

Figure 3. Adhesion of *B. longum* subsp. *infantis* ATCC 15697 to HT-29 cells following incubation with HMO, goat milk oligosaccharides (GMO), bovine milk oligosaccharides (BMO) and P95 using the miniaturised assay (represented as percentage of adherent cells = (CFU/mL of recovered adherent bacteria/CFU/mL of inoculum) × 100). Results are graphed as fold-change relative to percent adhesion of control, with error bars representing standard deviation.

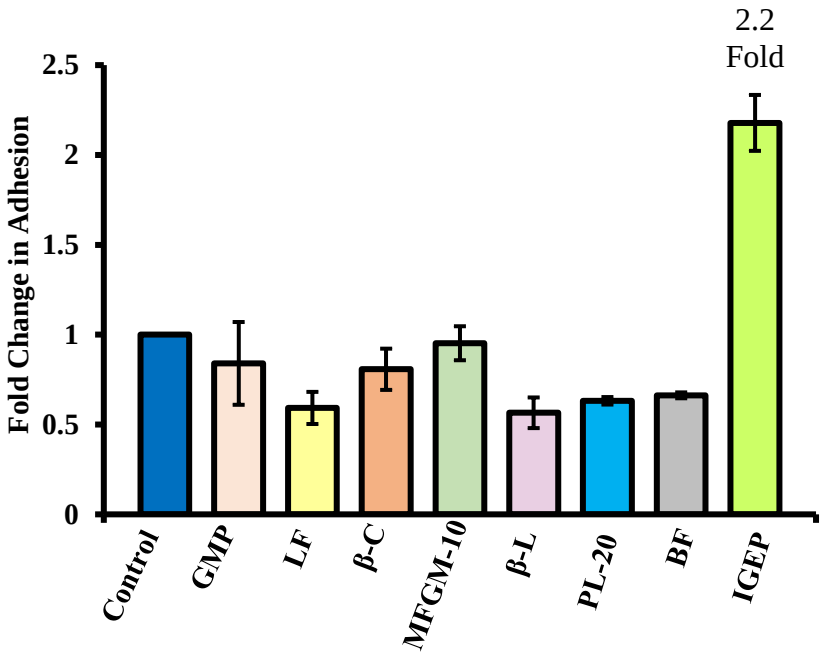

Figure 4. Adhesion of *B. longum* subsp. *infantis* ATCC 15697 to HT-29 cells following pre-treatment of bacteria with glycomacropeptide (GMP), lactoferrin (LF), *β*-casein (*β*-C), Lacprodan® MFGM-10 (MFGM-10), *β*-lactoglobulin (*β*-L), Lacprodan® PL-20 (PL-20), buttermilk fraction (BF) and immunoglobulin G enriched powder (IGEP) using miniaturised assay protocol (represented as percentage of adherent cells = [CFU/mL of recovered adherent bacteria ÷ CFU/mL of inoculum] × 100). Results are graphed as fold-change relative to percent adhesion of control, with error bars representing standard deviation.

Supplementary Information

Table SI: Major differences in growth of *B. infantis* following 1 hour incubation with the milk derived components.

Powder†	Percentage Difference
***β*-C**	65%
***β*-L**	-7%

Results for individual powders are represented as the average of three experimental replicates.

† HMO, GMO, BMO, 3' & 6'-SL P95, GMP, LF, MFGM-10, PL-20, BF and IGEP did not result in a major difference in growth of *B. infantis* following 1 hour incubation.

Table S2: Percentage adhesion of initial inoculum of *B. longum* subsp. *infantis* ATCC 15697 that attached to HT-29 cells following treatment with 3’ & 6’ SL and HMO in conventional and miniaturized assays (Corresponding to Figure 2).

Milk Derived Powder	
Conventional Assays	**Percentage Adhesion of Initial Inoculum**
Untreated *B. infantis*	0.188 ± 0.014
3' & 6' SL treated *B. infantis*	0.833 ± 0.167
HMO treated *B. infantis*	0.647 ± 0.093
Miniaturised assays	
Untreated *B. infantis*	0.081 ± 0.016
3' & 6' SL treated *B. infantis*	0.277 ± 0.006
HMO treated *B. infantis*	0.477 ± 0.032

Table S3: Percentage adhesion of initial inoculum of *B. longum* subsp. *infantis* ATCC 15697 that attached to HT-29 cells following treatment with oligosaccharides in miniaturised assays (Corresponding to Figure 3).

Oligosaccharide	***B. infantis* alone**	**Treated *B. infantis***
HMO	0.083 ± 0.010	0.477 ± 0.032
GMO	0.151 ± 0.009	1.262 ± 0.034
BMO	0.198 ± 0.020	0.181 ± 0.008
P95	0.019 ± 0.008	0.020 ± 0.010

Table S4: Percentage adhesion of initial inoculum of *B. longum* subsp. *infantis* ATCC 15697 that attached to HT-29 cells following treatment with milk-derived powders in miniaturised assays (Corresponding to Figure 4).

Milk Derived Powder	***B. infantis* alone**	**Treated *B. infantis***
GMP	0.153 ± 0.002	0.128 ± 0.035
LF	0.237 ± 0.015	0.140 ± 0.212
β-C	0.202 ± 0.149	0.158 ± 0.224
MFGM-10	0.116 ± 0.009	0.110 ± 0.011
β-L	0.411 ± 0.151	0.232 ± 0.035
PL-20	0.237 ± 0.007	0.150 ± 0.005
BF	0.554 ± 0.015	0.367 ± 0.009
IGEP	0.061 ± 0.021	0.133 ± 0.010

Chapter III

Bifidobacterium longum subsp. *infantis* ATCC 15697 and Goat Milk Oligosaccharides Show Synergism *In Vitro* as Anti-Infectives against *Campylobacter jejuni*

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Published in Foods (2020), *9* (3), 348.

Acknowledgements: Erinn Quinn performed all experimental work for this chapter with the exception of Dionex ICS-3000 Series HPLC which was conducted by Helen Slattery and metabolite analysis with was conducted by Dan Walsh. Rita Hickey assisted with manuscript preparation.

Abstract

Bifidobacteria are known to inhibit, compete with and displace the adhesion of pathogens to human intestinal cells. Previously, we demonstrated that goat milk oligosaccharides (GMO) increased the attachment of *Bifidobacterium longum* subsp. *infantis* ATCC 15697 to intestinal cells *in vitro*. In this study, we aimed to exploit this effect as a mechanism for inhibiting pathogen association with intestinal cells. We examined the synergistic effect of GMO-treated *B. infantis* on preventing the attachment of a highly invasive strain of *Campylobacter jejuni* to intestinal HT-29 cells. The combination decreased the adherence of *C. jejuni* to the HT-29 cells by an average of 42% compared to the control (non-GMO treated *B. infantis*). Increasing the incubation time of the GMO with the *Bifidobacterium* strain resulted in the strain metabolizing the GMO, correlating with a subsequent 104% increase in growth over a 24 h period when compared to the control. Metabolite analysis in the 24 h period also revealed increased production of acetate, lactate, formate and ethanol by GMO-treated *B. infantis*. Statistically significant changes in the GMO profile were also demonstrated over the 24 h period, indicating that the strain was digesting certain structures within the pool such as lactose, lacto-*N*-neotetraose, lacto-*N*-neohexaose 3′-sialyllactose, 6′-sialyllactose, sialyllacto-*N*-neotetraose c and disialyllactose. It may be that early exposure to GMO modulates the adhesion of *B. infantis* while carbohydrate utilisation becomes more important after the bacteria have transiently colonised the host cells in adequate numbers. This study builds a strong case for the use of synbiotics that incorporate oligosaccharides sourced from goat′s milk and probiotic bifidobacteria in functional foods, particularly considering the growing popularity of formulas based on goat milk.

1. Introduction

Bifidobacteria are considered one of the first colonisers of the human gastrointestinal (GI) tract and are suggested to confer positive health outcomes to the host, explaining their prevalence as probiotics [1]. These bacteria are particularly effective at protecting against infectious diseases, regulating immune responses and exerting effects against conditions ranging from irritable bowel syndrome, allergic diseases, ulcerative colitis, and immunoglobulin E associated diseases, to atopic dermatitis [2]. In terms of protecting against infection, bifidobacteria can operate through strain-specific antagonistic means for the competitive exclusion of pathogens [3]. A strain of *B. breve* was found to inhibit the growth of enterotoxigenic (ETEC) and enteropathogenic (EPEC) *Escherichia coli*, while other species were found to inhibit intestinal colonisation of pathogens such as *Salmonella, Shigella, Listeria monocytogenes*, and *Clostridium difficile* [4, 5, 6]. Microbe-associated molecular patterns (MAMPs) are recognized by the host′s intestinal pattern recognition receptors (PRRs), and these interactions play key roles in the association of pathogens with the intestinal epithelia. Probiotics also express molecular patterns which can recognize the same trans-membrane receptors as the pathogens, thus blocking the sites for pathogenic contact by competitive exclusion and, in some cases, displacing already-attached pathogens [7]. However, the health benefits associated with bifidobacteria are reliant on such strains colonising the host in sufficient numbers [8]. The important step in microbial colonisation of the intestinal epithelium is the attachment of bacterial surface lectins to intestinal sugar structures. Recent studies have suggested that milk oligosaccharides may enhance the specific ability of bifidobacteria to attach to the GI epithelium [9, 10, 11]. Indeed, our group investigated the ability of goat milk oligosaccharides (GMO) to increase the attachment of *Bifidobacterium longum* subsp. *infantis* ATCC 15697 to HT-29 cells. Exposure of the strain to the GMO resulted in an 8.3-fold increase in its adhesion to the intestinal cells [11].

As well as aiding the colonisation of bifidobacteria, GMO can also act as a prebiotic. The prebiotic potential of GMO recovered from whey has been identified *in vitro* where significant growth of *Bifidobacterium* spp. was observed on isolated GMO [12]. Oligosaccharide-enriched fractions prepared from both stage one and stage two goats′ milk-based infant formula were also recently shown to significantly enhance the growth of bifidobacteria *in vitro* and reduce the adhesion of *E. coli* NCTC 10418 and *Salmonella enterica* subsp. *enterica* serovar Typhimurium to Caco-2 cells [13], possibly by acting as analogues of epithelial receptors on the gut cells [14]. *In vivo* studies have also indicated that ingestion of GMO by mice during gestation and lactation increased the relative abundance of bifidobacteria in the colon of their

pups at weaning [15]. These studies suggest that a combination of both a probiotic and a prebiotic (GMO) could provide a synergistic effect and may be an effective strategy to enhance the persistence and metabolic activity of specific beneficial bifidobacterial strains. Therefore, the aim of the current study is to examine such a synbiotic combination *in vitro* and determine if GMO may have the potential to increase the competitiveness and metabolic activity of *B. infantis* in the intestinal tract. The ability of the synbiotic to competitively exclude an invasive strain of *Campylobacter jejuni* to intestinal cells is first examined. Fermentation of the GMO by the *B. infantis* strain is then investigated through growth studies, metabolite analysis and oligosaccharide depletion assays.

2. Materials and Methods

2.1 Oligosaccharides Standards

The oligosaccharide standards; 2′-Fucosyllactose (2′FL), Lacto-*N*-tetraose (LNT), 3′-Sialyllactose (3′SL), 6′-Sialyllactose (6′SL), Disialyllactose (DSL), Lacto-*N*-hexaose (LNH), *N*-Acetylneuraminic acid (Sialic Acid), LS-tetrasaccharide c (LSTc), Lacto-*N*-neotetraose (LNnT) and Lacto-*N*-neohexaose (LNnH) were purchased from Carbosynth Ltd. (Berkshire, UK) and lactose was obtained from VWR (Dublin, Ireland).

2.2 Isolation of Goat Milk Oligosaccharides

Goat milk oligosaccharides (GMO) were isolated and characterized as previously described [11]. In brief, mature milk from goats was kindly donated by Ardsallagh Goat Farm (Carrigtwohill, Co. Cork) and stored at −80 °C on arrival. To generate low molecular weight fractions, the milk was initially defatted and de-caseinated as per Quinn et al. [11]. Large peptides and whey proteins were removed by ultrafiltration and the permeates were freeze-dried as previously described [11]. To separate lactose from the oligosaccharides, a BioGel P2 size exclusion column (Bio-rad, Deeside, UK) was employed and the fractions collected were analysed for lactose, 3-SL and 6-SL using high pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) as detailed below, and the protein/peptide concentration was determined by the Bradford assay [16]. Peptide-free and low-trace lactose (<80 mg/L) fractions were pooled and freeze-dried to give an oligosaccharide-enriched fraction.

2.3 Milk Oligosaccharide Analysis

Oligosaccharide analysis of the pooled GMO was performed as previously described [11]. Oligosaccharide-enriched fractions were diluted in water and analysed in order to quantify levels of lactose, 2′FL, LNT, 3′SL, 6′SL, DSL, LNH, Sialic Acid, LSTc, LNnT and LNnH using a Dionex ICS-3000 Series system (Dionex Corporation, Sunnyvale, CA, USA) equipped with an electrochemical detector.

2.4 Bifidobacterium longum subsp. infantis Culture Conditions

Bifidobacterium longum subsp. *infantis* ATCC® 15697™ (*B. infantis*) was obtained from the American Type Culture Collection (ATCC, Middlesex, UK). Bacterial cultures were maintained as previously described [10, 11]. The strain was stored in deMan Rogosa Sharpe (MRS) (Difco, Sparks, MD, USA) broth containing 50% glycerol at −80 °C. The strain was cultured twice in MRS media supplemented with L-cysteine (0.05% *w/v*) (Merck, Dannstadt,

Germany) prior to use, and was routinely grown overnight at 37 °C under anaerobic conditions generated using an Anaerocult A system (Merck).

2.5 Campylobacter jejuni Culture Conditions

Campylobacter jejuni 81–176 (*C. jejuni*) is a well-characterized, mobile flagellated invasive strain which has been used in many previous studies [17, 18]. The pathogen was stored in Mueller–Hinton broth (Oxoid, Ireland c/o Fannin Healthcare, Dublin, Ireland) containing 50% glycerol at −80 °C and cultured directly from storage onto Mueller–Hinton agar plates. The pathogen was grown under microaerophilic conditions generated using CampyGen gas packs (Oxoid), for 48 h at 37 °C. Prior to pathogen inhibition assays, *C. jejuni* 81–176 was grown on Mueller–Hinton agar and then transferred to biphasic media in 25 cm^2 tissue culture flasks (Corning, NY, USA) consisting of Mueller–Hinton agar supplemented with *Campylobacter* selective supplement (Skirrow), (Oxoid) and 6 mL of McCoy′s 5A media (Merck) supplemented with 2% FBS. The flask was then incubated for 24 h under microaerophilic conditions at 37 °C.

2.6 Exposure of B. infantis to Goat Milk Oligosaccharides

Exposure of the bacteria to GMO was performed as previously described [11]. A final concentration of 5 mg/mL of GMO was used reflecting the concentration of oligosaccharides present in mature human milk [11, 19–27]. Bacterial suspensions were then incubated for 1 h at 37 °C under anaerobic conditions. Following this, bacteria were harvested by centrifugation (3850× *g*, 5 min), the supernatants removed, and pellets were washed three times in phosphate-buffered saline (PBS) and then re-suspended in non-supplemented McCoy′s media prior to use in the adhesion assays.

2.7 Mammalian Cell Culture Conditions

HT-29 cells were grown as previously described [11] and maintained in McCoy′s 5A modified medium (Merck) supplemented with 10% fetal bovine serum (FBS) using 75 cm^2 tissue culture flasks incubated at 37 °C in 5% CO_2 in a humidified atmosphere. Once the cells were nearing confluency (approximately 80%–90%), they were passaged into 48 well tissue culture plates (Sarstedt Ltd., Wexford, Ireland) at a density of 1×10^5 cells/mL between passages 15–21. The cells were then used once fully confluent (approximately 2×10^6 cells/ well). The media was changed every other day and supplemented with 2% FBS 24 h prior to use.

2.8 Adhesion Assays with B. infantis

Adhesion assays with *B. infantis* were performed as previously described [11]. HT-29 cells were washed twice with PBS, and 250 µL of the bacteria and media suspensions were added to the wells, corresponding to approximately 40 bacterial cells per human cell. Bacterial cells were incubated with the HT-29 cells after which they were washed with PBS to remove non-adherent bacteria. HT-29 cells were then lysed and the lysates were serially diluted and enumerated by spot-plating on MRS plates to enumerate bacterial colony forming units (CFU). The adhesion of the bacteria was determined as the percentage of original inoculum that attached, thus accounting for variations in the starting inoculum. Percentage adhesion = (CFU/mL of recovered adherent bacteria/ CFU/mL of inoculum) × 100.

2.9 Anti-Infective Assays and Exclusion Assay

Anti-infective assays were performed as previously described [18] with minor modifications. In brief, *C. jejuni* was incubated in the absence and presence of GMO (5 mg/mL) at a final OD_{600nm} of 0.3 (approximately 5.14×10^8 CFU/mL) in McCoys′s media and incubated under microaerophilic conditions for 1 h at 37 °C; 250 µL of the mix was then applied to three wells containing HT-29 cells, and allowed to incubate for 3h, after which the eukaryotic cells were washed five times with PBS, lysed with 250 µL 0.1% Triton X-100 (Merck) in PBS and spread plated onto Mueller–Hinton agar plates and incubated under microaerophilic conditions for 72 h at 37 °C to enumerate CFU. For exclusion assays, exposure of *B. infantis* to 5 mg/mL GMO was performed as described above, and this suspension was subsequently incubated with the HT-29 cells for 2 h. A non-supplemented control was also included. Non-adherent bacteria were removed from the cells as described above, after which the cell line was challenged with *C. jejuni*. To do this, the pathogen was harvested from the biphasic medium, washed twice in non-supplemented McCoy′s, and diluted to an OD_{600nm} of 0.3. From this suspension, 250 µL was then added to each well, and cells were incubated under anaerobic conditions for 3 h at 37 °C. Cells were then washed five times with PBS, lysed with 0.1% Triton X-100 (Merck) in PBS and plated onto supplemented Mueller–Hinton agar. Mueller–Hinton plates were incubated under microaerophilic conditions for 72 h at 37 °C, after which bacterial CFU were enumerated. The assays were performed in triplicate on three separate occasions. The exclusion of *C. jejuni* was determined as the average CFU/mL of recovered adherent bacteria. The percentage decrease in *C. jejuni* adhesion was calculated as the difference in *C. jejuni* CFU/mL between non-supplemented and GMO-supplemented bifidobacteria.

2.10 Effect of GMO on the Growth of B. infantis

B. infantis was grown in the absence and presence of 5 mg/mL GMO over a 72 h period under adhesion assay conditions. Aliquots of 150 µL of the bacterial suspensions were added to the individual wells of a 96 well microtiter plate. Other controls included a control containing no bacteria, and bacteria grown in deMan Rogosa Sharpe (MRS) (Difco, Sparks, MD, USA) broth supplemented with L-cysteine (0.05% *w/v*) (Merck). These experiments were performed in a concept 400 anaerobic chamber (Baker, ME, USA) and bacterial growth was monitored by determining OD_{600nm} using a Synergy-HT multidetector microplate reader driven by Gen5 reader control and data analysis software (BioTek Instruments Inc. Bedfordshire UK) at 0, 12, 24, 48 and 72 h. The microtitre plate was automatically shaken for 30 s prior to each measurement to achieve a homogenous suspension. The results are represented as the average OD_{600nm} of triplicate experiments performed on three separate occasions. The percentage increase in growth of *B. infantis* was calculated as the difference in OD_{600nm} between non-supplemented and GMO-supplemented bifidobacteria.

2.11 GMO Consumption by B. infantis

B. infantis was grown overnight under the optimal conditions outlined above and then re-suspended in McCoy′s media at an OD_{600nm} of 0.25 with a final oligosaccharide concentration of 5 mg/mL. A one milli-litre aliquot of this cell suspension was then dispensed into a sterile Eppendorf (Merck) after 0 h and 24 h of growth. A negative control containing no bacteria was also included. These solutions were then centrifuged for 5 min (3850× g) and the supernatants collected. The process was repeated a total of three times to ensure bacteria were not present in the supernatant. The sample was also treated with ultraviolet light for 30 min in a laminar flow hood to ensure no further metabolic activity occurred., 2′FL, LNT, 3′SL, 6′SL, DSL, LNH, Sialic Acid, LSTc, LNnT, and LNnH were quantified using HPAEC-PAD analyses to quantify oligosaccharide levels before and after 24 h of fermentation using a Dionex ICS-3000 Series system (Dionex Corporation). Reductions in the areas of the peaks after immediate exposure and over the 24 h period were calculated through comparative analysis as per Lane et al. [28]. These experiments were performed on three separate occasions.

2.12 B. infantis Metabolite Analysis

Supernatants collected for HPAEC-PAD analysis at 0 and 24 h were also assessed for changes in metabolite production using high-pressure liquid chromatography. Metabolic end products, lactate, acetate, formate and ethanol were measured using an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA) with a refractive index detector. Metabolite

peaks and concentrations were identified and calculated based on known metabolite retention times and standard solutions at known concentrations. A negative control of non-supplemented media was also included. A REZEX 8 m 8%H, organic acid column (300 × 7.8 mM Phenomenex, CA, USA) was used and the elution was performed for 25 min with a 0.01 M H_2SO_4 solution at a constant flow rate of 0.6 mL/min and a temperature of 65 °C. The standard solutions were prepared as per Table 1. These experiments were performed in triplicate. The ratios of acetate to lactate, acetate to formate, and lactate to ethanol were also calculated.

2.13 Statistical Analysis

Graphs were drawn using Microsoft Excel. The results are presented as the mean ± standard deviations of replicate experiments, and the Student *t*-test was used to determine statistically significant results. For all biological triplicate experiments, $p < 0.05$ was considered significant.

3. Results and Discussion

3.1 Characterisation of the Goat Milk Oligosaccharides

The batch of goat′s milk used in this study was previously characterised in terms of its oligosaccharide content [29]. Forty-one oligosaccharide structures were identified including both neutral and acidic structures, of which 64% of the acidic fraction were Neu5Gc linked [29]. In this study, we isolated oligosaccharide from the same batch of goats milk and quantified some of the major structures, which included LNnT, LNnH, 3′-SL, 6′-SL, LSTc and DSL (Table 2). In terms of the difference between human and animal milk composition, type I oligosaccharides predominate in human milk [27], while type II oligosaccharides are either exclusive or predominate over type I structures in animal milk. Regarding human milk oligosaccharide (HMO) structures, 70% are fucosylated, with lacto-*N*-biose (type I) structures (Gal(β1–3)GlcNAc) predominating over structures containing the *N*-acetyllactosamine (type II) (Gal(β 1–4)GlcNAc) [29]. In contrast, animal-derived oligosaccharides are predominantly sialylated, containing *N*-acetylneuraminic acid (Neu5Ac) and/or *N*-glycolylneuraminic acid (Neu5Gc) [30, 31]. However, it is important to note that the oligosaccharide yield obtained from animal milk is much lower than that of human milk. While changes in GMO composition occur across lactation [24], previous studies have indicated that a number of oligosaccharides, such as 3′ and 6′ sialylactose, *β*3′-galactosyllactose, *β*6′-galactosyllactose, 2′-fucosyllactose, Lactose-*N*-hexaose, 6′-*N*-acetylneuraminyllactose and 3′-*N*-acetylneuraminyllactose are present in both human and goat milk [32, 33, 34, 35]. In addition, a recent study by Leong et al. 2019 [13] investigated the presence of naturally occurring oligosaccharides in commercial goats′ milk-based stage one and stage two infant formulas and their prebiotic properties. Fourteen quantifiable oligosaccharides in goats′ milk-based infant formula were detectable by LC/MS, indicating that, structurally, GMO may represent an alternative to the human milk derivatives where breastfeeding is not possible. In addition, high purity and recovery of GMO consisting of 67.6% acidic and 34.4% neutral oligosaccharides have been demonstrated [36], indicating that potentially viable commercial production methods may be available in the not too distant future.

3.2 Combined Effect of GMO and B. infantis on C. jejuni Adhesion

Probiotic bacteria, such as *Lactobacillus acidophilus* UO 001 and *Lactobacillus gasseri* UO 002, are known to inhibit the growth of *Campylobacter* without interfering with the normal microbiota of the gastrointestinal tract, suggesting other probiotic bacteria may have similar effects [37, 38]. Murine fecal microbiota transplantation treatment was shown to alleviate

intestinal and systemic immune responses in *C. jejuni*-infected mice harbouring a human gut microbiota. These with mice displayed higher numbers of lactobacilli and bifidobacteria, further suggesting the beneficial effects of probiotics against pathogen colonisation [39]. However, a prerequisite for survival in the intestinal tract is the ability of probiotic strains to transiently adhere efficiently to the intestinal mucosa. Probiotic and pathogenic strains have been suggested to share similarities in terms of their surface adhesins and, thus, may compete for adhesion sites [3, 40]. Previously, we demonstrated an increase in *B. infantis* adherence to HT-29 cells after pre-exposure to GMO [11]. The HT-29 cells are extensively used as a model of the gastrointestinal tract, particularly as *in vitro* intestinal models of bacterial colonisation [41, 42]. These cells exhibit classical characteristics that model small intestinal absorptive epithelial cells upon reaching confluence [43] and are a useful indicator of the structural landscape of the intestinal epithelium [44]. In the current study, we also employed HT-29 cells and hypothesised that the increase in *B. infantis* adhesion following GMO treatment may provide a protective effect against *C. jejuni* colonisation of HT-29 cells. We pre-treated *B. infantis* with GMO and observed an average increase in adhesion of 4.4 fold (*P-value*; 0.23), demonstrating a clear increase in adhesion potential in each biological replicate experiment (Figure S1). Following this, anti-infective assays were conducted to investigate if GMO alone could prevent *C. jejuni* colonisation. Overall, an average inoculum of 5.14×10^8 CFU/mL was applied to the HT-29 cells, of which 1.35×10^6 CFU/mL of *C. jejuni* were demonstrated to adhere. The percentage of *C. jejuni* from the original inoculum which adhered to the HT-29 cells was 0.26% ± 0.05. Notably, both GMO alone and *B. infantis* alone had no protective effects against *C. jejuni* colonisation (Figure 1). Exclusion assays assessed the ability of *B. infantis* alone and pre-treated with GMO to prevent attachment and invasion of *C. jejuni* to HT-29 cells. The assays revealed a prophylactic protective effect following prior treatment of *B. infantis* with GMO (Figure 1) with an average significant decrease ranging from 42%–46% in *C. jejuni* adherence observed over triplicate experiments performed on three separate occasions. Interestingly, the non-supplemented *B. infantis* control demonstrated no protective effect against *C. jejuni* colonisation. This may suggest that a critical population level of attached bifidobacteria is required to result in the competitive exclusion of a pathogen. In contrast to the results demonstrated here, a previous study in mice with *L. johnsonii* indicated no reduction in lower intestinal *C. jejuni* colonization, however, a suppressed intestinal and systemic pro-inflammatory and enhanced anti-inflammatory immune response were both observed, indicating other potential benefits of the use of probiotics on *C. jejuni* infection [45].

Notably, no statistically significant increase in *C. jejuni* growth in the presence of GMO was observed under anti-infective and exclusion assay conditions.

The results presented here are particularly significant as *Campylobacter* is one of four key global causes of diarrhoeal diseases and is considered to be the most common bacterial cause of human gastroenteritis in the world [46] *C. jejuni*, in particular, poses a great risk worldwide due to its associated diarrhoeal disease and the risk of development of severe secondary diseases, such as irritable bowel syndrome and Guillain–Barré syndrome post-infection [17, 47]. A number of studies have suggested that probiotic bacteria may inhibit pathogens through competitive exclusion to pathogen adhesion sites and nutrients [48]. Commercial broiler chickens are a major reservoir for *C. jejuni* and consumption can lead to human infection [49]. However, swine and cattle can also act as zoonotic vectors in addition to ingestion of contaminated surface waters [50, 51]. Notably, strains such as *Lactobacillus* spp., i.e., *acidophilus*, *casei*, *crispatus*, *gasseri*, *helveticus*, *pentosus*, *plantarum*, *rhamnosus*, and *salivarius* have been suggested to exhibit anti-*Campylobacter* activities *in vitro* and *in vivo* [49]. In addition, Dec et al. [52], in screening *Lactobacillus* isolates for anti-*Campylobacter* activity, selected seven *Lactobacillus* isolates with potential applications in reducing *Campylobacter* spp. in chickens, which may have potential to prevent infections in both birds and humans [52]. *In vivo* studies in poultry using synbiotic combinations of *Bifidobacterium longum* PCB 133 and galactooligosaccharides in animal feed have been shown to reduce *C. jejuni* infection, demonstrating the potential to reduce transmission along the food chain, which is of fundamental importance for the safety of poultry meat consumers [53]. Microencapsulated *Bifidobacterium longum* PCB133 and xylooligosaccharides (XOS), when combined, have also been shown to have a synbiotic effect on improving the safety of poultry meat by protecting against *C. jejuni* infection (p-value: <0.01) at the beginning of life, while the microbiota is still developing [54]. Additionally, *in vivo* studies in chicks have demonstrated the ability of three chicken commensal isolates to induce a 1–2 log reduction in *Campylobacter* numbers. However, isolates were only capable of reducing *Campylobacter* levels in one out of three trials. Notably, follow–up experiments demonstrated *Lactobacillus salivarius* subsp. *salicinius*, in combination with 0.04% mannan oligosaccharides resulted in a 3-log decrease of cecal *Campylobacter* suggesting that, similar to the current study, the use of synbiotic combinations may be more effective at preventing *Campylobacter* colonisation when compared to a commercial strain alone [55]. The cumulative prebiotic and colonisation-promoting effect of the GMO on the *B. infantis* strain in the current study further supports the use of such synbiotics in foods aimed at preventing infection.

3.3 Prebiotic Effects of GMO

The ability of GMO to stimulate the growth of *B. infantis* was determined using growth curves (Figure 2). When GMO was incubated with *B. infantis* following the pre-treatment period, an average increase in growth of 104% was observed in the presence of GMO at 24 h. No significant increases in the growth of *B. infantis* occurred during earlier incubation periods (≤ 12 h). Thus, it is unlikely that this growth contributed to the observed increase in bifidobacterial adhesion to the HT-29 cells after the 1 h exposure.

Previous studies have demonstrated statistically significant increases in *Bifidobacterium* and *Bacteroides* growth following incubation with GMO [12]. Similarly, GMO has also been shown to increase levels of bifidobacteria using *in vitro* fermentation models and in *in vivo* mouse trials [15, 39]. The prebiotic effect observed here is not surprising given that *B. infantis* ATCC 15697 is particularly adept at the utilising human milk glycans due to the presence of a 43 kb gene cluster responsible for their transport and utilization [56–64]. Indeed, *Bifidobacterium longum* subsp. *infantis* has been described as the "champion colonizer of the infant gut" and is unique among gut bacteria in its prodigious capacity to digest and consume milk oligosaccharide structures [58, 59, 60]. Moreover, a number of bifidobacterial strains have the ability to utilize milk oligosaccharides as substrates for growth [65]. *In vitro* fermentation of human milk oligosaccharides by different bifidobacterial strains including *B. longum* subsp. *infantis* ATCC 15697, *B. longum* ATCC 15707, *B. breve* ATCC 27539, *B. adolescentis* ATCC 15703 and *B. bifidum* ATCC 29521 has been previously demonstrated [66].

The ability of *B. infantis* ATCC 15697 to use multiple specific carbohydrate structures (2′-FL, LNT, 3′-SL, 6′-SL, DSL, LNH, Sialic Acid, LSTc, LNnT and LNnH) within the pool was also investigated by generating HPAEC-PAD profiles of the oligosaccharides in the media before and after 24 h of bacterial growth (Figure 3). Notably, before the incubation period, a total of 24 peaks were detected in the GMO by the method used, of which six (3′-SL, 6′-SL, DSL, LSTc, LNnT and LNnH) were identified. Following 24 h incubation with *B. infantis,* 19 peaks were detected, of which 11 unidentified peaks were found in trace amounts that were not detected in the starting material. 2'-FL, LNT, LNH, and free sialic acid were not detected at either time point. Overall, our results demonstrated that the strain was capable of utilizing multiple structures. For instance, 3′ and 6′-SL were depleted by 94% and 71% respectively, while LNnT and LNnH, were 100% utilised. LSTc was depleted by 94%, DSL was depleted by 43.1%, and lactose was depleted by 52%. This may be expected as *B. longum* subsp. *longum* has been shown to utilize free LNnT, one of the dominant components of HMO, [67, 66]. In addition, *B. longum* subsp. *infantis* BRS8-2 and TPY1201 are known to degrade 2′-FL, 3-FL,

3′-SL and LNnT, while *B. longum* subsp. *infantis* DSM 20088 has been shown to utilise 2′-FL, 3-FL, and LNnT [68]. Therefore, it is likely that the prebiotic effect associated with GMOs may extend to other strains.

The presence of bifidobacteria in the gut are known to influence the production of formate, acetate, ethanol and lactate [69] and gut homeostasis is achieved through their production [70]. The inhibition of gram-negative bacteria through the production of metabolites has been shown [71]. Acetate is the most prominent short chain fatty acids (SCFA) [72] and accounts for over half the total SCFA content in stools [73, 74]. Acetate has been shown to improve protection against pathogen colonisation as it modulates the gut epithelium and induces anti-inflammatory and anti-apoptotic effects [75].

In this study, HPLC was implemented to determine the production of metabolites such as acetate, lactate, formate and ethanol by *B. infantis*, following growth on GMO (Table 3). The results indicated that GMO resulted in a 12-fold (p-value = 0.0009) higher concentration of acetic acid at 24 h in comparison to the non-supplemented control. Indeed, the concentration of lactic acid was 15-fold higher (p-value = 0.0019), while the concentration of formic acid was 8-fold (p-value = 0.0001) higher than the non-supplemented control at 24 h. Notably, ethanol was only detected in the GMO-supplemented sample at a concentration of 8mM at 24 h (Table 3). Notably, bifidobacterial strains can catabolize 2 moles of hexose resulting in the production of 2 moles of lactic acid and 3 moles of acetic acid using the fructose-6-phosphate phosphoketolase pathway, resulting in a theoretical yield of acetate:lactate ratio of 1.5:1 in hexose [76]. However, this ratio is rarely obtained and varies depending on the growth substrate and/or culture conditions [77]. For example, growth on substrates LNT, LNnT, inulin-type fructans and 2'-FL has been demonstrated to result in higher ratios of acetate to lactate [76, 78, 79]. Similarly, in this study, the acetate to lactate ratio of acetate to lactate produced by *B. infantis* was 3.3:1 following 24 h growth on GMO. Increased acetate production has been reported to occur in log-phase cells and relates to the phosphorolytic splitting of pyruvate derived from carbons four, five, and six of hexose and this could account for the additional levels of acetate we observed in this study [80]. The high ratio of acetate to lactate in substrated LNT and LNnT has been suggested to be in part a result of the deacetylation of the GlcNAc [76] and this, too, may have contributed the results found here. Notably, some bifidobacteria convert pyruvic acid into formic acid and ethanol rather than into lactic acid, thereby yielding an extra ATP [77]. In this study, we detected concentrations of ethanol (8.004 mM), formic acid (8.57 mM) and lactate acid (9.04 mM) after 24 h fermentation in GMO, which also may explain the lower than expected ratio of lactate to acetate. While bifidobacteria are incapable

of producing butyrate, within the host, the production of acetate and lactate can be converted to butyrate through cross-feeding pathways via indigenous colonic species, such as *Faecalibacterium prausnitzii* (clostridial cluster IV) and *Anaerostipes, Eubacterium*and *Roseburia* species (clostridial cluster XIVa) [70, 81, 82, 83, 84] and, thus, *in vivo*, may have additional beneficial effects.

4. Conclusion

Initial exposure of bifidobacteria to GMO resulted in a trending increase in attachment to HT-29 cells and, in turn, had a prophylactic effect against *C. jejuni* attachment and invasion of intestinal cells *in vitro*. Protection against pathogen colonisation through competitive exclusion is a key benefit of the increasing bifidobacterial colonisation and could be exploited in order to deal with the rising numbers of *Campylobacter* infections. While other pathogens were not assessed in this study, there is potential that GMO treatment could provide overall resistance to pathogenic colonisation if used prophylactically as a synbiotic with bifidobacteria. Longer exposures of *B. infantis* to GMO increased the growth of the strain, highlighting its ability to adapt to environmental factors and promote its overall survival and colonisation in the GI tract, further highlighting the benefits of using synbiotic combinations. Overall, this study highlights the potential benefits in combining oligosaccharides from goat milk with select probiotic strains for promoting a healthy gut ecosystem, whilst protecting the host against pathogenic disease. Moreover, the use of such synbiotics is not limited to gastro-intestinal disorders, as low numbers of bifidobacteria have been associated with many other disorders. Such disorders include periodontal disease, rheumatoid arthritis, atherosclerosis, allergy, multi-organ failure, asthma and allergic diseases in addition to inflammatory bowel diseases, such as Crohn′s disease, irritable bowel syndrome and ulcerative colitis [85, 86]. Synbiotics may, therefore, be more effective than either probiotics or prebiotics when used alone in the treatment and management of human health.

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Figures and Tables

Table 1. Volumes of standards used for metabolite analysis.

HPLC Standard	Per 100mL	Molecular weights
10 mM Lactic acid	0.09 g	90.08
10 mM Acetic acid	57 µL	60.05
10 mM Formic Acid	38 µL	46.03
10 mM Ethanol (99%)	58 µL	46.07

Table 2. Levels of different oligosaccharides present in goat milk oligosaccharides (GMO) pool.

Oligosaccharide Structures	**µg/mL**
Lacto-*N*-neotetraose (LNnT)	3.4
Lacto-*N*-neohexaose (LNnH)	3
3′-Sialyllactose (3′SL)	32.83
6′-Sialyllactose (6′SL)	33.05
LS-tetrasaccharide c	0.94
Disialyllactose (DSL)	33.46

Table 3. Production of metabolites by *B. longum* subsp *infantis* American Type Culture Collection (ATCC) 15697 after 0 and 24 h incubation with GMO.

	Control		**GMO**	
Concentration mM	**0 h**	**24 h**	**0 h**	**24 h**
Acetate	ND	2.42 [a]	0.07	30.14[a,b]
Lactate	ND	0.62[n.s]	ND	9.04 [a,b]
Formate	0.81	1.13 [a]	0.79 [b]	8.57 [a,b]
Ethanol	ND	ND	ND	8.00[a,b]

[a]indicates significance in comparison to time 0 and 24 h within the one group, and [b]indicates significance in comparison to the non-supplemented control. [n.s] indicates not significant.

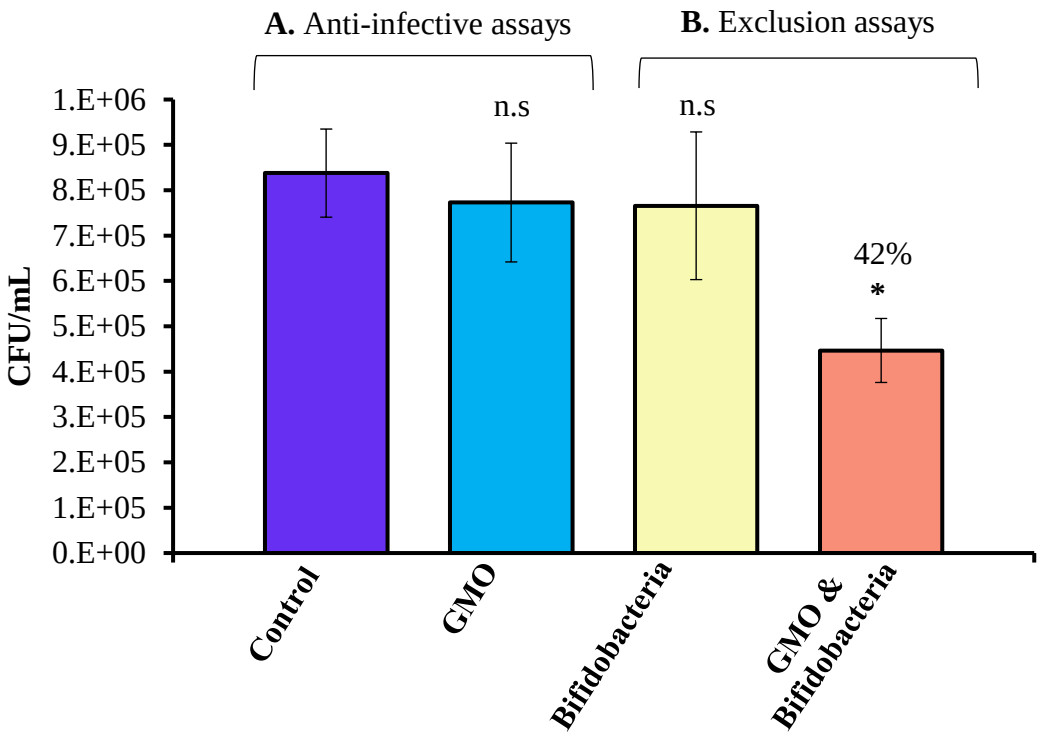


Figure 1. Anti-infective assays (A) demonstrating *Campylobacter jejuni* 81–176 adhesion in the absence and presence of GMO, and (B) competitive exclusion assays demonstrating *Campylobacter jejuni* 81–176 adhesion to HT-29 cells following pre-treatment of the HT-29 cells with *Bifidobacterium longum* subsp. *infantis* 15697 (yellow) and *Bifidobacterium longum* subsp. *infantis* 15697 pre-treated with GMO (orange). Results demonstrate the average colony forming units (CFU)/mL of adherent *Campylobacter jejuni* 81–176 of one representative triplicate experiment, with error bars representing standard deviation. The unpaired non-parametric *t*-test was used, *: *p*-value: <0.05, n.s: not significant.

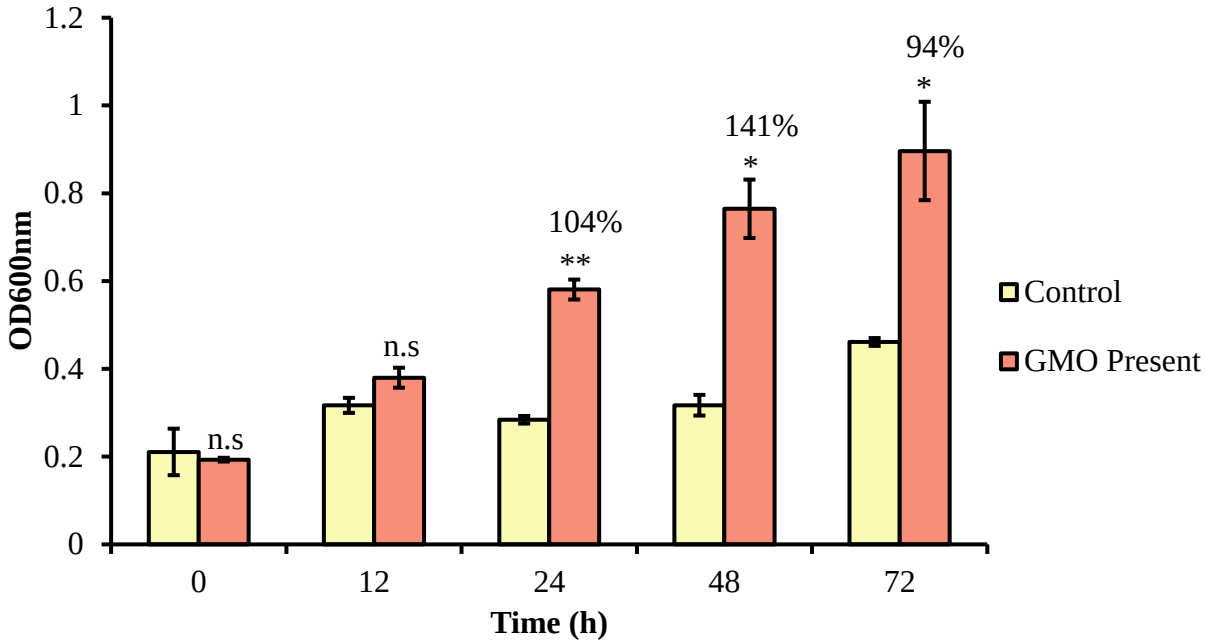
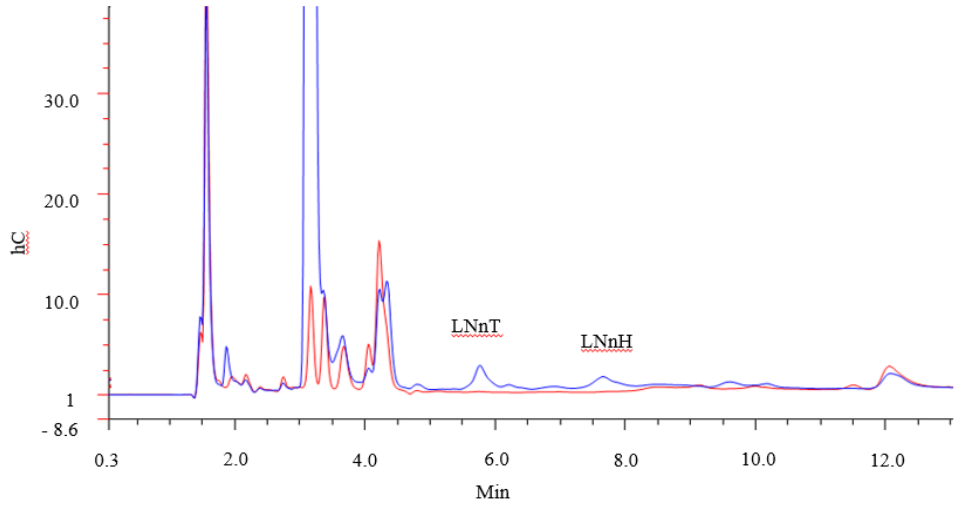
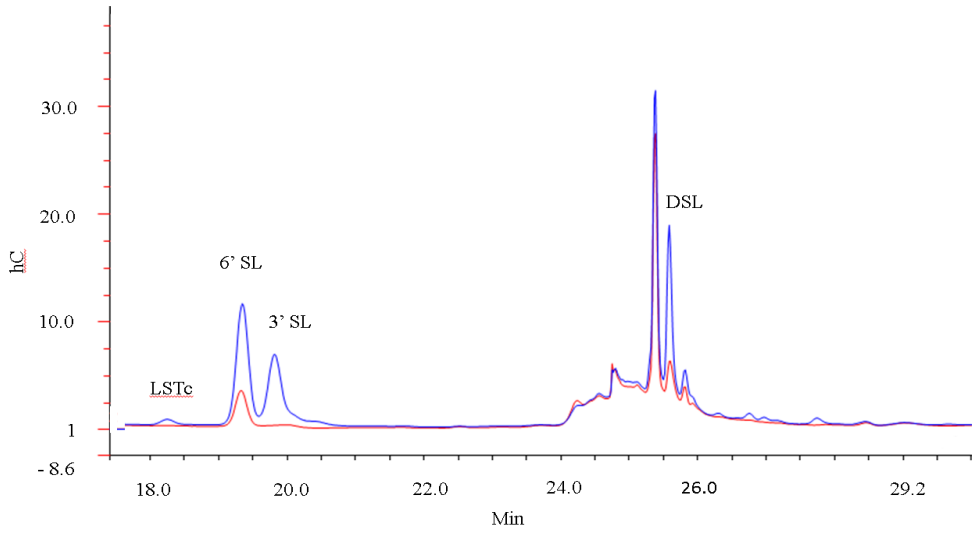


Figure 2. Growth of *B. longum* subsp. *infantis* in McCoy's media supplemented with GMO over 72 h. Optical density readings were taken at time 0, 12, 24, 48 and 72 h. Results are represented as the average of three biological replicates, with error bars representing standard deviation. The student *t*-test was used, *: *p*-value: <0.05, ** *p*-value: <0.005, n.s: not significant.



A.



B.

Figure 3. GMO profile of (A) neutral oligosaccharides and (B) acidic oligosaccharides after 0 h (blue) and 24 h (red) incubation with *B. infantis*.

Supplementary Materials

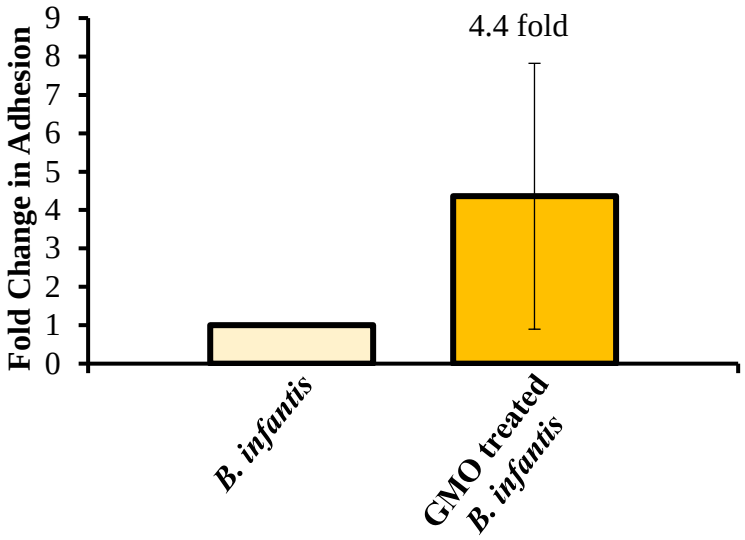


Figure S1. Adhesion of *B. longum* subsp. *infantis* ATCC 15697 to HT-29 cells following incubation with goat milk oligosaccharides; Results are represented as the average of triplicate experiments performed on three separate occasions and are presented as the percentage of adherent cells = [CFU/mL of recovered adherent bacteria ÷ CFU/mL of inoculum] × 100 and graphed as fold-change relative to percent adhesion of control, with error bars representing the standard deviation.

Table S1: Average percent adhesion of *B. longum* subsp. *infantis* ATCC 15697 initial inoculum which attached to HT-29 cells following incubation with goat milk oligosaccharides (Corresponding to Figure S1).

***B. infantis* alone**	***B. infantis* treated with GMO**
0.32 ± 0.26	1.02 ± 0.45

Chapter IV

A Whey Fraction Rich in Immunoglobulin G combined with *Bifidobacterium longum* subsp. *infantis* ATCC 15697 exhibits synergistic effects against *Campylobacter jejuni*

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International Journal of Molecular Sciences, (2020), *In Press.*

Acknowledgements: Erinn Quinn performed all experimental work for this chapter with the exception of metabolite analysis which was conducted by Dan Walsh. Rita Hickey and Michelle Kilcoyne assisted with manuscript preparation.

Abstract

Evidence that whey proteins and peptides have health benefits beyond basic infant nutrition has increased dramatically in recent years. Previously, we demonstrated that a whey-derived immunoglobulin G enriched powder (IGEP) enhanced adhesion of *Bifidobacterium longum* subsp. *infantis* ATCC 15697 (*B. infantis*) to HT-29 cells. In this study, we investigated the synergistic effect of IGEP-treated *B. infantis* on preventing the attachment of highly invasive *Campylobacter jejuni* 81-176 (*C. jejuni*) to intestinal HT-29 cells. The combination decreased the adherence of *C. jejuni* to the HT-29 cells by an average of 48% compared to the control (non-IGEP treated *B. infantis*). We also confirmed that treatment of IGEP with sodium metaperiodate, which disables the biological recognition of the conjugated oligosaccharides, reduced adhesion of *B. infantis* to the intestinal cells. Thus, glycosylation of the IGEP components may be important in enhancing *B. infantis* adhesion. Interestingly, an increased adhesion phenotype was not observed when *B. infantis* was treated with bovine serum-derived IgG, suggesting that bioactivity was unique to milk derived immunoglobulin-rich powders. Notably, IGEP did not induce growth of *B. infantis* within a 24 h incubation period, as demonstrated by growth curves and metabolite analysis. The current study provides insight into the functionality of bovine whey components and highlights their potential in positively impacting the development of a healthy microbiota.

1. Introduction

Whey derived from cows' milk contains many similar components to those found human milk and for this reason, is a key ingredient in a wide variety of infant formulas. While breastfeeding is preferred, infant formulas containing whey proteins are currently the best alternative when breastfeeding is not an option [1]. Emerging evidence from *in vitro*, animal, and a small number of human studies indicate that a variety of beneficial bioactivities are linked to whey protein and its derivative forms, i.e., concentrate, isolate, hydrolysate, and individual proteins and peptides [1]. Whey proteins are suggested to play a role in influencing the infant gut microbiota. Bifidobacteria are strong colonizers of the infant gut and an array of bifidobacterial strains can utilize milk glycans as substrates for growth [2, 3, 4, 5]. High levels of beneficial bacterial species such as bifidobacteria are present in the breast-fed infants gut, inhibiting the growth of pathogenic microorganisms, modulating the mucosal barrier function, and promoting the inflammatory and immunological responses [6]. Balmer et al. [7] showed that infants ingesting a whey-protein formula had higher levels of bifidobacteria in their stool compared to those who received a casein-predominant formula at two weeks of age [7]. Similarly, a German-based double-blind study randomized 102 healthy infants under two weeks of age and demonstrated that whey protein-fed infants had more abundant counts of bifidobacteria in their stools [8]. More recently, it has been shown that infants receiving a formula low in protein and phosphate with whey protein as the main constituent developed a bifidobacteria profile similar to that of the breast-fed infants [9]. These studies indicate that supplementation of infant formula with specific whey proteins may have the potential to emulate the bioactivities associated with human breast milk, including the development of microbiota rich in beneficial bifidobacteria.

Breast milk and its constituents naturally select for protective bifidobacteria, and specific glycans present in mammalian milk have been demonstrated to bind to and inhibit a range of enteric pathogens *in vitro* [10, 11, 12]. Specific glycosylated milk components have been suggested to not only confer prebiotic effects but also contribute to the colonization potential of *Bifidobacteria* spp. in the intestine by directly modulating the intestinal cells, or by enhancing the adherence properties of the bacteria. For instance, our group recently demonstrated a bovine milk-derived fraction containing 23.64 μg/mL IgG among other components, including 3' and 6' sialyllactose, β-lactoglobulin, and α-lactalbumin, was capable of modulating HT-29 cells which resulted in a subsequent increase in bifibacterial adhesion of up to 52-fold compared to non-treated HT-29 cells [13]. Transcriptomic, proteomic and glycomic analysis of the intestinal cells following treatment with the bovine milk-derived

fraction confirmed that the cell surface proteome and glycome were altered [13, 14]. Regarding enhancing the adherence abilities of bifidobacteria, growth of *B. longum* in defatted human milk up-regulated a putative type II glycoprotein binding fimbriae, which may be involved in attachment and colonization [15]. Chichlowski et al. [16] showed increased *B. longum* subsp. *infantis* ATCC 15697 (*B. infantis*) adherence to HT-29 intestinal cells following its growth on human milk oligosaccharides. Additionally, our group demonstrated that treatment of *B. infantis* with a combination of the milk oligosaccharides, 3′- and 6′-sialyllactose significantly increased bacterial adhesion to HT-29 cells up to 9.8-fold [17]. Previously, we also demonstrated that increased adhesion of *B. infantis* following exposure to a panel of oligosaccharides and a bovine whey derived powder enriched in IgG [18]. Subsequent studies with goat milk oligosaccharides (GMO) indicated a prophylactic protective effect against *C. jejuni* colonization when HT-29 cells were pre-exposed to GMO-treated bifidobacteria [19].

Taken together these studies indicate that glycosylated fractions of domestic animal milks may result in an increase in bifidobacteria colonizing the gut. The term synbiotic is used to describe the use of a combination of probiotics and prebiotics that together beneficially improve host health by modulating the development of a healthy gut microbiota. Therefore, the current study aimed to examine the effects of combining *B. infantis* with a bovine whey derived fraction rich in IgG on the attachment of *B. infantis* to intestinal cells, and in turn the ability of the combination to exclude an invasive strain of *C. jejuni* to intestinal cells *in vitro*.

2. Materials and Methods

2.1 Materials and Bacterial Strains

The IGEP was kindly provided by Upfront Chromatography (Copenhagen, Denmark). The protein content of the powder was determined by the Bradford assay [20]. *B. longum* subsp. *infantis* ATCC® 15697™ (*B. infantis*) was obtained from the American Type Culture Collection (ATCC, Middlesex, UK). *Campylobacter jejuni* 81-176 (*C. jejuni*) was kindly provided by Dr Marguerite Clyne's, University College Dublin.

2.2 Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis

Sample preparation and reduction was performed as per manufacturer's instructions (NuPAGE system, Life Technologies, Thermo Fisher Scientific Inc.). In brief, 7.5 µL of sample buffer, 3 µL of reducing agent and 12 µL of deionized water was added to 7.5 µL of sample at 10 mg/mL (by mass of powder) to give a final volume of 30 µL and a total of 15 µg of protein/well. The sample was then centrifuged and heated to 70 °C for 10 min and 10 µL of sample was added to each well of a 4-12% Bis-Tris gel (1.00 mm x 9 well, Life Technologies). A molecular weight standard solution (Invitrogen Mark12 Unstained Standard, Thermo Fisher Scientific Inc.) was prepared as per manufacturer's instructions (diluted 1:10 with Invitrogen LDS Sample Buffer) and was loaded onto the gel. Electrophoresis was performed at 200 V for 50 min using MOPS buffer supplemented with 0.25% NuPAGE Antioxidant (Life Technologies) in the upper chamber. Protein bands were visualised on the gels using Coomassie blue stain (Invitrogen SimplyBlue SafeStain) following the manufacturer's procedure.

2.3 Periodate Treatment of Powder

Sodium metaperiodate ($NaIO_4$) treatment of IGEP was performed to oxidize IGEP and produce IGEP-P as previously described [21]. In brief, IGEP (10 mg/ml) was incubated with 0.011 mM NaIO4 at 4 °C for 30 min. To remove excess NaIO4, the sample was centrifuged using a 3 kDa MWCO (Amicon) with three washes of phosphate-buffered saline at pH 7.4 (PBS). The retentate collected contained the IGEP and was lyophilized to produce a powder. A negative control containing no IGEP, and a control sample not treated with NaIO4 were also included.

2.4 Bacterial Culture

Bacterial cultures were maintained as previously described [17, 18]. *B. infantis* was stored in deMan Rogosa Sharpe (MRS) (Difco, Sparks, Maryland, USA) broth containing 50% glycerol at -80 °C. The strain was cultured twice in MRS media supplemented with L-cysteine (0.05%

w/v) (Merck, Dannstadt, Germany) prior to use, and was routinely grown overnight at 37 °C under anaerobic conditions generated using an Anaerocult A system (Merck).

C. jejuni 81-176 was stored in Mueller Hinton broth (Oxoid, Ireland c/o Fannin Healthcare, Dublin, Ireland) containing 50% glycerol at -80 °C and cultured directly from storage onto Mueller-Hinton agar plates. The pathogen was grown under microaerophilic conditions generated using CampyGen gas packs (Oxoid), for 48 h at 37 °C as previously described [22, 19]. Prior to pathogen inhibition assays, *C. jejuni* 81-176 was grown on Mueller-Hinton agar and then transferred to biphasic media in 25 cm^2 tissue culture flasks (Corning, New York, USA) consisting of Mueller Hinton agar supplemented with *Campylobacter* selective supplement (Skirrow), (Oxoid) and 6 ml of McCoy's 5A media (Merck) supplemented with 2% fetal bovine serum (FBS). The flask was then incubated for 24 h under microaerophilic conditions at 37 °C.

2.5 Exposure of B. infantis to IGEP for Adhesion Assays

Exposure of the bacteria to IGEP was performed as previously described [18, 19]. Bacteria were used at mid-exponential growth phase (18 h), and the OD_{600nm} was adjusted to 0.3 at the start of the assay, after which the cells were cultured for 1.5–2 h and used once an OD_{600nm} of 0.5 was reached (corresponding to approximately 1.6×10^8 colony-forming units (CFU) /mL). Bacterial cells were washed twice with PBS by centrifugation (3850× *g*, 5 min). Cell pellets were re-suspended to a final OD_{600nm} of 0.25 in non-supplemented, or IGEP supplemented McCoy's 5A tissue culture media. A final concentration of 5 mg/mL as per Quinn et al. was used [18]. Bacterial suspensions were then incubated for 1 h at 37 °C under anaerobic conditions. Following this, bacteria were harvested by centrifugation (3850 × *g*, 5 min), the supernatants removed, and pellets were washed three times in PBS and then re-suspended in non-supplemented McCoy's media prior to use in the adhesion assays.

2.6 Mammalian Cell Culture Conditions

HT-29 cells were grown as previously described [18, 19] and maintained in McCoy′s 5A modified medium (Merck) supplemented with 10% FBS using 75 cm^2 tissue culture flasks incubated at 37 °C in 5% CO_2 in a humidified atmosphere. Once the cells were nearing confluency (approximately 80–90%), they were passaged into 48 well tissue culture plates (Sarstedt Ltd., Wexford, Ireland) at a density of 1×10^5 cells/mL between passages 15–21. The cells were then used once fully confluent (approximately 2×10^6 cells/well). The media was changed every other day and supplemented with 2% FBS 24 h prior to use.

2.7 Adhesion Assays with B. infantis

Adhesion assays with *B. infantis* were performed as previously described [18, 19]. HT-29 cells were washed twice with PBS, and 250 µL of the bacteria and media suspensions were added to the wells, corresponding to approximately 40 bacterial cells per human cell. Bacterial cells were incubated with the HT-29 cells for 2 h at 37 °C under anaerobic conditions using an Anaerocult A system (Merck). The HT-29 cells were then washed five times with PBS to remove non-adherent bacteria. HT-29 cells were then lysed with 250 µL of 1% TritonTM X-100 (Merck) for 5 min at 37 °C. The lysates were serially diluted and enumerated by spot-plating on MRS plates to enumerate bacterial colony forming units (CFU). The adhesion of the bacteria was determined as the percentage of original inoculum which attached, thus accounting for variations in the starting inoculum. Percentage adhesion = (CFU/mL of recovered adherent bacteria/ CFU/mL of inoculum) × 100. Experiments were performed in triplicate on three separate occasions.

2.8 Anti-infective Assays and Exclusion Assay

Anti-infective assays were performed as previously described [22, 19]. In brief, *C. jejuni* was incubated in the absence and presence of IGEP (5 mg/mL) at a final OD_{600nm} of 0.3 (approximately 4.7 x 10^8 CFU/mL) in McCoys's media and incubated under microaerophilic conditions for 1 h at 37 °C, 250 µl of the mix was then applied to three wells containing HT-29 cells, and allowed incubate for 3 h, after which the eukaryotic cells were washed five times with PBS, lysed with 250 µl 0.1% Triton X-100 in PBS and spread plated onto Mueller Hinton agar plates and incubated under microaerophilic conditions for 72 h at 37 °C to enumerate CFU. For exclusion assays, exposure of *B. infantis* to 5 mg/mL IGEP was performed as described above, and this suspension was subsequently incubated with the HT-29 cells for 2 h. A non-supplemented control was also included. Non-adherent bacteria were removed from the cells as described above after which the cell line was challenged with *C. jejuni*. To do this, the pathogen was harvested from the biphasic medium, washed twice in non-supplemented McCoy's, and diluted to an OD_{600nm} of 0.3. From this suspension, 250µl was then added to each well, and cells were incubated under anaerobic conditions for 3 h at 37 °C. Cells were then washed five times with PBS, lysed with 0.1% Triton X-100 in PBS and plated onto supplemented Mueller-Hinton agar. Mueller-Hinton plates were incubated under microaerophilic conditions for 72 h at 37 °C after which bacterial CFU were enumerated. For anti-infective assays and competitive exclusion assays, all results are presented as the mean of replicate experiments, with error bars representing standard deviation, graphed as fold-change

relative to percent adhesion of the control. Percentage adhesion = (CFU/mL of recovered adherent bacteria ÷ CFU/mL of inoculum) × 100.

2.9 Effect of IGEP on the Growth of B. infantis

B. infantis was grown in the absence and presence of 5 mg/mL IGEP over a 48 h period under adhesion assay conditions and in carbohydrate-free growth medium; MRS supplemented with L-cysteine (0.05%w/v) prepared by first principles excluding glucose and meat extract [23, 24]. Carbohydrate-free MRS was unable to support bacterial growth above an OD_{600nm} of 0.2. The optical density readings between supplemented and un-supplemented carbohydrate-free media were noted and the difference between these was recorded as the turbidly of the sample, and this was added to all control sample readings to correct the difference in optical density. Aliquots of 150 μL of the bacterial suspensions were added to the individual wells of a 96 well microtiter plate corresponding to an optical density of 0.03. Other controls included media alone with no bacteria, and bacteria grown in MRS broth supplemented with L-cysteine (0.05% w/v) (Merck, Dannstadt, Germany). These experiments were performed in an anaerobic hood and bacterial growth was monitored by determining absorbance (OD_{600nm}) at 0, 12, 24 and 48 h. The microtitre plate was automatically shaken for 30 seconds prior to each measurement to achieve a homogenous suspension. Experiments under adhesion assay conditions were performed in triplicate on three separate occasions and in triplicate under carbohydrate-free MRS conditions. The percentage change in growth was calculated as the difference in OD_{600} between the supplemented and non-supplemented samples.

2.10 B. infantis Metabolite Analysis

Metabolite analysis was conducted as previously described [19]. *B. infantis* was grown overnight under the optimal conditions outlined above and then re-suspended in McCoy's media at an OD_{600nm} of 0.25 with a final oligosaccharide concentration of 5 mg/mL. A one milli-litre aliquot of this cell suspension was then dispensed into a sterile Eppendorf after 0 h and 24 h of growth. A negative control containing no bacteria was also included. These solutions were then centrifuged for 5 min (3,850 x g) and the supernatants collected. The process was repeated 3 times to ensure that bacteria were not present in the supernatant. The sample was also treated with ultraviolet light for 30 min in a laminar flow hood to ensure no further metabolic activity occurred [19]. The metabolic end products lactate, acetate, formate and ethanol in the supernatant were measured in triplicate using an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA) with a refractive index detector. A negative control of non-supplemented media was also included. A REZEX 8m 8 %H, organic acid column (300

x 7.8 mM Phenomenex, CA, USA) was used and the elution was performed for 25 min with a 0.01 M H_2SO_4 solution at a constant flow rate of 0.6 mL/min and a temperature of 65 °C. Metabolite peaks and concentrations were identified and calculated based on known metabolite retention times and standard solutions at known concentrations. The ratios of acetate to lactate, acetate to formate, and lactate to ethanol were also calculated.

2.11 Statistical Analysis

Graphs were drawn using Microsoft Excel. The results are presented as the mean ± standard deviations of replicate experiments, and the student *t*-tests were used to determine statistically significant results in comparison to the control. For all biological triplicate experiments, *p*-value <0.05 was considered significant.

3. Results

3.1 Characterisation of IGEP

IGEP was kindly provided by Upfront technologies. The IGEP contained 77% protein as determined by the Bradford assay [20]. SDS-PAGE analysis of the IGEP powder (Figure 1) revealed that it mainly comprised of IgG, with two protein bands corresponding to 55 and 20 kDa representing the IgG heavy chain and light chain, respectively.

3.2 Effect of IGEP on B. infantis Adhesion

Bacteria were incubated with IGEP (5 mg/mL) which was suspended in McCoy's 5A tissue culture media as per Quinn et al. [18]. IGEP-treated *B. infantis* displayed a 3-fold increase in adhesion to HT-29 cells (Figure 2). Bovine serum-derived IgG (S-IgG) had no effect on *B. infantis* adhesion. Treatment of IGEP with sodium metaperiodate (MP-IGEP) to disable biological recognition of the conjugated oligosaccharides in the sample abolished any increase in *B. infantis* adhesion [21]. Thus, the increase of adhesion of IGEP-treated *B. infantis* to HT-29 cells was mediated at least in part by IGEP glycosylation.

3.3 Combined Effect of IGEP and B. infantis on C. jejuni Adhesion

Anti-infective and competitive exclusion assays were employed to investigate if A) IGEP had a direct anti-infective effect on *C. jejuni* colonization, and B) to determine if the increase in *B. infantis* adhesion as a result of IGEP pre-treatment resulted in a protective effect against *C. jejuni* colonization. An average inoculum of 4.7×10^8 CFU/mL of *C. jejuni* were applied to the HT-29 cells, of which 3.1×10^5 CFU/mL (0.1% ± 0.01% of the original inoculum) attached to the HT-29 cells. We chose *C. jejuni* 81-176 for this study as our group has previously demonstrated that bovine milk oligosaccharides have anti-infective effects against this strain [22], additionally, it has been used in many other previous studies and is well characterised [21, 19]. A high inoculum of *C. jejuni* was used in order to increase the opportunity of pathogenic infection of the HT-29 cells. Anti-infective assays were implemented as previously described [22] to determine if IGEP had any direct anti-pathogenic effects. No direct anti-infective effect was observed (Figure 3A). Competitive exclusion assays were implemented in order to assess if the increased adhesion observed in IGEP treated *B. infantis* facilitated protection against pathogen colonization. First, the *B. infantis* was treated with IGEP for 1 h, after which the bacteria were washed and applied to the HT-29 cells for 2 h. The cell line was washed to remove non-adherent *B. infantis* and then challenged with *C. jejuni* for 3 h prior to enumeration of colonized *C. jejuni*. Notable, IGEP treated *B. infantis* revealed a reduction in

C. jejuni colonisation following prior treatment of the HT-29 cells with IGEP-treated *B. infantis* (Figure 3B), with an average significant decrease of 48% in *C. jejuni* adherence observed. Neither *B. infantis* nor IGEP alone had a protective effect against *C. jejuni* colonization (Figure 3A and B). Notably, no growth of *C. jejuni* in the presence of IGEP was observed under anti-infective and exclusion assay conditions. Overall, IGEP treated *B. infantis* appears to provide prophylactic protection against *C. jejuni* colonization (48% reduction), this is likely through the increased colonization potential of *B. infantis*. Therefore, the combined use of IGEP and *B. infantis* may have superior bioactive potential compared to either used alone.

3.4 B. infantis Growth in IGEP

In order to investigate if growth was implicated in the observed increase in adhesion and consequent anti-infective activities, the ability of IGEP to stimulate the growth of *B. infantis* was examined. When IGEP was incubated with *B. infantis* over 42 h, no increase in bacterial growth was observed (Figure 4). Thus, it is unlikely that increased numbers of *B. infantis* contributed to the observed increase in adhesion to the HT-29 cells after exposure to IGEP.

3.5 B. infantis Metabolite Analysis

The presence of bifidobacteria in the gut influences the production of bacterial metabolites including formate, acetate, ethanol and lactate [25]. In this study, HPLC analysis was used to quantify these metabolites in the supernatant following *B. in*fantis growth in the presence of IGEP (Table 1). There were no statistically significant changes in levels of acetate, lactate, formate or ethanol in IGEP-treated culture compared to no IGEP supplementation (control) following 24 h fermentation and the ratios of acetate to lactate (4:1) and acetate to formate (2:1) were the same for both the supplemented and non-supplemented sample. Ratios observed were likely due to carbohydrate (glucose) in the baseline media (McCoy's media) as these assays were performed under adhesion assay conditions. Slight ethanol production was observed in the IGEP supplemented sample (1:1), while no ethanol was detected in the non-supplemented sample, and thus, this was likely contributed to the IGEP composition itself. Overall, there was a slightly higher production of acetate, lactate and decreased formate in the presence IGEP compared to control (Table 1). Low levels of ethanol production were observed in the IGEP supplemented sample (1:1), while no ethanol was detected in the non-supplemented sample.

4. Discussion

In this study, IGEP increased the adhesion of *B. infantis* 3-fold to HT-29 cells. Notably, SDS-Page revealed that IgG was a dominant protein in the powder, although other immunoglobulins (Igs) such as IgA and IgM may also have been present. IgG is present in human (0.1 g/L) and bovine (1.8 g/L) milk [26, 27, 28] and Igs in general represent about 10-15% of whey proteins in both human and bovine milk [29, 30]. Igs are known for their potent immunological properties and ability to inhibit gastrointestinal pathogens such as bacteria, protozoa and viruses [31, 32]. IgG is the predominant Ig in bovine milk and is known to be heavily glycosylated [33] and in this respect, may also be expected to alter colonization of specific types of bacteria. Indeed, IgG is also the most common Ig in human blood serum [34]. IgG plays a crucial role in protective immunity and is involved in opsonization, agglutination, antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity activation [35, 36]. Three domain structures exist in IgG; two structures are involved in antigen binding (Fab region) and the other is the fragment crystalizable (Fc) that activates Fcγ receptors (FcγRs) of leukocytes and the C1 component of complement. One glycosylation site at asparagine 297 (*N*-linked) within the Fc region of IgG exists, in contrast to the vast array of other immunoglobulin isotypes which have more than one glycosylation site within the Fc region, [37]. The hypervariable region of the antigen-binding fragment (Fab) of IgG has also been shown to contain *N*-glycans [38]. Notably, SDS-Page on IGEP showed traces of bands between the 116 and 55 kDa markers that could be IgA components. Increased levels of secretory IgA (SIgA) in stools have been associated with infant formula supplemented with probiotics [39]. The commensal *Bacteroides fragilis* has been previously demonstrated to modulate binding of IgA to the intestinal mucosa suggesting that IgA may facilitate host-microbial symbiosis [40]. Additionally, SIgA has been shown to bind to mucin producing HT29-MTX (HT-29 cells treated with methotrexate). This binding ability was significantly less in non-mucin producing HT-29 cells [41]. In the current study, we did not use mucin producing HT-29 cells. Moreover, while trace levels of IgA may be present, IgG appeared to be the dominant Ig. Interestingly, while the percent of IgA in bovine blood serum is lower (1.6%) than that of bovine milk (9.9%), the actual concentration of IgA is lower in bovine milk (0.08 g/L) than in bovine blood serum (0.37 g/L) [42]. Thus, while further powder characterization of the IGEP is required to fully identify the active component, considering the concentrations of IgA present in bovine milk and the results obtained via SDS-Page, it may be more likely that IgG may be modulating the observed effects, however, isotyping is required to confirm this. Nonetheless, as periodate treatment abolished the increased adhesion of *B. infantis*, the glycan

component of IGEP is likely to be responsible at least in part for the strain's enhanced adhesion to the HT-29s cells.

The importance of milk protein glycosylation on biological function has been shown previously using glycomacropeptide (GMP). GMP, which promotes the growth of *B. infantis*, lost its prebiotic effect following periodate treatment [43]. Additionally, GMP's ability to prevent *Escherichia coli* and *Salmonella enteritidis* intestinal infection was reduced following sialidase treatment and abolished following periodate oxidation [44]. Notably, bovine IgG-glycans contain fucose, galactose, and mannose structures in addition to sialic acids such as *N*-acetylneuraminic acid (Neu5Ac) and the non-human *N*-glycolylneuraminic acid (Neu5Gc) [45]. While IGEP bound glycans may directly influence the adhesion of *B. infants*, it is important to note that *B. infantis* encodes an endoglycosidase, EndoBI-1 (glycosyl hydrolase family 18) which is capable of cleaving *N*-linked oligosaccharides on glycoproteins. This activity could potentially release oligosaccharides bound to glycoproteins in IGEP [46], resulting in the production of intact free oligosaccharides that could then directly influence the adhesion potential of the strain. Notably, in this study, bovine serum-derived IgG failed to induce any increase in the adhesion phenotype of *B. infantis*, suggesting that bovine milk-derived IgG may possess different glycan structures. Glycosylation is known to be cell type specific [47, 48] and IgG produced in different cells may contain differences in their oligosaccharide chains. In addition, the glycosylation pattern of milk-derived IgG is known change over lactation [45] and may in part explain the different activities observed between serum and milk-derived IgG.

IGEP may be influencing adhesion in a number of ways. For instance, IGEP may induce the increased expression of adhesion factors by the bacteria. Our group previously demonstrated that milk oligosaccharides resulted in the increased adhesion of *B. infantis* to HT-29 cells. Exposure to the milk oligosaccharides was associated with an up-regulation of genes involved in adhesion e.g. DNA-binding protein-ferritin, GroEL, DnaK and TadE and a down-regulation of genes involved in complex oligosaccharide metabolism [17]. IGEP may also aid the adhesion of *B. infantis* to the host cells by acting as a bridge between the bacteria and the host cells. For example, bovine lactoferrin has been shown to function as a molecular bridge for internalization of *Streptococcus uberis* into bovine mammary epithelial cells [49]. Less likely, is the possibility that residual IGEP is modulating the cell line itself. This is less likely as IGEP was washed from bacterial cells prior to exposure to intestinal cells. Our group has

however, recently shown that IgG isolated from bovine colostrum and milk does modulate the cell surface of HT-29 cells in turn enhancing the adhesion of bifidobacteria to HT-29 cells [50].

A variety of milk-derived proteins have been shown to possess anti-infective activities. For example, lactoferrin has antimicrobial activity [51] and displays anti-adhesive effects against pathogens such as *E. coli* and enteropathogenic Y*ersinia* [52, 53]. Lactoferrin also has been reported to promote the growth of various lactobacilli [54]. Previously, milk fat globule membrane glycoproteins have been shown to have anti-adhesive properties against a range of pathogens including rotavirus and various enteric bacteria [55, 56]. Notably, sialic acid on some milk glycoproteins have been implicated in binding *E. coli* and *Salmonella enteritidis* [57, 44], increasing growth of the bifidobacterial spp. such as *B. breve*, *B. bifidum, B infantis* [58] and preventing *Helicobacter pylori* colonization [59]. IgG in milk is responsible for agglutinating bacteria, neutralising toxins, deactivating viruses, binding pathogens such as *Shigella flexneri, E. coli, Clostridium difficile*, and rotavirus [60, 61] and developing an environment favourable for the growth of health-promoting bacteria [62]. In this study, the 0.1% ± 0.01 of *C. jejuni* from the original inoculum adhered to the HT-29 cells which is similar to the 0.25% ± 0.05 observed in a previous study [19]. Interestingly no protective effect was observed for IGEP alone which was unexpected as IgA in breast milk has been associated with lower rates of *Campylobacter* in children [63]. This may be a result of the short exposure period of the pathogen to the IGEP throughout the anti-infective assays.

C. jejuni infection is prevalent in commercial broiler chickens in addition to swine, cattle and contaminated water, and these sources act as a major reservoir that can result in human infection [64]. In this study, a combination of *B. infantis* and whey protein derived IGEP resulted in a 48% decrease in pathogen colonization following prior treatment of the HT-29 cells with IGEP treated *B. infantis.* Notably, the non-supplemented *B. infantis* control demonstrated no protective effect against *C. jejuni* colonization, in line with previous results that suggested a critical number of attached bifidobacteria are required to competitively exclude *C. jejuni* [19]. IGEP treated *B. infantis* may result in the appropriate numbers interacting with the cell which in turn may result in modulation of the HT-29 cells. *C. jejuni* is known to enter the gut epithelial cells and impair intestinal barrier function through cleavage of occludin by serine protease HtrA [65]. Notably, HMO grown *B. infantis* is associated with a higher adhesion rate to HT-29 cells and has been shown to influence the expression of the epithelial cell surface receptors and immune responses [16]. In addition, occludin expression in Caco-2 cells is higher when exposed to HMO grown *B. bifidum* [16]. Thus, protection

against *C. jejuni* colonisation could result from modulation of gene expression on the epithelial cells through exposure to IGEP-treated *B. infantis*. Previously we demonstrated that an increase in adhesion induced by GMO had a protective effect against *C. jejuni* colonization of HT-29 cells in pathogen exclusion assays [19]. While no direct anti-infective effect was observed in this study, the concept that probiotics and pathogenic strains compete in the gastrointestinal tract for host cells surface receptors and nutrients has been well documented [66, 67]. Strains such as *Lactobacillus* spp., i.e., *acidophilus, casei, crispatus, gasseri, helveticus, pentosus, plantarum, rhamnosus*, and *salivarius* have been suggested to exhibit anti-*Campylobacter* activities *in vitro* and *in vivo* [64, 68]. *In vivo* studies using galactooligosaccharides synbiotics have shown potential with *B. longum* PCB 133 in reducing *C. jejuni* infection in poultry [69]. In addition, a microencapsulated *B. longum* PCB133 and xylooligosaccharides synbiotic can protect against *C. jejuni* infection in poultry meat [70] and *L. salivarius* subsp. *salicinius* combined with 0.04% mannan oligosaccharides have demonstrated a decrease (3 log) in broiler chicken cecal *Campylobacter* levels [71]. While previous studies focused on pathogen protection using free glycans in synbiotics combinations, the use of milk glycoproteins also holds promise.

This study is particularly relevant considering the prevalence of *Campylobacter* and the fact that it is recognized as one of four key global causes of diarrheal diseases. Indeed, it is considered to be the most common bacterial cause of human gastroenteritis in the world and has an associated risk of secondary post-infection diseases such as irritable bowel syndrome and Guillain-Barre syndrome [72, 21, 73]. Further studies with other probiotic strains and pathogens are required to determine if this bioactivity can be extended to protect against other diseases.

IGEP did not demonstrate any significant influence on the growth of *B. infantis* or metabolite production following 24 h incubation with the strain and no statistically significant difference between the supplemented sample and non-supplemented sample were observed. However, metabolite analysis showed growth on IGEP resulted in slightly higher levels of acetate, lactate and decreased levels of formate (Table 1). Additionally, low levels of ethanol, which were not detected in the non-supplemented sample were also observed. This may suggest that the level of glycans present in IGEP are too low to support growth. Notably, previous work in our lab has shown that growth on oligosaccharides isolated from goats milk results in slight ethanol production by bifidobacteria [19]. Therefore, this result is not surprising as the presence of bifidobacteria in the gut has been demonstrated to influence the production of formate, acetate,

ethanol and lactate [25], and some bifidobacteria convert pyruvic acid into formic acid and ethanol as opposed to lactic acid, yielding an additional ATP [75]. Overall, these data indicate that IGEP did not significantly influence *B. infantis* metabolite production *in vitro* and that the altered adhesion of *B. infantis* when cultured in the presence of IGEP was due to another mechanism.

5. Conclusions

Exposure of *B. infantis* to IGEP resulted in an increased attachment to HT-29 cells and was at least impart mediated by IGEP glycosylation. This synbiotic combination ultimately led to protection against *C. jejuni* colonization of HT-29 cells *in vitro*. This is a particularly beneficial outcome as *Campylobacter* is recognized in both developed and developing counties as a leading cause of diarrhoeal diseases and is most common in children younger than five years of age [72, 76]. Animal reservoirs are the most common source of *Campylobacter* infection, with 60% to 80% of broiler chicken flocks estimated to be contaminated with *Campylobacter* at slaughter [77], and it has been suggested that 48% of *Campylobacter* infections are related poultry exposure [78]. Consequently, host protection via competitive exclusion by commensals has the potential to reduce the rising numbers of *Campylobacter* infections in humans and could be extended for use in livestock animal feed to reduce the rates of transmission from such reservoirs. Moreover, the use of synbiotics such as IGEP in combination with probiotic bacteria has potential to be used as a means to protect against other gastrointestinal pathogens. Overall, this study highlights the benefits of using a symbiotic to promote overall gut health and indicates that such combinations may hold superior health-promoting properties when compared with either probiotics or prebiotics used alone.

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Figures and Tables

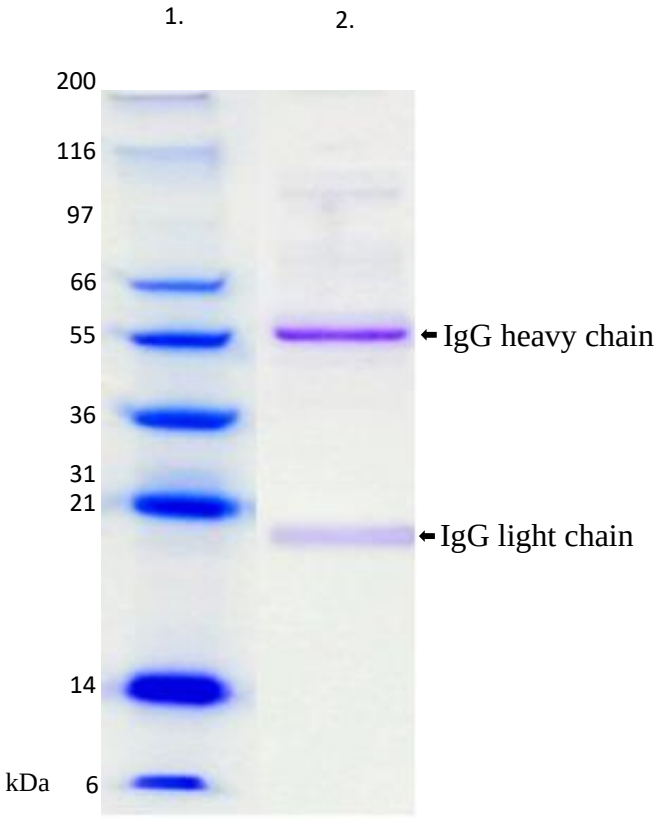

Figure 1. Protein characterization of the IgG-enriched powder (IGEP) by NuPAGE 4-12% SDS-PAGE separation with Coomassie Blue staining. Lane 1, molecular mass marker; lane 2, reduced IGEP.

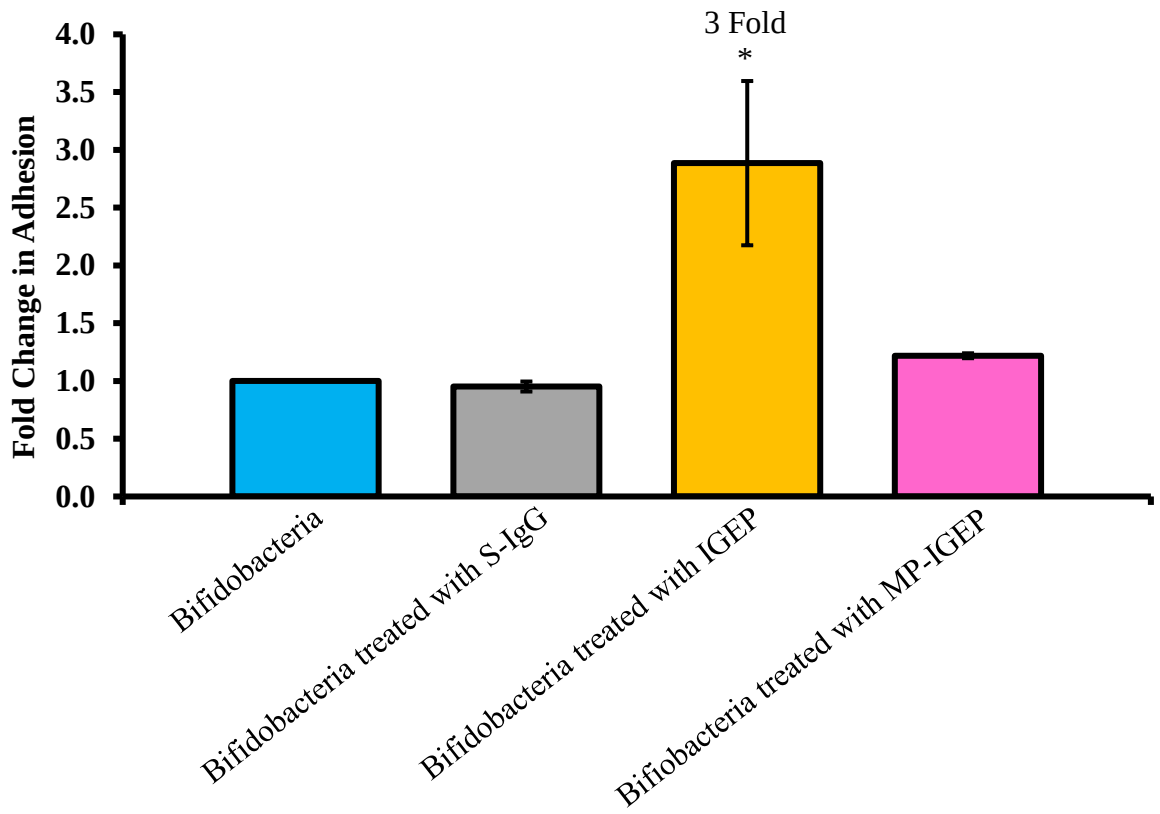

Figure 2. Adhesion of *B. infantis* to HT-29 cells following incubation with bovine serum-derived IgG (S-IgG), whey-derived Ig enriched powder (IGEP) and metaperiodate-treated IGEP (MP-IGEP). Results are from three biological replicate experiments each performed in technical triplicate, except for S-IgG and MP-IGEP treatment which were assayed in technical triplicate using one biological replicate. Results were calculated as the percentage of adherent cells = (CFU/mL of recovered adherent bacteria ÷ CFU/mL of inoculum) × 100 and bars represent the fold-change relative to percent adhesion of control, with error bars representing +/- one standard deviation. The student *t*-tests were used to test for significance in compassion to the control (Bifidobacteria). *, *p*-value <0.05; n.s., not significant.

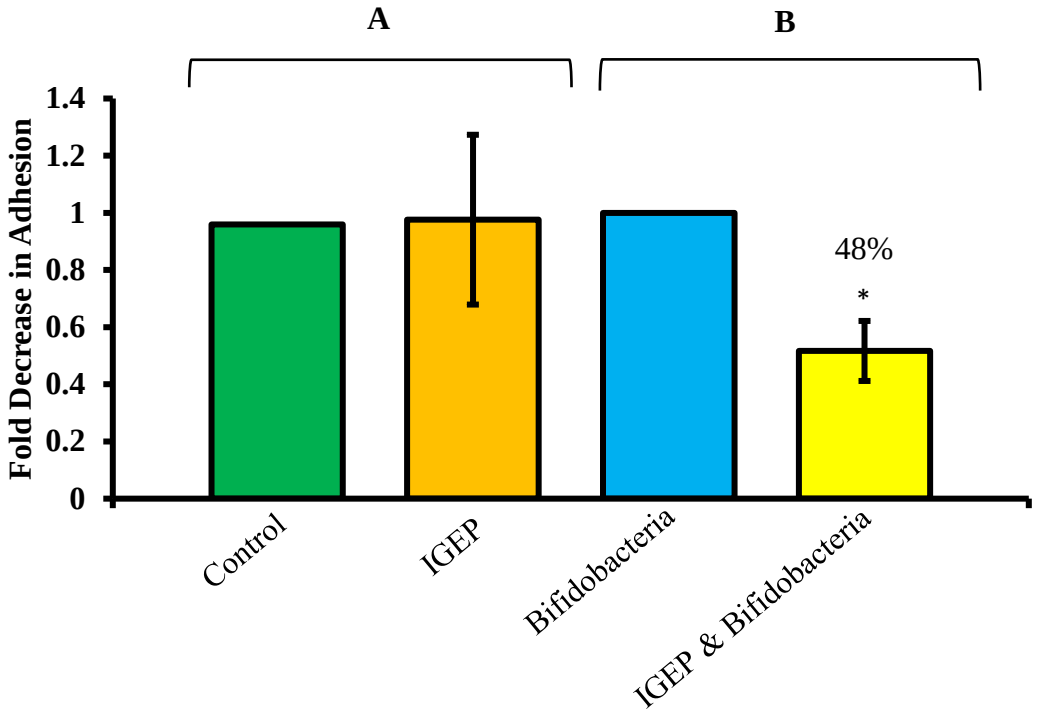

Figure 3. Anti-infective assays (A) demonstrating *C. jejuni* 81-176 adhesion in the absence (green) and presence (orange) of IGEP. (B) Competitive exclusion assays demonstrating *C. jejuni* 81-176 adhesion to HT-29 cells following pre-treatment of the HT-29 cells with *B. infantis* 15697 (blue) and *B. infantis* 15697 pre-treated with IGEP (yellow). Results are represented as the average of one biological replicate experiment performed in technical triplicate (A), and the average of three biological replicate experiments performed in technical triplicate (B) and are represented as a percentage of adherent cells = (CFU/mL of recovered adherent bacteria ÷ CFU/mL of inoculum) × 100 and graphed as fold-change relative to percent adhesion of control, with error bars representing +/- one standard deviation. The student *t*-tests were used to test for significance in comparison to the control (A), or in comparison to un-treated bifidobacteria (B). *, *p*-value <0.05; n.s., not significant.

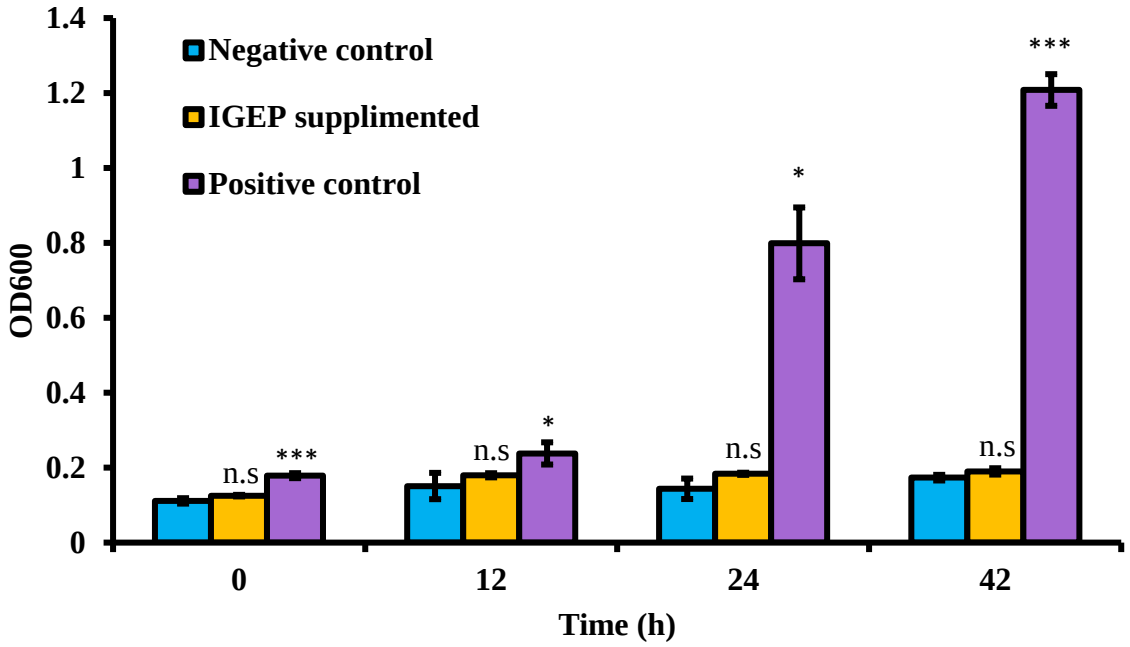

Figure 4. Growth of *B. infantis* in carbohydrate-free media supplemented with IGEP over 48 h (IGEP supplemented) compared to a positive control supplemented with glucose and a negative control of non-supplemented carbohydrate-free media. Optical density (OD_{600nm}) was measured at 0, 6, 12, 24 and 48 h post-inoculation. Results are represented as the average of three replicates, with error bars representing +/- one standard deviation. The student *t*-tests were used to test for significance in comparison to the negative control. *, *p*-value <0.05; ***, *p*-value <0.002; n.s., not significant.

Table 1. Metabolite analysis by *B. infantis* following 24 h incubation with IGEP.

Concentration (mM)	**Control**	**IGEP**
Acetate	2.42	2.70
Lactate	0.62	0.77
Formate	0.32	0.26
Ethanol	ND	1.15

Concentration values (mM) are the average of three biological replicates, each analyzed in technical triplicate. ND; Not detected.

Supplementary Materials

Table S1. Average percent adhesion of initial inoculum of *B. longum* subsp. *infantis* ATCC 15697 which attached to HT-29 cells following incubation with bovine serum-derived IgG (S-IgG), whey-derived Ig enriched powder (IGEP) and metaperiodate-treated IGEP (MP-IGEP) (Corresponding to Figure 2).

	Control	**Treated**
***B. infantis* treated with S-IGEP**	0.49 ± 0.03	0.47 ± 0.02
***B. infantis* treated with IGEP**	0.06 ± 0.02	0.20 ± 0.02
***B. infantis* treated with MP-IGEP**	0.22 ± 0.07	0.26 ± 0.01

Chapter V

A Low Molecular Weight Fraction of Camel Milk; Characterization and Bioactivity

Erinn M. Quinn, Helen Slattery, Lokesh Joshi and Rita M. Hickey

Acknowledgements: Erinn Quinn conducted all experiments with the exception of exclusion chromatography and Dionex ICS-3000 Series HPLC which was conducted by Helen Slattery. Jonathan Lane provided the camel milk fraction and King Faisal University (Saudi Arabia) supplied the camel milk. Rita Hickey assisted with manuscript preparation.

Abstract

Recent years have seen a major increase in commercial camel milk production, due in part to its nutritive, therapeutic and antimicrobial properties. In this study, we isolated and characterized a minimally processed low molecular weight fraction of camel milk (CMF) rich in lactose (77%), oligosaccharides and containing trace amounts of low molecular weight peptides. We then examined if the CMF had the ability to increase the adhesion of a range of bifidobacteria to intestinal HT-29 cells *in vitro*. Seven bifidobacterial strains that are found across the human lifespan were pretreated with CMF and their adhesion to HT-29 cells was evaluated. *Bifidobacterium longum* subsp. *infantis* ATCC 15697, *B. infantis* ATCC 15702 and *B. longum* ATCC 15708 were found attached to the mammalian cells in higher numbers with 1.4-, 1.8- and 3.3-fold increases in adhesion observed respectively after prior exposure to CMF. Further adhesion assays demonstrated lactose was not the causative agent for the increased adhesion observed for ATCC 15708 and ATCC 15697, indicating other components such as oligosaccharides present in CMF may be involved. We then aimed to exploit the increased adhesion as a mechanism for inhibiting pathogen association with intestinal cells. We examined the synergistic effect of CMF-treated bifidobacteria on preventing the attachment of a highly invasive strain of *Campylobacter jejuni* to intestinal HT-29 cells. Unlike CMF treated *B. longum* ATCC 15708 which did not affect *C. jejuni* adhesion, CMF treated *B. infantis* ATCC 15697 decreased the adherence of *C. jejuni* to the HT-29 cells by an average of 43% compared to the control. This study builds a case for the use of camel milk components as functional ingredients that can potentially increase numbers of health-promoting bacteria in the gut.

1. Introduction

In 2013, the United Arab Emirates (UAE) and Dubai were granted final approval to become the first Middle Eastern country to export camel dairy products into the European Union (EU). In terms of worldwide domestic animal dairy product production, camel milk is the fifth most produced and is typically farmed in hot regions [1]. In terms of yield, 3 to 10 kg of camel milk is produced per day during a lactation period of 12 to 18 months [2]. Dromedary camels make up approximately 95% of the population of large camelids (dromedary and Bactrian) [3] and the Food and Agriculture Organizations live animals statistics have reported that the worldwide camel population of ~35 million is mainly located in Somalia, Sudan, Niger, Kenya, Chad, Ethiopia, Mali, Mauritania, and Pakistan [4, 5, 1]. Dromedary camels are an excellent candidate species for milk production due to their unique physiology and ability to resist climate change [6]. Dromedary camel milking machinery has been developed and advanced over the last 20 years, resulting in increased production yield, in addition to improved milk hygiene [7, 8, 9]. Konuspayeva et al. [10] used meta-analysis to demonstrate that dromedary camel milk contains on average 3.35% protein, 3.82% fat, 4.46% lactose, 0.79% ash, and 12.5% dry matter, which is similar to levels present in goat and bovine milk. Notably, the water content of dromedary camel milk is comparable to that associated with human milk [11] and does not change under extreme heat-stress conditions [12, 13]. Global warming has been suggested to be implicated in the increase in camel rearing particularly in Africa [14, 4], and thus their popularity is likely to continue increasing. Numerous studies have indicated the benefits of dromedary camel milk, which has been implicated in the alleviation of diseases such as hepatitis, jaundice, tuberculosis, dropsy, asthma, anaemia, piles, type 1 diabetes, gastrointestinal ulcers and gastrointestinal viruses [15, 16, 17, 18, 19]. Additionally, its protein components have been suggested to protect against bacterial and viral infections [20, 21].

Indeed, mice fed camel milk have been shown to have a better survival rate against *Salmonella enterica* infection than those fed bovine milk, suggesting potential superior bioactivities [22]. Currently, most infant milk formulas are made from bovine milk which contains low levels of oligosaccharides (0.03 g/L) in comparison to human milk (5-25 g/L) [23, 24, 25]. Bovine milk and human milk share a minimum of 10 common oligosaccharide structures [25] which could imply common functionalities [26, 27, 28, 29] and camel milk also contains several oligosaccharides found in human milk. Forty-eight different oligosaccharide structures, 7 of which are fucosylated, a characteristic associated with human milk have been identified in dromedary camel milk [30]. Furthermore, human milk oligosaccharides (HMO)

with fucose linkage by α1,2-glycosidic bonds have been shown as prebiotic, facilitating the growth of *Bifidobacterium longum* subsp., *B. bifidum* subsp., and *B. breve* subsp., which are capable of hydrolyzing fucosylated HMO [31, 32].

High levels of beneficial bacterial species such as bifidobacteria are present in the breastfed infants gut and are known to inhibit the growth of pathogenic microorganisms, modulate the mucosal barrier function, and promote inflammatory and immunological responses [33]. Previously, we demonstrated increased adhesion of *B. infantis* to intestinal cells following exposure to a goat milk oligosaccharides and a bovine whey derived powder enriched in immunoglobulin G [22]. Subsequent studies with the goat milk oligosaccharides (GMO) indicated a prophylactic protective effect against *C. jejuni* colonisation when HT-29 cells were pre-exposed to GMO treated bifidobacteria [34]. These studies indicate that fractions derived from domestic animal milks may result in an increase in the levels of bifidobacteria colonising the gut. In the current study, we isolated and characterised a low molecular weight minimally processed fraction of camel milk (CMF). CMF was generated on the basis that production was less time-consuming and more economically viable when compared to isolating pure oligosaccharides. We then assessed the ability of CMF to improve the attachment of a variety of bifidobacteria intestinal cells. The proficiency of the CMF treated bifidobacteria to competitively exclude an invasive strain of *Campylobacter jejuni* to intestinal cells was then examined.

2. Materials and Methods

2.1 Oligosaccharides Standards

The oligosaccharide standards; 2'-Fucosyllactose (2'-FL), 3-Fucosyllactose (3-FL), LS-tetrasaccharide c (LSTc), 3'Sial-*N*-acetyllactosamine (3'-SNL), Disialyllacto-*N*-tetraose (DSLNT), Disialyllactose (DSL), Lacto-*N*-neohexaose (LNnH), Lacto-*N*-tetraose (LNT), Lacto-*N*-neotetraose (LNnT), Lacto-*N*-hexaose (LNH), *N*-Acetylneuraminic acid (Sialic Acid), 3'-Sialyllactose (3'-SL) and 6'-Sialyllactose (6'-SL) were purchased from Carbosynth Ltd. (Berkshire, UK). Lactose was obtained from VWR (Dublin, Ireland) and glucose and galactose were obtained from Merck (Darmstadt, Germany).

2.2 Generation of Camel Milk Fraction

To generate the camel milk fraction (CMF), mature milk from dromedary camels was kindly provided by King Faisal University (Saudi Arabia) and was stored at −80 °C. Extraction of the low molecular weight fraction was carried out as previously described with modifications [28, 22]. In brief, the milk was initially defatted by centrifugation at 4 °C, for 20 min at 3850 × *g*. Caseins were then precipitated by adjusting the pH to 4.6, followed by centrifugation at 3850× *g* at 25 °C for 20 min. After neutralisation (by adjusting the pH to 6.7), large peptides and whey proteins were removed by ultrafiltration using a 5 kDa molecular weight cut off (MWCO) membrane (Millipore Helicon S10 Spiral Cartridge; Millipore). The permeates were freeze-dried. The fraction was collected and analysed for monosaccharides, oligosaccharides, proteins and peptides as detailed below.

2.3 Characterisation of the Camel Milk Fraction

Carbohydrate content

Oligosaccharide analysis of CMF was performed as described previously [22] using a Dionex ICS-3000 Series HPLC system (Dionex Corporation, Sunnyvale, CA, USA) equipped with an electrochemical detector. Samples were separated on a CarboPac PA100 column (250 × 4 mm) equipped with a guard column using the following gradient; 95% 100 mM NaOH (Eluent A) and 5% 100 mM NaOH with 500 mM NaAc (Eluent B) for 3 min, 88% eluent A and 12% eluent B for 10 min, and 50% eluent A and 50% eluent B for 17 min for a 30 min separation at a flow rate of 1ml/min. The column was re-equilibrated for 15 min with 95% eluent A and 5% eluent B after each separation. Oligosaccharide components in the CMF were identified and quantified by comparing to a standard mixture of thirteen known oligosaccharides at different concentrations. Disaccharide and monosaccharide quantification was performed using

a Waters Alliance 2695 HPLC equipped with a Refractive Index Detector. Lactose, glucose, and galactose were quantified using an Aminex HPX-87C column (300 X 7.8 mm) (Bio-Rad, UK) using a refractive index detector at a flow rate of 1ml/min. The eluant used was 9mM sulphuric acid for 30 min [35].

Peptide content

Peptide analysis of CMF using size exclusion chromatography was performed using a TSK G2000sw column (300 X 7.5mm) and a TSK G2000swxl column(300 x 7.8) in series (Tosu Hass, Japan) attached to a Waters Alliance 2695 (Waters Corporation, Milford, MA, USA) HPLC system. (Waters Corporation, Milford, MA, USA) The flow rate was 1 mL/min using 30% (v/v) acetonitrile containing 0.1% as TFA mobile phase. The eluent was monitored constantly at A214 nm using a Waters model 441 fixed wavelength detector. The 20 µl sample at a concentration of 1 mg/mL was injected into the column. Data was collected and analysed using Empower 3RD data analysis system (Waters Corporation, Milford, MA, USA). A molecular weight calibration curve was made from the average retention times of standard proteins and peptides and this was used to determine the molecular weight of the peptides in the CMF sample.

Mineral content

Ash analysis was carried out on the CMF to determine the amount of mineral content in the sample. An ash crucible was heated at 104 °C in an oven to remove any moisture, cooled in a desiccator and weighed. 10 mL of the CMF sample at a concentration of 5 mg/ml was added to the crucible and the weight of both dish and sample was taken. The sample was placed on a hotplate and gently heated until all the water had evaporated. The crucible was then placed in a muffle furnace overnight at 550 °C where the CMF sample was turned to ash. The dish was again placed in a desiccator cooled and weighed. The percentage of ash was then calculated as follows: Ash % = [weight after furnace – weight of container] ÷ weight of sample X 100.

2.4 Bifidobacterial Strains and Culture

The bifidobacterial strains used in this study are listed in Table 1 and culture conditions were maintained as previously described [36, 22, 34]. Typically, strains were taken from -80ºC stocks and streaked onto DeMan Rogosa Sharpe (MRS) (Difco, Sparks, MD, USA) agar plates supplemented with 0.5% L-cysteine hydrochloride (Merck, Darmstadt, Germany). For all strains, a single colony was taken from each plate and inoculated into MRS broth. Bacteria were incubated anaerobically using the Anaerocult A system (Merck, Darmstadt, Germany) at

37 °C for 48 h. Stocks were made using 50:50 MRS broth and glycerol (Merck)and were stored at -20 °C or -80 °C.

2.5 Mammalian Cell Culture

HT-29 cells were prepared as previously described [22]. Typically, HT-29 cells were grown in McCoy′s 5A modified medium (Merck) supplemented with 10% fetal bovine serum (FBS), which were maintained in 75 cm^2 tissue culture flasks and incubated at 37 °C in 5% CO_2 in a humidified atmosphere. Once the cells were confluent (approximately 80–90%), cells were passaged into 12 well plates. For the assays, cells in 75 cm^2 flasks were trypsinised and seeded into a 12 well tissue culture plate (Sarstedt Ltd., Wexford, Ireland) at a density of 1×10^5 cells/mL between passages 15–21 and cells were used once fully confluent (approximately 4×10^6 cells/well at day 5–7). The media was changed every other day and supplemented with 2% FBS 24 h prior to use.

2.6 Exposure of Bacteria to Camel Milk Fraction

Exposure of the bacteria to CMF was performed as previously described [36, 22, 34]. Bacteria were used at mid-exponential growth phase (18 h) and the OD_{600nm} was adjusted to 0.3 at the start of the assay, after which the cells were cultured for 1.5–2 h and used once an OD_{600nm} of 0.5 was reached. Bacterial cells were washed twice with PBS by centrifugation. Cell pellets were re-suspended to a final OD_{600nm} of 0.5 in McCoy's 5A tissue culture media supplemented with 2% FBS and 5 mg/mL CMF. To determine if the high levels of lactose in the CMF may have contributed to the increased adhesion, *B. infantis* ATCC 15697 and *B. longum* ATCC 15708 were exposed to 3.85 mg/mL of lactose (the amount present in 5 mg/ml CMF) and the assays repeated as above. A non-supplemented negative control was also included for all assays. Bacterial suspensions were then incubated for 1 h at 37 °C under anaerobic conditions. Following this, bacteria were harvested by centrifugation (3850 × *g*, 5 min), the supernatants removed, and pellets were washed three times in PBS and then re-suspended in non-supplemented McCoy's media prior to use in the adhesion assays.

2.7 Adhesion Assays

Adhesion assays were performed as previously described [36, 22]. HT-29 cells were washed twice with PBS, and 500 µL of the bacteria and media suspensions were added to the wells. Bacterial cells were incubated with the HT-29 cells for 2 h at 37 °C under anaerobic conditions using an Anaerocult A system (Merck). The HT-29 cells were then washed five times with PBS to remove non-adherent bacteria. HT-29 cells were then lysed with 500 µL of 1% Triton™

X-100 (Merck) for 5 min at 37 °C. The lysates were serially diluted and enumerated by spot-plating on MRS plates. The adhesion of the bacteria was determined as the percentage of original inoculum which attached, thus accounting for variations in the starting inoculum. Percentage adhesion = (CFU/mL of recovered adherent bacteria/CFU/mL of inoculum) × 100.

2.8 Pathogen Strain and Culture

Campylobacter jejuni 81-176 (*C. jejuni*) is a well-characterized, mobile flagellated invasive strain which has been used in many previous studies [17, 18]. The pathogen was stored in Mueller Hinton broth (Oxoid, Ireland c/o Fannin Healthcare) containing 50% glycerol at -80 °C and cultured directly from storage onto Mueller-Hinton agar plates. The pathogen was grown under microaerophilic conditions generated using CampyGen gas packs (Oxoid), for 48 h at 37 °C. Prior to pathogen inhibition assays, *C. jejuni* 81-176 was grown on Mueller-Hinton agar and then transferred to biphasic media in 25 cm^2 tissue culture flasks (Corning, New York, USA) consisting of Mueller Hinton agar supplemented with *Campylobacter* selective supplement (Skirrow), (Oxoid) and 6 ml of McCoy's 5A media (Merck) supplemented with 2% FBS. The flask was then incubated for 24 h under microaerophilic conditions at 37 °C.

2.9 Exclusion Assay

The exclusion assay screen was performed as previously described [34] with minor modifications. Exposure of bifidobacterial strains; *B. infantis* ATCC 15697 and *B. longum* ATCC 15708 to 5 mg/mL CMF was performed as described above and this suspension was subsequently incubated with the HT-29 cells for 2 h. A non-supplemented control was also included. Non-adherent bacteria were removed from the cells after which the cell line was challenged with *C. jejuni*. To do this, the pathogen was harvested from the biphasic medium, washed twice in non-supplemented McCoy's, and diluted to an OD_{600nm} of 0.3. From this suspension, 500 µl was then added to each well, and cells were incubated under anaerobic conditions for 3 h at 37 °C. Cells were then washed five times with PBS, lysed with 0.1% Triton X-100 in PBS and plated onto supplemented Mueller-Hinton agar. Mueller-Hinton plates were incubated under microaerophilic conditions for 72 h at 37 °C after which bacterial CFU/mL were enumerated. The assays were performed in triplicate on one occasion. The exclusion of *C. jejuni* was determined as the average CFU/mL of recovered adherent bacteria. The percentage decrease in *C. jejuni* adhesion was calculated as the difference in *C. jejuni* CFU/mL between non-supplemented and CMF treated bifidobacteria.

2.10 Statistical Analysis

Graphs were drawn using Microsoft Excel. The results are presented as the mean ± standard deviations of replicate experiments, and the student *t*-tests were used to determine statistically significant results for experiments conducted in triplicate on three occasions. For all experiments, $p<0.05$ was considered significant.

3. Results and Discussion

3.1 Characterisation of the Camel Milk Fraction

The camel milk used in this study was previously characterized in terms of its oligosaccharide content [30] and was found to contain a total of forty-eight structures of which twenty-three belonged to the neutral oligosaccharide fraction and seven were identified as fucosylated [30]. In this study, we identified oligosaccharide from the same batch of camel milk and quantified some of the major structures, which included 3-FL, LSTc, 3'-SNL, DSLNT, DSL, LNnH, LNT, sialic acid, 3'-SL and 6'-SL, the levels of which are quantified in Table 2. Notably, fucosylation is a key feature of human milk oligosaccharide pools accounting for 70%, with lacto-*N*-biose (type I) structures (Gal(β1–3)GlcNAc) predominating over structures containing *N*-acetyllactosamine (type II) (Gal(β 1–4)GlcNAc) [29, 34]. Considering the presence of fucosylated structures in camel milk, it may have bioactivities similar to those associated with human milk. Ninety-eight percent of the acidic oligosaccharides characterized previously were decorated with Neu5Ac and trace quantities of a galactotriose decorated with hexuronic acid were also observed [30]. Animal-derived oligosaccharides are predominantly sialylated, containing *N*-acetylneuraminic acid (Neu5Gc) a non-human *N*-glycolylneuraminic acid, and/or *N*-glycolylneuraminic acid (Neu5Ac) [30, 31]. A key feature of the CMF was the presence of lactose which was identified as the primary component accounting for 77% of the powders total composition. This was expected as the powder was not highly processed and was not treated to remove lactose. While lactose is present in high concentrations, *in vivo,* lactose would be mostly digested, while oligosaccharides would remain undigested and thus reach the colon intact. Notably, ash analysis indicated that minerals potentially including; sodium (Na), potassium (K), calcium (Ca), magnesium (Mg), phosphorus (P), iron (Fe), zinc (Zn), copper (Cu), and manganese (Mn) accounted for 17.9% of the total powder. While levels of peptides within the sample were not quantified, various peptides of different molecular weights were detected using size exclusion chromatography and it was determined that 98.1% of the total peptide content was below 10 kDa (Table 3). These results are not surprising given that camel milk has previously been identified as being high in minerals (sodium, potassium, iron, copper, zinc and magnesium), vitamin C and protective proteins [37].

3.2 Adhesion Assays with Camel Milk Fraction

A variety of milk oligosaccharides have previously been demonstrated to increase the adhesion potential of *B. longum* subsp. *infantis* ATCC 19697 to HT-29 cells [36, 38, 22, 34]. This bioactivity is beneficial as colonization and survival of probiotic strains in the intestinal tract

is particularly reliant on their transient adhesion to the intestinal mucosa [34]. To date, studies have focused on the bioactivities associated with isolated and purified components such as oligosaccharides, and investigations into how less processed milk fractions may influence the adhesion of beneficial strains to the intestinal epithelial cells have been largely unexplored. In this study, we conducted a screen to assess the ability of the CMF to modulate the adhesion of a panel of different bifidobacterial strains (Table. 1) to HT-29 cells *in vitro*. HT-29 cells were used as a model of the gastrointestinal epithelial cells as they display classical characteristics of small intestinal absorptive epithelial cells once confluent [39, 40, 41, 42, 34]. Notably, out of the seven strains screened against CMF, three showed increases in adhesion (Figure 1**A**). The control strain, *B. infantis* ATCC 15697 displayed the lowest fold increase (1.4 fold). This increase was not expected as *B. infantis* ATCC 15697 is particularly adept at utilizing human milk glycans [43–51] and a variety of other studies have also indicated increased adhesion (up to 9 fold) following pre-treatment of this strain with milk-derived oligosaccharides [36, 22, 34]. The fact that the increase was lower may be due to the high level of lactose in the CMF as lactose has previously been indicated to inhibit the ability of oligosaccharides to increase adhesion of *B. infantis* ATCC 15697 *in vitro* [36]. To test this hypnosis, future studies should isolate purified oligosaccharide from camel milk and conduct adhesion assays to assess if an increase in adhesion occurs in the pure oligosaccharide sample. Furthermore, these adhesion assays should be conducted with the same quantity of camel oligosaccharide spiked with lactose to assess if lactose reduces any activity observed with purified camel milk oligosaccharide. In this study, other strains displaying an increase in adhesion included; *B. infantis* ATCC 15702 (1.8 fold) and *B. longum* ATCC 15708 (3.3 fold). Interestingly, *B. angulatum* ATCC 27535 indicated a decrease (-27%) in adhesion, while *B. animalis* BB12 also showed a trend of decreased adhesion (-37%), however, further biological replicates are required to confirm these results. It has previously been suggested that carbohydrate utilization results in a reduced adhesion phenotype of *B. infantis* ATCC 15697 [36, 22], and thus the increase in growth, may indicate that the strain is focusing on metabolism of carbohydrates. No statistically significant increase in growth was observed under adhesion assay conditions in the presence of CMF for the indicator strain (*B. infantis* ATCC 15697) or highest hit strain (*B. longum* ATCC 15708), however further biological replicate are required to assess the significance of the remaining strains (Supplementary information. Table S1). Additionally, as lactose was the primary component in CMF, we investigated the ability of the equivalent concentration of lactose in the CMF to modulate adhesion of *B. infantis* ATCC 15697 (control strain) and *B. longum* ATCC 15708 (strain showing the highest adhesion following CMF

pretreatment) to HT-29 cells (Figure 1B). Notably, no statistically significant change in adhesion of either strain resulted following pre-treatment with lactose, indicating that lactose was not contributing to the increased adhesion observed in the presence of CMF, and further supporting the possibility that the increased adhesion potential of these strains could be a result of other components in the CMF such as oligosaccharides as demonstrated in previous studies [36, 22, 34].

3.3 Synbiotic Effect of CMF and Bifidobacteria on C. jejuni Adhesion

Recently, a variety of studies have indicated that the adhesion potential of commensal bacteria can be increased using milk-derived components [22, 52, 34, 36, 53, 38]. Furthermore, our group has shown that such synbiotic combinations can be exploited to prophylactically protect against pathogen colonization [34]. In this study, a pathogen exclusion assay was conducted. Pre-treatment of *B. infantis* ATCC 15697 with CMF provided a decrease in pathogen colonisation in comparison to the non-supplemented control (-43%) but not in comparison to the *B. infantis* ATCC 15697 treated control (+8%), (Figure 2A). Surprisingly, although *B. infantis* ATCC 15708 displayed increased adhesion to HT-29 cells (3.3 fold) following treatment with CMF, both *B. infantis* ATCC 15708 and CMF treated *B. infantis* ATCC 15708 showed a decreased trend in pathogen colonisation in comparison to the non-treated control. Notably, no major decrease in pathogen colonisation occurred between the *B. infantis* ATCC 15708 (-21%) and CMF treated *B. infantis* ATCC 15708 (-25%) (Figure 2B) indicating that this trend was a result of direct competition of *C. jejuni* with *B. infantis* ATCC 15708. It may be that the treatment of bacteria with specific milk-derived components increased the expression of specific adhesion sites, and these may vary from strain to strain. In the case of *B. infantis* ATCC 15708, it may be possible that while increased adhesion did occur following treatment with CMF, the specific receptor site may not have been shared with *C. jejuni*. However, further investigations are required to confirm these results and assess the specific receptor sites that facilitated the increased adhesion phenotype of these strains and also to investigate if the increase in adhesion observed here can prevent the colonisation of other pathogens. Overall, it is likely that the pathogen protection observed with CMF treated *B. infantis* ATCC 15697 resulted from less availability of receptor sites for pathogen colonization. Notably, strains such as *B. infantis* ATCC 15697 have been shown to exploit oligosaccharides to modulate adhesion in turn providing prophylactic pathogen protection [34]. These results indicate the potential of CMF and *B. infantis* ATCC 15697 combinations to be used as a preventative of *C. jejuni* infection in risk groups such as infants and the elderly and builds a

case for the use of milk-derived fraction in a less processed form to be used as functional foods. Notably, fucosylated oligosaccharides present in human milk among others have exhibited the ability to bind to enteric pathogens such as *C. jejuni*, *E. coli* and *Streptococcus pneumoniae* [54–59]. Given that CMF is known to contain fucosylated oligosaccharides such as 3-FL (2.91**µg/ml)**, it was surprising that no protective effect against *C. jejuni* colonization was observed when anti-infective assays were performed (Supplementary information, Figure S1).

4. Conclusion

The current findings suggest that camel milk components may directly modulate the adherence of distinct species of bifidobacteria. Furthermore, the low molecular weight fraction of camel milk isolated in this study when combined with *B. infantis* ATCC 15697 provided prophylactic protection against *C. jejuni* colonisation. This study builds a case for the use of minimally processed, economically viable milk fractions in the functional foods industry as a means to increase the levels and variety of bifidobacterial strains in the gastrointestinal tract. Next steps should confirm results obtained in pathogen protection assays. Furthermore, a wider variety of commensal and pathogenic strains should be screened to assess if CMF can increase bifidobacterial adhesion and protect against pathogen colonisation. Moving forward, purified camel milk oligosaccharides should be isolated and their ability to modulate adhesion should be assessed and compared to that of CMF. The ability of CMF to modulate the intestinal microbiota should also be assessed using faecal fermentations. Animal trails with CMF would also provide further insight into the protective effects of such combinations, with the added benefit of lactose digestion which would occur *in vivo*. In this context, low molecular weight fractions may be capable of influencing microbiota homeostasis and promoting health. In the near future, climate change might decrease agricultural areas for animal production worldwide. In these conditions, extensive animal production, for example, bovine production, may become limited to some extent as a result of semi-arid rangelands. In such regions, camels may become an important milk source for humans and as such camel milk warrants further investigation in terms of bioactivity.

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Figures and Tables

Table 1. List of *Bifidobacterium* strains used in this study.

Species and strain	Abbreviation
Bifidobacterium longum subsp. *infantis* ATCC® 15697™	*B. infantis* ATCC 15697
Bifidobacterium longum subsp. *infantis* (Reuter) ATCC® 15702™	*B. infantis* ATCC 15702
Bifidobacterium breve Reuter ATCC® 15698™	*B. breve* ATCC 15698
Bifidobacterium longum Reuter ATCC® 15708™	*B. longum* ATCC 15708
Bifidobacterium angulatum ATCC® 27535™	*B. angulatum* ATCC 27535
Bifidobacterium longum subsp. *longum* Reuter ATCC® 15707™	*B. longum* ATCC 15707
Bifidobacterium animalis subsp. *lactis* BB12	*B. animalis* BB12

Table 2. Carbohydrate and mineral content of CMF.

Milk Component	Level present in CMF (µg/ml)
Lactose	770
3-Fucosylactose (3-FL)	2.9076
LS-tetrasaccharide c (LSTc)	0.2688
3’Sial-*N*-acetyllactosamine (3’SnL)	0.2166
DiSiallacto-*N*-tetraose (DSLnT)	8.6998
Disialyllactose (DSL)	10.3383
Lacto-*N*-neohexaose (LNnH)	0.0523
Lacto-*N*-Tetraose (LNT)	0.3288
Sialic acid	0.1384
3' Sialylactose (3’SL)	0.0027
6' Sialylactose (6’ SL)	0.0011
Minerals content	179.3

Table 3. Peptide profiling of Camel Milk Fraction.

CMF Peak	Molecular Weight	Retention Time	Area	% Area
1	>20kDa	10.950	6989	1.00
2	20-10kDa	14.167	1754	0.25
3	10-5kDa	16.300	4748	0.68
4	5-2kDa	18.000	21426	3.08
5	2-1kDa	19.074	36311	5.21
6	1-0.5kDa	20.167	41926	6.02
7	<0.5kDa	22.228	583435	83.76

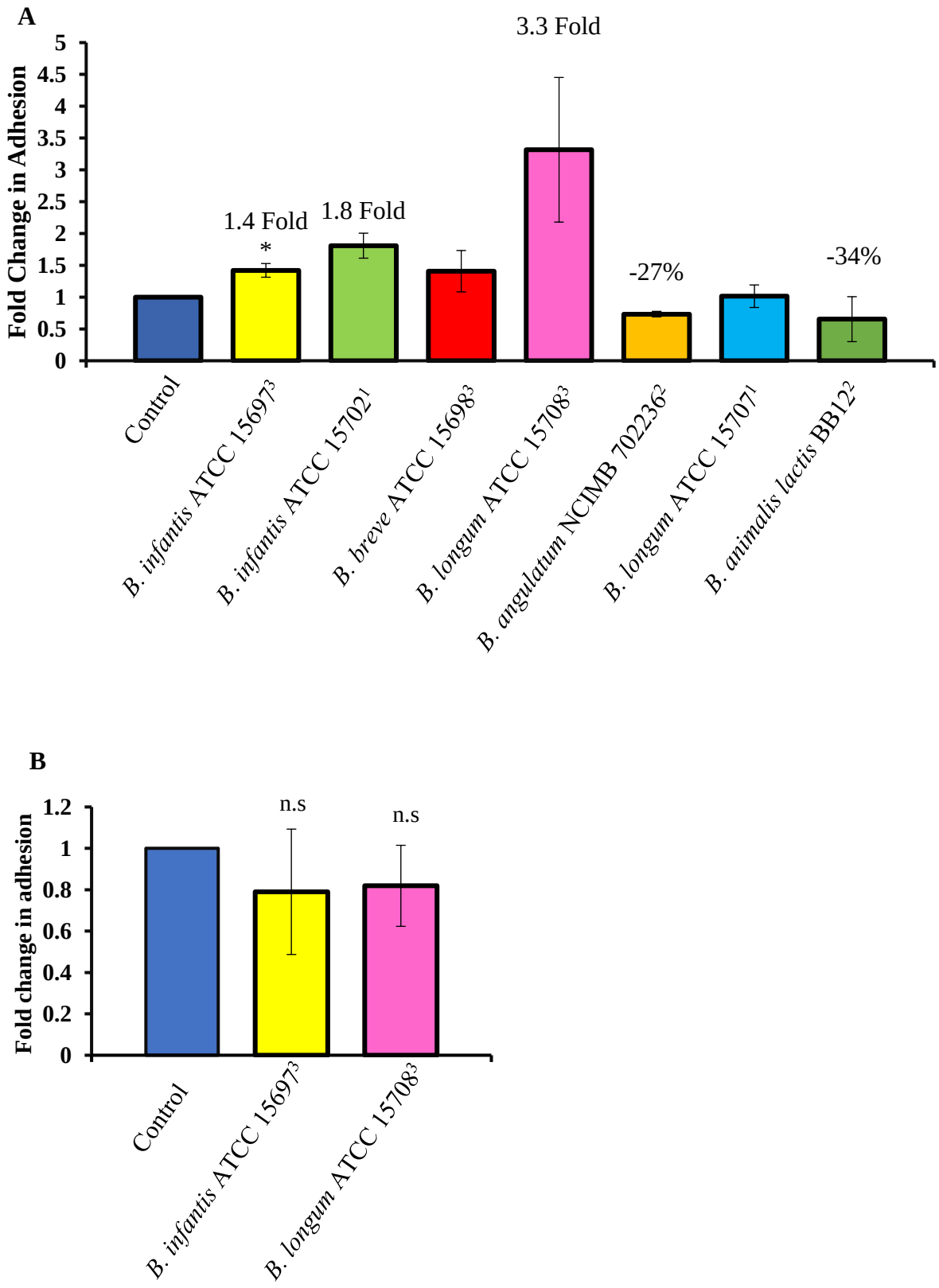

Figure 1. (A) Adherence of the following bifidobacterial strains to camel milk fraction (CMF) treated HT-29 cells (*B. infantis* ATCC 15697, *B. infantis* ATCC 15702, *B. breve* ATCC 15698, *B. longum* ATCC 15708, *B. angulatum* ATCC 27535, *B. longum* ATCC 15707, *B. animalis*

BB12). (B) Adherence of lactose treated *B. infantis* ATCC 15697 and *B. longum* ATCC 15708 to HT-29 colonic epithelial cells. Results are represented as the average of triplicate experiments performed on [1]one, [2]two or [3]three occasions. The control is represented as the normalized average fold and standard deviation of all individual experiments. All results are represented as the percentage of adherent cells = [CFU/mL of recovered adherent bacteria ÷ CFU/mL of inoculum] × 100 and graphed as fold-change relative to percent adhesion of control, with error bars representing the standard deviation. The student *t*-tests were used to determine significance of biological triplicate experiments, * *p*-value: <0.05, n.s: not significant.

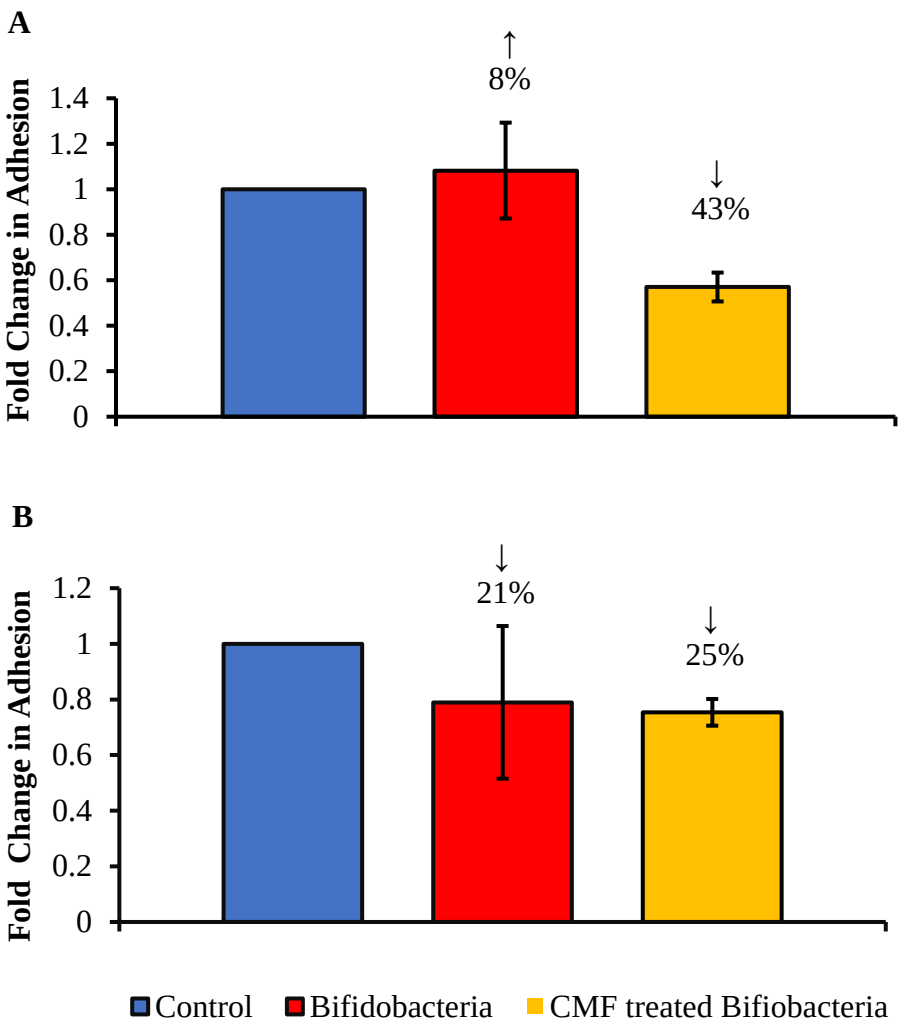

Figure 2. Adherence of *Campylobacter jejuni* 81-176 to HT-29 cells (A) alone (blue), following prior treatment of the HT-29 cells with *B. infantis* ATCC 15697 (red) and following prior treatment of the HT-29 cells with CMF treated *B. infantis* ATCC 15697 (orange) and (B) alone (blue), following prior treatment of the HT-29 cells with *B. longum* ATCC 15708 (red) and following prior treatment of the HT-29 cells with CMF treated *B. longum* ATCC 15708 (orange). Results are represented as the average of triplicate experiments performed on one occasion. All results are represented as the percentage of adherent cells = [CFU/mL of recovered adherent bacteria ÷ CFU/mL of inoculum] × 100 and graphed as fold-change relative to percent adhesion of control, with error bars representing the standard deviation.

Supplementary information

Table SI. Differences in growth (CFU/mL) of the different bifidobacterial strains under adhesion assay conditions following 1 h incubation with CMF. Results are represented as the average of triplicate experiments performed on one[1], [2]two or [3]three occasions.

Bifidobacterial strains	**Non-supplemented**	**Supplemented**
B. infantis ATCC 15697[3]	3.8 X 10^8	4.6 X 10^8
B. infantis ATCC 15702[1]	3.7 X 10^7	3.2 X 10^7
B breve ATCC 15698[3]	8.5 X 10^7	9.6 X 10^7
B. longum ATCC 15708[3]	5.8 X 10^7	3.1 X 10^7
B. angulatum ATCC 27535[2]	2.2 X 10^7	3.0 X 10^7
B. longum ATCC 15707[1]	4.7 X 10^7	4.4 X 10^7
B. animalis lactis BB12[2]	2.6 X 10^7	3.8 X 10^7

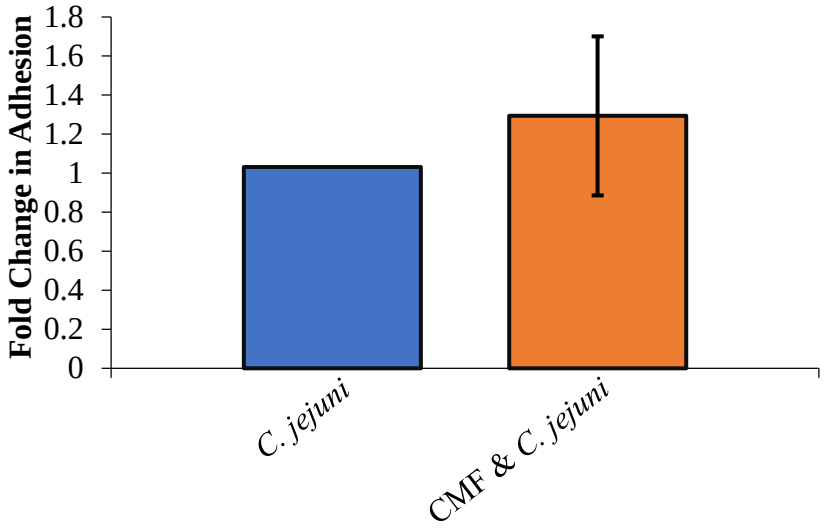

Figure S1. Anti-infective effect of CMF on *C. jejuni* colonization. Results are represented as the average of triplicate experiments performed on one occasion. The control is represented as the normalized average fold and standard deviation of all individual experiments. All results are represented as the percentage of adherent cells = [CFU/mL of recovered adherent bacteria ÷ CFU/mL of inoculum] × 100 and graphed as fold-change relative to percent adhesion of control, with error bars representing the standard deviation.

Chapter VI

Lactose Promotes *In Vitro* Attachment of *Bifidobacterium breve* UCC2003 to Intestinal Epithelial Cells

Erinn M. Quinn, Douwe Van Sinderen, Lokesh Joshi and Rita M. Hickey

Acknowledgements: Erinn Quinn performed all experimental work for this chapter. Rita Hickey and Douwe Van Sinderen assisted with manuscript preparation.

Abstract

The effect of lactose on adhesion and colonization of bifidobacteria has yet to be fully explored. In this study, we investigated the ability of lactose to influence adhesion of *Bifidobacterium longum* subsp. *infantis* ATCC® 15697™, *Bifidobacterium longum* Reuter ATCC® 15708™ and *Bifidobacterium breve* UCC2003 to intestinal HT-29 cells. Adhesion levels of lactose treated-*B. breve* UCC2003 to the cells increased 2.4 fold, while no significant change was observed for the other strains. The increased adhesion of *B. breve* UCC2003 in response to lactose was also found to protect HT-29 cells from *C. jejuni* colonization with a 42% reduction in cell numbers attaching to the cells. A transposon mutant of *Bifidobacterium breve* UCC2003, 101C6, which is unable to degrade lactose also displayed a 2-fold increase in adhesion in response to lactose. Therefore, lactose metabolism is unlikely to be implicated in the increased adhesion phenotype observed. This study suggests that lactose in milk may play a role in the promotion of a diverse and protective ecosystem of bifidobacterial strains within the gastrointestinal tract. Overall, the different microbial inhabitants of the gastrointestinal tract may exploit alternative milk components in order to successfully colonize the host.

1. Introduction

Human breastmilk contains 70 g/L lactose (galactose-β1,4-glucose) which is the most abundant carbohydrate component in breastmilk, followed by human milk oligosaccharides (HMO) [1, 2]. Lactose accounts for 40% of the caloric value ingested by the nursing neonate [3] and is also found in many milk-derived products as well as being used as a raw material in a variety of pharmaceutical products due to its chemical characteristics [4, 5, 6]. As a food ingredient, it benefits the consumer as it demonstrates no neurologically rewarding effect when ingested [3, 7] and has a low glycemic index [8]. Its relative sweetness is 40 in comparison to the 'Gold Standard' sucrose which has a relative sweetness of 100 [9]. Nonetheless, lactose has gained much negative press in recent times due to lactose intolerance as a result of host deficiency in the enzyme lactase or excessive intake of lactose (above 10-15 g/day). In addition, there is a lack of consumer awareness surrounding these conditions which has impacted the dairy market with consumers seeking lactose-free alternatives, even in cases of misdiagnosis where symptoms are incorrectly attributed to poor digestion of lactose. Despite these concerns surrounding lactose, lactase-deficient individuals are capable of consuming up to 11 g of lactose a day [10, 11, 3] and it is still currently recommended that individuals consume 2-3 servings of milk or dairy per day [12, 13,14].

Notably, lactose that escapes digestion has been suggested to promote the growth of commensal bacteria in the gut and increase saccharolytic activities including enhancing the production of fatty acids and lowering gastric pH [3, 15, 16, 17, 4]. Lactose contains a β-1,4 glycosidic bond between galactose and glucose, which is somewhat rare in nature, being present in milk, cellulose and chitin [6]. Milk is believed to elicit a protective effect on the gastrointestinal tract (GI tract) of the nursing neonate by selecting for lactose-fermenting bacteria. Previously, investigations have demonstrated a dose-related effect of dietary supplementation with lactose on intestinal bifidobacteria and lactobacilli, and an increase in total volatile fatty acids in faeces of weaning pigs [18]. In an infant study, lactose supplementation (38 g/L) of a hydrolyzed formula demonstrated significantly higher faecal counts of bifidobacteria and lactic acid bacteria and lower counts of *Bacteroides* and *Clostridium* species compared to those fed a lactose-free formula [19]. Additionally, adults with lactose malabsorption receiving a dietary intervention of whole milk (250 mL/day) over a four-week study demonstrated a higher abundance of faecal Actinobacteria, which include members of the genus *Bifidobacterium*, *Anaerostipes* (butyrate producers) and *Blautia* (acetogenic) in comparison to individuals that are able to digest lactose normally [20].

Moreover, media containing high levels of lactose has been reported to maximize adhesion of *B. bifidum* to hexadecane [21], while adhesion of lactobacilli to epithelial cells has been reported to increase when grown on MRS containing lactose [22]. These findings suggest that lactose may be a critical contributor to the development of a healthy microbial gut ecosystem by directly impacting on the adhesion potential of health-promoting bifidobacteria and lactobacilli. A variety of milk components, normally associated with prebiotic capabilities, have been shown to also modulate adhesion of commensal bacteria. Up-regulation of a putative type II glycoprotein binding fimbriae, thought to be involved in attachment and colonization *B. longum*, has been reported following its growth in defatted human milk [23]. Increased adhesion of *B. longum* subsp. *infantis* ATCC 15697 (*B. infantis*) to intestinal cells was demonstrated after exposing the bacteria to human milk oligosaccharides [24,25]. Additionally, we demonstrated increased adhesion of *B. infantis* to HT-29 cells following treatment of the bacteria with a variety of milk oligosaccharides and a bovine whey derived powder enriched in IgG [26]. Subsequent studies indicated that the increased adhesion of bifidobacteria can facilitate a prophylactic protective effect against *C. jejuni* colonization of the HT-29 cells [27]. Despite the observed beneficial effects of lactose on the gut microbiota, to the best of our knowledge, no study to date has explored its ability to influence bifidobacterial attachment to intestinal cells. In fact, pre-treatment of *Bifidobacterium longum* subsp. *infantis* ATCC® 15697™ (*B. infantis* ATCC 15697) with lactose has been proposed to inhibit the beneficial effects of human milk oligosaccharides on its adhesion phenotype [24]. Considering the variety of different bifidobacterial strains present in the gastrointestinal tract, we aimed to examine the ability of lactose to modulate adhesion of *B. infantis* ATCC 15697, *Bifidobacterium longum* Reuter ATCC® 15708™ (*B. longum* ATCC 15708) and *Bifidobacterium breve* UCC2003 (*B. breve* UCC2003) to HT-29 cells and determine their potential to competitively exclude an invasive strain of *C. jejuni* to intestinal cells *in vitro*.

2. Materials and Methods

2.1 Carbohydrate Standards

Lactose was obtained from VWR (Dublin, Ireland) and glucose and galactose were obtained from Merck (Darmstadt, Germany).

2.2 Bifidobacterial Strains and Cultivation

The bifidobacterial strains used in this study are listed in Table 1 and cultivation conditions were maintained as previously described [24, 26, 27]. Typically, strains were taken from -80ºC stocks and streaked onto DeMan Rogosa Sharpe (MRS) (Difco, Sparks, MD, USA) agar plates supplemented with 0.5% L-cysteine hydrochloride (antioxidant) (Merck). For all strains, a single colony was taken from each plate and inoculated into MRS broth. Bacteria were incubated anaerobically using the Anaerocult A system (Merck) at 37 °C for 48 h. Stocks were made using 50:50 MRS broth and glycerol (Merck) and were stored at -20 °C or -80 °C. The *B. breve* UCC2003 mutant strain (101C6) (*B. breve*^{TMut}) was kindly provided by Prof. Douwe Van Sinderen, University College Cork, Ireland. This mutant has been previously described by Ruiz et al., [28]. The mutant strain was routinely cultured as described above but broth and plates were supplemented with 10μg/ml of tetracycline (Tet).

2.3 Pathogenic Strain and Cultivation

Campylobacter jejuni 81-176 (*C. jejuni*) was stored in Mueller Hinton broth (Oxoid, Ireland c/o Fannin Healthcare) containing 50% glycerol at -80 °C and cultured directly from storage onto Mueller-Hinton agar plates. The pathogen was grown under microaerophilic conditions generated using CampyGen gas packs (Oxoid), for 48 h at 37 °C. Prior to pathogen inhibition assays, *C. jejuni* 81-176 was grown on Mueller-Hinton agar and then transferred to biphasic media in 25 cm^2 tissue culture flasks (Corning, New York, USA) consisting of Mueller Hinton agar supplemented with *Campylobacter* selective supplement (Skirrow), (Oxoid) and 6 ml of McCoy's 5A media (Merck) supplemented with 2% Fetal bovine serum (FBS). The flask was then incubated for 24 h under microaerophilic conditions at 37 °C.

2.4 Mammalian Cell Culture

HT-29 cells were prepared as previously described [26]. Typically, HT-29 cells were grown in McCoy′s 5A modified medium (Merck) supplemented with 10% fetal bovine serum (FBS), which were maintained in 75 cm^2 tissue culture flasks and incubated at 37 °C in 5% CO_2 in a humidified atmosphere. Once the cells were confluent (approximately 80–90%), cells were passaged into 12 well plates. For the assays, cells in 75 cm^2 flasks were trypsinised and seeded

into a 12-well tissue culture plate (Sarstedt Ltd., Wexford, Ireland) at a density of 1×10^5 cells/mL between passages 15–21 and cells were used once fully confluent (approximately 4×10^6 cells/well at day 5–7). The media was changed every other day and supplemented with 2% FBS 24 h prior to use.

2.5 Exposure of Bacteria to Carbohydrates

Exposure of the bacteria (Table 1) to lactose was performed as previously described [24, 26, 27]. Bacteria were used at mid-exponential growth phase (18 h) and the OD_{600nm} was adjusted to 0.3 at the start of the assay, after which cells were cultured for 1.5–2 h and used once an OD_{600nm} of 0.5 was reached. Bacterial cells were washed twice with PBS by centrifugation. Cell pellets were re-suspended to a final OD_{600nm} of 0.5 in McCoy's 5A tissue culture media supplemented with 2% FBS and 4 mg/mL lactose. Assessments with *B. breve*^{TMut} with 4 mg/mL lactose, galactose and glucose were also conducted. A non-supplemented negative control was also included for all assays. Bacterial suspensions were then incubated for 1 h at 37 °C under anaerobic conditions. Following this, bacteria were harvested by centrifugation (3850 × *g*, 5 min), the supernatants removed, and pellets were washed three times in PBS and then re-suspended in non-supplemented McCoy's media prior to use in the adhesion assays.

2.6 Adhesion Assays

Adhesion assays were performed as previously described [24, 26]. HT-29 cells were washed twice with PBS, and 500 µL of the bacteria and media suspensions were added to the wells. Bacterial cells were incubated with the HT-29 cells for 2 h at 37 °C under anaerobic conditions using an Anaerocult A system (Merck). The HT-29 cells were then washed five times with PBS to remove non-adherent bacteria. HT-29 cells were then lysed with 500 µL of 1% Triton™ X-100 (Merck) for 5 min at 37 °C. The lysates were serially diluted and enumerated by spot-plating on MRS plates. The adhesion of the bacteria was determined as the percentage of original inoculum which attached, thus accounting for variations in the starting inoculum. Percentage adhesion = (CFU/mL of recovered adherent bacteria/CFU/mL of inoculum) × 100.

2.7 Pathogen Exclusion Assay

The exclusion assay screen was performed as previously described [27] with minor modifications. Exposure of Bifidobacterial strains; *B. infantis* ATCC 15697, *B. breve* UCC2003 and *B. longum* ATCC 15708 to 4 mg/mL lactose was performed as described above and this suspension was subsequently incubated with the HT-29 cells for 2 h. A non-supplemented control was also included. Non-adherent bacteria were removed from the cells

after which the cell line was challenged with *C. jejuni*. To do this, the pathogen was harvested from the biphasic medium, washed twice in non-supplemented McCoy's, and diluted to an OD_{600nm} of 0.3. From this suspension, 500 µl was then added to each well, and cells were incubated under anaerobic conditions for 3 h at 37 °C. Cells were then washed five times with PBS, lysed with 0.1% Triton X-100 in PBS and plated onto supplemented Mueller-Hinton agar. Mueller-Hinton plates were incubated under microaerophilic conditions for 72 h at 37 °C after which bacterial CFU/mL were enumerated. The assays were performed in triplicate on one occasion. Results are presented as the mean of biological replicate experiments, with error bars representing standard deviation, graphed as fold-change relative to percent adhesion of the control. Percentage adhesion = (CFU/mL of recovered adherent bacteria ÷ CFU/mL of inoculum) × 100.

2.8 Statistical Analysis

Graphs were drawn using Microsoft Excel. The results are presented as the mean ± standard deviations of replicate experiments, and the student *t*-test was used to determine statistically significant results for biological triplicate experiments. For all experiments, $p<0.05$ was considered significant.

3. Results and Discussion

The possible role of lactose as a prebiotic was initially deduced from studies in animals showing that lactose stimulates bifidobacterial growth [15]. More recently, Francavilla et al. [19] demonstrated that the addition of lactose to an extensively hydrolyzed formula was able to positively modulate the composition of gut microbiota in infants by increasing the total faecal counts of lactobacilli and bifidobacteria. In infants, lactose is not completely hydrolyzed in the small intestine, it reaches the colon where it is salvaged by bacteria, resulting in the growth of bifidobacteria and lactobacilli. Notably, lactase production by the newborn goes from 30% to 98% by Day 5 post-parturition [6]. While lactose may pass into the colon at varying periods throughout life, it is not possible to predict how much lactose might survive digestion. Detection of lactose in stools is commonly conducted in suspected lactose-intolerant individuals using stool acidity tests which are not quantitative. However, some studies in preterm infants suggest that 90% of the lactose in human milk is absorbed [29]. For the purposes of this study, we estimated that approximately 10% of the lactose present in human milk may reach the colon intact. By selecting the lowest levels of lactose that might be found in human milk [30] we use a concentration of 4 mg/mL throughout the study to represent the minimum amount of lactose that might reach the colon intact. While this is relevant, it is important to note that during these assays *B. breve* was pre-treated with lactose and following a 1 h incubation period was removed prior to applying to the HT-29 cells. Thus, lactose was never in direct contact with the HT-29 cells. In terms of the mechanisms of action that may take place *in vivo*, it is possible that pre-treatment of *B. breve* with lactose occurs before digestion, in or on breast tissue. Notably, bifidobacteria have been isolated from breastmilk [31], indicating that pre-incubation in or on breast tissue may be possible. These assays indicate that pre-treatment of *B. breve* with lactose in potential probiotic products prior to encapsulation may enhance its ability to adhere to intestinal cells. This product would also be suitable for those who suffer from lactose mal-digestions, lactose intolerance and those following a low fermentable oligo-, di-, mono-saccharides and polyols (FODMAPs) diet as such products would be virtually lactose-free consisting of lactose treated bacteria only.

Here, lactose-treated *B. breve* UCC2003 was found attached in 2.4 fold higher numbers to HT-29 cells when compared to the non-treated control. No statistically significant change in adhesion to HT-29 cells was observed with lactose-treated *B. infantis* ATCC 15697 or *B. longum* ATCC 15708. The result reported for *B. infantis* ATCC 15697 is not surprising given that Kavanaugh et al. [24] demonstrated that lactose abolished the increased adhesion

phenotype associated with HMO-treated *B. infantis* ATCC 15697. Notably, in the same study, the increased adhesion phenotype was shown to be associated with an up-regulation of genes involved in adhesion and a down-regulation of genes involved in complex oligosaccharide metabolism [24]. In the presence of lactose, upregulation in genes involved metabolism may occur in *B. infantis* ATCC 15697 and *B. longum* ATCC 15708 at the expense of those genes involved in adhesion. The results observed for *B. breve* UCC2003 indicate that in contrast to *B. infantis* ATCC 15697 and *B. longum* ATCC 15708, this strain may be able to exploit lactose to promote its adhesion phenotype. Notably, no significant increase in growth was observed for any of the bifidobacterial strains within the 1 h pre-incubation period with lactose when compared to the control.

Colonization of *B. breve* UCC2003 in the neonatal gut has been described as a 'carbohydrate syntrophy' [32]. In terms of adhesion, bacterial pili are involved in its adhesion to host tissues. Notably, functional genome analysis of *B. breve* UCC2003 has shown that the strain encodes type IVb tight adherence (Tad) pili, which act as an essential and conserved host-colonization factor [33]. The fibre of the Tad pilus contains multiple copies of one or more different pilin proteins including the Flp pilin which forms the pilin shaft, and this is believed to be decorated with pseudopilins, TadE and TadF. TadE has been shown to be involved in the formation and assembly of type IVB pili which are capable of binding to carbohydrate moieties and are suggested to initiate the initial encounter with host cell receptors [34]. Furthermore, Kavanaugh et al. [24] demonstrated an increase in expression of TadE gene in *B. infantis* following pre-treatment with milk oligosaccharides and this has been suggested to potentially contribute to the enhanced adhesive response of *B. longum* subsp. *infantis* ATCC 15697. In fact, the tight adherence locus (tad) is homologous in members of [35] and is responsible for the formation and assembly of type IVB pili. Additionally, autoinducer-2, a member of a family of signalling molecules used in quorum sensing has been shown to play a crucial role in the colonization of *B. breve* UCC2003 [36]. It is possible that these *B. breve* UCC2003 adhesion factors were upregulated following exposure to lactose however, transcriptional studies are required to confirm their involvement in the increased adhesion phenotype observed.

To assess if lactose metabolism was involved in the increased adhesion phenotype observed following exposure of *B. breve* UCC2003 to lactose, we employed a *B. breve* UCC2003 mutant, *B. breve*^{TMut} which contains a transposon insertion in the gene (Bbr_0010) encoding lacZ1 β –galactosidase (Figure 2A) rendering the strain unable to utilise lactose [28]. In our investigation, the percentage of the original inoculum of the wild type of *B. breve*

UCC2003 which adhered to the HT-29 cells was 0.74% ± 0.22. This was significantly higher than that observed for the *B. breve*^{TMut} where 0.14% ± 0.11 of the original inoculum attached to the HT-29 cells. However, it is important to note, these levels are still within the normal range for bifidobacterial strains [37]. Overall, the adhesion ability the wild type of *B. breve* UCC2003 was 5.2-fold higher than *B. breve*^{TMut} (Figure 2B). Bacterial mutants have been reported to often lose some fitness associated with their typical growth properties and may fail to utilize a particular carbon or nitrogen source, fail to grow without a particular nutrient or acquire a new growth property (e.g. ability to grow in the presence of some toxic substance) [38]. Thus, the reduced adhesion capability observed for the mutant strain may be linked to an inherent reduction in fitness associated with many mutants, however, further studies are required confirm gene (Bbr_0010) encoding lacZ1 β –galactosidase not implicated in adhesion.

During digestion and bacterial metabolism, lactose is broken down into galactose and glucose by intestinal membrane-bound β-galactosidase [3] and bacterial β-galactosidases [39, 40, 41]. *B. breve*^{TMut} was pre-incubated individually with 4 mg/mL glucose, galactose or lactose to determine their effects on the adhesion of the strain to the HT-29 cells. A 1.2-fold increase in adhesion was observed when pre-incubated with glucose, while treatment with galactose resulted in an s 1.4-fold increase in adhesion. Pre-treatment of *B. breve*^{TMut} with lactose resulted in a 2-fold increased trend (*p-value*; 0.078) in adhesion (Figure 3) indicating that lactose metabolism may not be implicated in the increased adhesion phenotype. While the mechanism of action is unclear, two possible strategies may exist explaining the increase in adhesion. Lactose attachment to externally located transporters may aid the adhesion of *B. breve* UCC2003 to the host cells, acting as a bridge between the bacteria and the host's cells. Bovine lactoferrin has been shown to function as a molecular bridge for internalization of *Streptococcus uberis* into bovine mammary epithelial cells [42]. Alternatively, lactose it may induce the increased expression of adhesion factors by the bacteria.

Ruiz et al., [28] employed a Tn5-based random mutagenesis system in *B. breve* UCC2003 and identified Bbr_0010 as the main β-galactosidase responsible for lactose utilization in this strain. The LacZ1 β-galactosidase, encoded by the gene Bbr_0010 which is interrupted in *B. breve*^{TMut} (Figure 2A) cannot digest lactose but the strain still contains intact sugar-binding regions which may interact with lactose and be involved in the increased adhesion observed. In terms of next steps, proteomics studies could be used to evaluate the proteins produced by the wild type *B. breve* UCC2003 and the *B. breve*^{TMut} in response to lactose. It might be expected that proteins encoded by these genes may be produced at higher

levels. Notably, galactoside symporters (lacS), which have been predicted to function in the transport of lactose and galactooligosaccharides into the cell may be produced at higher levels following treatment with lactose and this may facilitate increased adhesion [43, 44]. Interestingly, a previous study demonstrated upregulation of seven *B. breve* UCC2003 genes during growth in mMRS medium supplemented with lactose as the sole carbohydrate [43]. These genes included IntP1, IntP2 which encode permease components of an ABC transporter system (corresponding to locus tags Bbr_0527 and Bbr_0528 respectively), IntA and LacZ6 encoding a GH42 and GH2 β-galactosidase (Bbr_0529 and Bbr_1552 respectively), IntS encoding a Solute-binding protein of ABC transporter system for sugars (Bbr_0530), a hypothetical protein (Bbr_1550) and lacS encoding a Galactoside symporter (Bbr_1551) [43]. Considering that LacZ1(Bbr_0010) which is interrupted in *B. breve*^{TMut} is intracellular, it may be possible that externally located transporters are involved in the increase in adhesion observed here. Solute-binding proteins may bind lactose with high affinity and deliver the membrane-bound components into the cytoplasm. Such solute-binding proteins may be expressed at higher levels in the presence of lactose. Overall, we can only speculate the mechanism of action and further studies such as a proteomics study is required to understand how lactose confers its effect on *B. breve* UCC2003 adhesion.

As a final objective, we aimed to exploit the effect of lactose on increasing the adhesion of *B. breve* UCC2003 as a mechanism for inhibiting pathogen association with intestinal cells. We examined the synergistic effect of lactose-treated *B. breve* on preventing the attachment of a highly invasive strain of *Campylobacter jejuni* to intestinal HT-29 cells. An overall average inoculum of 2.13 x 10^7 CFU/mL *C. jejuni* was applied to the HT-29 cells, of which 3.75 X 10^5 CFU/mL were demonstrated to adhere. *B. breve* UCC2003 alone provided a 20% reduction in *C. jejuni* colonization in comparison to the control (non-treated HT-29 cells infected with *C. jejuni*) (Figure 4A). Prophylactic protection against *C. jejuni* colonization was revealed following prior treatment of *B. breve* UCC2003 with lactose with a 41% reduction adhesion in comparison to the negative control and a 27% reduction in adhesion compared to the bifidobacterial control (Figure 4A). Assays performed with *B. infantis* ATCC 15697 (Figure 4B) indicated no difference and assays with *B. infantis* ATCC 15708 showed a decreased trend when the HT-29 cells were pre-treated with the *B. infantis* ATCC 15708 alone (26% reduction) and when pre-treated with lactose treated *B. infantis* ATCC 15708 (48% reduction), indicating that this trend was a result of direct competition of *C. jejuni* with *B. infantis* ATCC 15708 (Figure 4C). However, further assays are required to confirm the

significance of these results. Shared similarities between probiotic and pathogenic strain surface adhesins have been suggested to facilitate competition for adhesion sites [3, 44]. In *campylobacter*, genes such as cadF have been implicated in adhesion which is suggested to be involved in the synthesis of a protein which binds fibronectin on the outer membrane, facilitating the attachment to epithelial cells [45]. Homologs of the fibronectin type III domain protein genomic arrangements (among others) were found to be present in *B. breve* [46]. These findings suggest that *C. jejuni* and *B. breve* may share similarities in the mechanisms they use to attach to host cell surface receptors and could explain how competitive exclusion occurs in this instance. As a next step, gentamicin protection assays could be contacted to determine if there is any protection against pathogenic invasion. Furthermore, proteomic studies may give insight into the mechanism of action by which a trend in pathogen protection was achieved and may facilitate rapid identification of other pathogens which may be inhibited by such combinations.

4. Conclusion

In this study, lactose treated *B. breve* UCC2003 and a mutant *B. breve*^{TMut} which was unable to utilise lactose displayed an increased adhesion phenotype following exposure to lactose. No increased adhesion was observed in any other strains under investigation. Moreover, the increase in adhesion phenotype of lactose treated *B. breve* UCC2003 provided prophylactic protection against *C. jejuni* colonization. This study suggests that lactose treated *B. breve* UCC3002 may facilitate its increased colonization in the gut and positively impact the gut microbiota in general. Further investigations are required to assess the specific mechanism of action and identify the specific receptor sites that facilitate the increased adhesion of this strain. Additionally, further investigations into the potential of lactose treated *B. breve* UCC2003 in preventing the colonisation of other pathogens is warranted. This is the first study to provide evidence that lactose may increase adhesion of *B. breve* UCC2003 to intestinal cells.

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Figures and Tables

Table 1. List of *Bifidobacterium* strains used in this study.

Species and strain	**Abbreviation**
Bifidobacterium longum subsp. *infantis* ATCC® 15697™	*B. infantis* ATCC 15697
Bifidobacterium longum Reuter ATCC® 15708™	*B. longum* ATCC 15708
Bifidobacterium breve UCC2003 (NCIMB 8807)	*B. breve* UCC2003
Bifidobacterium breve UCC2003 transposon mutant (101C6)	*B. breve*^{TMut}

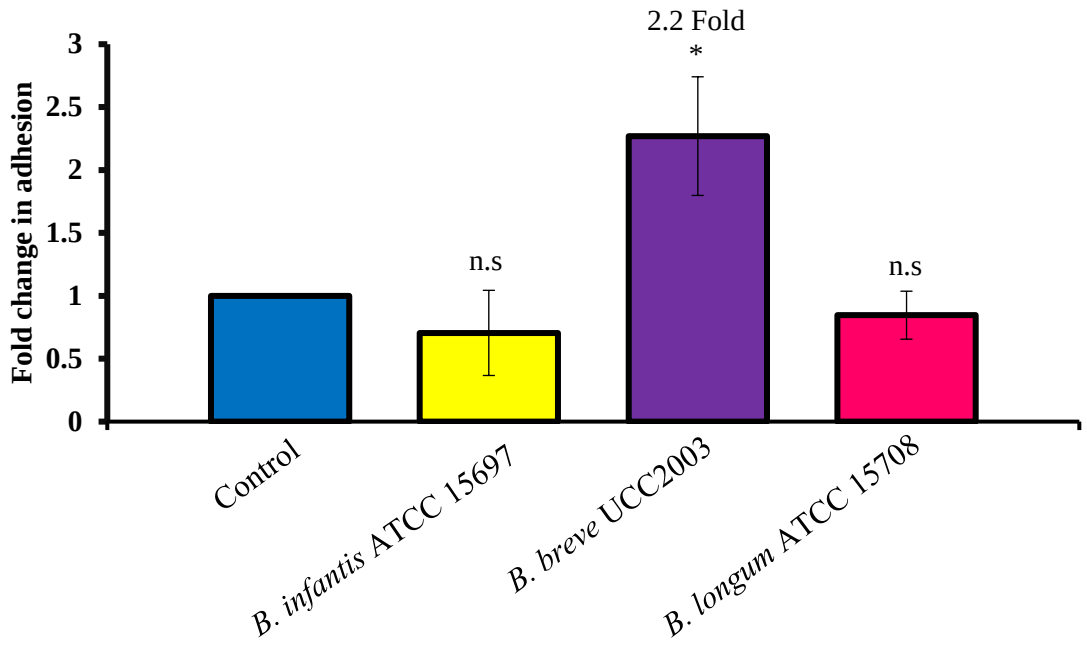
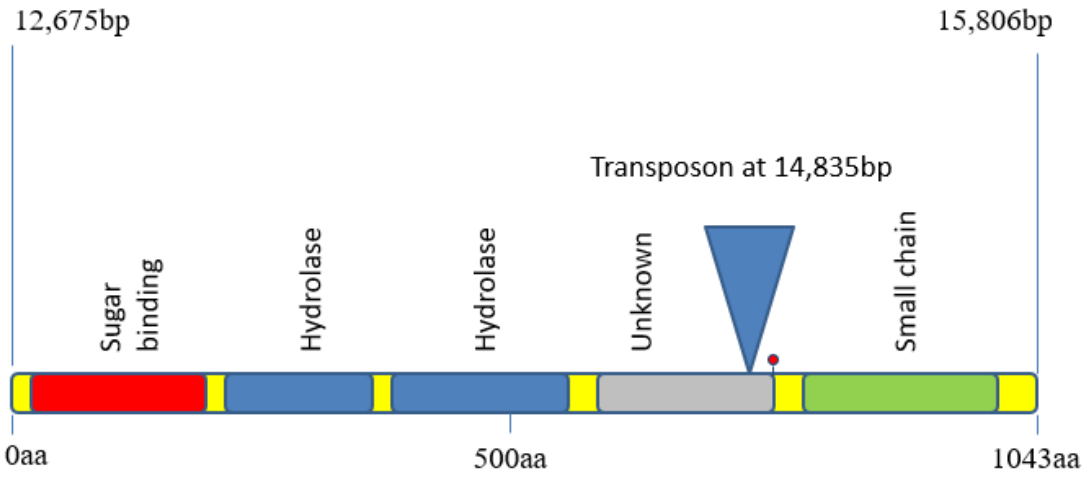


Figure 1. Adhesion of *Bifidobacterium longum* subsp. *infantis* ATCC® 15697™, *Bifidobacterium longum* Reuter ATCC® 15708™ and *Bifidobacterium breve* UCC2003 to HT-29 cells following 1 h exposure to 4 mg/mL lactose. The results are represented as the average of triplicate experiments performed three occasions. The control is represented as the normalized average fold and standard deviation of all individual experiments. All results are represented as the percentage of adherent cells = [CFU/mL of recovered adherent bacteria ÷ CFU/mL of inoculum] × 100 and graphed as fold-change relative to percent adhesion of control, with error bars representing the standard deviation. The student *t*-tests were used to determine significance, * *p*-value: <0.05, n.s: not significant.

A.



B.

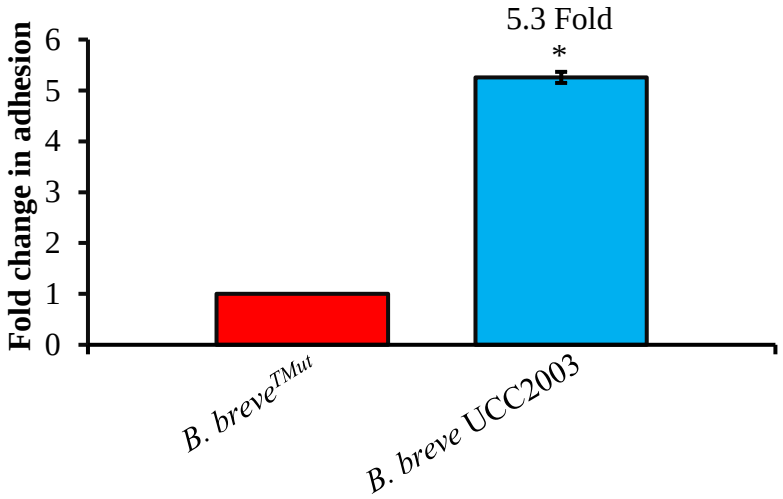


Figure 2. (A) Gene Bbr_0010 of *B. breve* UCC2003, encoding lacZ1 Beta-galactosidase. The gene contains a sugar-binding region, which is situated before the transposon insertion. A stop codon is indicated immediately after the transposon. (B) Adhesion of *B. breve* UCC2003 and *B. breve* UCC2003 transposon mutant (101C6) to HT-29 cells. Results are represented as the average of triplicate experiments performed on three occasions. All results are represented as the percentage of adherent cells = [CFU/mL of recovered adherent bacteria ÷ CFU/mL of inoculum] × 100 and graphed as fold-change relative to percent adhesion of control, with error bars representing the standard deviation. The student *t*-tests were used to determine significance, * *p*-value: <0.05, n.s: not significant.

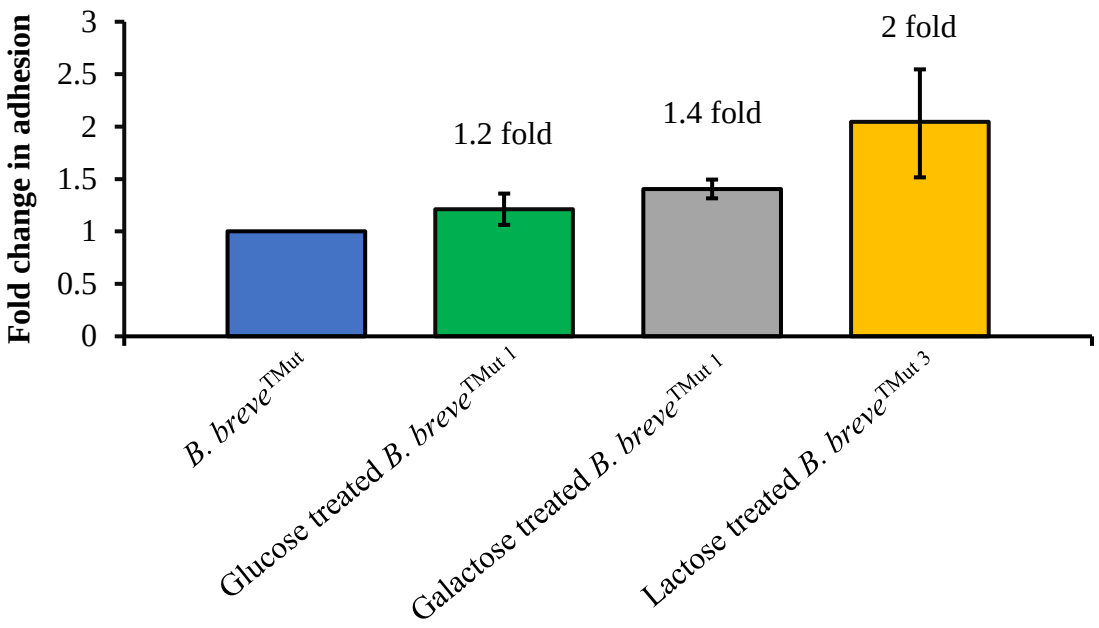
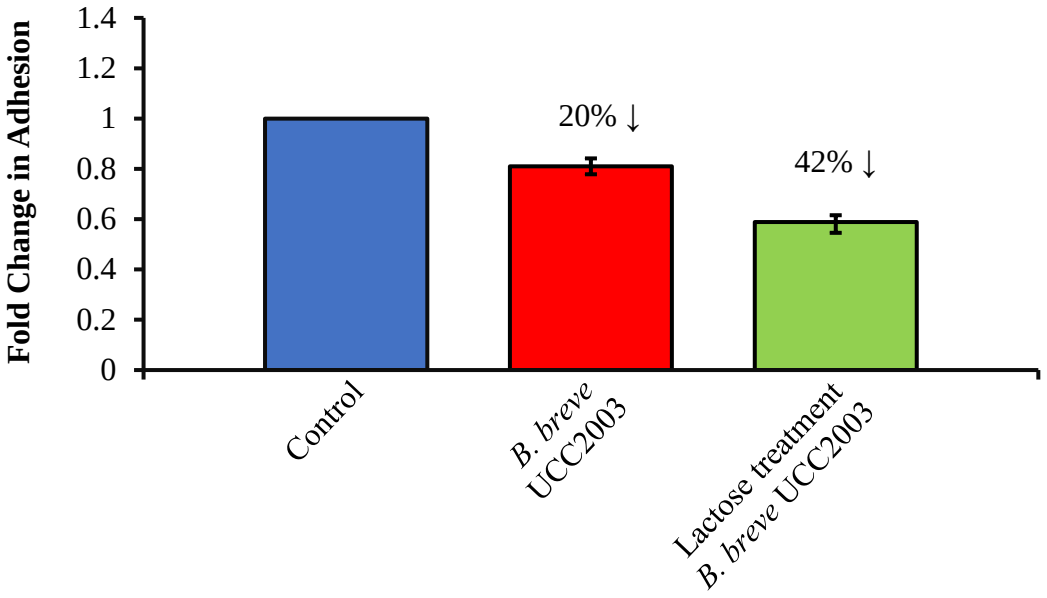
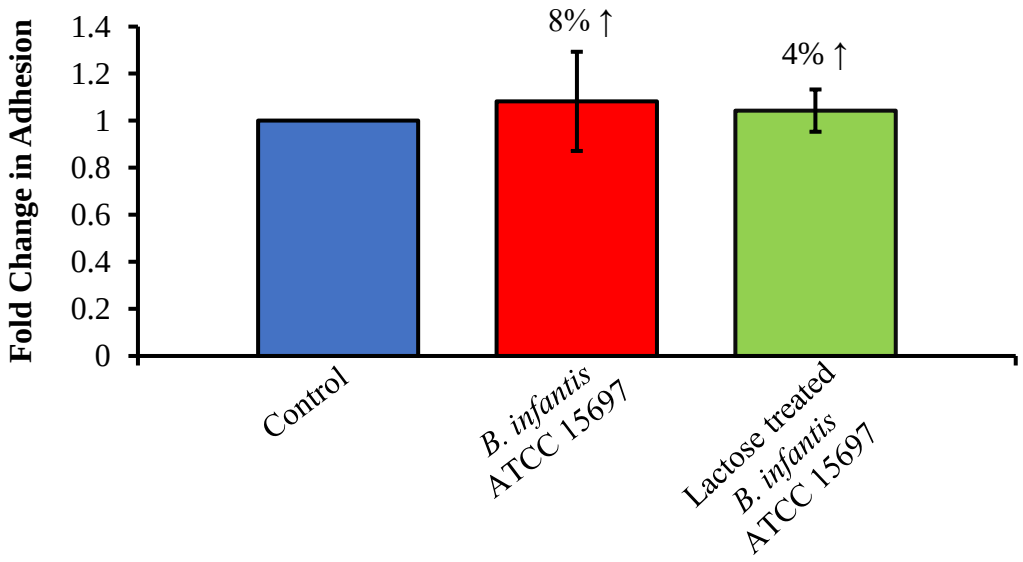


Figure 3. Adhesion of *B. breve* UCC2003 transposon mutant (101C6) to HT-29 cells following incubation with glucose (green), galactose (grey) or lactose (orange). Results are represented as the average of triplicate experiments performed on [1]one, [2]two or [3]three occasions. The control is represented as the of the average of the fold and standard deviation for all experiments All results are represented as the percentage of adherent cells = [CFU/mL of recovered adherent bacteria ÷ CFU/mL of inoculum] × 100 and graphed as fold-change relative to percent adhesion of control, with error bars representing the standard deviation.

A.


Fold Change in Adhesion
1.4
1.2
1
0.8
0.6
0.4
0.2
0
20% ↓
42% ↓
Control
B. breve UCC2003
Lactose treatment B. breve UCC2003

B.


Fold Change in Adhesion
1.4
1.2
1
0.8
0.6
0.4
0.2
0
8% ↑
4% ↑
Control
B. infantis ATCC 15697
Lactose treated B. infantis ATCC 15697

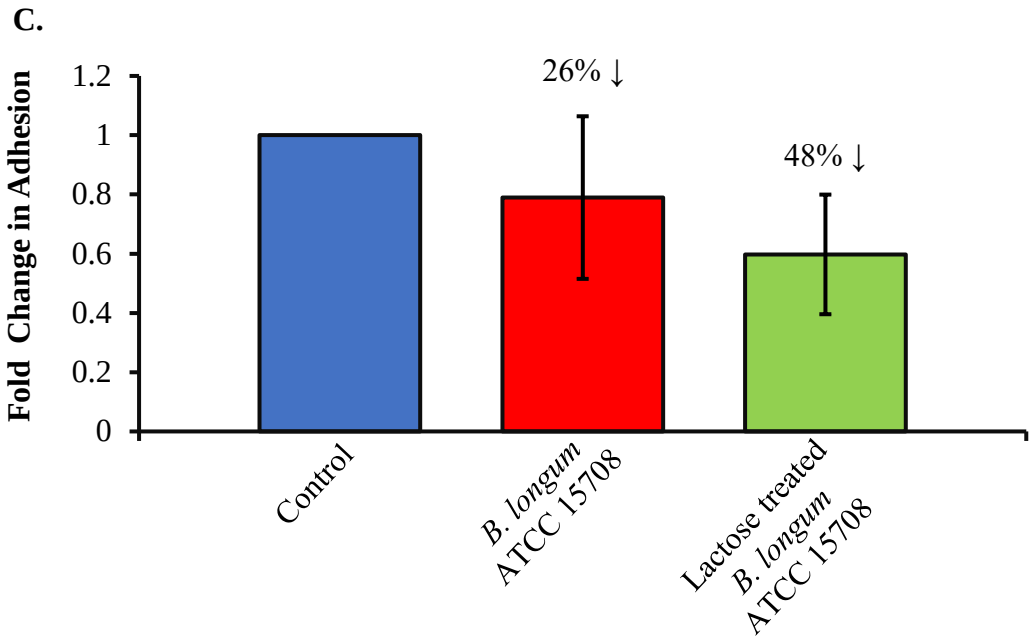


Figure 4. Adhesion of *Campylobacter jejuni* 81-176 to HT-29 cells (blue) or following prior cell line treatment with (A) *B. breve* UCC2003, (B) *B. infantis* ATCC 15697, or (C) *B. longum* ATCC 15708 alone (red) or exposed to lactose (4 mg/ml) (green). Results are represented as the average of triplicate experiments performed on one occasion. All results are represented as the percentage of adherent cells = [CFU/mL of recovered adherent bacteria ÷ CFU/mL of inoculum] × 100 and graphed as fold-change relative to percent adhesion of control, with error bars representing the standard deviation.

General Summary and Discussion

It is well established that a "healthy" gut microbiota is important in maintaining wellness, and that undesirable changes in the gut microbiome have been linked to several gastrointestinal (GI) disorders. Taking probiotics such as bifidobacteria to restore the normal, protective microbiome and to "balance" the body's flora is becoming increasingly common. Indeed, a plethora of studies have demonstrated the positive outcomes of probiotic supplementation on disorders, such as antibiotic-associated diarrhoea, irritable bowel syndrome, necrotizing enterocolitis, ulcerative colitis, lactose intolerance, and colorectal cancer [1]. Several articles have reviewed their mechanisms of action, which include modulation of the immune system, induction of anti-inflammatory and anti-oxidant responses, competitive pathogen exclusion, as well as production of anti-microbial substances [2, 3]. In terms of probiotic health benefits, exclusion of pathogens is highly topical considering the current increase in antibiotic-resistant pathogens. For probiotics to be effective not only do they need to survive GI transit, they also need to colonize the GI tract. Evidence of probiotic gut mucosal colonization efficacy remains scarce and contentious. Studies have repeatedly shown that probiotics are more like tourists than tenants as they pass through the GI tract without prolonged attachment. An effective probiotic must reside at its target sites in the GI tract long enough and at sufficient concentrations to elicit health-promoting effects. Adherence to the intestinal surface lengthens the retention time of a probiotic, which is important in the small intestine because of the short transient time of intestinal material. However, recent studies suggest that although probiotic-consuming individuals displayed probiotics in their stool, only some had probiotics present in their GI tract [4,5].

Recent studies have suggested that components in milk may contribute to the selective growth (prebiotic effect) of commensal bacteria, but also may enhance their specific ability to adhere to the GI epithelium through the differential regulation of bacterial colonisation factors [6,7,8]. Our group have also recently demonstrated, using *in vitro* assays, that colostrum influences the intestinal cell surface, and in turn the attachment of health-promoting microorganisms [9]. More recently, this activity was shown to be linked to IgG and its associated glycosylation [10] which based on preliminary evidence, influences the glycosylation pattern of HT-29 cells [11]. It therefore becomes clear, that milk, the sole dietary source for the first months of infancy, has a large influence on the microbal colonisation of the newborn gut.

Human milk is a highly complex matrix of macronutrients and bioactive compounds that may influence colonisation and it is the gold standard for infant nutrition. Hormones, antimicrobials, oligosaccharides, immunoglobulins, glycoproteins and glycolipids are all found

in abundance [12]. Notably, breastfed infants have higher levels of *Bifidobacterium longum, B. infantis, B. breve,* and *B. bifidum*, and less episodes of diarrhoea and ear infections in children [13,14] when compared to their formula-fed counterparts [15]. Where breastfeeding isn't an option, alternatives are desirable. However, the possibility of commercial production of many of these human milk bioactives is unlikely due to the limited availability of donor milk and the complexity of synthesis at large-scale. For this reason, domestic animal milk is seen as an alternative source of such bioactive components despite the many obstacles to overcome in order to meet the market needs. Currently, colostrum accounts for approximately 0.5% of bovine annual milk production [16] and this quantity represents a viable stream for further processing to produce high-value products [17]. Thus, despite the commercial production obstacles the implementation of breeding programs and new filtrations technologies means animal milks remain an alternative source of such bioactive components. In this respect, using *in vitro* model systems, this thesis sets out to explore components in domestic animal milks as alternatives to human milk bioactives which can positively influence host-microbial interactions.

Increased Adhesion of Bifidobacterial

In Chapter I of this thesis, we reviewed the structural and bio-functional similarities between domestic animal milk glycoconjugates and their human milk-derived counterparts, focusing in part on their ability to impact both commensal and pathogen colonisation. This set the scene for the following investigations and highlighted the potential of domestic animal milks to act as alternatives to human milk.

In chapter II, we collected and isolated a panel of 13 milk-derived components (Chapter II, Table 1). Additionally, we also isolated a low molecular fraction from camel milk (Chapter IV, Table 1) and included lactose in our studies (Chapter VI). The selected powders are characterised in Chapters III, IV and V. In chapter II, a high throughput technique which can quickly define the colonising ability of bacterial strains to human cells *in vitro* was developed. Following this, we screened a variety of fractions isolated from different milk sources for their ability to increase the adherence of *Bifidobacterium longum* subsp *infantis* ATCC 15697 (*B. infantis*) to intestinal cells. In our experiments, the percentage adherence of bacterial strains varied throughout the chapters of this thesis, which was not unexpected considering the inherent variability associated with such assays. Previous studies have indicated that the percentage adhesion of bifidobacterial strains such as *B. bifidum* DSM20239 to HT-29 cells can be as low as 0.13%. While other strains such as *B. bifidum* LMG13195 demonstrate high

adhesion rates up to 74.79. This highlights strain to strain variability in adhesion [18]. Notably, *B. infantis* has been shown to have a lower adhesion rate to that of *B. bifidum* when grown on lactose [6]. In Chapter II, we screened for increased adhesion of *B. infantis* ATCC 15697 in the absence and presence of various milk-derived components. Overall, the percentage of the original inoculum which adhered to the HT-29 cells under untreated conditions was 0.49%, which is similar to adhesion rates observed for this strain previously (0.40% ± 0.18%) by Kavanaugh *et al*., [8]. While bifidobacteria attach at a low percent, any increase in this adhesion stimulated by milk-derived components may have profound beneficial effects and may improve immune modulation, induce anti-inflammatory and antioxidant responses, facilitate competitive pathogen exclusion and produce anti-microbial substances [2,3], however further studies are required to investigate and confirm such potential benefits. Overall, our screening study demonstrated the increased adhesion of the indicator strain, *B. infantis* to HT-29 cells following pre-treatment with an immunoglobulin enriched fraction from bovine whey, goat milk oligosaccharides and the positive controls, human milk oligosaccharides and a combination of 3' and 6' sialyllactose. Furthermore, similar investigations were conducted in Chapters V and VI using conventional assays to determine the effect of a camel milk fraction (CMF) and lactose respectively on bifidobacterial adhesion to HT-29 cells. However, in this case, a panel of bifidobacterial strains found across the human life span were also assessed (Chapter V and Chapter VI). CMF was found to increase the adherence of three strains; *B. infantis* ATCC 15697 (1.4 fold), *B. infantis* ATCC 15702 (1.8 fold), and *B. longum* ATCC 15708 (3.3 fold) from the seven bifidobacterial strains examined. Lactose was not the causative agent suggesting other components in the CMF may be involved. Chapter VI, revealed an increase in lactose-treated *B. breve* UCC2003 adherence (2.4 fold) to HT-29 cells. In addition, a transposon mutant of *B. breve* UCC2003, 101C6, which is unable to degrade lactose also displayed a 2-fold increase in adhesion in response to lactose indicating that lactose metabolism was not implicated in the increased adhesion phenotype observed.

One interesting observation in this thesis was that increased adhesion typically occurred before or in the absence of bacterial growth, suggesting that an up-regulation of genes involved in adhesion may be associated with a down-regulation of genes involved in complex oligosaccharide metabolism as reported previously [8]. Moreover, previous studies have suggested that an increase in gene expression associated with adhesion correlates with a decrease in gene expression associated with carbohydrate utilisation [8]. Consequently, to further distinguish this adherence promoting activity from prebiotic effects, transcriptomic

studies over different exposure times and in the presence of the various milk components should be examined. A shift from adhesion-related gene expression to carbohydrate metabolism might be expected in the treated bacteria. Alternatively, profiling the expression of specific genes associated with increased adhesion of *B. infantis* ATCC 15697 such as DNA-binding protein-ferritin, GroEL, DnaK and TadE [8] could be conducted using RT-qPCR to assess transcriptional changes occurring over time in response to the milk components.

HMO grown *B. infantis* is known to influence the expression of the epithelial cell surface receptors [6]. A separate proteomics study could be used to evaluate the proteins produced by the treated and untreated strains in response to the various milk components investigated in this thesis. Specific proteins might be produced at higher or lower levels following the pre-treatments and thus this would give insight into the mechanism by which increased adhesion is achieved in some of the treated bifidobacterial cells. Furthermore, a proteomics study could also be conducted to determine the effect of treated and non-treated bifidobacteria on the production of membrane proteins of the intestinal surface. This would prove interesting as the receptors bifidobacterial utilise are largely unknown. This could be conducted using label-free quantitative proteomic analysis.

Chichlowski *et al.,* [6] used real-time quantitative PCR to measure adhesion and translocation of *B. infantis* and *B. bifidum* on Caco-2 and HT-29 cells, however, this method does not give insight into the viability of the attached and internalised bacteria. In this thesis, we wanted to capture the viability of the bacterial cells and thus employed the colony counting method in order to enumerate viable bacterial numbers. The colony counting method is commonly used in adhesion and colonisation assays. Morrin *et al.,* [9], used colony forming units (CFU) counts during adhesion assays to demonstrate bovine colostrum modulates the intestinal epithelial cells in turn allowing increased commensal colonisation. Lane *et al.,* [19], showed anti-infective effects of bovine colostrum oligosaccharides against *Campylobacter jejuni* invasion of HT-29 cells again using CFU counts. Ross *et al.,* [20] used colony counting to demonstrate the ability of defatted bovine milk fat globule membrane to inhibit enterohaemorrhagic *Escherichia coli* O157:H7 attachment to HT-29 cells. Notably, quantifying bacteria using colony counting on solid agar has been quoted as being the ‘gold standard method’ for enumeration of bacteria bound to or internalized within eukaryotic cells, despite such methods being laborious [21]. Furthermore, a new high-throughput method for the quantification of bacterial-cell interactions using virtual colony counts (VCC) has recently been developed, and this method was validated by demonstrating VCC correlated with CFU

counting after plating [21]. VCC is based on the capturing quantitative growth kinetics, VCC relates the time to reach a given absorbance threshold to the initial cell count using a series of calibration curves. While the colony counting method was the best method to enumerate viable colonising bacteria in this thesis, VCC could be implemented in future assays as this method is a high-throughput, label-free, compatible alternative to standard colony counting, requiring minimized manual input for the quantification of bacteria in in vitro colonisation assays. It is important to note that adhesion assays are not without their limitations. In addition to these assays being highly labour intensive, inherent assay to assay variations can occur. In this thesis, different values in the percent adherence of initial inoculum to HT-29 cells of untreated bacteria varied. For this reason, data was presented as the percentage of adherent cells and graphed as fold-change relative to percent adhesion of control. This variation has the potential to impact the interpretation of the results as alternating levels of bifidobacterial adhesion may indicate the bacteria are not in a consistent phase of growth during adhesion assays, although every effort was made to ensure the consistency. This may be one reason for the inherent assay to assay variation associated with these methods. Notably, the surface properties of beneficial bacteria such as *Lactobacillus rhamnosus* GG have been shown to change during growth and this can influence its adhesion ability to Caco-2 cells [22] demonstrating the importance of stage of growth in adhesion assays. This may also occur for bifidobacteria and indeed transcriptional analysis has shown that a variety of pili genes can be expressed under *in vitro* conditions and can be regulated in response growth phase (in addition to the substrates present and environmental stresses) [23]. In this thesis, transcriptomic analysis was not conducted, so it is impossible to confirm if bacteria were consistently in the exact same phase of growth during replicate assays and this may have influenced the adhesion properties of the bacteria. Variations in the levels of the bacteria attaching to the cells may also be influenced by quorum sensing. As the bacterial population density increases, gene expression may be collectively altered. Functions controlled by quorum sensing include antibiotic production, biofilm formation, and virulence factor secretion [24] and thus, having different levels of bacteria attaching may impacted such factors and influenced results observed. Another consideration is the alterations that occur in bacterial strains under laboratory conditions. Laboratory cultivation of bacterial strains has been suggested to result in loss of bacterial fitness, for example, laboratory adaptation of *C. jejuni* has been shown to cause rapid loss of its flagellar motility [25]. This may also occur in bifidobacterial strains, thus, in future experiments transcriptomics would be beneficial in demonstrating consistency of growth stage before and after exposure to different treatments. Future studies should also consider including strains freshly isolated from

the host that have not undergone numerous rounds of sub-culturing to help ensure competitive fitness of the strain. As an alternative to transcriptomics, parallel microscopy during adhesion assays may aid in identifying any day to day to variations in bacterial adhesion are recorded. Moreover, pilus-like structures have been observed on several bifidobacteria by electron and atomic force microscopy, and this might be a viable option for observing the variations that may occur in strains that have been sub-cultured on multiple occasions [23].

The models used to date for assessing the impact of milk components on host-microbe interactions, as such as the HT-29 cells used here have limitations. These single-cell models do not mimic the composition of the normal intestinal monolayer, which contains more than one cell type, and they also lack a fully formed mucus layer to separate the epithelial cell layer from the luminal content [26]. For this reason, it is important that in future studies, a robust model system is used to study the interactions between the gastrointestinal epithelium and colonizing microbes. Organoids allow for restoration of the physiological composition of intestinal epithelial cells. They represent suitable models to study the mechanisms of development and differentiation or the molecular basis of functions in specific types of cells, such as goblet, paneth, tuft and M cells. Intestinal organoids are becoming more common as model systems to investigate the dynamic processes occurring at the host–microbe interface. Moreover, multi-omics frameworks have been applied to organoids to obtain a holistic view of the molecular mechanisms that drive differential gene and protein expression [27]. When moving from *in vitro* to *in vivo* models, pigs are more similar to humans when compared to rodents, in terms of anatomy, physiology, microbiota, and genetics, providing a more attractive model for elucidating the influence of milk components on host-microbe interactions.

Bifidobacterial growth

In chapter III and IV, we explored the effect of the selected milk components on bifidobacterial growth over time. Notably, over longer exposure periods *B. infantis* metabolized the GMOs correlating with a 104% increase in growth over a 24 h period when compared to the negative control. Metabolite analysis in the 24 h period also revealed increased production of acetate, lactate, formate and ethanol by GMO-treated *B. infantis*. Statistically significant changes in the GMO profile were also demonstrated over the 24 h period, indicating that the strain was digesting certain structures within the pool. IGEP had no effect on the growth of *B. infantis* over a 24 h incubation period, although a slight increase in ethanol production was revealed through metabolite analysis (Chapter IV, Table 1) indicating that trace levels of digestible glycans may be present in the powder. Overall, these results suggest that early exposure of *B.*

infantis to these milk-derived powders modulates the adhesion of the strain, while carbohydrate utilisation (in the case of GMO) becomes more important after the bacteria have transiently colonised the host cells in adequate numbers. Notably, oligosaccharides were the best at promoting colonisation from all the components examined, and also demonstrated prebiotic effects. This is in line with other reports that suggest that growth on human milk oligosaccharides (HMOs) does not typically commence until nearly 20 hours following initial exposure [28].

Pathogen Exclusion

There are two main mechanisms of action that milk-derived glycans facilitate pathogen protection. Glycans can attach to bacterial cell receptors, neutralising their ability to bind gut cell receptors [29]. Alternatively, glycans may act as analogues for epithelial cell carbohydrate receptors and thus competitively exclude pathogens [30]. Notably, probiotic strains have also been shown to exhibit adhesive properties that inhibit the adhesion of bacterial pathogens to host cells [31] and multiple studies have indicated protection from pathogens through competitive exclusion [32]. A further aim of this thesis was to explore if increasing the colonisation of bifidobacteria might lead to preventing pathogenic colonisation. *Campylobacter jejuni* was selected as a case study given that globally, *Campylobacter* a major global cause of human gastroenteritis [33] and is associated with the development of severe secondary diseases [34,35,36]. Currently, *Campylobacter* is considered a drug-resistant threat due to the rising numbers of resistant infections in the community [37]. Considering this, finding alternatives to reduce the risk of infection is highly relevant.

In Chapter III, IV, V and VI we showed a synbiotic combination of *B. infantis* with GMOs, IGEP and CMF respectively, reduced attachment of a highly invasive strain of *Campylobacter jejuni* to intestinal HT-29 cells by 42%, 48% and 43% respectively. Similarly, a synbiotic combination of *B. breve* and lactose reduced attachment of *C. jejuni* to the mammalian cells by 42%. The adherence ratios of *C. jejuni* to *B. infantis* under treated and untreated conditions in the chapters of this thesis are not directly comparable as these assays were conducted independently of each other. However, in chapter III, under untreated conditions we estimated the adherence ratios of *C. jejuni* to *B. infantis* to be approximately 1:1, while under treated conditions the ratio was 1:3.4. Similarly, in Chapter IV, we estimated the adherence ratios of *C. jejuni* to *B. infantis* to be 1:0.6 under untreated conditions, and 1:2 in treated conditions. This might explain why there were reductions in *C. jejuni* colonisation in these assays. However, to confirm these estimations, adhesion and exclusion assays should be conducted as

one single experiment, such that both *C. jejuni* to *B. infantis* adhesion is captured in parallel. While a reduction in pathogen colonisation using a synbiotic combination of oligosaccharides (GMOs) is not surprising, the results obtained for IGEP, CMF and lactose were unexpected. However, it should be noted that result obtained with IGEP may be glycan driven also as the metaperiodate treatment abolished activity, however further experiments where IGEP is treated with an endoglycosidase and filtered to separate the protein and released oligosaccharide fraction should be conducted to confirm that glycans and not the protein fraction is implicated in the observed activities. Notably, the CMF contained oligosaccharides which may have been implicated in the observations of this thesis.

Remarkably, the expression of genes encoding of DNA-binding protein-ferritin, GroEL, DnaK and TadE, which have increased expression following growth on HMO [8] are also associated with pathogen colonisation [38-46], which may facilitate the competitive exclusion of pathogens. The dps-ferritin type family has been suggested to be involved in the formation of fine tangle pili in *Haemophilus ducreyii* [38] and may contribute to adhesion and protection against oxidative stress in *Helicobacter pylori* [39]. GroEL is known to be expressed on the surface of pathogens including *Clostridium difficile* and *Escherichia coli*, and has been suggested to be involved in adhesion and immunomodulation [40-45]. Hsp70 (DnaK) may initiate pathogen survival processes, activating the hosts immune response [47] and TadE has been implicated in the formation and assembly of type IVB pili [48], and *Salmonella enterica* serovar *Typhi* has been reported to implement type IVB pili in order to enter human intestinal epithelial cells [49]. Additionally, CadF of *campylobacter* has been suggested to be involved in adhesion as the gene encodes a protein with a fibronectin-binding domain, which is thought to aid in the attachment to epithelial cells [50]. Interestingly, *B. bifidum* strains can adhere to fibronectin [51] and homologs of the fibronectin type III domain proteins are present in other *Bifidobacterium* species such as *B. adolescentis* and *B. breve* [52]. Thus, increased expression of such factors may facilitate competitive exclusion of campylobacteria. For example, it is possible that bifidobacteria and campylobacter may both compete for the fibronectin on the cell surface. While this remains a possible mechanism of action, the fact that adhesion rates of bifidobacteria are low might suggest that competitive exclusion through blockage of pathogen binding sites may not be the mechanism of action.

Another possible mechanism is that treated bifidobacteria may have activated *B. infantis* such that it influenced the expression of epithelial cell surface receptors resulting in pathogen protection. *C. jejuni* has been shown to enter the gut epithelial cells and impair intestinal barrier

function through cleavage of occludin by serine protease HtrA [53]. Notably, occludin expression in Caco-2 cells has been demonstrated to be higher when incubated with HMO grown *B. bifidum* [6]. Thus, by increasing expression of certain cell surface receptors may make it more difficult for pathogens to impair intestinal barrier function and colonise the host. Alternatively, treated bifidobacteria may facilitate the production of bacterial anti-microbial biproducts. The presence of bifidobacteria in the gut can modulate the production of formate, acetate, ethanol and lactate [54]. Oligosaccharides have been shown to increase bifidobacterial production of such compounds [55]. The inhibition of gram-negative bacteria through the production of metabolites has been shown [56]. Treated bifidobacteria may produce increased levels of short chain fatty acids and this may facilitate direct pathogen protection or may modulate the epithelial cells such that they have resistance to pathogenic colonisation. However, further investigations are required to understand the intricacies of how protection is achieved.'

Future studies employing 96-well plates coated with fibronectin [57,58] should be implemented and treated with bifidobacteria and campylobacter to determine if binding occurs. Proteomic studies should also be conducted to evaluate the proteins produced by the different strains in response to the different milk powders. Furthermore, proteomics studies could be used to determine the effect of treated and non-treated bifidobacteria on the array of receptors and membrane proteins of the intestinal surface. Additionally, future studies such also investigate if there are any major differences in the supernatants of HT-29 cells following exposure to treated and untreated bifibacteria to assess any differences in the metabolites and proteins present.

Commercial availability of Milk Bioactives

We are beginning to see how the microbiota of the human gastrointestinal tract (GIT) can drive the development of new products and services to benefit human health and wellbeing. This thesis identifies a new application for components from a variety of milks. Compositions containing such components have the potential for supplementation in many products such as infant formula or as food supplements for toddlers, with the aim of improving the discrepancy of *Bifidobacterium* counts found between breastfed and formula-fed infants. Such compositions may also have potential in products aimed at benefiting the elderly, immuno-compromised individuals, individuals on antibiotics or indeed as a means for treating or preventing diseases associated with lower counts of commensal bacteria such as inflammatory bowel diseases (Crohn's disease, irritable bowel syndrome, ulcerative colitis), periodontal

disease, rheumatoid arthritis, atherosclerosis, allergy, multi-organ failure, asthma, and allergic diseases [59]. The outputs of this thesis may be exploited by a number of sectors. Apart from the dairy industry, companies which market probiotics may be interested in these results. According to global market analysis, the global probiotic market size is predicted to exceed 3.5 billion US dollars by 2026 [60]. The *Bifidobacterium* market size prediction suggests it will increase close to 6% until 2024 [61]. However, there are concerns that most microbial supplements are unable to establish themselves in the gut and fail to exert an effect on the resident community. Companies supplying probiotics will no doubt be interested in any natural means of increasing establishment of these microbes in individuals.

Limitations relating to the powders used in this thesis were plentiful, and despite the labour-intensive isolation techniques and variety of characterisation methods used, it is not possible to guarantee the purity of the powders under investigation. The low molecular weight fraction of camel milk contains multiple components including unidentified peptides, the IGEP predominantly contained IgG, but may also contain other components such as IgA and IgM at very low levels. The oligosaccharide powders were composed predominantly of oligosaccharides, but lactose was still present at low levels and other contaminants such as low molecular weight peptides may also have escaped the filtration process. Thus, as a next step, powders should be fully characterised by mass-spectrometry. One of the most important considerations of this work was the potential of such components to be produced on an industrial scale. Notably, all animal milks under investigation are currently commercially produced. The annual production of bovine, goat and camel milk between 2019 and 2017 was approximately 522, 18.7 and 2.9 million metric tons respectively [62,63]. Powders such as lactose can be obtained at high yield from bovine milk dairy streams and thus are commercially viable. Bovine milk is the most widely commercially available source of bioactive components investigated in this thesis. The Irish bovine dairy industry produced approximately 6.65 billion liters of milk in 2016, making up around to 1% of global production [64,65] with approximately 85% of dairy products being exported globally. The abolishment of milk quotas further supports the commercial availability of bovine milk in Ireland. Lactose is a natural disaccharide obtained from the milk of most mammals and a waste product of cheese and casein manufacturing. Due to its physiological and functional characteristics, it is used in a large number of foodstuffs as well as in the pharmaceutical industry [66]. Lactose is produced from whey by crystallizing an oversaturated solution of whey concentrate. Global demand for lactose has grown appreciably over the last 20 years and ways of adding value to lactose such as those described in this thesis will no doubt be welcomed by the dairy industry. Reviewing the current

literature on lactose digestion, it became clear that estimating the amounts of lactose that might reach the colon intact proves difficult. Further research on tracking lactose through gastric transit is required to fully understand the extent to which lactose influences host-microbe interactions.

Ingredients enriched in IgG similar to IGEP isolated from bovine milk could be also easily manufactured with the use of membranes [67] and are readily available in bovine colostrum and milk (49.27g/L and 0.64g/L respectively) [68]. Maintenance of this biological integrity is of the utmost importance for the inclusion of Igs in functional foods. Igs are considered relatively heat-labile, in which antigen-binding sites are more heat-sensitive than the other areas [69]. Exposure of Ig to heat treatments can directly alter the conformational integrity, which in turn influences Ig functionality. Many authors have suggested that minimum exposure to heat treatments should be achieved when manufacturing Ig based functional foods or immune supplements [69-71]. Studies have shown that the structure of Ig and its functions are usually affected by heat treatments of above 65 °C [70,72] and as such future studies evaluating the effect of the various processing steps on IgG's ability to promote bifidobacterial adhesion to intestinal cells is required.

Purified milk-derived bioactives are expensive and time-consuming to isolate and multiple processing steps can lead to loss of bioactivity. For this reason, products produced from minimal processing such as the CMF described in this thesis may be an effective alternative. *In vivo*, it is likely that the lactose present in the CMF would be largely digested, and the oligosaccharides present therefore enriched, further enhancing biological activities. However, such a scenario requires investigation in animal trials or dietary intervention studies.

Purified oligosaccharides isolated from goats milk demonstrated a dual effect acting as both a tool to increase the adhesion of *B. infantis* to intestinal cells as well as a means to increase overall bifidobacterial numbers by acting as a prebiotic. However, while goats milk oligosaccharides showed the most beneficial effects, purified oligosaccharides are the most expensive and difficult to isolate. Considering the limitations associated with yields and isolations, currently, it is not realistic to produce powders such as GMO and CMF on an industrial scale. However, membrane filtration technology is continuously improving. In fact, Martinez-Ferez *et al*., [73] described the use of membrane technology for the isolation of oligosaccharides from animal milk using a two-stage tangential filtration process. The final product contained 80% of the initial oligosaccharides. Furthermore, Oliveria *et al*., [74] also used ultrafiltration to remove proteins and fat globules from caprine whey to yield an

oligosaccharide rich fraction. Thus, it is likely that commercially viable isolation technology for oligosaccharides from animal milks will be possible in the future, but for now remains a limitation. Another limitation in terms of ingredient preparations was the presence of potential allergens in the milk. For example, humans lack the ability to synthesize the common sialic acid, *N*-glycolylneuraminic acid (Neu5Gc). However, these are present in animal milk and meat [75]. Humans can synthesise polyclonal antibodies against Neu5Gc-glycans when they are ingested [76,77]. Neu5Gc can be metabolically incorporated into newly synthesized glycans which are present on human cells [78,79]. The combination of Neu5Gc-containing epitopes and circulating anti-Neu5Gc antibodies are suggested play a role in chronic inflammation-mediated diseases [79]. Thus, any ingredient preparation would have to be clear of such components to remove the risk of host health implications. This could be done using membrane filtration technology or enzymatic digestions. As the synthesis of oligosaccharides at large scale becomes more commonplace, deciphering which oligosaccharides in the GMO pool may be enhancing colonisation of bifidobacteria could prove important and allow the manufacture of individual bioactive structures.

Conclusion

The results from this thesis demonstrate that animal-derived milk components have the potential to be used as bioactive alternatives to human milk components. These beneficial effects include increasing the adhesion of specific bifidobacterial strains to intestinal cells as observed with *B. infants* treated with GMO, IGEP and CMF, and lactose treated *B. breve*. This increase in adhesion may represent a novel bioactivity, distinct from prebiotic, which specifically increases the adhesion potential of probiotic strains, effectively acting as 'adhesin-biotics'. One of the most interesting findings in this thesis was the associated protection against pathogen colonisation observed when synbiotic combinations were introduced. While this thesis raises questions in terms of the mechanism of action of these bioactives, it also highlights the economic value of milk components that are in some cases seen as waste-products. In addition, the latest trend in probiotic research, next-generation probiotics, should be considered going forward. Such strains go beyond traditional terminology and include microorganisms with beneficial actions that are not members of the commonest probiotic genera, lactobacilli and bifidobacteria. This is a factor which should be considered in future studies in the selection of bacteria to be used to evaluate the milk-derived products. Overall, this thesis represents a step towards a better understanding of host-microbe interactions that take place in the infant gut, which in the future can be applied to promote gut health.

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