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**Bifidobacterial interactions with human milk
oligosaccharides and their combined potential for
the advancement of infant nutrition**

**A Thesis Presented to University College Cork for the Degree
of Doctor of Philosophy (Ph.D) in the School of Microbiology**

January 2025

By

Clodagh Walsh, B.Sc, M.Sc^{1,2,3}

¹ Teagasc Food Research Centre, Moorepark, Fermoy, Cork, Ireland

² APC Microbiome Ireland and School of Microbiology,
University College Cork, T12YT20, Ireland

³ Health and Happiness Group, H&H Research, Cork, P61K202, Ireland

**Research Supervisors: Dr. Rita M. Hickey^{1,3}, Prof. Douwe van
Sinderen², and Dr. Jonathan A. Lane^{1,3}**

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DECLARATION

I hereby certify that this material which I now submit for assessment on the programme of study leading to the award of PhD is entirely my own work, and has not been submitted for another degree, either at University College Cork or elsewhere.

Signed: *Clodagh Walsh*

Date: 08/05/2025

ABSTRACT

The primary aim of this research was to investigate the role of human milk oligosaccharides (HMOs) in shaping the infant gut microbiota and in supporting immune health. HMOs are complex carbohydrates found at high concentrations in human breast milk, where they confer various critical functions. Despite their inability to directly provide calories or nutritional value, HMOs are believed to play a crucial role in supporting infant gut health as well as in strengthening their immune system. The unique structural diversity of HMOs, including fucosylated and sialylated forms, may enhance their ability to interact with a wide range of microbial and host factors, and may influence overall host health. Given the increasing reliance on infant formula in populations where breastfeeding is not feasible, understanding the beneficial potential of HMOs to mimic the advantages of breast feeding is crucial. The research presented in this thesis aimed to investigate immunomodulatory effects of different HMO combinations, cooperative metabolism of bifidobacteria, the impact of these factors on gut microbiota composition and functionality, and the ability of HMOs to enhance bifidobacterial intestinal adhesion. Addressing these topics is of importance in order to advance infant nutrition, as the generated knowledge is expected to provide insights into how dietary components can foster a healthy gut environment, potentially reducing the risk of diseases associated with dysbiosis in early life.

The central research questions guiding this thesis were: How do HMOs influence the development of the infant gut and infant gut microbiome, and what mechanisms underlie these interactions?

The specific objectives included: Acquisition of a comprehensive understanding of the functional biology of HMOs, detailing their importance in infant development and gut health (Chapter I), gain insights into the characterization, production, and commercialization of HMOs, addressing the challenges of availability and synthesis that have historically limited their clinical applications (Chapter II), explore the specific adaptations of bifidobacteria to allow HMO metabolism (Chapter II), investigate the immunomodulatory effects of different HMO combinations (Chapter IV), characterize the cooperative metabolism of bifidobacteria on HMO substrates (Chapters V and VI) and assess the *in vitro* effects of HMOs on the adhesion of bifidobacteria to intestinal epithelial cells (Chapter VII).

To achieve these objectives, a comprehensive and multidisciplinary methodology was employed, integrating both experimental and analytical approaches. The initial phase involved establishing an *in vitro* co-culture model using Caco-2 intestinal epithelial cells and THP-1 macrophages to simulate the intestinal environment. This model enabled the examination of immune responses under controlled conditions. The research design incorporated quantitative methodologies, including multiplex ELISA, to assess changes in inflammatory cytokines following exposure of inflamed intestinal cells to various HMO combinations. To address the research questions related to bifidobacterial interactions with HMOs, the metabolic activities of bifidobacteria were investigated through co-cultivation experiments, where different strains were grown on various HMO substrates. The research specifically delved into the characteristics of four distinct commercial bifidobacterial strains: *Bifidobacterium bifidum* R0071, *Bifidobacterium longum* subsp *infantis* R0033, *Bifidobacterium breve* M-

16V, and *Bifidobacterium longum* subsp. *infantis* M-63 in the presence of HMOs. High-performance liquid chromatography (HPLC) and metabolomic analyses were employed to evaluate the consumption of HMOs and the production of beneficial metabolites, such as SCFAs, during bacterial growth. Whole-genome sequencing, *in silico* analysis, and label-free proteomic techniques were used to characterize strain-specific differences in HMO utilization and to identify proteins involved in these metabolic processes. Following on from this, the adherence of these probiotic strains in the presence of HMOs was evaluated using HT29-MTX human tumorigenic cell lines to mimic bifidobacterial adhesion to intestinal epithelial cells (IECs). The HT29-MTX cell line, which is capable of constitutively producing mucin, has more physiologically relevant characteristics when compared to the use of the HT29 cell line due to mucus layer formation, and has therefore been proposed as a more suitable cell line for studying host-microbe interactions. By combining these methodologies, the research aimed to provide a thorough examination of the complex interactions between HMOs, bifidobacteria, and the human host contributing to a deeper understanding of their roles in developmental health.

The findings of this research yielded several significant insights into the role of HMOs in promoting gut health. One of the key discoveries was that specific combinations of HMOs, particularly 2'-fucosyllactose (2'-FL) and 6'-sialyllactose (6'-SL), were effective in modulating immune responses. These HMOs significantly reduced pro-inflammatory cytokine levels in the co-culture human cell model, indicating their potential to mitigate inflammation in the infant gut. Moreover, the investigation into the cooperative metabolism of bifidobacteria revealed that interspecies

interactions significantly enhance HMO utilization. Co-cultivation of four commercial infant-derived bifidobacteria strains demonstrated that the presence of multiple strains led to increased bifidobacterial populations and the production of beneficial metabolites, suggesting that cooperative behaviour among strains is crucial for establishing a healthy gut microbiota. Another important finding was that HMOs enhanced the adhesion of bifidobacteria to intestinal epithelial cells. The research indicated that exposure to HMOs increased the adherence of the various *Bifidobacterium* strains by up to 90%, underscoring the role of HMOs in promoting colonization and reinforcing gut health. HMOs may prime infant gut-associated *Bifidobacterium* for colonisation to intestinal epithelial cells by influencing the expression of various colonization factors. The adhesive potential of bifidobacterial strains can be substantially increased through treatment with milk oligosaccharide components.

Overall, the research covered in this thesis has provided valuable insights into the role of HMOs in promoting gut health and shaping the infant gut microbiota. By addressing critical questions regarding the immunomodulatory effects of HMOs, the cooperative metabolism of bifidobacteria, and the mechanisms underlying bacterial adhesion, this research contributes to a deeper understanding of how dietary components can influence health during infancy. The findings not only support the existing knowledge of HMOs but also emphasize the potential of HMOs and commercially available bifidobacteria as key ingredients in formulating advanced infant nutrition products that mimic the health benefits of human milk, ultimately supporting the development of healthier infant gut microbiota and improved health outcomes.

PUBLICATIONS

Walsh, C., Lane, J. A., van Sinderen, D., & Hickey, R. M. (2020). Human milk oligosaccharides: Shaping the infant gut microbiota and supporting health. *Journal of Functional Foods*, 72, 104074.

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Walsh, C., Lane, J.A.; van Sinderen, D.; Hickey, R.M. (2024). Tailored Combinations of Human Milk Oligosaccharides Modulate the Immune Response in an In Vitro Model of Intestinal Inflammation. *Biomolecules*, 14, 1481.

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Walsh, C., Owens, R. A., Bottacini, F., Lane, J. A., van Sinderen, D., & Hickey, R. M. Interrogation of human milk oligosaccharide utilization pathways in an infant bifidobacterial community by integrated genomics and proteomics. 6th World Congress of Pediatric Gastroenterology, Hepatology and Nutrition, 2020.

Walsh, C., Owens, R. A., Bottacini, F., Lane, J. A., van Sinderen, D., & Hickey, R. M. Human milk oligosaccharide promote adhesion of commercial bifidobacteria to human intestinal cells. 54th Meeting of the European Society for Paediatric Gastroenterology, Hepatology and Nutrition. Copenhagen, 2022.

Walsh, C., Lane, J. A., van Sinderen, D., & Hickey, R. M. Is more HMO a key factor in closing the ‘immune-gap’ between formula-fed and breast-fed infants?. 55th Annual Meeting of the European Society for Paediatric Gastroenterology, Hepatology, and Nutrition. Vienna, 2023.

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Walsh, C., Owens, R. A., Bottacini, F., Lane, J. A., van Sinderen, D., & Hickey, R. M. Interrogation of human milk oligosaccharide utilization pathways in an infant bifidobacterial community by integrated genomics and proteomics. 6th World Congress of Pediatric Gastroenterology, Hepatology and Nutrition, 2020.

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Tingmin Hoe, based on interview with **Walsh, C.** and Lane, J. “HMObiotics”: H&H deepens research on specific HMOs, probiotic pairings for health benefits. Nutraingredients, March 2022.

AWARDS

Nominated by WCPGHAN committee for The Annual Jean Rey Prize at the 6th World Congress of Pediatric Gastroenterology, Hepatology and Nutrition, 2020.

Teagasc Walsh Scholar of the Year Finalist 2021

ABBREVIATIONS

1,2-PD	1,2-propanediol
2'-FL	2'-fucosyllactose
3'-SL	3'-Sialyllactose
3-FL	3-fucosyllactose
6'-SL	6'-Sialyllactose
ABC	ATP-Binding cassette
ANOVA	One-way analysis of variance
ATCC	American Type Culture Collection
BM-HMO	Breastmilk-derived HMO
CBM	Carbohydrate binding molecule
CE	Capillary Electrophoresis
cfMRS	Carbohydrate free De Man-Rogosa-Sharpe
CFU	Colony forming units
CLA	Conjugated linoleic acid
CMA	Cow's milk protein allergy
ct	Cycle threshold
DC	Dendritic cells
DC-SIGN	DC-specific intercellular adhesion molecule-3-grabbing non-integrin
DFL	Difucosyllactose
DMEM	Dulbecco's Modified Eagle's Medium
DP	Degrees of polymerization
DSLNT	Disialyllacto- <i>N</i> -tetraose
DTT	Dithiothreitol
ECACC	European Collection of Authenticated Cell Cultures

EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
ESI	Electrospray Ionization
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FHMO	Fucosyllated HMO
FOS	Fructooligosaccharides
FOS	Fructooligosaccharides
FSLNH	Fucosyl-lacto- <i>N</i> -hexaose
FSLNnH	Fucosyllacto- <i>N</i> -neohexaose
Fuc	Fucose
Gal	Galactose
GBS	Group B Streptococci
GH	Glycosylhydrolase
GI	Gastrointestinal
GL	Galactosyllactose
Glc	Glucose
GlcNAc	<i>N</i> -acetylglucosamine
GO	Gene ontology
GOS	Galactooligosaccharides
GRAS	Generally Regarded as Safe
GT	Glycosyltransferase
h	Hour
HBGA	Histo-blood group antigens
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus

HMO	Human Milk Oligosaccharide
HPAEC	High-pH anion-exchange chromatography
HPLC	High Performance Liquid Chromatography
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IEC	Intestinal epithelial cell
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMS	Ion Mobility Spectrometry
kDA	Kilodaltons
kg	Kilogram
L	Liter
LAB	Lactic acid bacteria
LDFT	Lactodifucotetraose
LDH	Lactate dehydrogenase
LIF	Laser-Induced Fluorescence
LNB	Lacto- <i>N</i> -biose
LNDFH	Lacto- <i>N</i> -difucohexaose
LNFP	Lacto- <i>N</i> -fucopentaose
LNH	Lacto- <i>N</i> -hexose
LNnH	Lacto- <i>N</i> -neohehexose
LNnT	Lacto- <i>N</i> -neotetrose
LNT	Lacto- <i>N</i> -tetrose
LPS	Lipopolysaccharide
LST	Sialyllacto- <i>N</i> -tetraose

MAPK	Mitogen-activated protein-kinase pathways
MCP	Monocyte chemoattractant protein
MIP	Macrophage inflammatory protein
Mr	Molecular weight
MRD	Maximum Recovery Diluent
MRS	De Man-Rogosa-Sharpe
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
NaCl	Sodium chloride
NEC	Necrotizing enterocolitis
Neu5Ac	<i>N</i> -acetylneuraminic acid
NF-κB	Nuclear factor (NF)-κB
NMR	Nuclear Magnetic Resonance
NP-LC	Normal Phase Liquid Chromatography
NS-HMO	Non-secretor HMO
OD	Optical Density
PBS	Phosphate-buffered saline
PGC	Porous Graphitic Carbon
PMA	Phorbol 12-myristate 13-acetate
qPCR	Quantitative polymerase chain reaction
QPS	Qualified Presumption of Safety
QqQ	Triple Quadrupole
RI	Refractive index
RP-LC	Reversed Phase Liquid Chromatography
RPMI	Roswell Park Memorial Institute Medium
RV	Rotavirus

SBP	Solute binding proteins
SCFA	Short-chain fatty acid
SDP	Sortase-dependent protein
SEC	Size Exclusion Chromatography
S-HMO	Secretor HMO
SIgA	Secretory Immunoglobulin A
Siglecs	Sialic acid-binding immunoglobulin-like lectins
SPE	Solid Phase Extraction
Srt	Sortase
TEER	Trans-epithelial electrical resistance
TFA	Trifluoroacetic acid
T1D	Type-1 diabetes
TLR	Toll-like receptor
TNF	Tumour Necrosis Factor
TOF	Time of Flight
WHO	World Health Organisation

ACKNOWLEDGEMENTS

As I reflect on the journey of this PhD, two major thoughts come to mind.

Firstly; I think of the paradox of knowledge or as Einstein once said “The more I learn, the more I realize how much I don't know”. From an early age, I was encouraged to have a curiosity about the world and seek truth wherever I wandered. Throughout this PhD, I have been bestowed with the tools that will allow me to do exactly this. But perhaps one of the greatest tools I have been granted is to realise that there is no such thing as an absolute truth because human knowledge is always evolving, limited by our cognitive and sensory abilities, as well as the context within which we operate. This PhD has made me come to realise the importance of arguments of philosophers of science, like Karl Popper and Thomas Kuhn, that science is not about discovering ultimate, unchangeable truths but about developing progressively more accurate and comprehensive explanations of the world. Science can bring us as close to the truth as possible by continuously refining and improving our understanding through empirical observation, experimentation, and logical reasoning. It is this process of ongoing inquiry and adjustment that allows science to provide ever more accurate approximations of how the world works. I want to acknowledge all my early teachers, lecturers, and those who imparted a curiosity within me, and also the forefathers of science; from the Ancient Greek and Hellenistic Scholars to Renaissance Thinkers to Early Modern Scientists to the early HMO researchers who I continue to admire and learn from.

The second major thought or philosophy that comes to mind is that of the Ubuntu philosophy; "I am because we are." A person is shaped by the people they surround themselves with and I have certainly been shaped by an endless number of people during my PhD journey. My time in Moorepark Research Centre is segregated into eras which are defined by the people, but also the specific place in the canteen where we sat. The early days were spent in the old canteen at the top table with Sarah, Anne-Marie, ‘Old’ Kev and company. Then came the adventurous, wilder era with Eibhlin, Ruth, Edel, Becky, and the Italians, followed by the days sitting in the interim COVID-era canteen with Leonie, ‘Young’ Kev, and Ashok trying to speak to each other through Perspex screens and getting our daily dose of entertainment by watching people in the carpark out the conference-room windows. And these days, I find myself sitting in some of the most uncomfortable seats in the swanky new Moorepark canteen

with the Health & Happiness crew. A journey of endless conversations about life, death, food, politics, sport, and everything in between.

Throughout this time in Moorepark, I also dedicated my time to leading the sports and social committee with some beautiful creative souls (Chloe and Amy in particular come to mind!). The social and charity events we organised during this time provided an avenue for escapism but also created environments where people could network, debate, and celebrate each other's talents and cultural differences. Moorepark is a constantly changing, dynamic, multicultural, and innovative space with some of the most ambitious and learned minds. It is also a place where everyone is willing to lend a helping hand (Michelle O'Donnell in my early days particularly comes to mind). I believe this is the case for two reasons; because we understand that life is a game of give and take but also, Moorepark is a place where people understand that the sharing of knowledge and time contributes to the advancement of scientific explorations. Either way, it creates an environment where everyone can find space to flourish. This is thanks, in no small part, to the leaders and directors of the Teagasc organisation, the Food Research Centre and the Walsh Scholar post-graduate program. I want to also acknowledge University College Cork, particularly the staff in APC and the School of Microbiology, for their continuous support throughout this PhD program. I have been a UCC student throughout all the formative years of my life; from my BSc in Biochemistry to my MSc in Biotechnology, to this current PhD program. UCC is a centre of educational excellence and I have taken tremendous pride in representing the university throughout my educational journey. Throughout this PhD program I also took to the sporting field for UCC and played a part in bringing silverware back to Leeside. Donning the skull and crossbones throughout my academic life with UCC is something I will cherish forever.

It would be remiss of me not to dedicate a significant proportion of these acknowledgements to the Hickey Glycobiology lab group and my supervisors who have constantly encouraged me to be better, explore further, dig deeper. Thank you all for your support, training, and guidance throughout. To Helen, who has become more like a maternal figure and friend than a lab manager or technical support, I will cherish the endless conversations we had about golf, wine, cats, and of course the LGBTQ+ community in Cork. To Sinead, post-doc and friends, who was there from the very start to offer guidance and support and

to remind us that we were capable and deserving of great things. To Geoff and Leonie and all the others who came through the doors of the office and lab (whether for a week or a year), thank you for the endless laughs and occasionally overly long conversations about Love Island. I like to believe that we created an environment in the lab where we could all prosper and become better versions of ourselves. To Jonny, a man who has been perpetually in my life for over seven years; from my Masters internship, throughout my PhD, and continuing into my life with H&H. You have not only acted as a supervisor during this time; you have offered me endless opportunities for development, you have been a confidant, a source of inspiration, and a soundboard for new ideas and conversations about sport, politics, and everything in between. To Douwe, one of the most exceptional researchers in Ireland and always at the cutting edge of new and exciting bifidobacterial science. Thank you for the time you gave this research, for the remarkably quick turnaround of corrections (will never understand how you do this!), and for constantly provoking further curiosities in me! And finally, to Rita. Slightly at a loss of words here to capture how grateful I am to have had you as a supervisor but you have been a rock to me over the course of this PhD, always finding time for me when I needed. I will always remember the days where one-hour meetings for data analysis or experimental design turned into three hours in your office talking about family and recounting days gone by in Moorepark. I will always be in awe of your abilities for ideation and innovation, but also how you approach your work as a supervisor with such compassion and care.

And a very last (but certainly not least) dedication to my family, friends, and partner Jean for igniting a deep curiosity of the world within me, for being by my side throughout this journey, for attempting to understand this very niche area of science and making it feel even more worthwhile, for being compassionate when I skipped out on important events and holidays, and for always pushing me to achieve my potential. Now mother... you can finally stop asking me when I am planning on submitting!

THESIS AIMS

Tailored combinations of human milk oligosaccharides (HMOs) can modulate immune function and selectively promote the growth and metabolic activity of bifidobacteria. These interactions can be leveraged to improve infant nutrition formulations by replicating the beneficial effects of human milk. The aims of this thesis therefore were to:

- 1) Identify structure-function relationships between HMOs and immune-modulatory effects.
- 2) Identify and compare the metabolic pathways used by different bifidobacterial strains to degrade and utilize HMOs using genomic and proteomic approaches.
- 3) Assess how cross-feeding and trophic interactions between multi-strain bifidobacterial consortia can influence HMO breakdown and metabolite production.
- 4) Evaluate whether HMO exposure enhances bifidobacterial adhesion and identify the adhesion-related proteins upregulated in response to HMO priming.
- 5) Translate findings into potential applications for infant nutrition:

By bridging microbiome research with mechanistic studies, this thesis aims to provide a deeper understanding of bifidobacteria-HMO interactions, paving the way for more effective nutritional interventions in infant health.

THESIS INTRODUCTION

Infant nutrition is critical during the early stages of human development, influencing both immediate and long-term health outcomes (Langley-Evans, 2015). The composition of an infant's diet profoundly affects growth, immune function, and the establishment of a healthy gut microbiota (Martin et al., 2010). Human milk is recognized as the optimal source of nutrition and protection for infants, providing a unique blend of nutrients and bioactive components tailored to meet their specific needs (Martin et al., 2016). Among these components, human milk oligosaccharides (HMOs) have emerged as a significant focus of research due to their multifaceted role in shaping the infant gut microbiota and supporting overall health (Hill et al., 2021). HMOs are a diverse group of complex carbohydrates constituting the third-largest solid component in human milk, after lactose and lipids (Chen, 2015). While indigestible by infants, HMOs are believed to play a crucial role as prebiotics, selectively promoting the growth of beneficial gut bacteria, particularly bifidobacteria (Wiciński et al., 2020). Specific members of the *Bifidobacterium* genus dominate the gut microbiota of breastfed infants and are associated with various health benefits, including protection against pathogens, immune modulation, and enhancement of gut barrier function (Servin, 2004). In addition to fostering beneficial bacteria colonization, HMOs also protect the infant gut by occupying niches and acting as anti-adhesive antimicrobials against pathogens (Bode & Jantscher-Krenn, 2012; Zuurveld et al., 2020). This selective prebiotic activity and close evolutionary relationship between bifidobacteria and HMOs result in a symbiotic interaction wherein these bacteria have developed specific metabolic

pathways to efficiently utilize HMOs as a primary energy source (Marcobal et al., 2010).

The increasing recognition of the importance of early-life microbiota in long-term health further underscores the need for a deeper understanding of HMO-bifidobacteria interactions. Dysbiosis, or the imbalance of gut microbiota in early life, has been linked to various health issues, including allergies, obesity, and autoimmune diseases (Gholizadeh et al., 2019; Zhuang et al., 2019). Elucidating the factors that promote a healthy gut microbiota in infancy, particularly through diet, is paramount. Despite the clear importance of HMOs and bifidobacteria in infant health, various knowledge gaps remain. Recent studies have demonstrated that HMOs not only act as prebiotics promoting beneficial gut microbiota, but also possess direct immunomodulatory properties (Boll et al., 2024; Kong et al., 2020). Specific combinations of HMOs have been shown to modulate immune responses, decreasing pro-inflammatory cytokine levels *in vitro* (Kim et al., 2023; Zhang et al., 2021).

Nevertheless, knowledge on the impact of tailored HMO combinations on inflammation-related diseases remains limited, necessitating further investigation into their structure-function relationships and mechanisms of action. Although it is established that bifidobacteria can metabolize HMOs, the specificity of these interactions and the variability in HMO utilization among different bifidobacterial species and strains are not fully understood. Furthermore, the potential for translating these interactions into practical applications, such as developing infant formulas that mimic the benefits of human milk, remains underexplored. The complexity of HMO structures combined with the diversity of bifidobacterial strains present

significant challenges in delineating the precise mechanisms by which these interactions occur and how such information can be harnessed to improve infant nutrition. To address these gaps, this thesis aims to bridge the divide between broad microbiome studies and detailed mechanistic research by focusing on the specific metabolic pathways by which bifidobacteria utilize HMOs. Understanding the molecular mechanisms of these interactions provides the foundational knowledge necessary for developing more effective, targeted interventions for infant nutrition

This thesis seeks to address these challenges by investigating the intricate dynamics between HMOs, selected bifidobacterial strains, and models of the infant intestine aiming to understand how these interactions contribute to establishing a healthy gut microbiota and overall infant health. By investigating the metabolic pathways utilized by bifidobacteria to metabolize HMOs, this research aims to identify key bacterial species and strains that are particularly effective in utilizing these oligosaccharides. Additionally, the thesis will aim to establish *in vitro* models to elucidate the mechanisms by which bifidobacteria interact with HMOs to enhance their colonisation potential and investigate which HMO combinations can modulate immune responses and potentially mitigate intestinal inflammation. The mechanistic approach adopted in this research contrasts with broader microbiome studies by investigating the underlying biological processes rather than merely cataloguing microbial populations or associations. While sequencing studies largely focus on the composition of the infant gut microbial community, they generally do not capture how HMOs influence host physiology (including gut barrier enhancement and immune cell activation) or impact host-microbiome crosstalk (including the production of

short-chain fatty acids [SCFAs], sialic acid release, or fucosylated metabolites that influence systemic health). This research therefore delves into the molecular details of how specific HMO combinations influence microbial growth, metabolism, and host health by studying specific pathways, receptors, and cellular responses. This mechanistic approach enables a more targeted understanding of how these interactions could be harnessed in practical applications.

The research is guided by several key questions. Can tailored HMO formulations replicate the protective effects associated with natural breast milk against inflammatory responses? What are the specific metabolic pathways utilized by different bifidobacterial species and strains to metabolize HMOs? How do specific combinations of HMOs and multi-strain bifidobacterial consortia influence the composition and function of the infant gut microbiota? What are the implications of bifidobacterial-HMO interactions for the development of advanced infant nutrition products? Finally, how can insights from genomics and proteomics studies be used to enhance the efficacy of bifidobacteria in commercial infant nutrition formulations?

The scope of this study is carefully defined to focus on the interactions between HMOs and bifidobacteria, emphasizing how these interactions influence the composition and function of the infant gut microbiota. Key delineations include focusing specifically on bifidobacterial interactions with HMOs, excluding other gut microbes and dietary components, and selecting specific bifidobacterial species and strains prevalent in the infant gut. The research will primarily utilize *in vitro* models, such as cell culture, bacterial cultures, and gut microbiota simulation systems, complemented by *in silico*

analyses, while excluding clinical trials or direct assessments of infant health outcomes. The research focuses largely on a subset of commercially relevant and clinically proven strains: namely *Bifidobacterium bifidum* R0071, *Bifidobacterium longum* subsp. *infantis* R0033, *Bifidobacterium breve* M-16V, and *Bifidobacterium longum* subsp. *infantis* M-63. This research is based on several key assumptions necessary to address its research questions and provide meaningful insights into bifidobacterial-HMO interactions. Extrapolation of the *in vitro* findings to *in vivo* contexts is assumed, as well as the consistency of bifidobacterial strain behaviour in utilizing HMOs across different experimental conditions. Genomic and proteomic data are expected to provide reliable indicators of bifidobacterial metabolic pathways and functional capabilities.

This study's significance lies in its potential to advance our understanding of the role of diet in shaping the infant gut microbiota and supporting health, particularly regarding bifidobacterial-HMO interactions. Additionally, demonstrating the capacity of tailored HMO combinations to mitigate intestinal inflammation could have profound implications for formulating infant formulas that better emulate the protective effects of human milk, especially for populations where breastfeeding is not feasible. Findings from this study may inform the development of microbiome-targeted therapies for a range of conditions, including gastrointestinal disorders, metabolic diseases, and immune-related conditions. By providing a detailed understanding of the mechanisms through which HMO and bifidobacteria work to support gut health, this research could facilitate the development of new products that meet the needs of various populations, including infants, children, and adults.

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CHAPTER I

Human Milk Oligosaccharide: shaping the infant gut microbiota and supporting health

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Abstract

Human milk oligosaccharides (HMO) are complex sugars which are found in breast milk at significant concentrations and with unique structural diversity. These carbohydrates are the fourth most abundant component of human milk after water, lipids, and lactose and yet provide no direct nutritional value to the infant. Recent research has highlighted that HMOs have various functional roles to play in infant development. These sugars act as prebiotics by promoting growth of beneficial intestinal bacteria thereby generating short-chain fatty acids which are critical for gut health. HMOs also directly modulate host-epithelial immune responses and can selectively reduce binding of pathogenic bacteria and viruses to the gut epithelium preventing the emergence of a disease. This review covers current knowledge related to the functional biology of HMOs and their associated impact on infant gut health.

1. Introduction

Breast milk is the optimal nutrition for infants, and its composition has been shaped by thousands of years of human evolution in order to provide tailor-made nutrition and protection to the developing neonate, all at an energetic cost to the mother (Ballard & Morrow, 2013; Garwolińska et al., 2018). Recent advances in analytical tools and the development and integration of “-omics” technologies have offered valuable insights into the composition of human breast milk, while reinforcing our understanding of the health benefits associated with breast feeding (O’Sullivan et al., 2018). One of the most remarkable features of breast milk is the diversity and abundance of complex sugars (glycans) including human milk oligosaccharides (HMOs), which are indigestible to the infant and for this reason reach the colon intact (Kunz et al., 2000). For decades, the presence of this indigestible material challenged scientists since milk was unlikely to have retained superfluous content that did not benefit the infant. A prebiotic function for these human milk glycans was first proposed by Gyrogy and co-workers in 1954 (Gyrogy, Norris, et al., 1954), and in the subsequent 60-plus years, investigators have propositioned numerous biological, physiological and protective functions which can be ascribed to HMOs (Bode, 2015). The abundance and structural diversity of these compounds would certainly allow for more than one function (Smilowitz et al., 2014).

Major innovations in the field of glycomics have enabled the identification of over 200 HMO structures, with the concentration of oligosaccharides in human milk reaching levels 100-1000 times higher than in the milk of any domesticated farm animal (Oliveira et al., 2015). **Table 1** outlines the structure of the core HMOs found in human milk. Concentrations

of HMO are highest in colostrum (20 g/L) and decrease along the lactation period, with 16 g/L detected in 30 day mature milk (Coppa et al., 1999). HMOs vary in size, structure, and complexity and are composed of 5 monosaccharide building blocks. Units of D-glucose (Glc), D-galactose (Gal), *N*-acetylglucosamine (GlcNAc), L-fucose (Fuc), *N*-acetylneuraminic acid are conjugated via glycosidic linkages to form a myriad of distinct HMO structures. All HMOs contain a lactose core at the reducing end, which is elongated via the addition of GlcNAc units (German et al., 2008). Elongation occurs through the addition of GlcNAc units via β 1-3 linkages (linear structures) or β 1-6 structures (introduces branching). Type 1 HMOs contain lacto-*N*-biose subunits, while type II HMOs contain *N*-acetyllactosamine disaccharide units at their core (**Table 1**). Further structural diversity is brought about by fucose and sialic acid decorations at various positions. HMO composition in milk varies between lactating mothers, and is impacted significantly by maternal genetics. This variability is largely driven by polymorphisms in the fucosyltransferases *FUT2* (*Se*) and *FUT3* (*Le*). If both genes are active then all fucose-borne HMO species can be synthesised. Mothers with an inactive *FUT2* gene cannot synthesise α 1-2-fucosylated HMOs (such as 2'-fucosyllactose [2'-FL] and lacto-*N*-fucopentaose I [LNFP I]) while mothers with an inactive *FUT3* gene cannot synthesise α 1-3- or α 1-4-fucosylated HMOs (such as LNFP II and LNFP III). Based on these fucosyltransferase polymorphisms, four milk groups can be defined: 1) Lewis positive secretors (Le^+Se^+), 2) Lewis negative secretors (Le^-Se^+), 3) Lewis positive non-secretors (Le^+Se^-), 4) Lewis negative non-secretors (Le^-Se^-) (Thurl et al., 2010).

Table 1: Nomenclature and Structures of Core HMOs

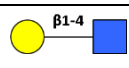
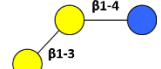
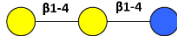
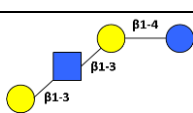
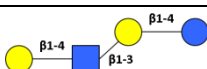
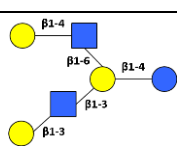
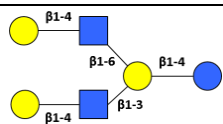
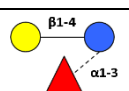
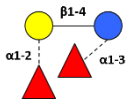
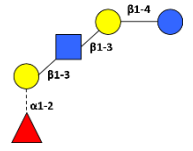
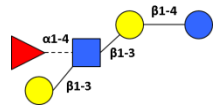
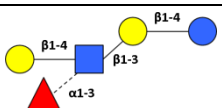
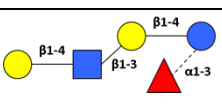
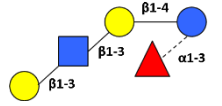
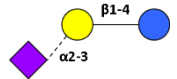
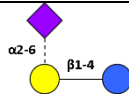
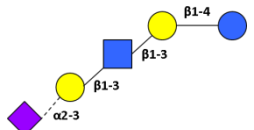
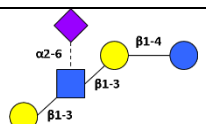
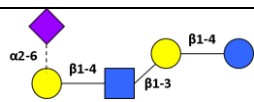
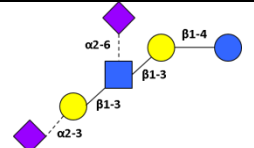
	Name	Abbreviation	Structure/Symbol
HMO Backbone	D-Glucose	Glc	
	D-Galactose	Gal	
	<i>N</i> -Acetylglucosamine	Neu5Ac	
	L-Fucose	Fuc	
	<i>N</i> -Acetylneuraminic acid (sialic acid)	Neu5Ac	
	Lactose	Lac	
	Lacto- <i>N</i> -biose	LNB	
	<i>N</i> -acetyllactosamine	–	
Colostrum HMOs	Galactosyllactose	3'GL	
		4'GL	
		6'GL	
Neutral HMOs	Lacto- <i>N</i> -tetraose	LNT	
	Lacto- <i>N</i> -neotetraose	LNnT	
	Lacto- <i>N</i> -hexaose	LNH	
	Lacto- <i>N</i> -neohexaose	LNnH	
Neutral	2'-Fucosyllactose	2'-FL	
Fucosylated HMOs	3-Fucosyllactose	3-FL	

Table 1: Nomenclature and Structures of Core HMOs

Name	Abbreviation	Structure/Symbol
Difucosyllactose	DFL	
Lacto- <i>N</i> -fucopentaose	LNFP I	
	LNFP II	
	LNFP III	
	LNFP IV	
	LNFP V	
Sialylated HMOs	3'Sialyllactose	
	6'Sialyllactose	
	LST a	
	LST b	
	LST c	
	Disialyllacto- <i>N</i> -tetraose	

The differences in gut microbiota composition observed between formula-fed and breastfed infants have, at least partially, been attributed to the absence (until recently) of HMOs in infant formula milk (Guaraldi & Salvatori, 2012; van den Elsen et al., 2019). A solution for narrowing the compositional gap between human milk and formula milk is the supplementation of infant formula with commercially available oligosaccharides (Reverri et al., 2018). Until recently, research in this field has been hampered by the lack of HMOs on pre-commercial and commercial scale. However, the (bio)technology for large-scale production of complex carbohydrates has advanced greatly, and although technical challenges remain, more and more HMO structures are being manufactured at industrial scale (Faijes et al., 2019). These advancements have paved the way for the first infant safety, growth and tolerance studies with particular HMOs, and have resulted in regulatory approval and commercialization of two major HMOs: 2'-FL, and lacto-*N*-neotetraose (LNnT) (Vandenplas et al., 2018). The commercial availability of these HMOs has allowed for a deeper appreciation of the functions of HMOs, many of which are believed to be exerted through interactions with the gut microbiota (Sela & Mills, 2010). It is expected that microbiome modulation will become a major translational asset in the near future, establishing the microbiome as a malleable and versatile therapeutic target (McBurney et al., 2019). It is important, therefore, that large-scale clinical studies are conducted to fully elucidate the mechanistic link between oligosaccharide structural variation and infant intestinal health. Such studies should aim to characterise the role of HMOs in gut microbiota composition and function, protection against pathogens, and support of barrier and immune functions. Determining how to standardize and

integrate large multi-omic, annotated data sets will likely require multidisciplinary approaches between chemists, glycobiologists, food scientists, microbiologists, bioinformaticians and paediatricians. In this review, the importance of HMOs in the developmental physiology of infants will be discussed. The review highlights the milestones in HMO research, from early research to our current understanding of their biological role in infant health.

2. Pioneers in HMO Research

The discovery of HMOs in the mid-1900s was the result of accumulative and collaborative efforts between paediatricians, microbiologists and chemists. It was noted at the beginning of the 20th century, that infant mortality rates were lower in breastfed infants when compared to those of bottle-fed infants, with decreased occurrences of infectious disease. Pioneers in the field, such as Escherich, Moro, and Tissier, noticed variances in the bacterial composition between breast-fed and bottle-fed infant faeces, substantiating claims that feeding regime and composition of intestinal bacteria were linked to infant health (Escherich, 1886; Moro, 1900). Moro, at the time, postulated that human milk contained a factor which stimulated bacterial growth in the gut. However, it took chemists another 50 years to identify and characterize this growth-promoting factor. Interest in HMOs from a chemical perspective had sparked in 1888 when Eschbach had found “a different type of lactose” in human milk (reviewed in (Montreuil et al., 1993)), and in the 1930s Polonowski and Lespagnol designated this carbohydrate fraction “gynalactose” (Polonowski & Montreuil, 1954; Polonowski & Lespagnol, 1930, 1931). The real breakthrough in HMO research came when paediatrician Paul Gyorgy, and chemist Richard Kuhn hypothesised a link between “gynalactose” and Moro’s “growth factor”. In 1954, thanks to this

partnership between a paediatrician and chemist, the bifidus factor in human milk responsible for enrichment of bacteria in breastfed infants was identified as *N*-acetylglucosamine (GlcNAc) (Gauhe et al., 1954; Gyorgy, Kuhn, et al., 1954; Gyorgy, Norris, et al., 1954).

Since 1954, analytical tools and technologies which allow for structural characterisation of human milk glycans have been developed. Through the employment of these methods, investigators have identified over 200 distinct HMO structures (Oliveira et al., 2015). As a result of four decades of research and development, it is now possible to precisely differentiate and quantify many HMO components in complex matrices. This bestows opportunities upon researchers to examine the structure-function relationships between HMOs consumed in early life and infant intestinal development.

3. Functions of Human Milk Oligosaccharides

Data from *ex vivo* and *in vivo* studies, including recent intervention studies in humans, highlight the importance of HMOs in the infant gastrointestinal tract (summarised in **Figure 1**). Many infant gut bacteria, particularly members of the genus *Bifidobacterium*, possess the genetically encoded ability to consume HMOs, providing rationale for their predominance in the gut of breast-fed infants (Milani et al., 2017). HMOs serve as prebiotic components supplying metabolic substrates necessary for beneficial bacteria to thrive (Bode, 2009; Gibson et al., 2017). Genetic adaptations of these health-promoting bacteria represent an intriguing example of host-microbe coevolution. However in recent years, thanks to accumulating evidence from cell culture studies, animal studies and mother-infant association studies, it

has become apparent that HMOs are more than just “food for bugs” (Bode, 2012). HMOs also function as soluble decoy receptors, preventing pathogen attachment to mucosal surfaces and thereby reducing the risk of microbial infections (Laucirica et al., 2017). It has also been shown that HMOs improve gut barrier function (Chichlowski et al., 2012), promote immune development and tolerance (Donovan & Comstock, 2016), modulate intestinal cell responses (Kong et al., 2019), and lower the risk of contracting necrotizing enterocolitis (NEC) (Autran et al., 2018; Good et al., 2016). In addition, HMOs are believed to provide the infant with a source of sialic acid, an essential nutrient in brain development and cognition (Jacobi et al., 2016; Tarr et al., 2015). Thus, HMOs afford multiple layers of protection and support for the functionally naïve newborn (**Figure 1**). The functions that HMOs impart on a developing infant are as diverse as their structural composition, and these molecules are major contributors to breast milk being considered ‘the gold standard in infant nutrition’ (Walker, 2010). While the future for this field is bright, there is much yet to explore. In the sections below, the biological, physiological and protective roles associated with these molecules will be discussed and the mechanisms by which HMOs are thought to exert these functions.

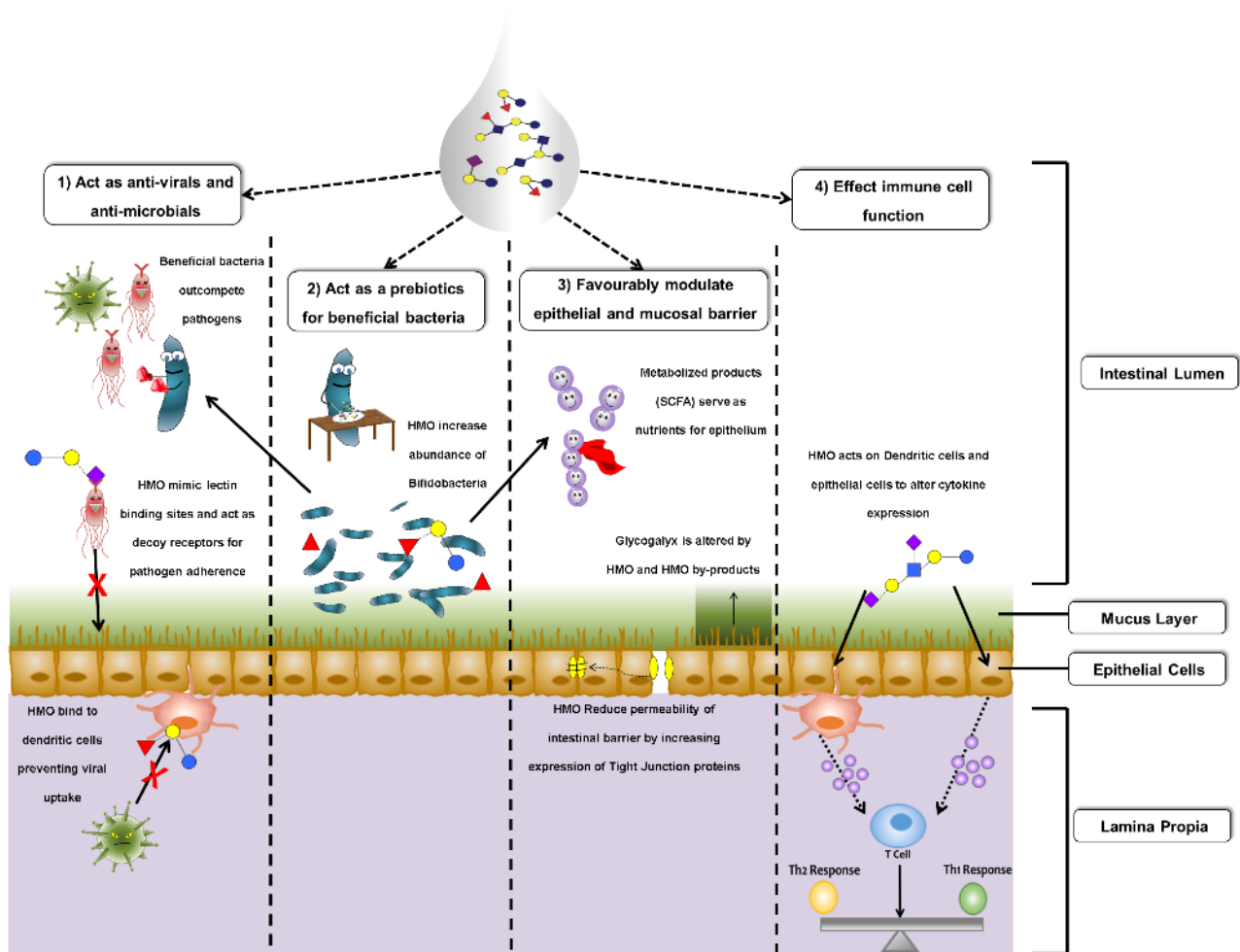


Figure 1: Schematic summary of the beneficial roles human milk oligosaccharides (HMOs) play in the digestive tract of breast-fed infants. (1) HMOs have been shown to act as antivirals and antimicrobials by binding to pathogens and toxins and by directly binding to epithelial receptors. Adhesion and infection by invading pathogens is subsequently blocked. HMOs also prevent viral uptake by binding C-lectin receptors on dendritic cells (DCs) (2) HMOs serve as prebiotic agents, selectively enriching bifidogenic gut bacteria. This confers a selective advantage to bifidobacterial species over pathogens, providing further protection against infectious disease. Metabolites produced during microbial fermentation of HMO have a role to play in developmental physiology of infants. (3) Short Chain Fatty Acids, which are the major metabolites of HMO fermentation, have been shown to influence maturation of intestinal epithelial cells (IECs). HMOs improve barrier function by modulating expression of tight junction proteins, thereby reducing permeability of the intestinal barrier. Expression of proteins at the glycocalyx and mucus layers is also altered by HMOs and HMO metabolites. (4) HMOs interact with immune cells (DCs, T Cells, B Cells) and affect expression of pro/ anti-inflammatory cytokines. HMOs thereby play a role in maintaining immune homeostasis.

3.1. HMOs as Growth Factors for Beneficial Bacteria

Bifidobacterial strains are frequently found as the most abundant colonizers in the gut of breastfed infants, generally accounting for 50-90% of the total bacterial population detected in the faeces of breast-fed infants (Hao et al., 2019; Milani et al., 2017). This is believed to be due to their remarkable capacity to metabolize and consume prebiotic components in human milk (**Figure 1**). Genomic analyses of particular infant-derived bifidobacterial strains have revealed an assortment of transporters, carbohydrate binding proteins and glycosyl hydrolases (GHs), all of which are involved in the import and processing of HMOs (Garrido et al., 2012; Thomson et al., 2018). These HMO-degrading enzymes are essentially limited to (strains of the species) *B. breve*, *B. bifidum*, *B. longum* subsp. *longum* (*B. longum*), *B. longum* subsp. *infantis* (*B. infantis*), and, in some cases, *B. pseudocatenulatum* (Bottacini et al., 2014; Gotoh et al., 2018). Recently, a genetically-encoded pathway for the metabolism of fucosyllactose has also been described in a *B. catenulatum* subsp. *kashiwanohense* isolate (James et al., 2019).

The presence of these genetic loci suggests a coevolution between the human host, HMOs, and the microbes enriched during the lactation period (Chichlowski et al., 2011). The underlying molecular mechanism involved in HMO utilization remains elusive since it is a complex process involving multiple gene clusters. Moreover, the catabolic processes involved vary depending on the species and between strains of the same species (Lawson et al., 2019).

While HMO metabolism is observed in various members of the *Bifidobacterium* genus, it is by no means a characteristic observed in all bifidobacterial isolates. In fact, it is misleading to refer to HMOs as bifidogenic given that they promote growth of some, but certainly not all, bifidobacteria. For example, Ward et al. (Ward et al., 2007) assessed the growth of various *Bifidobacterium* isolates on HMOs purified from breast milk or individual HMO structures and found that non-infant-borne strains such as *B. adolescentis*, *B. dentium*, and *B. animalis* subsp. *lactis* were incapable of digesting HMOs. In contrast, *B. infantis* and *B. bifidum* are considered avid HMO consumers, containing all the GHs required for catabolizing a whole spectrum of HMO linkages (Underwood et al., 2015). Generally, individual strains of *B. longum* subsp. *longum* and *B. breve* display poor growth on HMOs, but are capable of utilizing more simple structures such as lacto-*N*-tetraose (LNT) and lacto-*N*-neotetraose (LNnT) (James et al., 2016; Ruiz-Moyano Seco de Herrera et al., 2013).

Two distinct HMO degradation strategies have been described in infant-borne bifidobacteria. The first involves importation of intact HMOs into the cytoplasm through ATP-Binding Cassette (ABC)-type transporters, whereupon the oligosaccharides are hydrolysed by intracellular glycosidases. The second approach relies on cell wall-anchored secretory GHs which hydrolyse HMOs extracellularly releasing mono- and di-saccharides (Garrido et al., 2015; Kitaoka, 2012). Through genomic and phenotypic analysis it has become evident that *B. infantis* and *B. breve* use oligosaccharide transporters, while *B. bifidum* utilizes extracellular hydrolases and processes the liberated lacto-*N*-biose (LNB - the core structure of type 1 HMO- **Table 1**) intracellularly (Thomson et al., 2018). Interestingly, the strategy used by *B.*

longum is strain dependent and reliant on the presence of the gene encoding lacto-*N*-biosidase (LnbX). LnbX-positive *B. longum* use extracellular hydrolases, while LnbX-negative utilize oligosaccharide transporters to assimilate HMO derivatives internally (Yamada et al., 2017). While the overall mechanistic model varies, all four infant gut-associated species encode a gene cluster which is dedicated to the assimilation of LNB. Genes within this GNB/LNB cluster are upregulated several fold during growth on HMOs (Garrido et al., 2015). The gene cluster contains an ABC transporter and cytoplasmic phosphorylase which are responsible for internalisation and metabolism of LNB, respectively (Nishimoto & Kitaoka, 2007).

Although divergent mechanisms are used for HMO catabolism in each species, the HMO-derivatives enter the same central fermentative pathway, termed the 'bifid shunt'. The bifid shunt is a *Bifidobacteriaceae*-specific metabolic pathway and encompasses the Leloir, fructose-6-phosphate phosphoketolase, and amino-sugar metabolising pathways (Pokusaeva et al., 2011). Proteomic analysis by Kim et al. indicated upregulation in the activity of enzymes involved in the bifid shunt pathway in the presence of HMOs, resulting in the release of large amounts of acetate and lactate (Kim et al., 2013). For every 2 moles of hexose that enter in this pathway five moles of ATP, three moles of acetate and two moles of lactate are produced. Flux through the bifid shunt pathway allows bifidoabacteria to produce more energy in the form of ATP compared to ATP levels produced through fermentative pathways in lactic acid bacteria (LAB) (reviewed in detail by (Pokusaeva et al., 2011)). Production of organic acids via bifid shunt pathways offers potential links between bifidobacterial physiology and infant nutritional and health outcomes (Alexander, Swanson, Fahey, & Garleb,

2019; Hidalgo-Cantabrana et al., 2017). The organic acids produced reduce the intestinal pH, and can thereby inhibit pathogenic bacteria. It has also been suggested that these metabolites improve intestinal barrier function and contribute to immune system development (Tan et al., 2014).

The various catalytic mechanisms used by bifidobacterial strains for HMO consumption may be linked to niche partitioning among bifidobacteria within the developing infant gastrointestinal tract (Pereira & Berry, 2017; Sela & Mills, 2010). Interestingly, the ability of a bifidobacterial strain to consume HMOs does not necessarily correlate with their dominance in the gut of breast-fed infants. Despite being recognised as avid HMO consumers, *B. bifidum* and *B. infantis* are typically detected in low numbers in the faeces of breast-fed infants, while *B. longum* and *B. breve* are often found as the dominant species in infant stools (Turrone et al., 2012), even though they demonstrate poor or at best selective *in vitro* HMO assimilation. Thus, simply understanding the HMO assimilation pathways does not provide a full picture of how bifidobacteria strains utilize HMO to colonize the nursing infant colon. In order to fully appreciate the role HMOs play in the establishment of a healthy infant microbiome, consideration must be given to the multi-faceted syntrophic relationships that exist between members of the infant microbiota (Gotoh et al., 2018; Milani et al., 2015).

Besides infant gut-associated bifidobacteria, HMO assimilation pathways have been described for a number of gut microbes belonging to other taxa. Numerous *Bacteroides* species were shown to metabolise 2'-FL, 3-FL, 3'-SL, 6'-SL, and LDFT (Yu, Chen, & Newburg, 2013) while *Lactobacillus casei* utilizes the HMO-derivatives LNB and lacto-*N*-triose II (Bidart, Rodríguez-Díaz, Monedero, & Yebra, 2014). In fact, a specific

phosphotransferase system for import of LNB has been identified in *Lactobacillus* species (Bidart et al., 2014). Although the capacity of these gut microbes to consume HMOs is generally quite limited, these minority species play a role in the gut ecology of breast-fed infants until weaning and may potentially influence the microbiota population in adulthood. It is also interesting to note that in a recent study by Salli et al., 2019, using a semi-continuous colon simulator, only slight changes in the microbiota were caused by the commercially available and most prevalent HMO, 2'-FL. This was reflected by the intermediate production of short chain fatty acids (SCFAs) with lower production of acetate and lactate observed when compared with fermentations in the presence of lactose or galacto-oligosaccharides (GOS). The results highlight the specificity of 2'-FL as energy source for only certain microbes over GOS and lactose in this simulated gut model. It maybe that 2'-FL is more important in directly interacting with the host cells and tissues (discussed below) rather than indirectly through its modulatory action on the gut microbiota composition.

3.2. Effect of HMOs on the colonisation of bifidobacteria

The ability of probiotic strains to adhere to the intestinal epithelium plays a key role in influencing the composition of the gut microbiota. Microbes in surface-associated populations can out-compete other species solely by being more adhesive (McLoughlin et al., 2016). In recent years, a number of studies have indicated that HMOs could enhance the adhesion ability of probiotic strains by influencing the expression of bacterial adhesins (Morrin & Hickey, 2020). This enhanced adhesion may prolong gut transit time of the probiotic and increase host-microbe and/or microbe-microbe interactions, thereby enabling greater health benefits to the host (Monteagudo-Mera et al., 2019).

HT-29 and Caco-2 cell lines are routinely employed to mimic the adhesion of probiotic bacteria to intestinal epithelial cells (Chichlowski et al., 2012). An *in vitro* study by Chichlowski et al. demonstrated that cultivation of *B. infantis* ATCC 15697 on HMOs significantly increased adherence of the strain to intestinal cells (Chichlowski et al., 2012). Likewise, incubation of the same strain (ATCC 15697) in the presence of 3'-SL and 6'-SL substantially increased *B. infantis* adherence to HT-29 cells (Kavanaugh et al., 2013). Subsequent transcriptomic analysis revealed that this increased adherence is associated with upregulation of chaperone proteins, transcription factors, adhesion-related proteins and glycosyl hydrolases (Kavanaugh et al., 2013). Additional studies which incorporate transcriptomic and proteomic analyses will further increase our understanding of the HMO-related factors which promote adherence of beneficial microbes to the mucosal surface. Nevertheless, it is plausible that HMO-containing nutritional products will be used in the future as a tool to increase the residence time of probiotic bacteria in the gut.

3.3. Anti-infective properties of HMO

By acting as prebiotic agents, HMOs confer a selective advantage to beneficial microbes over pathogens, thereby providing protection to the infant from infectious diseases (Figure 1). Since pathogenic bacteria are less capable of metabolising HMO species, symbionts can grow and outcompete harmful invaders through competitive exclusion (Hoeflinger et al., 2015). Moreover, bifidobacterial HMO metabolism results in the production of organic acids which creates an acidic environment wherein growth of pathogenic bacteria is hindered (Tan et al., 2014). In addition to indirect pathogenic deflection, HMOs also directly block pathogenic entry by acting as soluble receptor

decoys (Figure 1). Many viral, bacterial and protozoan pathogens must adhere to the ‘glycocalyx’ in order to invade the host and cause disease (Costerton et al., 1981). The glycocalyx is the term given to the carbohydrate-rich layer which coats epithelial cells, and consists of glycans conjugated to proteins or lipids (Kavanaugh et al., 2015). The chemical structures of HMOs are similar to the glycans pathogenic bacteria exploit to attach to the epithelial cell surface (Newburg & Grave, 2014). Pathogens and toxins that recognise and bind to HMOs rather than cell-surface glycans will therefore pass harmlessly through the gastrointestinal tract without causing infection (Craft & Townsend, 2018). Oligosaccharides can also inhibit pathogens by competitive binding with the host cell surface receptor (Morrow et al., 2005). HMOs have been shown to provide stereospecific protection against a wide spectrum of pathogens, including those associated with diarrheal, respiratory, and urinary infections and even human immunodeficiency virus (HIV). As outlined in Table 2, a significant number of *in vitro* and *in vivo* studies have been carried out in recent years, the findings of which demonstrate the anti-bacterial, anti-viral and anti-amoebic properties of HMOs. These studies provide insight into their potential as valuable candidates for future therapeutic developments against pathogenic infection (Craft & Townsend, 2019).

Table 2: Studies with HMO against enteric pathogenic infections

Pathogen	HMO	Model	Findings	Ref
<i>Campylobacter jejuni</i>	α 1,2-fucosylated HMO	Murine Model & <i>ex-vivo</i> human Intestinal mucosa	H-2 antigen necessary for campylobacter binding, which is blocked by α 1,2-fucosylated HMO	72
	2'-FL	Hep-2 & HT-29 epithelial cells	2'-FL inhibited invasion of <i>C. jejuni</i> which was associated with a reduction in IL-8 expression	73
	Breast-milk samples	Observational clinical study in mother-infant dyads	Inverse relationship between concentrations of α 1,2-fucosylated HMO and susceptibility to <i>C. jejuni</i> caused diarrhea	74
<i>Candida albicans</i>	Pooled HMO	H4 premature intestinal epithelial cells (pIECs)	Pooled HMO reduced invasion of pIECs by <i>C. albicans</i>	89
<i>Entamoeba histolytica</i>	Pooled HMO	HT-29 epithelial cells	HMO-enriched fractions reduced adherence and cytotoxicity of <i>E. histolytica</i> <i>in-vitro</i>	90
Enteropathogenic <i>E. coli</i> (EPEC)	Pooled HMO	Murine Model & HeLa and T84 epithelial cells	Reduced adhesion of EPEC in epithelial cells, which was reflected in reduced EPEC infection in mice	75
	HMO fractions	Caco-2 epithelial cells	All HMO fractions (acidic, low MW neutral, and high MW neutral) reduced adhesion of <i>E. coli</i> O119 to epithelial cells	76
	2'-FL and LNFP I	<i>in-vitro</i> binding assay	2'-FL and LNFP I bind extensively to heat labile enterotoxin type 1	77
<i>Helicobacter pylori</i>	Acidic HMOs	HuTu-80 human duodenal cells	3'-SL demonstrated strongest inhibition of <i>H. pylori</i>	121

Table 2: Studies with HMO against enteric pathogenic infections

Pathogen	HMO	Model	Findings	Ref
			adherence. 6'-SL less active in reducing <i>H. pylori</i> colonisation of HuTu-80 cells	
<i>Listeria</i> <i>Monocytogenes</i>	Pooled HMO	Caco-2 epithelial cells	Pre-incubation of Caco-2 cells with HMO reduced <i>L. Monocytogenes</i> adherence by 50%	78
Norovirus	Breast-milk samples	Saliva samples	Human milk blocked genogroup I genotype 1 (GI.1) and genogroup II genotype 4 (GII.4) binding to saliva	81
	HMO Structures	Surface plasmon resonance imaging (SPRi) of glycan microarrays	GI.1 (Norwalk) and GII.4 (VA387) capsids show greater affinity for fucosylated over non-fucosylated HMOs	84
	Fucosylated HMOs	Porcine Gastric Mucin or Saliva Samples	2'-FL binds GII.17 variants, while 2'-FL and 3-FL block GII.10 noroviruses.	85
Rotavirus (RV)	Breast-milk fractions	Human Kidney epithelial cells (MA-104) and Mouse Model	A sialic acid-containing Human Milk component inhibited RV replication <i>in-vitro</i> and reduced RV gastroenteritis <i>in-vivo</i>	87
	Pooled HMO Fractions	Human Kidney epithelial cells (MA-104) and Piglet animal models	Sialic acid containing HMO inhibited RV infection <i>in-vitro</i> . Neutral and Acidic HMO reduced RV replication <i>in-vivo</i>	88
	2'-FL, 3'-SL, 6'-SL, GOS	Human Kidney epithelial cells (MA-104)	All HMOs reduced infectivity of RV strains. G1P[8] infectivity was inhibited most significantly by 2'-FL. Combination of	31

Table 2: Studies with HMO against enteric pathogenic infections

Pathogen	HMO	Model	Findings	Ref
			3'-SL + 6'-SL had the greatest effect on G2P[4] variants	
<i>Salmonella fyris</i>	2'-FL/ 3-FL & pooled breast milk	Caco-2 Intestinal epithelial cells	Low MW fractions of HMO were most effective for inhibiting adhesion of <i>S. fyris</i> . 3FL and 2'-FL reduced infectivity of <i>S. fyris</i>	122
Group B <i>Streptococcus</i> (GBS)	Breast-milk samples	Observational clinical study in mother-infant dyads	Infants born to Lewis positive mothers had lower incidences of GBS infection. Branched chain HMOs reduced GBS growth <i>in-vitro</i>	123
	Neutral HMO (LNT, LNFPI)	<i>in-vitro</i> growth	LNT and LNFPI significantly inhibited growth of GBS	80
	Pooled HMO	<i>in-vitro</i> growth	HMO inhibits growth and alters biofilm formation of GBS, in a milk-group dependent manner	79

3.3.1 Bacterial infections

One example of a pathogenic bacterium inhibited by oligosaccharides from human milk is *Campylobacter jejuni*, which is recognised as one of the most common causes of diarrhoea worldwide. HMOs have been shown to inhibit *C. jejuni* pathogenesis in mice and in *ex vivo* cultures of human intestinal mucosa (Ruiz-Palacios et al., 2003). Subsequent *in vitro* studies demonstrated that α 1-2 fucosylated carbohydrate moieties inhibit adherence of *C. jejuni* to the H-2 antigen on intestinal cells (Ruiz-Palacios et al., 2003). A more recent investigation found that 2'-FL not only inhibits binding to the mucosal surface, but also reduces invasion of *Campylobacter* by 80% (Yu et al., 2016). Anti-Campylobacter effects have also been observed in murine models, with

2'-FL reducing invasion and intestinal inflammation associated with *Campylobacter* infection (Yu et al., 2016). The findings of these studies align with results from a prospective study of 93 Mexican breastfeeding mother-infant dyads (Morrow et al., 2004). This latter study reveal the existence of an inverse relationship between concentrations of α 1-2 fucosylated HMOs in maternal milk and susceptibility to *C. jejuni*-caused infant diarrhea, and concluded that α 1-2 fucosyloligosaccharides are key components in human milk that contribute to protection against infectious pathogens (Morrow et al., 2004).

Oligosaccharides present in human milk also function to protect against *Escherichia coli* pathogenesis. Enteropathogenic *Escherichia coli* (EPEC) cause serious diarrheal disease with high mortality rates in infants. Pre-incubation of EPEC with pooled HMO fractions significantly reduced pathogenic colonization of cultured epithelial cells (Manthey et al., 2014) while HMO-treated mice demonstrate significantly less EPEC infection than control or galacto-oligosaccharides (GOS)-treated pups (Manthey et al., 2014). These findings are consistent with work from Coppa et al. where infectivity of *E. coli* serotype O119 was shown to be inhibited by acidic HMO fractions as well as high- and low-molecular-weight HMO fractions (Coppa et al., 2006). Additional to reducing adhesion of whole pathogens, 2'-FL and Lacto-*N*-fucopentaose-I (LNFP-I) may also reduce infectivity by binding to heat-labile enterotoxin type 1 (El-Hawiet et al., 2015). Fucosylated HMO structures are also effective in mitigating adhesion and infectivity of enteropathogenic *Salmonella* (Weichert et al., 2013), while sialylated structures such as 3'-SL exhibit strong inhibition of *Helicobacter pylori*

adhesion to gastrointestinal epithelial cells (Simon, Goode, Mobasser, & Zopf, 1997).

Aside from reducing adhesion and invasion of pathogenic bacteria, HMOs have also been shown to reduce infection by modifying gene expression on the epithelial surface. Pre-incubation of Caco-2 cells with HMO alters gene expression of intestinal cells, resulting in a significant reduction in *Listeria monocytogenes* adhesion (Chen et al., 2017). HMO pretreatment of IECs reduced infectivity of *L. monocytogenes* through induction of the unfolded protein response and eIF2 signalling (Chen et al., 2017). HMOs can also elicit anti-infective effects by directly inhibiting growth of pathobionts such as Group B Streptococci (GBS) (Ackerman et al., 2017). Considerable inhibitory effects against GBS were demonstrated for the HMO structures LNT and LNFP-I (Lin et al., 2017)

3.3.2 Viral infections

HMO-mediated protection has also been described for many viruses such as rotaviruses or noroviruses, which are some of the leading causes of gastroenteritis outbreaks worldwide (Widdowson, Steele, Vojdani, Wecker, & Parashar, 2009; Robilotti, Deresinski, & Pinsky, 2015). HMOs function as antivirals by mimicking receptor sites and blocking entry into the cell, but also by preventing viral replication within the cell. One of the earliest studies demonstrating the potential of HMOs to reduce infectivity of Norovirus found that human milk blocked genogroup I genotype 1 (GI.1) and genogroup II genotype 4 (GII.4) binding to saliva (Jiang et al., 2004). Several studies have identified histo-blood group antigens (HBGA) as key binding sites for norovirus adhesion (Schroten et al., 2016). Some HMOs demonstrate

homology to HBGA, with Norovirus binding most avidly to high-mass HMO rich in α -fucose (Hanisch et al., 2018). Surface plasmon resonance imaging of glycan microarrays revealed that affinity of Norovirus genotypes to HMO structures is strain specific. The results show both GI.1 (Norwalk) and GII.4 (VA387) capsids have greater affinity for fucosylated glycans over non-fucosylated HMOs (Shang et al., 2013). GII.4 capsids demonstrated preferential binding to glycans with H type 2 (Gal β 1-4GlcNAc) or H type 6 (Gal β 1-4Glc) moieties (such as LNFP-III and 2'-FL). In contrast, GI.1 capsids were shown to display broader binding-specificity, recognising H type 1 (Gal β 1-3GlcNAc) linkages (such as LNFP-I or LNDFH-I) as well as H type 2/6. Moreover, GI.1 variants were shown to display affinity for glycans in the form of glycoproteins, but also in the form of free oligosaccharides, while GII.4 variants bound preferentially to glycans conjugated to proteins. The results of this study suggest that fucosylated HMOs mimic HBGA binding sites and thereby reduce adherence of norovirus (Shang et al., 2013). Studies from Hansman's group at the University of Heidelberg employed X-ray crystallography techniques to reveal that 2'-FL binds to GII.17 variants, while both 2'-FL and 3-FL were capable of blocking GII.10 noroviruses to HBGA, verifying that fucosylated glycans function as antiviral agents against Norovirus by binding GI and GII HBGA sites (Koromyslova et al., 2017; Weichert et al., 2016).

Milk oligosaccharides have also been shown to function as antiviral agents against Rotavirus (RV) infection. A sialic acid-containing component in human milk was shown to inhibit virus replication *in vitro* and to reduce symptoms of gastroenteritis in animal models (Yolken et al., 1992). The results of this study were consistent with subsequent experiments which

found that 3'-SL and 6'-SL act as antivirals against porcine RV (Hester et al., 2013). *In vivo* studies in piglet models revealed that both acidic and neutral fractions significantly reduced infectivity of RV (Hester et al., 2013). Most recently, HMOs were demonstrated to reduce infectivity of 2 dominant RV strains in monkey kidney epithelial cells, with notable strain-specific differences. G1P[8] infectivity was inhibited most significantly by 2'-FL while a combination of 3'-SL and 6'-SL had the greatest effect on G2P[4] variants (Laucirica et al., 2017).

In addition to the protective effects which have been demonstrated for HMOs against intestinal viruses, studies have also shown that human milk glycans can serve as protective agents against pathogenesis caused by respiratory viruses (Zevgiti et al., 2012). Sialylated oligosaccharides have been shown to reduce infectivity of influenza viruses, while 2'-FL reduced viral load of respiratory syncytial virus *in vitro* (Duska-Mcewen, Senft, Ruetschilling, Barrett, & Buck, 2014). Moreover, intervention of 2'-FL in an influenza-specific murine model resulted in improved adaptive and innate immunity (Xiao et al. 2018). It is plausible to speculate, that HMOs could act as anti-viral agents against members of the coronavirus family including severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), the causative pathogen of the COVID-19 pandemic. Many mammalian viruses have evolved to use glycans as host cell receptors (Thompson, de Vries, & Paulson, 2019) and potential associations between SARS-CoV-2 and glycans expressed on the cell surfaces cannot be disregarded. Investigating the potential of HMOs to act as soluble decoy receptors for the COVID-19 virus may be of great interest for future research.

3.3.3 Fungal infection

Although only a small number of publications are available which assess the anti-fungal effects of HMOs, the findings from these studies suggest that pooled HMOs can reduce infection by fungal species such as *Candida albicans*. In an *in vitro* study using H4 premature IECs, Gonia et al. found that HMOs altered hyphal induction in *C. albicans*, thereby preventing invasion of the intestinal epithelium (Gonia et al., 2015).

3.3.4 Protozoan parasite infections

Beyond anti-viral and anti-bacterial roles, studies are limited which detail the activity of HMOs against protozoan parasites. One example, however, is *Entamoeba histolytica*, an anaerobic amoebozoan which causes an estimated 55,000 deaths a year globally (Shirley, Farr, Watanabe & Moonah, 2018). Jantscher-Krenn et al. demonstrated that isolated HMO-enriched fractions reduced adherence and cytotoxicity of *E. histolytica* in an *in vitro* model (Jantscher-Krenn et al., 2012). By resembling Gal/GalNAc lectin binding sites, non-fucosylated HMOs act as soluble decoy receptors against amoebic infection.

3.4. HMOs Modulating Epithelial Cell Responses

The intestinal epithelium lining in the small intestine and colon is considered the first line of defence in innate immunity, since it acts as a physical, rate-limiting barrier between the gut lumen and the circulatory system (Maldonado-Contreras & McCormick, 2011). Intestinal crypts and villi are covered by a single layer of absorptive enterocytes and secretory cells (including goblet, paneth and enteroendocrine cells). Multipotent stem cells found in the basal portion of the intestinal crypt divide to form daughter cells,

which differentiate as they migrate along the villus-crypt axis (Umar, 2010). Permeability of the epithelium is dependent primarily on the structure and expression of tight junctions. The absorption of nutrients through this epithelium occurs trans-epithelially (through cells) or paracellularly (between tight junctions). Perturbations in barrier integrity result in irregular immune responses including gastrointestinal infections, inflammatory bowel diseases, and allergies (Vancamelbeke & Vermeire, 2017). Reliable measurements of intestinal barrier function during clinical intervention studies are rare. Therefore, *in vitro* studies using cultures of HT-29, Caco-2, or other intestinal cell lines are commonly used to understand the mechanisms by which nutrients and microbial metabolites affect epithelial integrity. Researchers have demonstrated that HMOs promote tight junction protein expression (**Figure 1**), and increase differentiation along the crypt-villus axis (Holscher et al., 2014). By modelling the varying differentiation patterns along the crypt-villus axis with pre-confluent HT-29 and Caco-2Be cells, and post-confluent Caco-2Be cells, Holscher et al. assessed the effects of three HMO structures (LNnT, 2'-FL, and 6'-SL) on epithelial cell differentiation, apoptosis, and proliferation (Holscher et al., 2014). The results demonstrated that 2'-FL significantly increases differentiation in HT-29 cells when alkaline phosphatase was used as a marker of differentiation. The authors observed reduced cell proliferation after exposure to LNnT and 6'-SL, with 2'-FL demonstrating weak anti-proliferative effects. LNnT was the only HMO structure capable of reducing permeability across the epithelial barrier (Holscher et al., 2014). The results, which are consistent with previous work by Kuntz and colleagues, signify that the function of HMOs is stereospecific (Kuntz et al., 2008).

Grabinger et al. recently demonstrated that 2'-FL reduced the progression of colitis in an interleukin-10 null (IL10^{-/-}) murine model. Intestinal permeability was determined by measuring serum FITC-dextran levels and it was shown that supplementation with 2'-FL significantly increased epithelial integrity (Grabinger et al., 2019). These effects were associated with increased abundances of the commensal *Ruminococcus gnavus* and elevated levels of propionate in the cecum. This implies that by promoting the growth of beneficial bacteria, HMOs function to favourably modulate the epithelium barrier by creating a healthier balance of SCFAs (**Figure 1**).

The glycocalyx acts as a skeleton of proteoglycans which provides binding sites for microorganisms and can function as a physical barrier preventing invasion of toxins. Defective maturation of the glycocalyx layer can therefore result in various gastrointestinal conditions. It has been shown through *in vitro* studies that HMOs support intestinal barrier function by altering expression of various glycocalyx components (**Figure 1**). For example, it was revealed through the use of microarray glycan profiling that HMOs can alter patterns of glycosylation on the epithelial cell surface (Angeloni et al., 2005). Again, these effects were found to be structure-dependent with exposure to 3'-SL significantly reducing the number of sialic acid residues found on the surface of Caco-2 cells. Alterations in glycocalyx were caused by reduced expression of the sialyltransferases ST3Ga11, ST3Ga12, and ST3Ga14, and was associated with a reduction in adhesion of enteropathogenic *E. coli* to the Caco-2 cells (Angeloni et al., 2005). More recently, Kong and colleagues demonstrated that fucosylated HMOs significantly enhanced glycocalyx development in Caco-2 cells (Kong et al.,

2019). The study revealed that 2'-FL and 3-FL significantly enhanced the thickness of absorbed albumin, while 3-FL increased the area coverage of albumin, heparan sulphate, and hyaluronic acid in the glycocalyx of the Caco-2 cells. Increased adsorption of albumin is associated with improved stability of the glycocalyx and decreased adherence of pathogenic bacteria (Kong et al., 2019). Heparan sulphate and hyaluronic acid form the major glycosaminoglycan chains in the glycocalyx and are linked to the core proteins of proteoglycans. Gastrointestinal disorders such as inflammatory bowel disease (IBD) are associated with alterations in the expression of these glycosaminoglycan chains. Improved expression of heparan sulphate and hyaluronic supports enhanced colonic epithelial repair and homeostasis of innate immunity (Kong et al., 2019). HMOs also have a positive effect on mucin glycoproteins expression in the mucus barrier. Narrowing of this mucus layer is linked to increased permeability of the intestine and a predisposition to gastrointestinal disorders such as NEC (Hodzic et al., 2017).

Recently, fractions of HMOs isolated from breast milk were shown to increase the expression of *MUC2* and decrease intestinal permeability in a neonatal mouse model of NEC (Wu et al., 2019). HMOs induced expression of mucins through direct action on chaperone proteins such as protein disulphide isomerase (Wu et al., 2019). Results of this animal study are in line with findings from an *in vitro* study (Cheng et al., 2019). Here, the effects of HMOs on expression of goblet cell secretory genes in a colorectal cell model were assessed. 2'-FL enhanced *MUC2* expression during IL-13 exposure, while 3-FL up regulated both *MUC2* and *TFF3* gene expression during TNF α and IL-13 exposure (Cheng et al., 2019). Further studies, particularly intervention studies in infants, are required to fully elucidate the

role of HMOs in the maturation of intestinal cells and mucosal surfaces. Results from *in vitro*, *ex vivo* and *in vivo* studies, however, suggest that HMOs positively influence gut barrier function, and thereby may prevent gut dysfunction and NEC through improved expression of tight junction proteins, glycoprotein components, and mucins.

3.5. HMO Effects on Immune Cell Populations and Cytokine Expression

While the vast majority of HMOs pass undigested into the colon, a small proportion is absorbed intact and excreted in the urine. The presence of intact HMO in the urine of breast-fed infants was first described in 1996 by Rudloff and colleagues (Rudloff et al., 1996), with subsequent clinical studies reporting absorption rates of 1-5% HMO from the GI tract to the circulatory system (Goehring et al., 2014; Rudloff & Kunz, 2012; Ruhaak et al., 2014). These findings suggest that, in addition to their role in an infant's intestinal lumen, HMO may also contribute to the development and physiological functions of other organs and systemic cell systems. Studies have shown that HMOs modulate immune cell-cell interactions, thereby facilitating maintenance of balanced inflammatory cell responses (**Figure 1**) (Donovan & Comstock, 2016). This HMO signalling is mediated by receptor molecules which are expressed predominantly on the surface of immune cells (Triantis et al., 2018). Several classes of lectins and toll-like receptors (TLRs) with HMO binding specificity have been described in the literature and are summarised in **Table 3**.

Table 3: Receptors Involved in Biological Recognition of HMO

Type	Expression	Function	Name	HMO related ligand	Reference
C-type lectins	Antigen presenting cells	Internalisation of antigens which contain saccharide epitopes. Play a role in IgA transcytosis and interact with various pathogens	DC-SIGN	2'-FL, 3-FL, LNFP-III, LNFP IV, LNDFH-I	124
Galectins	T cells/ Intestinal Epithelial cells	Regulation of immune cell homeostasis and inflammation, Participate in regulation of T-cell function	Galectin-9	scGOS	125
			Galectin-3	3'-SL, LNFP I, II, III, LNDFH	126
Selectins	Leukocytes/ Endothelial cells	Cell adhesion molecules that facilitate leukocyte trafficking.	P- selectin	HMO with sialyl Lewis X epitopes	127 128
			E-selectin		
Siglecs	Neutrophils, monocytes, dendritic cells	Inhibit cell activation and proliferation, modulate macrophages/ dendritic cell production of cytokines, regulate B-lymphocytes and T-cell function	Siglec-4	2,3-sialic acid	129
			Siglec-2	2,6-sialic acid	129
			Siglec-5	Sialyl-Lewis c	124
			Siglec-9	Numerous sialylated HMO	124
TLRs	Macrophages and dendritic cells,	Activate NF-kB and interferon regulatory factor pathways, which results in secretion of pro-inflammatory factors.	TLR2	3-FL, 3'-SL, 6'-SL	130 131
			TLR3	3'-, 4'-, 6'-GL	108
			TLR4	LNFP I, III, 2'-FL	108 131

In the period directly after birth, a neonate's cellular immune system undergoes rapid development. Multiple immune factors, such as neutrophils, macrophages, monocytes, natural killer (NK) cells, T cells, and dendritic cells (DCs), play a critical role in fighting against invading pathobionts (Kelly & Coutts, 2000). At birth, an imbalance between Th1/17 and Th2 phenotypes is noted in T-helper cell populations. A neonate's immune system is directed more towards the Th2 phenotype which promotes humoral immunity and confers protection against extracellular pathogens. On the other hand, Th1/Th17 pathways target intracellular pathogens and are less prevalent in the developing neonate (Adkins, 2000). The immature immune system is also characterized by overexpression of inflammatory markers and inadequate feed-back regulation of immune signalling. It is hypothesised, therefore, that breast-milk oligosaccharides favourably modulate neonatal innate immune responses by controlling expression of inflammatory markers involved in cell trafficking and affecting cytokine and chemokine networks that regulate Th1/Th2 lymphocyte balance (**Figure 1**) (Kulinich & Liu, 2016). However, information regarding the role of HMOs and specificities of their action in inflammation is limited and is largely dependent on findings from *in vitro* and *ex vivo* studies.

Several studies have focussed on assessing the immunomodulatory effects of HMO mixtures isolated from human milk. For example, He et al. assessed the impact of colostrum HMOs on intact immature human intestinal mucosa (He et al., 2014). The authors found that colostrum HMO reduced levels of pro-inflammatory cytokines (such as IL-1 β , IL-6 and IL-8) while stimulating expression of cytokines associated with tissue repair and homeostasis. The group also found that exposure to HMOs elevated

expression of Th1 polarization, and shifted the balance of Th1/ Th2 cytokines towards that of more mature tissues (He et al., 2014). More recently, isolated HMOs were found to stimulate semi-maturation of human monocytes-derived DCs which was associated with elevated levels of anti-inflammatory cytokines such as IL-6, IL-10, and IL-20 (Xiao et al., 2019). HMOs were also found to reduce LPS-induced production of pro-inflammatory cytokines such as IL-12p70 and TNF- α , by preventing the interaction of LPS and TLR4 (Xiao et al., 2019). Zhang et al. provided further mechanistic insight by demonstrating that a neutral HMO fraction influenced inflammatory cell populations via nuclear factor (NF)- κ B and mitogen-activated protein-kinase pathways (Zhang et al., 2019). Interestingly, a recent animal model study has demonstrated that HMOs may be protective against the development of type-1 diabetes (T1D) (Xiao et al., 2018). Dietary supplementation of HMOs was shown to reduce T1D incidence and to suppress development of pancreatic insulinitis in non-obese diabetic (NOD) mice. These effects were associated with changes in cytokine profiles and microbiota composition (Xiao et al., 2018). These findings suggest that HMO-supplementation may hold therapeutic potential in the treatment of gut-related autoimmune diseases.

Certain fucose-containing HMO species have been shown to alter expression of inflammatory cell populations, with the majority of studies indicating that fucosylated-HMOs play a role in Th2-promoting activities. The anti-inflammatory effects of 2'-FL were demonstrated in T84 and H4 intestinal epithelial cells, wherein 2'-FL quenched LPS-induced inflammation during infection with type I pili *E.coli* strains (He et al., 2016). Exposure to 2'-FL reduced CD14 expression, a factor which mediates interaction of LPS and TLR4 (He et al., 2016). Similar effects for Fuc-borne

HMO were observed by Sotgiu et al. wherein LPS-activated mononuclear cells taken from multiple sclerosis patients were exposed to 2'-FL and LNFP I. The oligosaccharides were found to reduce proliferation of the mononuclear cells, which was associated with a reduction in IL-12 and IFN- γ , while expression of IL-10 increased significantly (Sotgiu et al., 2006). Other fucosylated HMO have been shown to play a role in stimulating Th2 responses including LNFP III, which stimulates macrophage and NK cell activity *in vitro*, while increasing secretion of IL-10, TNF α , and prostaglandin E2 (Atochina & Harn, 2005). Immunomodulation by fucosylated HMO has also been demonstrated in animal studies. In a murine model of food allergy, 2'-FL reduced food allergy symptoms and induced significant increases in IL10⁺CD4⁺CD25⁺ Tregs from Peyer's patches (Castillo-Courtade et al., 2015). Similar effects were observed for 6'-SL albeit through different mechanisms to 2'-FL. A limited number of observational studies in breast-fed infants have found associations between fucosylated HMO and the development of allergy. For instance, Seppo et al. evaluated associations between concentrations of HMO and the development of cow's milk allergy (CMA). The researchers here found that the concentration of LNFP-III was lower in milk consumed by infant's who developed CMA, suggesting that HMO structures may play a protective role against the development of certain food allergies (Seppo et al., 2017).

In contrast to fucosylated HMOs, the effects of sialylated HMOs are bidirectional with studies demonstrating stimulation of both Th1 and Th2 responses in *in vitro* models. In a 2010 study by Eiwegger et al., cord-blood derived mononuclear cells were exposed to pooled sialylated HMO (Eiwegger et al., 2010). Exposure to acidic HMO fractions promoted the

production of IFN- γ and IL-10, resulting in downregulation of Th2 responses which shifted the immune response towards a more balanced Th1-Th2 profile. In this same investigation, sialylated HMOs were also shown to reduce IL-4 production in lymphocytes derived from adults with peanut allergy (Eiwegger et al., 2010). Moreover, 3'-SL has been shown to exhibit proinflammatory attributes by stimulating mesenteric lymph node CD11c⁺ dendritic cells, releasing cytokines that increase Th1 and Th17 immune cell populations (Kurakevich et al., 2013). In contrast to these findings, anti-inflammatory effects for 3'-SL were reported during an Caco-2 cell *in vitro* study with exposure to 3'-SL resulting in reduced expression of IL-8, IL-12 and TNF α (Zenhom et al., 2011). In addition to *in vitro* findings, results from clinical observational studies have identified associations between concentrations of certain sialylated HMO species in breast milk and development of NEC in preterm infants (Autran et al., 2018). In particular, low concentrations of DSLNT in breast milk were associated with development of NEC with authors suggesting that the DSLNT content in breast milk could serve as a potential non-invasive marker to identify infants at risk of developing NEC (Autran et al., 2018) (Bode, 2018). Despite increasing evidence that HMOs contribute to balanced inflammatory responses, it remains to be seen whether individual HMOs will become novel prophylactic and therapeutic agents for the treatment of allergy, auto-immune, and inflammatory diseases in the future. Further comprehensive studies, particularly large placebo controlled infant studies, are required to elucidate the potential of HMO in immunomodulation.

4. Conclusions

HMOs are thought to play a central role in the development of the neonatal immune system by promoting healthy microbial diversity, preventing pathogen attachment, stimulating maturation of intestinal epithelial surface and by modulation of immune cells. Further research is required however to reveal whether HMOs may also have a therapeutic rather than a protective effect in infant gut health. Numerous *in vitro* and animal experiments have been performed with HMO but to date; these bioactives have not been tested in many placebo controlled infant studies. In addition, HMOs have mostly been studied in isolation, yet they exist as a diverse pool and interact in the mammary gland and the infant gut. Novel combinations of methodologies will be essential in future studies to unravel the multifaceted interactions, specific or otherwise, between HMOs and the infant gut microbiota and their collective impact on immune function and prevention of multiple disease states.

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CHAPTER II

**From lab bench to formulated ingredient: Characterization,
production, and commercialization of human milk
oligosaccharides.**

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Abstract

Human milk oligosaccharides (HMOs) are known to positively influence infant health. Extensive variation exists in the levels, diversity, and complexity of oligosaccharides in the milk of a lactating mother. Until recently, limited availability of HMOs hampered their use in clinical applications. Most HMOs are unique to human milk, and have proven difficult and expensive to isolate and synthesize. Added to that, analysis of these complex glycans in milk samples requires state-of-the-art analytical instruments and associated technologies. The current review provides a critical overview of methods used in HMO analysis, and highlights the importance of understanding the factors which influence their composition and structural diversity. We also discuss recently employed strategies to overcome the availability of HMOs at industrial scale including microbial metabolic engineering and chemoenzymatic techniques. Finally, we examine how these recent advancements have opened up new avenues for future research and nutraceutical applications.

1. Introduction

Human breast milk is recognised as the gold standard in infant nutrition (Walker, 2010), with the World Health Organization (WHO) and the United Nations Children's Fund recommending exclusive breastfeeding up to at least 6 months of age (Organization, 2001). According to a systematic review of over 400 studies, breastfeeding was found to reduce the likelihood of atopic dermatitis, asthma, respiratory tract infections, gastrointestinal infections, type 1 and 2 diabetes, obesity, and necrotizing enterocolitis in preterm infants (Ip et al., 2007). The properties and chemical composition of human breast milk have been researched since the beginning of the 20th century, in an attempt to understand how specific structures in breast milk contribute to its diverse functionalities (Elgood, 1910; Gerstenberger et al., 1919). The composition of maternal milk changes constantly throughout lactation and adapts in order to fulfil the nutritional needs of the neonate for healthy growth and development (Andreas et al., 2015). The human infant enters the world with a functionally naïve immune system, which leaves the newborn at risk of infection. Human milk affords protection to the infant during this initial period by providing bioactive compounds which facilitate immune tolerance (Turfkruyer & Verhasselt, 2015), prevent pathogenic infection (Newburg et al., 2005), reduce intestinal permeability (Taylor et al., 2009), and act as highly specific prebiotics for particular commensal micro-organisms (Zivkovic et al., 2011). However, in cases where breast-feeding is not possible, or when the nutrient demands of the infant surpass what is provided by the lactating mother, fortified infant formulas which aim to mimic the functions of breast-milk have been developed. Targeting the prebiotic components in human milk which guide progression of infant's microbiota

represents an important strategy aimed at replicating the beneficial effects of breast-feeding (van den Elsen et al., 2019). One of the most important components imparting this prebiotic function is believed to be represented by the human milk oligosaccharide (HMO) fraction. In addition to guiding the progression of the infant intestinal microbiota, HMOs also demonstrate antimicrobial properties against Group B *Streptococcus* and other pathogens (Ackerman et al., 2017; Ackerman et al., 2018; Lin et al., 2017). HMOs have even recently been shown to act adjuvants to potentiate the function of antibiotics (Craft, Caddy & Townsend, 2018; Chambers et al., 2020). These molecules also act as soluble decoy receptors reducing colonisation of and infection by pathogenic bacteria (Laucirica, Triantis, Schoemaker, Estes, & Ramani, 2017). Studies have also demonstrated that HMOs support immune development (Donovan & Comstock, 2016), moderate intestinal permeability (Chichlowski, De Lartigue, German, Raybould, & Mills, 2012), influence intestinal cell responses (Kong et al., 2019), and reduce occurrences of necrotizing enterocolitis (Autran et al., 2018; Good et al., 2016). Substantiation of a cause-effect relationship between HMOs and infant health has encouraged the design of more functional formulas, such as through the inclusion of complex oligosaccharide structures (Smilowitz et al., 2014). Recent studies by Steenhout et al. and Puccio et al. have shown that consumption of a formula supplemented with two HMOs (2'-fucosyllactose [2'-FL] and lacto-*N*-neotetraose [LNnT]) is safe and well-tolerated, but most importantly results in gut microbiota progression similar to that witnessed in breastfed infants (Puccio et al., 2017; Steenhout et al., 2016). However, it is unlikely that the introduction of one or two HMOs will be sufficient in fully mimicking all beneficial effects of the total HMO pool. It is anticipated that

incorporation of more and more distinct HMOs, as they become available at commercial levels, will revolutionize the landscape of paediatric nutrition, and will offer opportunities to improve infant health worldwide.

Such a scenario cannot be achieved without accurate measurements of individual HMO structures and examination of how these HMO components are degraded/ metabolized in the gastrointestinal tract. While powerful tool sets of metagenomics, metatranscriptomics, and metabolomics have been employed routinely over the past decades, glycomics still has its technological challenges and may thus be considered a critical technology/knowledge gap in dairy, nutrition and clinical research. The concentrations of HMOs reported in the literature vary significantly (Thurl et al., 2017) which to a considerable degree can be explained by the influence of lactation stage, genetic/ethnicity factors (such as secretor status/lewis factor), and environment on the expression of HMOs (Erney et al., 2000; Xu et al., 2016). However, one major contributor to discrepancies in literature is the wide range of non-standardized analytical methods used across various laboratories. A recent systematic review by Thurl et al. emphasised this lack of standardization, stating that “nearly every study used a unique methodology with regard to the preparation, derivatization, and separation of samples, and the detection and quantification of HMOs” (Thurl et al., 2017). Advancements and standardisation of analytical methods, which allow for HMO quantification that is rapid enough for routine and high-throughput applications, will be crucial to understand how the mother-infant dyad functions to protect the vulnerable and developing neonate. Considering the multitude of functions assigned to HMOs, and often to specific HMO structures, it is very important to have robust methods to accurately determine

the levels of these structures in human milk. The aims of this review are to give a critical overview of the methods used in the analysis of human milk oligosaccharides, while also highlighting the importance of understanding the factors which influence the composition and structural diversity of HMOs. The review also aims to provide a summary of recent strategies which have been employed to overcome the lack of availability of HMOs and the regulatory framework involved to allow such artificial HMOs to be incorporated in commercial milk formulas. Finally, we examine the importance of placebo-controlled clinical intervention trials in supporting and furthering our understanding of HMO biology and the significance of these compounds in infant nutrition. As a semi-quantitative review and based on publically available information at the time of writing, PubMed databases were searched according to a predetermined search strategy using the following keywords: Human milk oligosaccharides, synthesis, secretor, lactation. Original studies and review articles published in English involving HMOs were eligible for inclusion.

2. HMO characterization

Analytical characterization of HMOs has proven problematic, in particular due to the complex stereochemistry of these compounds, as well as their branched-chain structures (Grabarics et al., 2017). Early analyses on the glycome of human milk were directed towards identifying and measuring a limited number of individual sugars, and the technology platforms applied only captured an incomplete snapshot of the whole picture (Mizoguchi et al., 1982). The past decade has witnessed major advancements in glycomics technology platforms, which allow for in-depth rapid, sensitive, and reproducible characterization of structural diversity in complex biofluids such

as milk (Yan et al., 2017). Such technologies are capable of resolving many individual HMO structures in a single analytical run, with (semi) quantitative assays preferred over basic qualitative analysis (Mantovani et al., 2016). Documenting and understanding the inter-individual and intra-individual variations of HMO concentrations is of utmost importance not only in basic biological research, but also for the development of infant nutritional products.

The majority of published HMO analytical methods incorporate similar isolation and purification processes in order to generate a highly pure HMO fraction from human milk. Initial centrifugation at low temperatures removes lipids, while precipitation and C-18 solid phase extraction (SPE) eliminates proteins and peptides from the sample. This is generally followed by porous graphitized carbon SPE, which removes lactose, monosaccharides, and salts from the sample. Once extracted, the HMOs must be separated in order to perform quantification analysis. Gel Permeation was one of the earliest separation techniques to be employed for the separation of HMOs (Thurl et al., 1991). Gel matrices such as Sephadex G-25 (Obermeier et al., 1999), Bio-Gel P-4 (Donald & Feeney, 1988) and TSK HW40 (Thurl et al., 1993b) separate HMOs on the basis of acidity and size. Although gel permeation is still used in purification of enriched HMO fractions, it is no longer employed for analysis due to its limited resolution.

Rapid developments in chromatography have taken place in recent years and are summarised in a review by O'Sullivan et al. (O'Sullivan et al., 2018). These methods are accurate and sensitive, and are capable of resolving many HMO structures including configurational isomers. Traditionally used methods for this purpose are reverse phase (RP) or normal phase (NP) high

performance liquid chromatography (HPLC) coupled to UV detectors (Chaturvedi et al., 1997; Sumiyoshi et al., 2007). Oligosaccharides in their native form have poor UV absorption and therefore derivatization of HMOs is required which not only facilitates their separation and retention but also increases sensitivity when using UV detectors (Sumiyoshi et al., 2007). Derivatization methods employed for chromatographic analysis of HMO are summarised in **Table 1**, which include reduction of HMOs with sodium borohydride to their alditol form (Bao et al., 2013). However, these approaches have disadvantages; namely, sample clean-up is required which is time-consuming and considerable disparity has been observed between analyses. As an alternative to derivatization and UV detection, high-pH anion-exchange chromatography (HPAEC) is more commonly used for quantification of HMO fractions (Kunz et al., 1996; Viverge et al., 1990). One of the earliest applications of HPAEC described in literature was in 1996 when Thurl et al. employed SEC for pre-fractionation, and pulsed amperometric detection (PAD) for quantification of neutral and acidic structures (Thurl et al., 1996). Although RP/NP HPLC and HPAEC are regarded historically as important chromatographic techniques, researchers have also recently developed methods outside of standard liquid chromatography. These include Nuclear Magnetic Resonance (NMR) methods (Smilowitz et al., 2013) and electrophoresis techniques such as capillary electrophoresis (CE) (Olivares et al., 2015).

Table 1: Derivitization, Separation, and Detection Techniques Employed for Analysis of HMOs

Separation	Detection	Derivitization	HMO Analytes	Location	Gestation	Secretor status	N	Reference
HPAEC	PAD	No derivitization	Neutral/ Acidic	France	Term	NS	15	Viverge et al., 1990
HPAEC	PAD	No derivitization	Neutral/ Acidic	Germany	Term	NS	2	Kunz et al., 1996
HPAEC	PAD	No derivitization	Neutral/ Acidic	NS	NS	NS	NS	Thurl et al., 1996
RP-LC	UV	Perbenzoylation	Neutral	Mexico	Term	NS	50	Chaturvedi et al., 1997
HPAEC	PAD	No derivitization	Neutral/ Acidic	Italy	Term	NS	18	Coppa et al., 1999
NP-LC/ RP-LC	MALDI-TOF MS/MS	2-aminoacridone	Neutral/ Acidic	UK	Term	+/-	3	Charlwood, Tolson, Dwek, & Camilleri, 1999
HPAEC	PAD	No derivitization	Neutral	Global (10 countries)	Term	+	435	Erney et al., 2000
CE	LIF	2-aminoacridone	Neutral/ Acidic	China	Term	NS	25	Song, Weng, Wu, & Xia, 2002
RP-LC	UV	Pyridylamination	Neutral	Japan	Term	NS	16	Sumiyoshi et al., 2003
RP-LC	UV	No derivitization	Acidic	Spain	Term	NS	12	Martín-Sosa, Martín, García-Pardo, & Hueso, 2003
PGC-Nano-LC	TOF-MS	Reduction with borohydride	Neutral/ Acidic	USA	Term		5	Ninonuevo et al., 2008
CE	UV	No derivitization	Acidic	USA	Term	NS	13	Bao, Zhu, & Newburg, 2007
RP-LC	UV	PMP	Acidic	Japan	Term	NS	20	Asakuma et al., 2007
RP-LC	UV	PMP	Neutral	Japan	Term	+	12	Asakuma et al., 2008

Table 1: Derivitization, Separation, and Detection Techniques Employed for Analysis of HMOs

RP-LC	UV	2-Anthranilic Acid	Neutral	Samoa	Term	NS	12	Leo et al., 2009
PGC- HPAEC	PAD	No derivitization	Neutral/ Acidic	Germany	Term	+/-	30	Thurl et al., 2010
CE	LIF-MS	APTS	Neutral	Netherlands	32 week	NS	5	Albrecht et al., 2010
NP-LC	Fd-EI-MS	2-aminoacridone	Neutral/ Acidic	Italy	Term	+/-	NS	Galeotti et al., 2012
PGC-LC	MS	Reduction with borohydride	Neutral	USA	Term	+	20	Bao et al., 2013
NMR		No dervitization	Neutral	USA	Term	+/-	52	Smilowitz et al., 2013
LC and GC	MS and MS/MS	No derivitization	Neutral/ Acidic	USA	Term	+/-	17	Goehring, Kennedy, Prieto, & Buck, 2014
NP-LC	ESI-MS	Reduction with borohydride	Neutral/ Acidic	USA	Term	+/-	20	Hong et al., 2014
GC	ESI-MS	O-trimethylsilyl- oxime	2'-FL, 3-FL	Hungary	Term	+	2	Balogh, Szarka, & Béni, 2015
CE	LIF	APTS	Neutral	Netherlands	Term	+/-	12	Olivares et al., 2015
NMR		No dervitization	Neutral	USA	Term/ Preterm	NS	28	Spevacek et al., 2015
RP-LC	MS/MS	Reduction with borohydride & permethylation	Neutral	NS	Term	NS	NS	Oursel, Cholet, Junot, & Fenaille, 2017
UPLC	QqQ-MS	No derivitization	Neutral/ Acidic	US/ Malawi	Term	+/-	45/88	Xu et al., 2017
PGC-LC	ESI-MS	Reduction with borohydride	Neutral/ Acidic	Brazil	Term	NS	41	Tonon et al., 2019

NS= not specified

Mass Spectrometry (MS) determines molecular weights of eluting compounds and is therefore capable of analysing individual HMO structures. This allows for measurement of HMOs with more specificity versus detection using UV or amperometric techniques. In recent years, MS has begun to play an increasingly important role in HMO characterization (Mirgorodskaya et al., 2018; Remoroza et al., 2018; Tonon et al., 2019). Low resolution tandem mass spectrometric analysers such as triple quadrupole (QqQ) have been employed for quantification of HMOs (Xu et al., 2017). Analysis of HMOs using LC-QqQ methods is robust, sensitive, cost-effective and accurate. However, detection requires the use of analytical standards and doesn't allow for the detection of unknown compounds. Technological improvements in high-resolution mass spectrometric analysers have offered new opportunities to analyse glycans. The resolving power of instruments such as time of flight (TOF) and Orbitrap analysers is up to 10 times higher than that of QqQ and low resolution instruments (O'Sullivan et al., 2018). One of the major advantages of high-resolution mass spectrometers is the identification of unknown compounds. The development of novel, combinational techniques using MS has allowed for the analysis of multiple HMO structures in large sample numbers. These methods include NanoLC-Chip/Q-TOF MS methods (Totten et al., 2014), capillary electrophoresis with laser-induced fluorescence and MS (CE-LIF-MS) (Albrecht et al., 2010), and liquid chromatography electrospray ionization MS (LC-ESI-MS) (Tonon et al., 2019). Ion mobility spectrometry (IMS) techniques have been developed in recent years and offer improved separation and identification of structurally similar HMO structures (Hofmann et al., 2017). IMS coupled to MS

instruments has also been employed recently for analysis of HMOs and other glycan structures (Ben Faleh et al., 2019; Khanal et al., 2018)

During the last ten years the glycomics field has gone through an extraordinary expansion in terms of technology developments, which have allowed detailed and a more comprehensive understanding of the structure of individual HMOs as well their concentrations in human breast milk at different lactation stages and across geographical distinct areas. Accurate measurement of the physiological intakes of HMOs across various population groups has furthered our understanding of the factors that contribute to variations in HMO composition.

3. Variations in HMO Structure and Concentration in Human Milk

Inter-individual variation of HMO concentrations is pronounced, and is strongly dependent on maternal genetics and allelic variation in the Secretor (*Se*) and Lewis (*Le*) genes (Thurl et al., 2010). Besides the influence of *Se* and *Le*, substantial differences between HMO profiles occur in the milk produced by women with the same genetic background. Therefore, non-genetic factors must also influence HMO composition (Samuel et al., 2019). Although studies are limited, it is thought that maternal factors such as parity and body mass index (BMI) (Azad et al., 2018; Jantscher-Krenn et al., 2019), as well as environmental factors such as geographical location, influence the HMO composition of human milk (Erney et al., 2000; van Leeuwen et al., 2018). An observational study of breastfeeding mothers in Brazil has also described apparent associations between maternal allergic disease and infant's sex on the one hand and HMO concentrations on the other (Tonon et al., 2019). Moreover, results from a recent intervention study have demonstrated that

HMO composition can be influenced by maternal probiotic supplementation during the late stages of pregnancy. The study, conducted by Seppo et al. in Finland, analysed data from 81 women who were fed either a placebo or a twice-daily dose of a combination of *B. breve* Bb99, *L. rhamnosus* GG, *L. rhamnosus* LC705 and *P. freudenreichii* subsp. *shermanii* JS. Significantly higher concentrations of 3-Fucosyllactose (3-FL) and 3'-Sialyllactose (3'-SL) were found in the colostrum of the probiotic group when compared to the control group (Seppo et al., 2019). While it has long been established that HMOs shape microbial communities, the latter study suggests that microbes play a role in modifying HMO composition and thus revises the hypothesis that interactions occurring between HMOs and the human microbiome is a one-way street.

Additional to inter-individual variations, intra-individual variations are pronounced with HMO composition altering constantly throughout the nursing period (N. Sprenger et al., 2017). Concentrations of HMOs alter throughout breast-feeding according to lactation stage (decreasing from 20-25 g/L in colostrum to 5-20 g/L in mature milk), time of day, and mother's diet while continuously adapting to the requirements of the infant (Austin et al., 2016; Thurl et al., 1993a; Xu et al., 2017). A number of studies assessing the HMO concentrations in the milk of mother's who had delivered preterm infants have highlighted that significant differences in HMO composition exist when compared to term milk. (De Leoz et al., 2012; Gabrielli et al., 2011; Nakhla et al., 2007). Most notably, a recent study analysed HMO concentrations in 500 samples from 28 mothers nursing term infants, and 25 mothers who had delivered very preterm neonates. The study revealed elevated concentrations of sialylated HMOs were present in preterm milk,

particularly 3'-SL (Austin et al., 2019). These observations align with the theory that the composition of milk adapts to satisfy the nutritional requirements of the neonate, though the underlying mechanistic process that drives these compositional changes is as yet obscure.

Oligosaccharides in human milk are characterised by remarkable structural diversity (Ayechu-Muruzabal et al., 2018). This diversity is brought about by 12 glycosidic linkage options between the monosaccharide building blocks of Glucose (Glc), Galactose (Gal), *N*-acetylglucosamine (GlcNAc), Fucose (Fuc), and *N*-acetylneuraminic acid (Neu5Ac). Chemical structure of monosaccharide building blocks are illustrated in **Supplementary Figure 1**. HMO synthesis occurs in the lactating mammary gland and is under the control of a series of glycosyltransferases, including fucosyltransferases, sialyltransferases, galactotransferases, and acetylglucosaminyltransferases (Bode, 2006; Urashima et al., 2011). The pathways for HMO synthesis are summarised in **Figure 1**. All HMOs follow a basic blueprint and contain a lactose molecule (Gal β 1-4Glc) at their reducing end (Bode, 2012). Linear chains form when the monosaccharide GlcNAc attaches to the galactose moiety through a β 1-3 linkage, while chain branching is introduced via β 1-6 glycosidic linkages. Linear structures are described as “para-HMO”, while those with chain branching are termed “iso-HMO”. Human milk is particularly rich in type 1 oligosaccharides, with lacto-*N*-biose (LNB; Gal β 1-3GlcNAc-) as the building unit, while the less prevalent type 2 oligosaccharides contain *N*-acetyllactosamine as the building block (Gal β 1-4GlcNAc) (**Figure 1** & **Supplementary Figure 1**) (Smilowitz et al., 2014). Lactose, linear, and branched oligosaccharides can be sialylated by α 2-3 or α 2-6 linkages and/or fucosylated by α 1-2, α 1-3, or α 1-4 linkages (**Figure 1**).

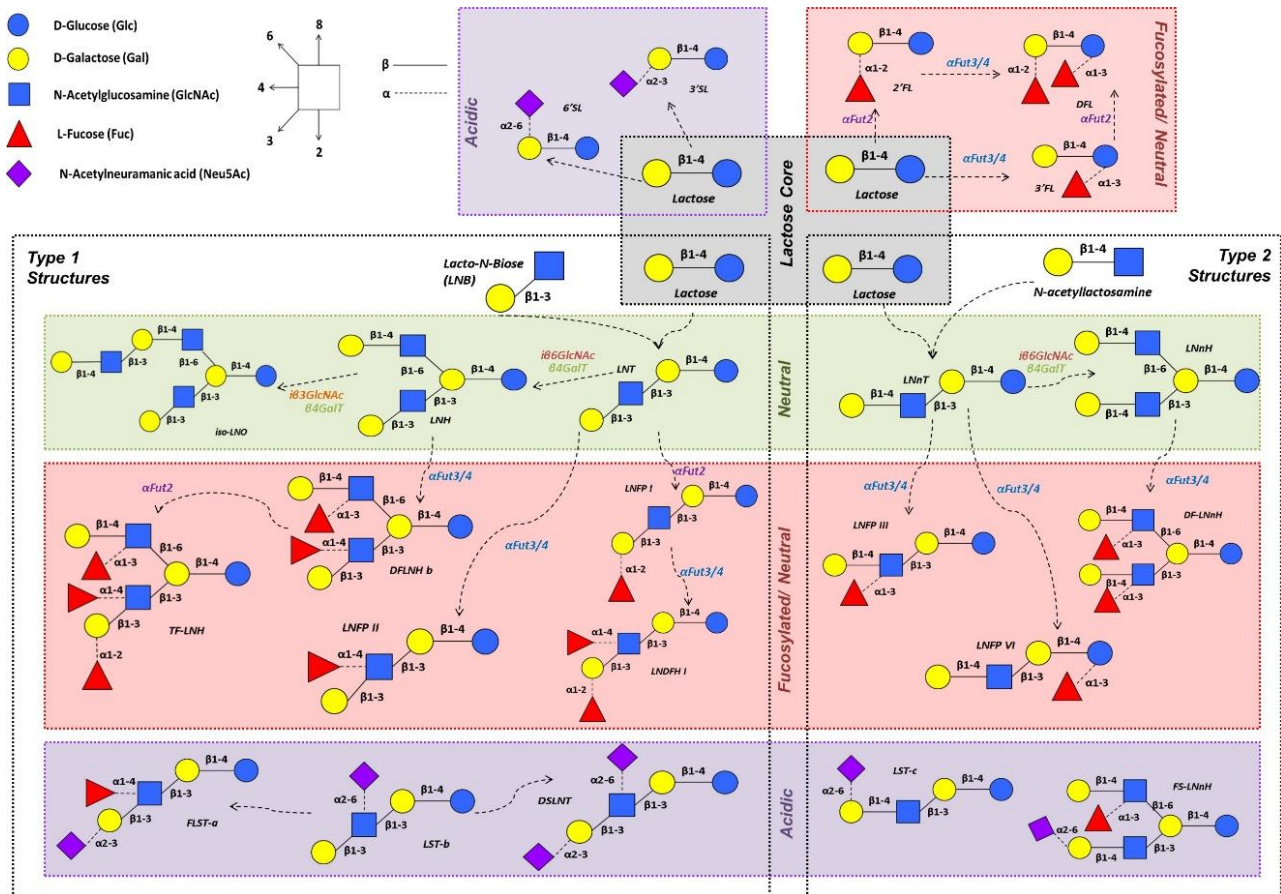


Figure 1: Biosynthetic pathways for assembly of complex human milk oligosaccharide (HMO) structures. Diversity is brought about by conjugation of 5 monosaccharide building blocks via 12 potential glycosidic linkages. Synthesis occurs in the mammary gland and is directed by a series of glycosyltransferases including galactotransferases (β 4-GalT), acetylglucosaminyltransferases (α 1-3- and α 1-6- GlcNAc), fucosyltransferases (α -Fut2 and α -Fut3/4) and sialyltransferases. All HMOs contain lactose at their core. Type 1 HMO chains incorporate lacto-*N*-biose (LNB) disaccharide units, while type 2 HMO chains incorporate *N*-acetylglucosamine disaccharide units. The simplest sugars are formed through the addition of fucose to the lactose core to form the trisaccharides 2'- and 3'- fucosyllactose (2'-FL and 3'-FL) or addition of the sialic acid, Neu5Ac, to lactose to form the trisaccharides 3'- and 6'-sialyllactose (3'-SL and 6'-SL).

The smallest oligosaccharides are formed when the sialic acid, NeuAc, attaches to the lactose core to form the sialylactoses, 3'-SL and 6'-SL (**Supplementary Figure 1**), or when fucose is added to lactose to generate the fucosyllactoses, 2'-FL and 3-FL (**Supplementary Figure 1**) (Bode, 2012). The presence of sialic acid means that HMOs are often separated into two groups on the basis of their chemical charge: Neutral oligosaccharides which contain no charged monosaccharides (Glu, Gal, *N*-acetylglucosamine, Fucose) and acidic oligosaccharides which contain negatively charged residues of NeuAc. Acidic HMOs make up 12-14 % of the total HMO, while 35-50 % are fucosylated structures with the remaining 42-55 % structures being non-fucosylated (Thurl et al., 2017). Decoration of HMOs with fucose is dependent on the mother's Secretor and Lewis blood group status (**Figure 2**). Polymorphisms of Lewis and Secretor genes have one of the greatest impacts on the phenotypic profile of HMOs present in human milk (Blank et al., 2012).

3.1 Secretor Status

While the biological variation (denoted as coefficient of variation or CV) for total HMOs is as high as 28 % (Xu et al., 2017), even greater disparity is observed for certain HMO structures such as 2'-FL, 3-FL, difucosyllactose (DFL), lacto-*N*-fucopentaose (LNFP) I, and LNFP II. The CV for these structures in mature breast milk ranges from 42 % to 84.3 % (Smilowitz et al., 2014). Variations in these fucosylated HMO structures can be largely accounted for by allelic variations in the expression of specific glycosyltransferases. HMO composition mirrors maternal blood group characteristics (Newburg et al., 1999), whereby expression of Secretor and Lewis genes in the mammary gland determines the structure and abundance

of HMOs (Blank et al., 2012; Thurl et al., 1997). The Secretor gene (*Se*) encodes the α 1-2 fucosyltransferase (FUT2) enzyme, which attaches fucose residues via an α 1-2 linkage to acceptor HMO chains, while the Lewis gene (*Le*) encodes for fucosyltransferase FUT3, which attaches fucose residues via an α 1-4 linkage to subterminal GlcNAc of type 1 HMO chains (Kunz et al., 2017). Four milk groups can be assigned based on the *Se/Le* blood group system, with each resulting in a different phenotypic HMO pattern as summarised in **Table 2**.

Table 2: Milk groups according to Lewis and Secretor Status

Milk Group	Classification	<i>Se</i> (FUT2)	<i>Le</i> (FUT3)	Fucose linkages	HMOs Secreted
1	Lewis Positive, Secretors	+	+	α 1-2, α 1-3, α 1-4	All HMOs
2	Lewis Positive, Non-Secretors	-	+	α 1-3, α 1-4	3-FL, LNFP-II, LNFP-III
3	Lewis Negative, Secretors	+	-	α 1-2, α 1-3	2'-FL, 3-FL, LNFP-I, LNFP-III
4	Lewis Negative, Non-Secretors	-	-	α 1-3	3-FL, LNFP-III and LNFP-V

Mothers with a functional *FUT2* allele produce milk which is abundant in α 1-2 fucosylated HMOs and are described as “Secretors”. Secretor milk contains significant amounts of 2'-FL, lacto-*N*-difucohexaose (LNDFH I), and lacto-*N*-fucopentaose I (LNFP I) (**Figure 2**). In contrast, mothers who are classified as non-secretors are homozygous for a non-active secretor gene, and their milk has low or undetectable levels of α 1-2 fucosylated oligosaccharides. The milk of non-secretor mothers also contains significantly lower amounts of total oligosaccharides, but higher abundances of lacto-*N*-tetraose (LNT), LNFP II, LNFP III, and LNDFH II (**Figure 2**).

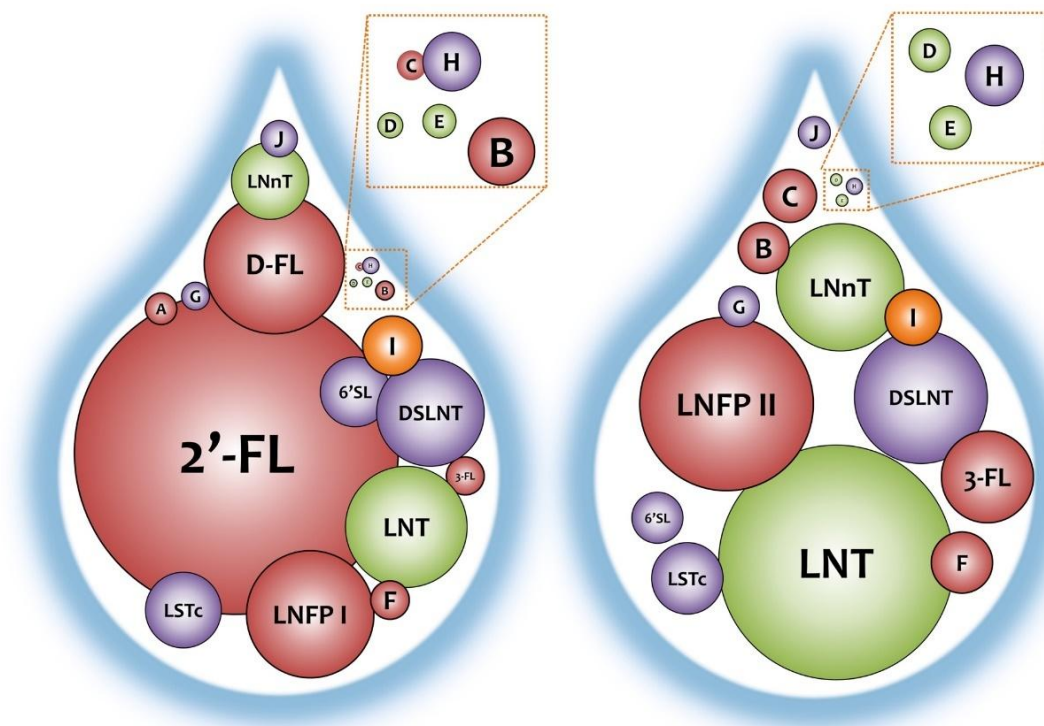


Figure 2: Schematic representation of human milk oligosaccharide (HMO) profile in the milk of secretor mothers (left) and non-secretor mothers (right). Secretor milk contains large amounts of α 1-2 fucosylated oligosaccharides, while non-secretor milk has undetectable levels of α 1-2 fucosylated oligosaccharides. Non-secretor milk also contains lower concentrations of total oligosaccharides but has higher concentrations of LNT, LNFP II, and LNFP III. Diameters of each circle depict the concentration of quantified HMO. Concentrations adapted from (Thurl, Munzert, Boehm, Matthews, & Stahl, 2017). Neutral non-fucosylated HMO = green. Neutral, fucosylated HMO = red. Acidic, non-fucosylated HMO = purple. Acidic, fucosylated HMO = orange. **A**= LNFP II, **B**= LNFP III, **C**= LNDFHII, **D**= LNH, **E**= LNnH, **F**= FLNH II, **G**= LST a, **H**= LST b, **I**= FSLNnH I, **J**= 3'-SL

Globally, a substantial number of allelic variants of the secretor phenotype have been detected. In a study by Ferrer-Admetlla et al. the coding region of the *FUT2* gene was re-sequenced in 732 individuals from 39 human populations (Ferrer-Admetlla et al., 2009). The researchers identified 55 single-nucleotide polymorphisms (SNPs) in the *FUT2* gene. Non-secretor

phenotypes were found to be present in all populations, but inter-population differences in secretors and non-secretors were notable (Ferrer-Admetlla et al., 2009).

Cross-sectional, observational studies have been conducted aimed at exploring the relationship between maternal genetics and HMO concentrations across various geographical locations. Perhaps the first comprehensive study of this kind was conducted by Erney et al. where HMO concentrations were measured in the milk of 435 lactating mothers from ten different countries. Significant differences in the concentrations of 2'-FL were observed, which was quantifiable in all milk samples from Mexican and Swedish mothers, but detected in only 46 % of the milk samples collected in the Philippines (Erney et al., 2000). Expanding on this work, McGuire et al. analysed the HMO composition in the milk of 410 breastfeeding women from 11 distinct cohorts (McGuire et al., 2017). More advanced analytical methods and sampling approaches were used when compared to those used in the earlier study, but similar distribution trends were observed. The number of secretor mothers ranged from 98 % in the Peruvian cohort to 65 % in the Rural Gambian and Rural Ethiopian cohort. The percentage of secretors in Sweden, Spain, Urban Ethiopia, Kenya, and Urban Gambia was similar ranging from 76 % to 85 %. The Hispanic cohort in the USA was similar to that of Peru (95 % secretors), while the other cohorts in the USA (of unspecified ethnicity) contained just 68 % secretors. The authors speculated that the high percentage of secretors in Peruvian and US-Hispanic cohorts was due to historical evolutionary pressures that conferred α 1-2 fuc related health benefits to these populations (McGuire et al., 2017). Perhaps the most interesting finding from this study was the divergence in secretors between

rural and urban populations in both Ethiopia and Ghana. It would be expected that the populations would be genetically related, implying that environmental influences might also hold significance.

The precise reason for the geographical disparities is unknown, but scientists have speculated that the phenomenon could be the result of evolutionary forces acting on different continental groups (Ferrer-Admetlla et al., 2009). Since pathogens are powerful selective agents, it is probable that different pathogenic environments have acted on the *FUT2* locus throughout human history, driving the varying haplotypes observed across populations. Results from population-based observational studies in infants verify that a possible relationship exists between *FUT2* gene expression and exposure to infectious diseases. Morrow et al. was one of the first groups to observe that α 1-2 fucosylated glycans in breast milk confer protection against pathogens (Morrow et al., 2005). Consumption of secretor milk was shown to correlate with a lower incidence of infant diarrhoea caused by *Campylobacter*, calciviruses, and stable toxin of enterotoxin *E. coli* (Newburg et al., 2003). Conversely, it has been reported that non-secretor milk confers protection against Norwalk-like virus (Jiang et al., 2004), while a non-functional *FUT2* allele is also linked with slower development of HIV-1 infection (Kindberg et al., 2006). It is clear, therefore, that specific HMO structures act as anti-adhesion agents either directly, by competing with epithelial cell-receptor binding (Morrow et al., 2005; Ruiz-Palacios et al., 2003) or indirectly, by promoting the colonization of beneficial bacteria in the gut (Garrido et al., 2015; James et al., 2019; Kavanaugh et al., 2013)

Strong associations have been observed between maternal genetics, HMOs and the type of commensal communities found in the infant gut (van

den Elsen et al., 2019). Specifically, secretor positivity of mothers is associated with higher abundances of *Bifidobacterium* in the infant gut microbiome (Lewis et al., 2015; Smith-Brown et al., 2016). Conversely, studies have shown delayed colonisation of these beneficial microbes in the gut of infants fed non-secretor milk. Higher numbers of *Clostridium* and *Enterobacteria* were also observed in the fecal microbiome of infants fed non-secretor milk (Mottram et al., 2017). It is therefore apparent that differences in HMO profiles between secretor and non-secretor mothers results in differences in the microbial communities of their infants. This highlights a huge level of stereospecific activity, with the different HMO structures and conformations accounting for their various biological functions.

4. Oligosaccharide production

Until recently manufacturing of HMOs in large quantities was a tedious and costly process which resulted in insufficient yields for clinical applications. For over two decades, paediatric nutritional products were fortified with alternative, non-human sources of complex oligosaccharides such as galactooligosaccharides (GOS) and fructooligosaccharides (FOS) in an attempt to close the gap between breastmilk and formula (Vandenplas et al., 2014). Chains of GOS consist of 3-10 galactose molecules, frequently with a glucose unit at the reducing end, and are synthesised through the enzymatic action of β -galactosidase, while FOS can be isolated in large quantities from chicory roots (Davani-Davari et al., 2019). Although a vast body of pre-clinical and clinical studies exist assessing the properties of GOS and FOS, the beneficial effects are indeterminate. The EFSA Panel on Dietetic Products, Nutrition and Allergies has subsequently stated that there is no

necessity to add GOS and FOS to infant formulae (Bode et al., 2016; Products, 2014).

When compared to human milk, the oligosaccharides found in the milk of domestic farm animals are present in significantly lower concentrations and have less structural diversity when compared to HMOs (Urashima et al., 2001). Despite these facts, milk from farm animals such as cows and goats, represent attractive alternatives for obtaining large quantities of oligosaccharides (reviewed by Quinn et al. 2020). Milk oligosaccharides are found in whey permeate, which is a lactose-rich effluent remaining after protein extraction from milk-derived cheese whey, an abundant dairy waste. A number of studies have highlighted the potential use of whey permeate as an alternative source of oligosaccharides. (Bode et al., 2016; Mehra et al., 2014). Bode et al. calculated a theoretical yield of 220,000 kg of bovine milk oligosaccharides annually from US dairy industry waste by-products (Bode et al., 2016). However, bovine milk contains trace amounts of fucosylated oligosaccharides, while HMOs are predominantly fucosylated. The differences in oligosaccharides profiles could potentially be resolved through the use of chemoenzymatic synthesis to construct fucosylated and elongated glycans. Bovine milk oligosaccharides therefore offer themselves as precursors for chemoenzymatic processes (Yu & Chen, 2019).

Considering the difficulties associated with large-scale synthesis of HMOs, characterisation and lab scale research of HMOs has traditionally been conducted using fractions isolated from human milk. The advantage of this was that HMO structures were obtained in their natural form. The challenge was that they could not be isolated in high enough quantities sufficient for clinical trials due to limited access to human donor milk.

Recently, development of new synthetic methods have begun to emerge that are able to solve this scale issue (Faijes et al., 2019). These methods, which include chemoenzymatic techniques and microbial metabolic engineering processes, have resulted in the production of unprecedented quantities of certain HMO structures, and have furthered our understanding of the biological role of specific HMOs in clinical settings. Moreover, progress in HMO manufacturing allows for design of HMO-containing products for pharmaceutical and nutraceutical applications (G. A. Sprenger et al., 2017).

4.1 Chemical Synthesis

The first HMOs (2'-FL and LNnT) registered as “novel foods” in Europe were produced by Glycom A/S through the use of *in vitro* chemical synthesis. In comparison to other biopolymers, significant challenges are encountered during chemical synthesis of carbohydrate molecules. Due to the structure-function relationships of HMOs and other oligosaccharides, correct branching and stereospecific linkages are required for biological function to be preserved (Smilowitz et al., 2014). Therefore, issues such as regioselective protection of hydroxyl groups on acceptor monosaccharides and stereospecific construction of glycosidic linkages must be overcome (Kulkarni et al., 2018). Despite these challenges, stepwise solid-phase methods have been applied for the synthesis of a significant number of complex HMO structures such as 2'-FL, 3-FL, DFL, LNT, LNnT, LNFP I, and LNFP III (Arboe Jennum et al., 2014). Methods for chemical synthesis of sialylated molecules have also been described (Fair et al., 2015; Lai et al., 2015). Most recently, Bandara et al. have developed methods for the chemical synthesis of LNnH using a convergent synthetic strategy (Bandara, Stine, & Demchenko, 2020). New synthetic protocols continue to be examined and

refined and allow for HMOs to be obtained in homogenous fashion (Bandara, Stine, & Demchenko, 2019). Thus, chemical synthesis can serve to enhance our understanding of the role of HMOs and guide larger scale production. However, it is often a laborious and inefficient process, resulting in low yields of the targeted HMO. To address these problems, methods are being developed whereby automated chemical synthesis is used in combination with enzymatic glycan synthesis (Wen et al., 2018).

4.2 Chemoenzymatic Synthesis

The enzymes used for chemoenzymatic synthesis of HMOs are largely glycosyltransferases (GTs) from pathogenic bacteria such as *Helicobacter*, *Neisseria*, or *Pasteurella* (G. A. Sprenger et al., 2017). To date, researchers have exploited fucosyltransferases, sialyltransferases, galactosyltransferases, and *N*-acetyl-glucosaminyltransferases for enzymatic synthesis of HMOs. Commonly employed chemoenzymatic pathways have been described in detail in many recent reviews (Zeuner et al., 2019). Pioneering works in this field revealed that recombinant β 1,3-*N*-acetyl-glucosaminyltransferase (LgtA) and β 1,4-galactosyltransferase (LgtB) from pathogenic *Neisseria* species can be employed to synthesize LNT with product yield of more than 85 % (Johnson, 1999). More recent work by Zeuner and colleagues demonstrated synthesis of LNT using glycosidases from natural sources. Here, the enzymatic action of β -galactosidase cloned from *Bacillus circulans* was exploited to add lactose moieties to lacto-*N*-triose acceptor molecules (Zeuner et al., 2016). Enzymatic synthesis of the core HMO structure, lacto-*N*-biose (LNB) has also been described. Nishimoto and Kitoaka used a collection of sucrose phosphorylase (from *B. longum*), UDP-glucose, UDP-glucose-4-epimerase, and LNB phosphorylase (from *Bifidobacterium*

bifidum) to produce LNB in kilogram quantities (Nishimoto & Kitaoka, 2007). Other methods to construct neutral HMO structures include the use of LNB phosphorylase from *Bifidobacterium longum* subsp. *infantis*, resulting in the synthesis of β 1,3-linked molecules including LNB and LNT (Yao et al., 2015; Yu et al., 2010).

Fucosylation of HMO structures can also be carried out using GTs cloned from microbial sources. Early work from Albermann et al. demonstrated that 2'-FL could be produced with a 65 % yield through a stepwise synthetic pathway (Albermann et al., 2001). This involved conversion of GDP-Fucose from mannose using recombinant *E. coli* enzymes, before transfer of GDP-Fucose to a lactose acceptor. This final enzymatic step was catalysed by recombinant α 1,2-FucT cloned from *H. pylori* (Albermann et al., 2001). FutT glycosyltransferases from *Helicobacter* strains are commonly used for synthesis of Fuc-borne HMO. However, the enzymes demonstrate higher affinity for LNB and LacNAc than lactose which hampers their use in the production of 2'-FL, 3-FL, and DFL. Choi et al. overcame these challenges by introducing mutations in the C-terminal domain of a 1,3-FucT, thereby significantly increasing yields of 3-FL (Choi et al., 2016). Mutagenesis techniques have also been applied in the engineering of α 1,2-fucosynthase from a glycosidase. Mutations in a 1,2- α -fucosidase from *B. bifidum* abolishes the enzyme's hydrolase activity, while retaining its transferase activity. The use of this mutagenesis method has been described for the synthesis of 2'-FL (lactose acceptor), 3-FL (lactose acceptor), and DFL (2'-FL acceptor) using β -L-fucopyranosylfluoride as the donor molecule (Sugiyama et al. 2016; Wada et al., 2008).

Chen et al. employed a one-pot multi-enzymatic approach to produce 148 mg of LNnT with a 81% yield (Chen et al., 2015). Further enzymatic steps resulted in the decoration of this LNnT receptor with fucose or sialic acid. In this study, the enzymatic action of α 1,3-FucT from *H. pylori* resulted in the production of LNFP III, while the sialyllacto-*N*-tetraoses LST-a and LST-c were synthesised by addition of sialic acid residues using an α 2,3-sialyltransferase (from *Pasteurella multocida*) and α 2,6-sialyltransferase (from *Photobacterium damsela*), respectively (Chen et al., 2015). Sialyltransferases from *P. multocida* have also been employed in the production of the trisaccharides 3'-SL and 6'-SL (Guo et al., 2015).

A more recent study from Chen's group at UC Davis employed similar one pot multi-enzyme (OPME) glycosylation processes to produce milligram quantities of LNT (McArthur, Yu, & Chen, 2016). Here, McArthur et al. identified a β 3GalT from *Chromobacterium violaceum* (CV β 3GalT) which was homologous to a previously assessed β 3GalT from *E. coli* but demonstrated much higher efficiency in catalysing the formation of LNT from lacto-*N*-triose II. Yields of 99.3% LNT from lactose were achieved after two OPME processes in sequence (McArthur et al., 2016). The high titres achieved using this CV β 3GalT facilitated the subsequent fucosylation and/ or sialylation of LNT to produce HMO structures such as LNFP II, LNDFH, and LNFP V (McArthur et al., 2016).

Despite an ever-increasing number of studies in this area, these approaches can still not satisfy the HMO demands associated with commercial applications. Therefore, use of HMO produced through chemoenzymatic pathways is more suitable for development of elaborate libraries of structurally diverse HMOs. Milligram quantities of HMO

produced during chemoenzymatic synthesis can be used for small-scale analysis of structure-activity relationships, and may ultimately guide the development of HMO production in larger scale biotechnological processes (Bode et al., 2016).

4.3 Microbial Metabolic Engineering

The potential use of microbial host organisms for large-scale HMO production was first demonstrated nearly two decades ago and since then, microbial fermentation processes have been employed routinely resulting in the production of commercially viable yields of 2'-FL and LNnT, with production of several other HMO structures imminent (Bych et al., 2019). Indeed, 42 HMO structures (including building blocks) have been produced using the cell factory approach to date (Faijes et al. 2019). To the best of our knowledge, no microbial organism naturally produces HMO. Therefore, metabolic engineering must be applied, and the appropriate glycosyltransferases expressed in microbes in order to generate HMOs. Owing to their extensive use as a research and cloning tool, non-pathogenic *E. coli* strains have become the preferred host organism for HMO synthesis (Sprenger et al., 2017). Despite this, there is no evidence that *E. coli* is the optimal microbial host, and efforts to develop alternative hosts, such as *Saccharomyces cerevisiae* (Yu et al., 2018), *Bacillus* species, and *Corynebacterium glutamicum* (Seo et al., 2018) are on-going. The production of HMOs via enzymatic and cell factory approaches has been reviewed in detail recently (Bych et al., 2019; Sprenger et al., 2017).

Beneficial properties associated with host organisms include efficient uptake of precursors (such as lactose), proficient export of HMO end-

products, and suitable pathways for production of nucleotide-activated sugar donors (such as GDP-Fuc). In addition, preferred host organisms have generally regarded as safe (GRAS) or qualified presumption of safety (QPS) status, have high growth rates, and possess features which reduce downstream processing costs and eliminant ingredient quality issues. Challenges in HMO production now lie with the development of efficient, cost-effective downstream processing lines to produce high quality HMO. Low levels of contaminants (such as endotoxins, DNA and protein), low biomass production, and low levels of viscosity are preferable for downstream processes. *E. coli* satisfies some of these requirements, but certainly not all (Bych et al., 2019). Until now, research has mostly focused on the large-scale production of 2'-FL since it is the most abundant HMO in human breast milk (in secretor women), and one of the simplest structures. Regardless of the chosen host strain, three elements are required for biosynthesis of 2'-FL: a) intracellular transport of lactose into the cell, b) overproduction of nucleotide-activated L-fucose (GDP-Fuc), and c) a fucosyltransferase enzyme capable of fucosylating lactose via α 1-2 linkages.

Although wild-type *E. coli* is able to take up lactose via lactose permease (LacY), it also has metabolic pathways dedicated to the degradation of lactose. Therefore, for the purpose of 2'-FL synthesis, research groups have engineered *E. coli* strains with altered or deleted β -galactosidase (LacZ) activity (Chin et al., 2016; Dumon et al., 2001). Currently, the preferred method is to feed lactose produced during whey processing directly into the fermentation system (Bych et al., 2019). However, low solubility of lactose is an issue, as is the isomerization of lactose to lactulose. Small amounts of lactulose present in the fermentation system results in the production of 2'-

fucosyllactulose. Therefore, researchers have proposed *in vivo* synthesis of lactose as a potential alternative (Bode et al., 2016).

GDP-L-fucose is produced naturally in *E. coli* strains as a precursor to colonic acid via the *de novo* pathway (Stevenson et al., 1996), but is required at much higher concentrations for 2'-FL production. Overproduction of enzymes along the *de novo* pathway has been investigated including ManB and ManC to increase flux towards GDP-D-mannose production, as well as increased expression of GDP-D-mannose-4,6-dehydratase (Gmd) and GDP-L-fucose synthetase (WcaG) to increase conversion of GDP-D-mannose to GDP-L-fucose (Wang et al., 2019). In addition, genes downstream of GDP-L-fucose along *de novo* pathway can be switched off to prevent conversion to colonic acid. A second pathway, the salvage pathway, present in mammalian and some *Bacteroides* species can be introduced to *E. coli* strains to increase concentration of GDP-L-fucose. Introduction of the *fkp* gene encoding fucokinase/GDP-L-fucose pyrophosphorylase (Fkp) from *Bacteroides fragilis* results in conversion of extracellular fucose to GDP-Fucose (Chin et al., 2015; Chin et al., 2016). Despite the high cost of supplying fucose to the system, the salvage pathway is still a viable option for overproduction of GDP-fucose.

The final element required for production of 2'-FL using cell factories is the introduction of an exogenous fucosyltransferase to catalyse addition of fucose to the lactose acceptor. Fucosyltransferases such as FutC from *H. pylori* are commonly employed, although a more favourable by-product profile is observed using the α 1,2-fucosyltransferase, WcfB, from *B. fragilis* (Chin et al., 2017), which results in reduced production of DFL (Engels & Elling, 2013). Through the employment of the engineering strategies

described above, whole-cell microbial synthesis of substantial quantities of 2'-FL has been achieved. In 2012, Lee et al demonstrated production of 2'-FL from lactose through engineering of the *E. coli* strain JM109. The wild-type JM109 is incapable of consuming lactose and, through overexpression of the *de novo* pathway genes *manB*, *manC*, *gmd*, and *wcaG*, 1.2 g/L of 2'-FL was produced after consumption of 14.5 g/L of lactose (Lee et al., 2012). Baumgartner et al. introduced salvage pathway genes to the strain in addition to the *de novo* genes, and produced significantly higher yields of 2'-FL when compared with overexpression of the *de novo* pathway genes alone (Baumgärtner et al., 2013). Knockout of the fucose degradation genes *fucI* and *fucK* by Chin et al. resulted in production of 20.6 g/L of 2'-FL from the consumption of 27 g/L of L-fucose and 53.6 g/L lactose (Chin et al., 2016). To date, the highest yield reported for the production of 2'-FL is 180 g/L according to <http://jennewein-biotech.de/cms/assets/uploads/2017/11/EDMbuntNov17.pdf>.

In 2014, Baumgartner et al. described the use of engineered strains for production of LNT. The protein products of two genes, *lgtA*, which encodes β 1,3-*N*-acetylglucosaminyltransferase, and *wgbO*, which specifies a β 1,3-galactosyltransferase, were overexpressed in *E. coli*. Lactose and glucose were used as substrates, and 0.219 g/L of LNT was produced. This was increased to 0.81 g/L when glucose was replaced with galactose as an energy source (Baumgärtner et al., 2014). Up-scaling to fed-batch fermentation resulted in formation of 12.7 g/L of LNT. Similar methods were applied by Glycom A/S for microbial production of L_NT. These methods included introduction of *lgtA* from *Neisseria meningitidis* and a *galT* from *H. pylori* into *E. coli* strains and utilisation of lactose, glycerol and D-glucose as

substrates (Bajza et al., 2015). LNnT produced by Glycom through these recombinant technologies received regulatory approval in the US and EU in 2016 (**Figure 3**). Despite decisive advancements in metabolic engineering practices for production of specific HMOs, significant limitations still exist. Harnessing the benefits of HMOs for inclusion in infant food products will require further developments for the large-scale synthesis of multiple HMO structures. For more complex HMOs, fermentation yields are low or the products are not exported to the external environment rendering purification difficult. Of particular significance is the identification of an appropriate β 1,6-*N*-acetylglucosaminyltransferase for manufacture of branched chain HMOs.

5. Regulatory Framework and Nutraceutical Applications

HMOs manufactured through the synthetic processes described above display exact structural identity to those found in human breast milk. Irrespective of this fact, introduction of these ingredients into European, American and Asian commercial arenas require regulatory approval (summarised in **Figure 3**). The regulatory framework in the EU involves designation of Novel Food status by the European Food Safety Authority (EFSA) following mandatory safety assessment, while in the US, HMOs must receive GRAS notification in order to be used as food ingredients. GRAS approval is granted by the Food and Drug Administration (FDA) (Salminen, 2017) and strong competition has ensued between manufacturers since the first regulatory approval of HMO in 2015. While Glycom A/S were the first to receive GRAS notification for their chemically synthesised 2'-FL and LNnT, Jennewein were the first company to receive regulatory approval for 2'-FL produced by microbial fermentation in November 2015 (**Figure 3**). In 2016, Glycom A/S received Novel Food status in the EU and GRAS notification in the US for their microbially

fermented 2'-FL and LNnT. More recently, Glycosyn/ Friesland Campina Domo and Inbiose/ DuPont have entered into HMO production with both companies receiving GRAS notification for 2'-FL. Genechem have recently received a positive response from the FDA for GRAS notification of 3'-SL produced by chemoenzymatic synthesis. Most recently, Novel Food status has been granted to Glycom A/S for their combination of 2'-FL and DFL in a ratio close to the composition naturally found in breast milk (Bych et al., 2019).

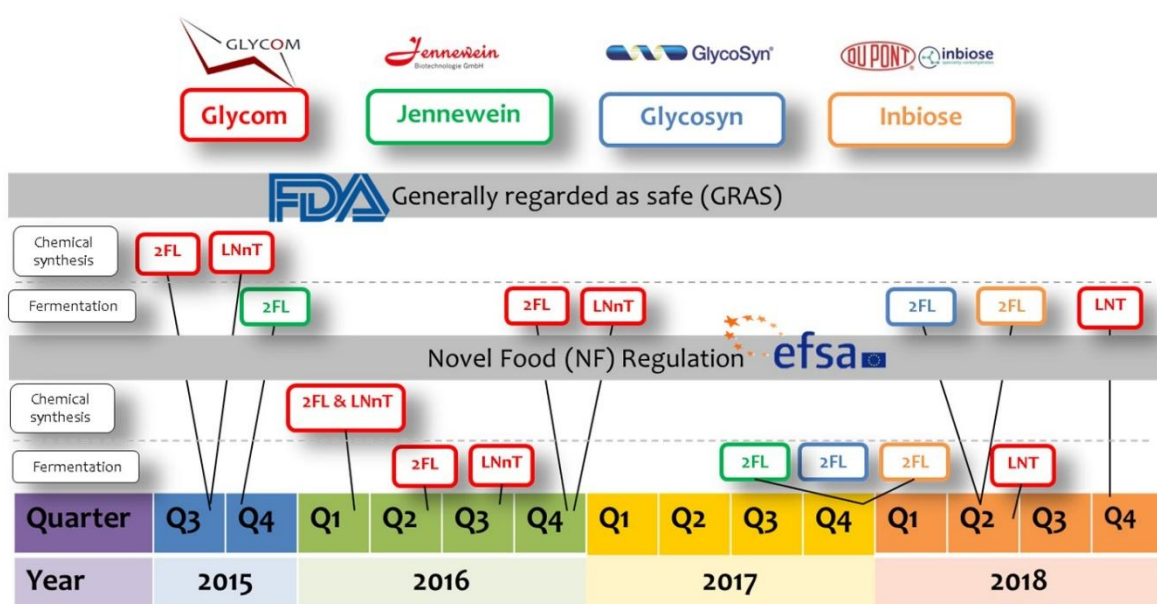


Figure 3: Summary of the regulatory landscape, at the time of writing, for HMOs. Adapted from (Bych et al., 2019). Timeline includes overview of the major HMO manufacturers, the technology utilized to synthesise HMO structures, the regulatory bodies in the US (FDA which grants GRAS status) and EU (EFSA which grants NF regulation), and highlights the progress which has been made over past number of years in achieving regulatory approval for synthetically manufactured HMO structures.

6. Clinical Intervention Trials

After decades of research in the field, HMOs are now available in quantities and at prices accessible for a broad range of applications. 2'-FL and/or LNnT are currently found in infant products in more than 30 countries and non-infant products are beginning to emerge (Zeuner et al., 2019). A limited number of intervention studies in infants have been performed to assess the safety and efficacy of HMO-fortified infant formulae (Vandenplas et al., 2018). These studies are summarised in **Table 3** and show that formula milk containing 2'-FL and LNnT is safe and well-tolerated while beneficial effects on the microbiota have also been reported.

The clinical data available to date indicates potential health benefits for the inclusion of 2'-FL and LNnT in formula, but it remains to be seen whether a limited number of HMO structures will close the compositional and physiological gap between breast milk and formula (Vandenplas et al., 2018). Many more prospective, intervention trials in infants are required to establish benefits for addition of HMO to formula. According to <https://clinicaltrials.gov/>, and as of the end of 2019, eight intervention studies assessing the impact of HMO on infants are active and/or recruiting with one further study planned which targets adult populations. Companies such as Nestle and Jennewein are both undertaking trials to assess the safety of new HMO formulations which contain a blend of five HMO structures, while others attempt to expand on previous studies involving 2'-FL and LNT/LNnT. Targeted outcome measures in these studies include colonisation of *Bifidobacterium*, iron absorption, and frequency of illness. It is anticipated that findings from these studies will expand our view of HMO as bioactive compounds.

Table 3: Outcomes from Clinical Intervention Studies in Infants

Population	Formula(e)	Outcomes	Reference
FF (n=189)	1. GOS (2.4g/L)	Consumption of 2'-FL is safe	Marriage, Buck,
BF (n=65)	2. GOS (2.2g/L) + 2'-FL (0.2g/L)	and well-tolerated. Growth of	Goehring,
	3. GOS (1.4g/L + 2'-FL (1g/L)	FF infants similar to those	Oliver, &
		who are BF	Williams, 2015
FF (n=317)	1. GOS (2.4g/L)	Compared to GOS alone,	Goehring et al.,
BF (n=107)	2. GOS (2.2g/L) + 2'-FL (0.2g/L)	infants who consumed	2016
	3. GOS (1.4g/L) + 2'-FL (1g/L)	formula with 2'-FL had	
		lower levels of immune	
		biomarkers comparable to	
		BF infants	
FF (n= 88)	1. Control (no oligosaccharide)	Formula with 2'-FL safe for	Kajzer, Oliver,
BF (n=43)	2. FOS (1.8g/L) + 2'-FL (0.2g/L)	consumption. Stool	& Marriage,
		consistency of infants fed 2'-	2016
		FL appears more similar to	
		BF infants.	
FF (n=175)	1. Control (no oligo)	Formula with 2'-FL and	Steenhout et al.,
BF (n= 38)	2. 2'-FL(1g/L) + LNnT (0.5g/L)	LNnT shifts stool microbiota	2016
		and metabolic profile of	
		infants closer to BF infants	
	1. Control (no oligo)	Formula with 2'-FL and	Puccio et al.,
FF (n=175)	2. 2'-FL(1g/L) + LNnT (0.5g/L)	LNnT supports age	2017
		appropriate-growth, and	
		results in lower incidences of	
		infection and medication use	
		vs control group	

7. Conclusions

Recent expansion of technologies for the characterization, purification, and production of HMOs promises successful translation of fundamental discoveries into commercial and therapeutic applications. Although there have been substantial breakthroughs in our understanding of HMOs, numerous uncertainties still remain. Owing to the fact that the beneficial effects of HMOs are highly structure-specific, it is pertinent that levels of these compounds in human milk are quantified with utmost precision. Furthermore, the maternal, genetic, and environmental factors that drive the inter-individual variations in HMO composition need to be fully defined. As researchers in the field look to verify the beneficial effects of HMOs in human populations and expand their use in nutraceutical applications, it is important to be specific about which structures are responsible for each function and to avoid assigning all of the observed benefits of HMOs to one or two structures. Future research should examine whether dietary supplementation of HMOs not present in the milk of some mothers may be counterproductive or even have potentially negative effects on their infants. Further well-designed clinical studies, both observational and placebo-controlled interventions, are necessary to further substantiate and grow our understanding of HMO biology and its significance for the advancement of infant nutrition. Overall, we are still a long way from artificially replicating the specified complexity of HMOs and many questions about their clinical implications remain unanswered. Findings from HMO research should therefore serve, above all else, as yet another compelling motive to encourage and support breastfeeding as the “gold standard” in infant nutrition.

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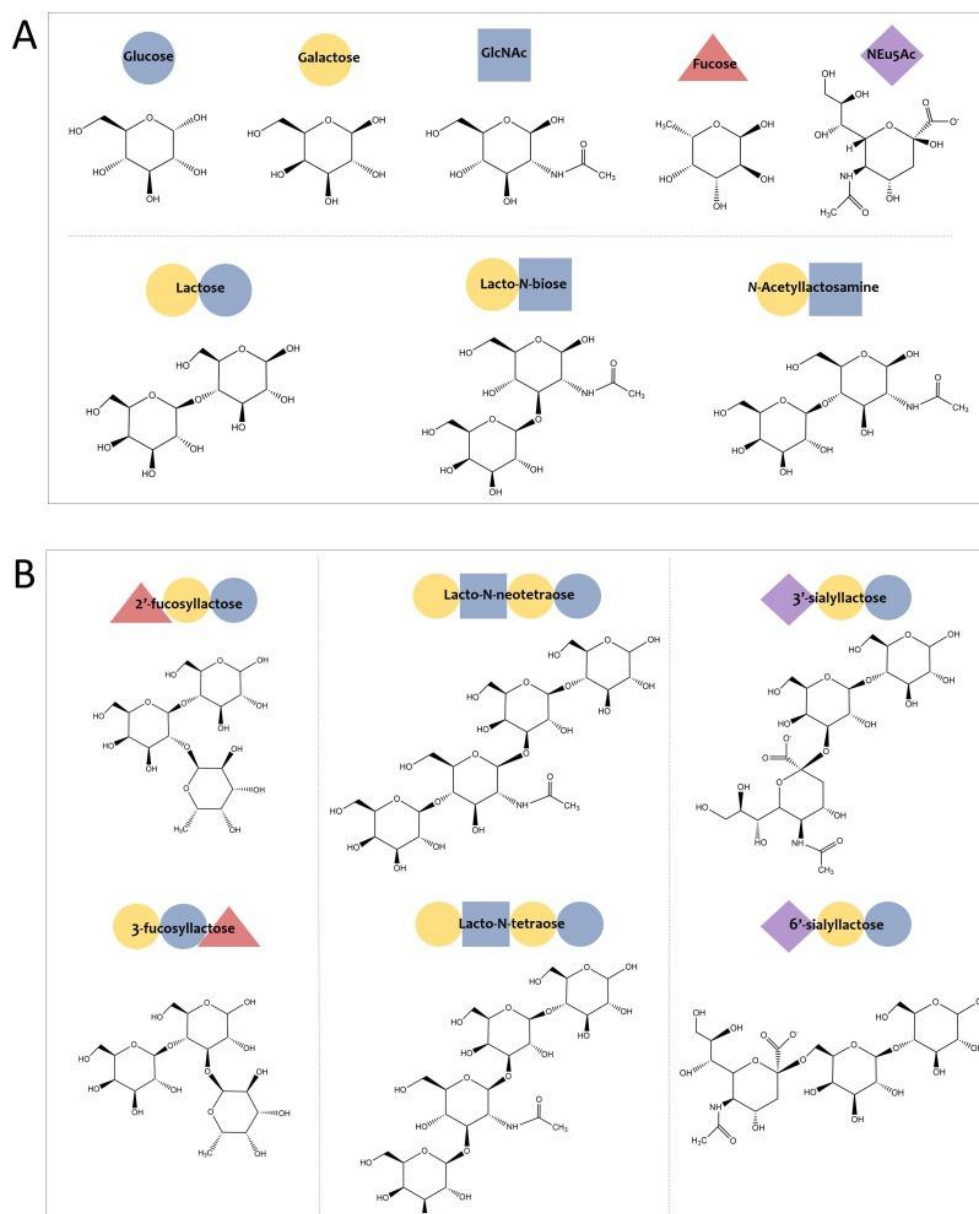
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Supplementary Materials



Supplementary Figure 1: Chemical structures of the major HMO-associated mono-, di-, and oligo-saccharides.

CHAPTER III

Microbiota-accessible carbohydrates in milk and bifidobacteria

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Abstract

One of the most remarkable features of breast milk is its diversity and abundance of complex sugars (glycans), in particular human milk oligosaccharides (HMOs), which are indigestible to the infant and thus reach the colon intact. HMOs are growth substrates for several species of gut bacteria and act as prebiotics, promoting the growth and metabolic activity of beneficial intestinal flora and shaping the infant gut microbiome. Short-chain fatty acids, generated through the saccharolytic breakdown of HMOs by the gut microbiome, are critical for intestinal health. Bifidobacteria are frequently found as the most abundant colonizers in the gut of breast-fed infants, generally accounting for 50-90% of the total bacterial population detected in the faeces of breast-fed infants. This is believed to be due to their remarkable capacity to metabolize and consume HMOs. This chapter describes the current knowledge related to the genetic adaptation of particular members of the genus *Bifidobacterium* that enable them to metabolize various HMOs.

1. Introduction

Breast-fed and formula-fed infants differ in their gut microbiota composition (Forbes et al., 2018) which has been linked to differential health outcomes, e.g., increased incidences of allergies, asthma, and obesity in formula-fed infants (Forbes et al., 2018; Ly et al., 2011). One of the major differences between the feeding regimes is the high abundance of conjugated and free complex carbohydrates in breast milk, the latter known as human milk oligosaccharides (HMOs) which, until recently, have been absent in infant formula and supplements. To date, over 200 different HMO structures have been identified (Smilowitz et al., 2014). Variability in the chemical and structural conformations of HMOs is biologically relevant. Indeed, there is a clear structure-function relationship, the potential of which needs to be fully defined. Oligosaccharides are the third most abundant group of solid compounds in human milk after lactose and fat, at levels that vary from 5 to 23 g/L, with very substantial compositional variations observed between women and across the lactation period (Ballard & Morrow, 2013). Remarkably, HMOs do not provide any direct nutritional value to the infant. Instead, these oligosaccharides promote the growth and colonization of beneficial bacteria, resulting in a *Bifidobacterium*-dominant microbiota, thereby supporting immune development and the well-being of the infant host (Thomson et al., 2018).

HMOs consist of a lactose backbone that is elongated into a variety of oligomeric structures by the addition of β 1-3- or β 1-6-linked lacto-*N*-biose (Gal β 1-3GlcNAc-, type 1 chain) or *N*-acetyllactosamine (Gal β 1-4GlcNAc-, type 2 chain) (Bode, 2012; Plaza-Díaz et al., 2018). Moreover, HMOs are classified as neutral non-fucosylated (*e.g.*, lacto-*N*-tetraose and lacto-*N*-

neotetraose) or fucosylated [*e.g.*, 2'-fucosyllactose (2'-FL) and 3-fucosyllactose (3-FL)], and acidic non-fucosylated [*e.g.*, 3'-sialyllactose (3'-SL) and 6'-sialyllactose (6'-SL)] or fucosylated [*e.g.*, FS-LNH, which corresponds to one Fuc and one NeuAc on a lacto-*N*-hexaose core] (Bode, 2012; Plaza-Díaz et al., 2018). A major breakthrough in infant milk formulation was recently made with the introduction of 2'-FL and/or lacto-*N*-neotetraose (LNnT) to various infant products in more than 30 countries (Zeuner et al., 2019). Humans lack the digestive enzymes required to assimilate complex HMOs such as sialidases and fucosidases. As a result, HMOs pass undigested into the colon where they are metabolized by bacteria present in the intestinal microbiota. Since not all bacteria possess the saccharolytic features necessary to consume HMOs, these glycan species are believed to serve as selective regulators of infant gut microbiota development. This prebiotic effect facilitates the establishment of a microbial ecosystem dominated by bifidobacteria, while indirectly impeding growth of other gastrointestinal species (Bode, 2009; Morrow et al., 2005). These early patterns of colonization are crucial for a healthy microbiome and are hypothesised to prevent or reduce the risk of disease later in life (Bokulich et al., 2016; Timmerman et al., 2017).

Bifidobacteria are Gram positive, hetero-fermentative, strict anaerobes, with a saccharolytic metabolism of the phylum Actinomycetota. Bifidobacteria isolated from distinct sources display differences in genomic traits such as genome size and accessory gene composition and particular traits related to amino acid biosynthesis and degradation of carbohydrates (Rodriguez & Martiny, 2020). *Bifidobacterium* genomes are known to contain a large set of genes for carbohydrate utilization (Khoroshkin et al.,

2016; Milani et al., 2016), indicating a preference for complex carbohydrates. Moreover, some of these genomes display a clear adaptation to the nursing period when the diet of their host may be solely composed of (HMO-rich) breast milk (Sela et al., 2008). Advances in genomics, transcriptomics and glycomics have been very useful to study the molecular basis of bifidobacterial enrichment by HMOs, contributing to our understanding of the beneficial effects of breast milk on the gut microbiome establishment.

Bifidobacteria were first isolated from infant feces by Tissier in 1900 (Tissier, 1900). Since this time human-associated bifidobacteria have come to be recognised as key members of the gut microbiome and are believed to confer numerous health benefits upon their host (Mitsuoka, 1990; Turroni et al., 2020). Bifidobacteria are believed to contribute to gut homeostasis through host–microbe interactions (Milani et al., 2017; Ruiz et al., 2016) and microbe-microbe cross-talk (Turroni et al., 2018; Turroni et al., 2015). Colonization of the gut with *Bifidobacterium* supports proper immune development and helps prevent the development of certain gastrointestinal disorders (Alessandri et al., 2019). Most significantly, bifidobacteria have an antagonistic role against pathogenic bacteria, viruses, and toxins through competitive exclusion (Aw & Fukuda, 2019) and are associated with the production of a number of possible health promoting metabolites such as bacteriocins (Balciunas et al., 2016), short chain fatty acids (SCFA) (Ratajczak et al., 2019), and conjugated linoleic acid (CLA) (Coakley et al., 2003). Differences in the composition of *Bifidobacterium* subspecies are noted when comparing healthy and diseased subjects. For example, bifidobacterial abundance in patients suffering from gastrointestinal disorders, such as allergy (Akay et al., 2014; Hevia et al., 2016), necrotizing

enterocolitis (NEC), obesity (Gao et al., 2015), colic (Savino et al., 2017), and irritable bowel syndrome (IBS) (Taverniti & Guglielmetti, 2014), is significantly reduced compared to healthy controls. It is not clear whether these observed perturbations in bifidobacterial populations are a cause of disease development or a consequence of it (Arbolea et al., 2016). Due to their reported health-promoting effects, bifidobacteria have been incorporated into many functional foods and supplements as probiotic ingredients (Di Gioia et al., 2014; Sarkar & Mandal, 2016). The therapeutic potential of *Bifidobacterium* has been demonstrated for treatment of metabolic disorders (Michael et al., 2020), inflammatory bowel disease (IBD) (Allen et al., 2017), allergies (Del Giudice et al., 2017; Dennis-Wall et al., 2017), infections (Park et al., 2017; Taipale et al., 2016), dyslipidemia (Guardamagna et al., 2014), and cancer (Niu et al., 2019; Wei et al., 2018). Psychobiotic effects have also been reported for certain bifidobacterial strains (Pinto-Sanchez et al., 2017; Savignac et al., 2014).

Since the publication of the first bifidobacterial genome in 2002, there has been a continual rise in the number of publicly available bifidobacterial genome sequences (**Figure 1A**) (O'Callaghan & van Sinderen, 2016). As of April 2020, the NCBI database holds 1155 publicly available bifidobacterial genome sequences, of which 146 represent complete genome sequences (**Figure 1**, source; <https://www.ncbi.nlm.nih.gov/genome/browse#!/prokaryotes/bifidobacterium>, April 2020). In the *Bifidobacterium* genus, four species, i.e. *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium longum* ssp. *infantis* (*B. infantis*), and *Bifidobacterium longum* ssp. *longum* (*B. longum*), make up over 50% of the available genome sequences (**Figure 1B**).

The high number of completed sequences for these species reflects their abundance and prevalence in infant faeces but perhaps more significantly, bifidobacteria belonging to these taxa represent commercially viable probiotic agents.

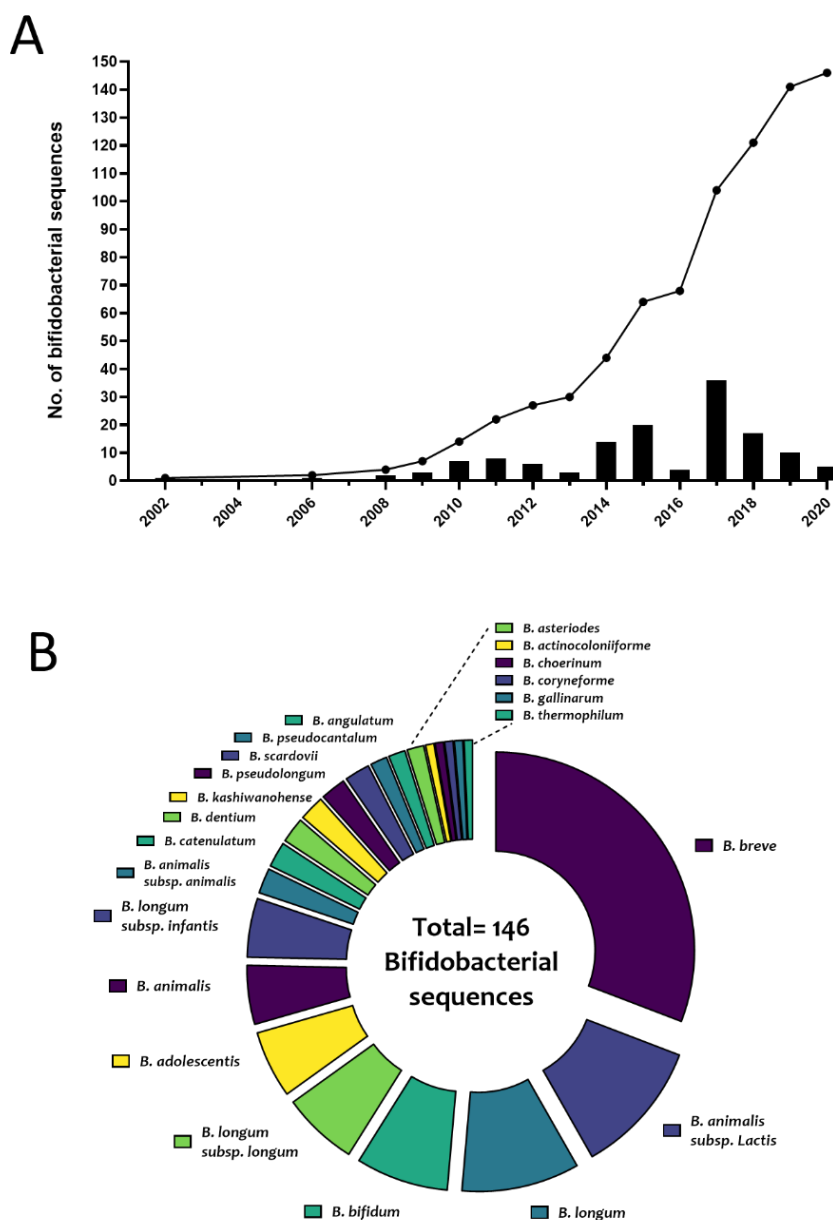


Figure 1: Number of publicly available bifidobacterial sequences according to <https://www.ncbi.nlm.nih.gov/genome/browse#!/prokaryotes/>.

A Bar graph specifies the year in which sequencing was completed, with line graph indicating the cumulative number of sequences available. **B** Pie chart denotes number of bifidobacterial sequences at the subspecies level.

2. General features of bifidobacteria HMO metabolism

Bifidobacterium colonize their host rapidly following birth, although their relative abundance decreases following weaning and with ageing (Kato et al., 2017; Turrone et al., 2020). One of the reasons as to why bifidobacteria are such dominant and prevalent members of the (early life) gut microbiota is their remarkable capacity to metabolize and consume HMO. Genomic studies on several infant-derived bacterial isolates have found that bifidobacterial strains possess numerous transporters, carbohydrate binding proteins and glycosyl hydrolases (GHs), all of which are involved in the import and assimilation of HMOs (Garrido, Barile, et al., 2012; Thomson et al., 2018). The bifidobacterial glycosidases implicated in HMO metabolism are classified into 8 GH families; 1,2- α -L-fucosidase (GH95), 1,3/1,4- α -L-fucosidase (GH29), sialidase (GH33), lacto-*N*-biosidase (GH20 & GH136), β -galactosidase (GH2 & GH42), β -*N*-acetylglucosaminidase (GH20), and GNB/LNB phosphorylase (GH112). The salient features of these GH enzymes are summarised in **Table 1** including their prevalence in various strains/species across the *Bifidobacterium* genus. These HMO-degrading enzymes (**Table 2**) are found most frequently in *B. breve*, *B. bifidum*, *B. longum*, *B. infantis*, and, in some cases, *B. pseudocatenulatum* (Bottacini et al., 2014; Gotoh, Katoh, Sakanaka, Ling, Yamada, Asakuma, Urashima, Tomabechi, Katayama-Ikegami, Kurihara, et al., 2018). A pathway for the metabolism of fucosyllactose has also been recently reported in a *Bifidobacterium catenulatum* ssp. *kashiwanohense* isolate (Bunesova et al., 2016; James et al., 2019).

Table 1 Prevalence of Glycosyl Hydrolases with specificity for HMO and HMO derivatives across *Bifidobacterium* genus

Species	Strain	Genome Size (Mb)	GC Content (%)	Accession no.	GH29	GH95	GH33	GH20	GH136	GH2	GH42	GH112
					α -1,3/1,4-fucosidase	α -1,2-fucosidase	α -2,6- / α -2,3-sialidase	lacto-N-biosidase	lacto-N-biosidase	β -1,4-Galactosidase	β -1,3-Galactosidase	GNB/ LNB phosphorylase
<i>B. adolescentis</i>	ATCC 15703	2.08964	59.2	AP009256.1	0	0	0	0	0	3	2	0
<i>B. animalis ssp. lactis</i>	DSM 10140	1.93848	60.5	CP001606.1	0	0	0	0	0	2	2	0
<i>B. bifidum</i>	JCM 1255	2.21104	62.7	AP012323.1	0	1	2	4	1	3	2	2
<i>B. bifidum</i>	PRL2010	2.21466	62.7	CP001840.1	1	1	3	4	1	3	2	2
<i>B. breve</i>	BR3	2.26941	58.9	AP012324.1	1	1	1	1	0	2	3	1
<i>B. breve</i>	JCM 1192	2.42268	58.7	CP000303.1	0	1	1	1	0	2	2	1
<i>B. breve</i>	UCC2003	2.42601	59.1	CP010413.1	0	1	1	1	0	5	2	1
<i>B. catenulatum</i>	JCM 1194	2.07952	56.2	AP012325.1	0	0	0	0	0	4	3	0
<i>B. catenulatum ssp. kashiwanohense</i>	APCKJ1	2.44541	56.2	CP026729.1	2	1	0	1	0	4	3	0
<i>B. kashiwanohense</i>	PV20-2	2.37098	56.1	CP007456.1	0	1	0	1	0	2	3	0
<i>B. dentium</i>	JCM 1195	2.63567	58.5	AP012326.1	1	0	0	1	0	6	4	0
<i>B. longum</i>	CACC 517	2.28166	59.8	CP048001.1	0	0	0	0	1	3	3	1

Table 1 Prevalence of Glycosyl Hydrolases with specificity for HMO and HMO derivatives across *Bifidobacterium* genus

Species	Strain	Genome Size (Mb)	GC Content (%)	Accession no.	GH29	GH95	GH33	GH20	GH136	GH2	GH42	GH112
					α -1,3/1,4-fucosidase	α -1,2-fucosidase	α -2,6- / α -2,3-sialidase	lacto-N-biosidase	lacto-N-biosidase	β -1,4-Galactosidase	β -1,3-Galactosidase	GNB/ LNB phosphorylase
<i>B. longum</i>	NCTC 11818	2.38516	60.3	LR134369.1	0	0	0	1	1	2	2	1
<i>B. longum ssp. infantis</i>	ATCC 15697	2.83275	59.9	CP001095.1	3	1	2	3	0	2	3	1
<i>B. longum ssp. infantis</i>	BT1	2.57811	59.4	CP010411.1	3	1	2	3	0	2	2	1
<i>B. longum ssp. longum</i>	JCM 1217	2.38516	60.3	AP010888.1	0	0	0	1	1	2	2	1
<i>B. longum ssp. longum</i>	JDM 301	2.47784	59.8	CP002010.1	1	1	0	1	0	2	3	1
<i>B. pseudocatenulatum</i>	JCM 1200	2.31375	56.4	AP012330.1	0	1	0	1	0	4	3	0
<i>B. pseudolongum</i>	UMB-MBP-01	2.0081	63.4	CP022544.1	1	0	0	0	0	3	1	0
<i>B. scardovii</i>	JCM 12489	3.15835	64.6	AP012331.1	2	0	1	3	0	7	6	1
<i>B. thermophilum</i>	RBL67	2.29164	60.1	CP004346.1	0	0	0	0	0	0	1	0

While HMO catabolic processes have been described in several (sub)species across the genus, HMO consumption abilities vary significantly among different *Bifidobacterium* species and even between strains of the same (sub)species (**Figure 2**) (LoCascio et al., 2007). Hierarchical clustering of species based on the number of glycosidases present within the genomic architecture highlights this strain-to-strain variation (**Figure 2**). This pronounced variability in HMO assimilation genotype has been validated in phenotypic growth studies. For instance, Ruiz-Moyano et al. (2013) (Ruiz-Moyano et al., 2013) assessed growth of several *Bifidobacterium* isolates on HMO purified from breast milk or individual HMO structures and reported that strains derived from adult gut/mouths or from dairy sources, such as *B. adolescentis*, *B. dentium*, and *B. animalis* subsp. *lactis*, were unable to metabolise HMO. In contrast, infant gut-associated *Bifidobacterium* such as *B. infantis* and *B. bifidum* have been shown to assimilate many HMO structures, including HMOs decorated with fucose and sialic acid residues, and are considered the most efficient HMO utilizers (Gotoh, Katoh, Sakanaka, Ling, Yamada, Asakuma, Urashima, Tomabechei, Katayama-Ikegami, & Kurihara, 2018; Sela et al., 2008; Underwood et al., 2015). Generally, *B. longum* and *B. breve* in pure culture show limited ability to metabolise complex HMO structures, but demonstrate growth on mono- and di-saccharide HMO degradants such as lacto-*N*-biose (LNB) (Ward et al., 2007). More recent studies have identified strains of *B. longum* and *B. breve* with competitive HMO assimilation phenotypes and pathways for utilisation of lacto-*N*-tetraose (LNT) and lacto-*N*-neotetraose (LNnT) have been described in *B. breve* strain UCC2003 (James et al., 2016).

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Table 2 Summary of Glycosyl Hydrolase Proteins discussed in chapter

Abbreviation	Locus Tag	GH function	GH family	Strain	Accession no.
<i>Bifidobacterium longum subsp. infantis</i>					
–	Blon_2336	α -1,3/1,4-L-fucosidase	GH29	ATCC 15697	ACJ53394.1
–	Blon_2335	1,2- α -L-fucosidase	GH95	ATCC 15697	ACJ53393.1
NanH1	Blon_0646	exo- α -sialidase	GH33	ATCC 15697	ACJ51753.1
NanH2	Blon_2348	α -2,6/2,3-sialidase	GH33	ATCC 15697	ACJ53406.1
–	Blon_0459	β -N-acetylhexosaminidase	GH20	ATCC 15697	ACJ51575.1
–	Blon_0732	β -N-acetylhexosaminidase	GH20	ATCC 15697	ACJ51836.1
–	Blon_2355	β -N-acetylhexosaminidase	GH20	ATCC 15697	ACJ53413.1
Bga2A	Blon_2334	β -1,4-galactosidase	GH2	ATCC 15697	ACJ53392.1
Bga42A	Blon_2016	β -galactosidase	GH42	ATCC 15697	ACJ53083.1
Bga42B	Blon_2123	β -galactosidase	GH42	ATCC 15697	ACJ53184.1
Bga42C	Blon_2416	β -galactosidase	GH42	ATCC 15697	ACJ53470.1
	Blon_2174	GNB/LNB phosphorylase	GH112	ATCC 15697	ACJ53235.1
<i>Bifidobacterium bifidum</i>					
AfcB	BBPR_1360	α -1,3/1,4-L-fucosidase	GH29	PRL2010	ADP36408.1
AfcA	BBPR_0193	1,2- α -L-fucosidase	GH95	PRL2010	ADP35322.1
SiabB1	BBPR_1793	exo- α -sialidase	GH33	PRL2010	ADP36806.1
SiabB2	BBPR_1794	exo- α -sialidase	GH33	PRL2010	ADP36807.1
BbhI	BBPR_1529	β -N-acetylhexosaminidase	GH20	PRL2010	ADP36558.1
LnbB	BBPR_1438	lacto-N-biosidase	GH20	PRL2010	ADP36472.1
BbgIII/ LacZ	BBPR_0482	β -1,4-galactosidase	GH2	PRL2010	ADP35589.1
BbgIV/ LacZ6	BBPR_1460	β -galactosidase	GH2	PRL2010	ADP36493.1
LnpA1/ LnbP	BBPR_1055	GNB/LNB phosphorylase	GH112	PRL2010	ADP36122.1
LnpA2/ LnbP2	BBPR_0233	GNB/LNB phosphorylase	GH112	PRL2010	ADP35361.1
<i>Bifidobacterium breve</i>					
–	RY69_518	α -1,3/1,4-L-fucosidase	GH29	BR3	ALE12777.1
–	Bbr_1288	1,2- α -L-fucosidase	GH95	UCC2003	ABE95964.1
NahA/ NagZ	Bbr_1556	β -N-acetylhexosaminidase	GH20	UCC2003	ABE96227.1
LacZ2	Bbr_0285	β -galactosidase	GH2	UCC2003	ABE94991.1
LacZ6	Bbr_1552	β -galactosidase	GH2	UCC2003	ABE96223.1
LntA/ gosG	Bbr_0529	β -galactosidase	GH42	UCC2003	ABE95226.1
LnbP	Bbr_1587	GNB/LNB phosphorylase	GH112	UCC2003	ABE96256.1

Table 2 Summary of Glycosyl Hydrolase Proteins discussed in chapter

Abbreviation	Locus Tag	GH function	GH family	Strain	Accession no.
<i>Bifidobacterium longum</i> subsp. <i>longum</i>					
–	BLJ_0326	α -1,3/1,4-L-fucosidase	GH29	JDM301	ADG99808.1
–	BLJ_0327	1,2- α -L-fucosidase	GH95	JDM301	ADG99809.1
ChB	BLLJ_1391	β -N-acetylhexosaminidase	GH20	JCM1217	BAJ67058.1
LnbX	BLLJ_1505	lacto-N-biosidase	GH136	JCM1217	BAJ67172.1
LacZ1	BLLJ_1486	β -galactosidase	GH2	JCM1217	BAJ67153.1
LacA1	BLLJ_0443	β -galactosidase	GH42	JCM1217	BAJ66112.1
LacA2	BLLJ_0352	β -galactosidase	GH42	JCM1217	BAJ66022.1
LnpA1/ LnpP	BLLJ_1623	GNB/LNB phosphorylase	GH112	JCM1217	BAJ67290.1

Bifidobacterium strains of infant origin assimilate HMOs via two distinct saccharolytic approaches. The first approach involves translocation of intact HMOs through ATP-Binding cassette (ABC) transporters into the cytosol wherein the oligosaccharides are assimilated by intracellular glycosyl hydrolases (GH) (Asakuma et al., 2011; Sakanaka et al., 2020). The second strategy employs cell wall-anchored secretory glycosidases which hydrolyse the HMOs extra-cellularly releasing mono- and di-saccharides (Asakuma et al., 2011; Sakanaka et al., 2020). Combined genomic, proteomic and phenotypic studies have demonstrated that *B. infantis* and *B. breve* use oligosaccharide transporters (Garrido et al., 2011; James et al., 2016; Sela et al., 2008), while *B. bifidum* utilizes extra-cellular hydrolases and processes liberated lacto-N-biose (LNB) intra-cellularly (Wada et al., 2008). The strategy employed by *B. longum* is strain dependent and dependent on the presence of the gene encoding lacto-N-biosidase (LnbX) (Sakurama et al., 2013). LnbX-positive *B. longum* utilize an extracellular GH, while LnbX-negative strains employ oligosaccharide transporters to metabolise HMO

internally (Yamada et al., 2017). Despite variances in the mechanisms used by bifidobacterial strains to assimilate HMO, all four infant gut-associated species harbour a gene cluster dedicated to the hydrolysis of LNB (the core structure of type 1 HMO). Garrido et al. (2016) demonstrated that activity of enzymes within this GNB/LNB cluster is upregulated several fold in the presence of fucosylated HMO (Garrido et al., 2016). Within this gene cluster an ABC transporter and cytoplasmic phosphorylase are found specific to internalisation and metabolism of LNB (Kitaoka et al., 2005; Xiao et al., 2010).

Although different pathways are used for HMO degradation in each species, the glucose, galactose, sialic acid, and *N*-acetylglucosamine derivatives enter into the same central fermentative pathway, termed the ‘bifid shunt’ (Desjardins et al., 1990). Conversely, fucose derivatives are metabolized through a distinct metabolic pathway, which results in the production of 1,2-propanediol (1,2-PD) (James et al., 2019). The bifid shunt is a *Bifidobacteriaceae*-specific metabolic pathway and encompasses the Leloir, fructose-6-phosphate phosphoketolase, and amino-sugar metabolising pathway (Lanigan et al., 2019). Movement of molecules through the bifid shunt pathway results in the release of large amounts of acetate and lactate (Kim et al., 2013). For every 2 moles of hexose that enter in this pathway, 3 moles of acetate and 2 moles of lactate are produced (Pokusaeva et al., 2011). Flux along the bifid shunt results in the production of higher levels of ATP, when compared to other carbohydrate fermentative pathways, such as those found in lactic acid bacteria (LAB) (Pokusaeva et al., 2011). The short chain fatty acids (SCFA) and intermediary metabolites produced through the bifid shunt pathway reduce intestinal pH, thereby inhibiting adhesion of pathogenic

bacteria (den Besten et al., 2013). Acetate and lactate produced by bifidobacterial carbohydrate metabolism are converted in butyrate by other members of the gut microbiota (Rivière et al., 2016). Similarly, cross-feeding between infant gut symbionts is implemented for the production of propionate from the intermediate metabolite 1,2-propanediol (1,2-PD) (Cheng et al., 2020). These cross-feeding relationships offer further links between bifidobacterial fermentative pathways and infant nutritional and health outcomes since butyrate represents the primary energy source for colonocytes, in addition to exerting anti-inflammatory effects and improving gut barrier function (Bedford & Gong, 2018; Li et al., 2018; Zhan et al., 2018).

Remarkably, the saccharolytic capacity of a bifidobacterial species to consume HMO is not reflected in their dominance in the gut of breast-fed infants. Prototypical HMO consumers, *B. bifidum* and *B. infantis*, are detected in low numbers in the feces of breast-fed infants while *B. longum* and *B. breve* are regularly found as the dominant species in infant stools, even though they demonstrate minimal growth on HMO *in vitro* (Turroni et al., 2012). Thus, simply understanding the HMO assimilation phenotypes does not provide a full picture of how bifidobacterial strains utilize HMOs. In order to fully appreciate the role that HMOs play in the creation of a healthy infant gut microbiome, consideration must be given to the cross-feeding relationships that exist between members of the infant microbiota (Milani et al., 2015). Cross-feeding studies have indicated that HMO degradation by bifidobacterial strains permits the sharing of resources across the infant gut ecosystem (Lawson et al., 2020; Turroni et al., 2018; Turroni et al., 2015). *In vitro* growth experiments by Lawson et al. (2019) using HMO users and non-HMO users on ‘conditioned’ media or in direct co-culture highlighted the

cooperative nature of *Bifidobacterium*. Authors concluded that *Bifidobacterium* strains from the same infant employ overlapping, but distinct HMO utilisation strategies. It is postulated from this study that *Bifidobacterium* may function as a foundation genus in early life, serving to maximise nutrient availability from breast milk, specifically HMOs (Lawson et al., 2020).

In recent years, our understanding of bifidobacterial biology has advanced significantly. This is partly due to advancements in next generation sequencing, as well comparative and functional genomic analyses. These combined approaches have enabled detailed assembly of the genomic architecture of all known members of the *Bifidobacterium* genus (Milani et al., 2015; Milani et al., 2016). Functional analysis of the bifidobacterial pan-genome suggests that approximately 14% of the identified bifidobacterial genes encode enzymes involved in carbohydrate metabolism (Rodriguez & Martiny, 2020; Turrone et al., 2018). The profiles of these carbohydrate-active enzymes vary depending on the environment from which they are isolated (Rodriguez & Martiny, 2020). Thus, it can be assumed that the various catalytic mechanisms used by bifidobacteria strains for HMO consumption may be linked to niche partitioning among bifidobacteria within the developing infant gastrointestinal tract.

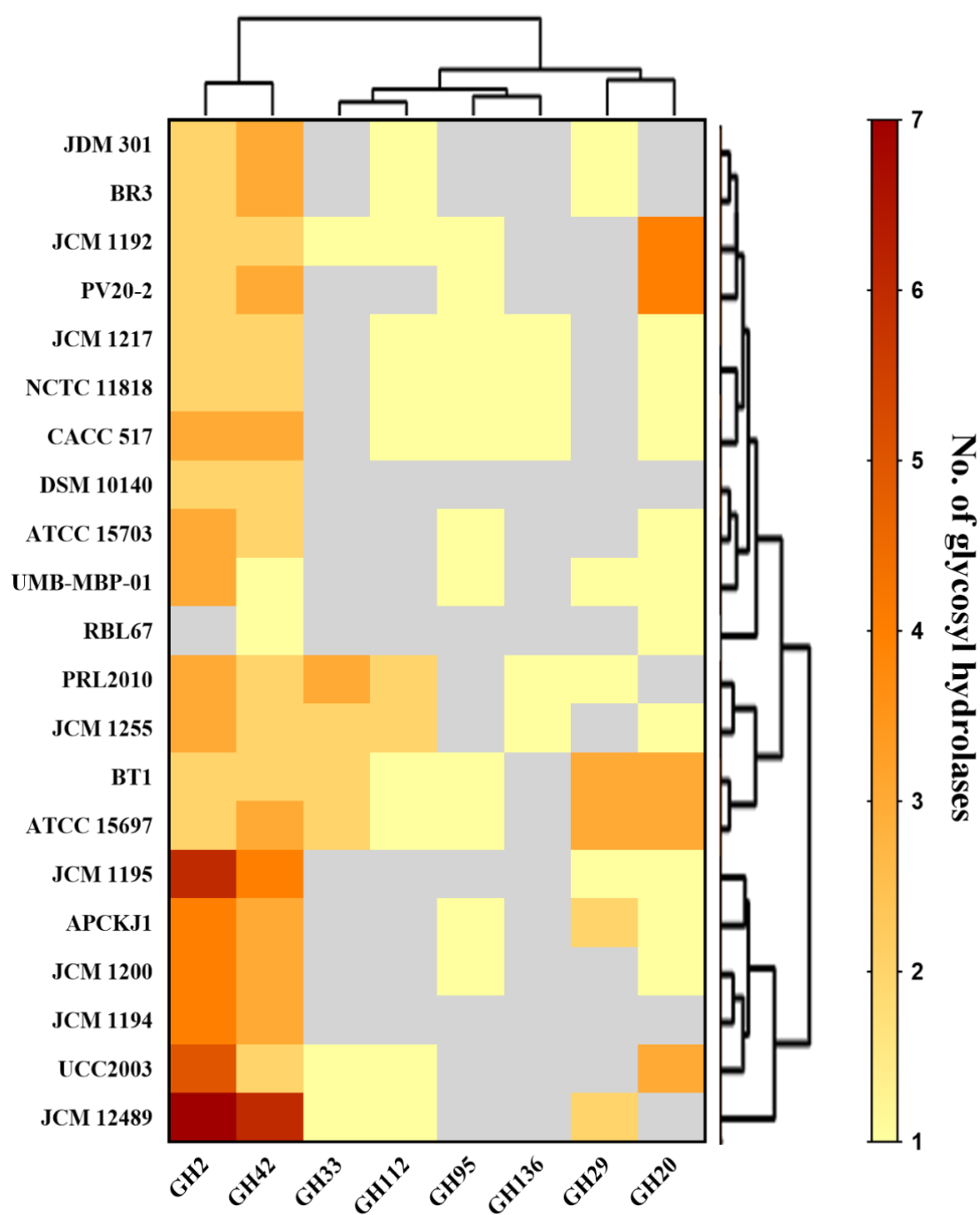


Figure 2: Hierarchical clustering of strains across the *Bifidobacterium* genus based on number of glycosidase proteins present within proteome. Glycosyl hydrolases classification according to: <http://www.cazy.org/Glycoside-Hydrolases.html>.

2.1. *Bifidobacterium longum* subsp. *infantis*

For well over a decade, *B. longum* subsp. *infantis* (*B. infantis*) has been considered as the prototypical HMO utilising strain, capable of catabolizing the whole spectrum of HMO linkages and molecules present in breast milk. This HMO assimilating capacity is conserved across the entire subspecies. The vigorous consumption of HMOs by *B. infantis* was first demonstrated by Ward et al., (2006) (Ward et al., 2006) and LoCascio et al., (2007) (LoCascio et al., 2007) when the groups performed *in vitro* cultivations of several bifidobacterial isolates on HMOs as the sole carbon source. The researchers observed inter-species variation, with *B. infantis* reaching a cell density three times that of any other bifidobacterial species. Analysis of HMO concentrations in the spent media revealed that *B. infantis* preferentially consumed HMO species with degrees of polymerization (DP) <7. Expanding on these previous studies, Marcobal et al, (2010) showed that *B. infantis* strain ATCC 15697 can consume HMOs with a DP of up to 12 (Marcobal et al., 2010). Genome sequencing of *B. infantis* has facilitated a comprehensive understanding of the mechanisms employed for hydrolysis of HMO (Sela et al., 2008). Most notable, perhaps, is UC Davis's early work on the ATCC 15697 strain wherein a novel 43kb gene cluster was identified which contained all the GHs required to cleave HMOs (sialidase, fucosidase, galactosidase, and hexosaminidase) as well as an assortment of transport-related genes. The proximity of genes within this "HMO Island" suggests co-regulation and involvement in a common metabolic pathway (Sela et al., 2008). Since that time, similar HMO-utilizing gene clusters have been described in other *B. infantis* isolates (LoCascio et al., 2010; Zabel et al., 2019).

As described previously, the postulated mechanistic model for HMO consumption in *B. infantis* involves bacterial import of intact HMOs into the cells (**Figure 3**). This transmembrane transport is mediated by several solute binding proteins (SBPs) and ABC transporters (Garrido et al., 2011). Transcriptome analysis by Garrido et al. (2011) revealed that SBP and ABC genes are significantly upregulated in the presence of HMOs, while also identifying that the HMO-degrading enzymes required for complete HMO assimilation are located within the cytosol, verifying that consumption of HMOs takes place within the cell. In this way, *B. infantis* can be considered a “selfish” subspecies, since it limits the number of HMO degradants available to other species within the microbiota. *B. infantis* ATCC15697 encodes three putative GH20 intracellular β -*N*-acetylglucosaminidases (Blon_0459, Blon_0732 and Blon_2355) (**Table 2**). Small-mass HMOs are internalised and hydrolysed into lacto-*N*-triose and galactose by two of these β -galactosidases. One β -galactosidase targets LNT (type 1 HMO chains), while the other acts on LNnT (type 2 HMO chains) (**Figure 3**). The liberated lacto-*N*-triose is subsequently acted upon by *N*-acetylhexosaminidase and hydrolysed into lactose and *N*-acetylglucosamine (GlcNAc). Activity by the other β -galactosidase hydrolyses this lactose into glucose and galactose. The liberated monosaccharides released by GH activity in *B. infantis* then enter into the bifid shunt central metabolic pathway resulting in the production of large amounts of acetate and lactate. Studies by Bunesova et al. (2016) and Zabel et al. (2019) have also revealed the presence of a L-fucose degradation pathway in *B. infantis* which results in the production of the propionate precursor, 1,2-PD (Bunesova et al., 2016; Zabel et al., 2019). A 2017 study by Schwab et al. demonstrated that *Eubacterium hallii* (one of the first

butyrate producers in the infant gut), was capable of utilizing intermediates of *B. infantis* HMO fermentation to produce butyrate, propionate and formate (Schwab et al., 2017). Specifically, *E. halli* consumed the bifid shunt products acetate, lactate and 1-2PD. This implies that although *B. infantis* does not partake in cross-feeding activities with other bifidobacteria species, trophic interactions with other members of the gut microbiota are prevalent (Schwab et al., 2017).

In terms of removing sialic acid from HMOs, *B. infantis* ATCC 15697 harbors two sialidase-encoding genes (*nanH1* and *nanH2*) (**Table 2**). Gene *nanH1* is located in a gene cluster dedicated to sialic acid catabolism, whereas *nanH2* is located with a putative *N*-acetylneuraminate lyase-encoding gene (*nanA2*) within the 43 kb HMO cluster (Sela et al., 2011). Both enzymes lack export signals, suggesting that they are located intracellularly. Both enzymes are known to cleave α -2,3 and α -2,6-linked sialosides with preference for the α -2,6 linkage (Sela et al., 2011). However, when assayed with purified sialylated HMOs sialyllacto-*N*-tetraose and di-fucosylated sialylated lacto-*N*-hexaose-like oligosaccharide and, α -2,3- and α -2,6-linked disialyl-lacto-*N*-tetraose, NanH1 was unable to hydrolyze any of the structures, while rapid degradation took place in the presence of NanH2 (Sela et al., 2011). Added to that, expression of NanH1 was not induced by HMOs whereas NanH2 expression was up-regulated by HMOs (Sela et al., 2011). Sela et al. therefore suggested that NanH1 may be active on other sialylated substrates.

The release of α -linked fucose residues is catalysed by α -fucosidases and *B. infantis* ATCC 15697 harbors five α -fucosidases: Blon_0248 (GH29), Blon_0426 (GH29), Blon_2335 (GH95), Blon_2336 (GH29), and Blon_0346 (GH151) (**Table 2**) (Sela et al., 2012; Zúñiga et al., 2018). Paralogs

Blon_0248 and Blon_0426 are 95% identical and Blon_2335 and Blon_2336 are located in the HMO utilization gene cluster (Sela et al., 2012). Blon_0346 contains a pfam01120 α -fucosidase domain at the 5' terminus, a middle β -galactosidase trimerization domain (pfam08532), which suggests that this enzyme may be active as a trimer, and it is quite different from the other α -fucosidases (Sela et al., 2012). The activity of these enzymes have been characterised by Sela et al. (2012). Blon_0248 and Blon_0426 were shown to cleave α -1,3 linkages. Blon_2335 cleave α -1,2 linkages and were also active on α -1,3 linkages, while Blon_2336 preferentially cleaves α -1,3 linkages and to a lesser extent α -1,2 linkages. Blon_0346 displayed some activity on Fuc α 1-2Gal and no activity on 2'-FL or 3-FL (Sela et al., 2012).

Studies on HMO transporters are still behind compared to the aforementioned glycosidases involved in the hydrolytic decomposition of HMOs. Indeed, only three transporters for LNB, FL, and LNT have been physiologically characterized. *B. infantis* ATCC 15697 possesses two paralogous FL transporters with their SBPs sharing 60% identity. Biochemical and structural analyses, combined with genetic analysis, unequivocally revealed that the functions of the two transporters are overlapping but distinct (Sakanaka et al., 2019). One (designated FL transporter-1 [SBP name: FL1-BP]) imports 2'-FL and 3-FL only, while the other (FL transporter-2 (SBP name: FL2-BP)) is capable of internalizing LDFT and LNFP I in addition to 2'-FL and 3-FL. A recent review by Sakanaka et al. (2020) examined the varied pathways of infant gut-associated bifidobacteria to assimilate HMOs by data mining (Sakanaka et al., 2020). Overall, the authors found the conservation of HMO-related genes in *B. infantis* genomes (21 examined) is very high, except for FL1-BP (57%),

LNnT-BP (NahS) (48%), and β -N-acetylglucosaminidase (NahA) (48%) whose prevalence is moderate. However, the absence of FL1-BP and NahA was compensated for by the presence of FL2-BP (86% occurrence) and the other two GH20 β -N-acetylglucosaminidases (100% for Blon_0732 and 86% for Blon_2355) (Sakanaka et al., 2020).

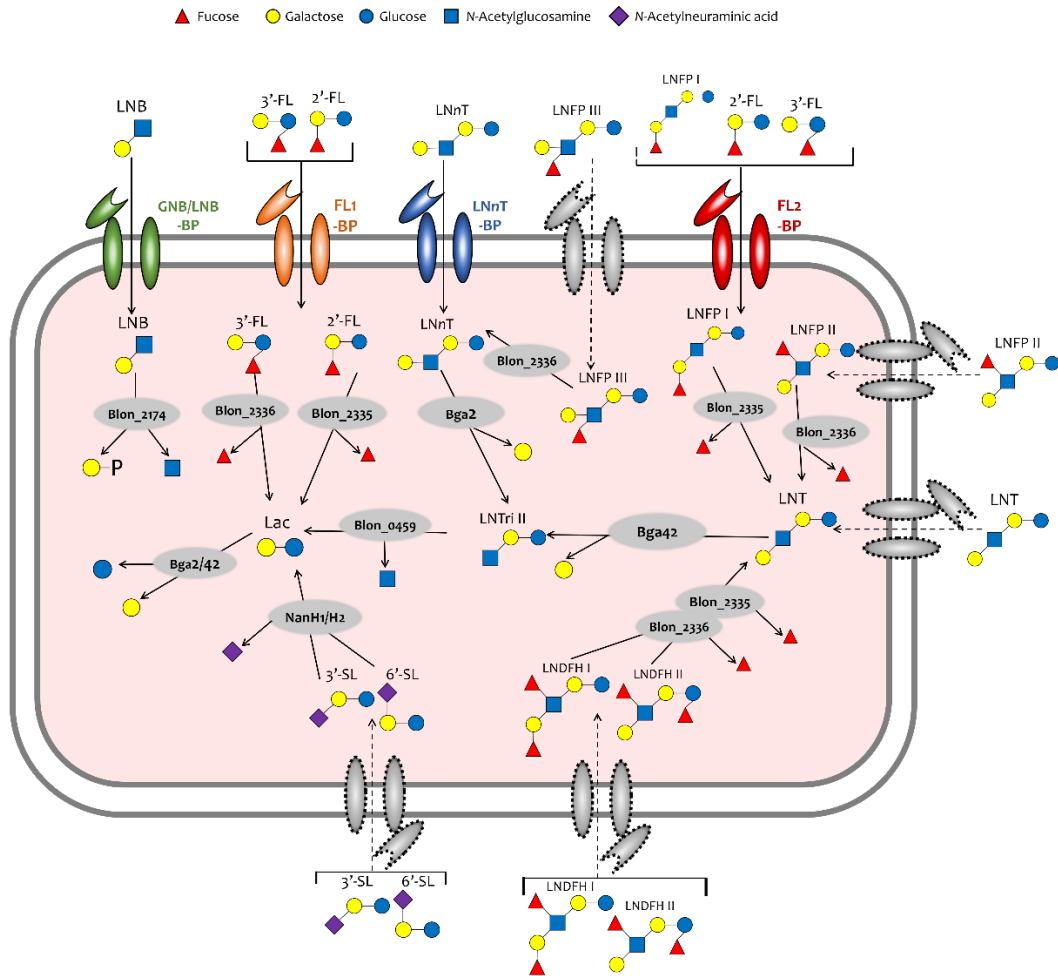


Figure 3: Schematic summary of HMO-assimilation pathways in *Bifidobacterium longum* subsp. *infantis*. *B. infantis* internalizes intact HMO through ATP-Binding cassette (ABC) wherein HMOs are assimilated by intracellular glycosyl hydrolases (GH). *B. infantis* strains are capable of hydrolysing the majority of HMO glycosyl linkages and are considered a ‘prototypical HMO user’. Transporters not yet characterised are shown in grey with black dotted lines. GH enzyme abbreviations and locus tags according to Table 2.

2.2. *Bifidobacterium bifidum*

Despite limited HMO consumption by a few specific isolates, *B. bifidum* can generally be considered an avid HMO user, with a conserved presence of HMO-degrading glycosyl hydrolases and transporters (Sakanaka et al., 2020). In contrast to the intracellular degradation mechanism described for *B. infantis*, isolates of *B. bifidum* rely on a unique set of cell wall-anchored extracellular GHs to metabolise HMOs (**Figure 4**) (Asakuma et al., 2011; Turroni et al., 2010). Studies have revealed that six extracellular enzymes have a key role to play in HMO metabolism along with one transporter, and one intracellular phosphorylase. 1,2- α -L-Fucosidase (AfcA), 1,3-1,4- α -L-fucosidase (AfcB), lacto-*N*-biosidase (LnbB), β -1,4-galactosidase III (BbgIII), β -1,3-*N*-acetylglucosaminidase I (BbhI), and sialidase II (SiaBb2) are the secretory enzymes which degrade HMOs extracellularly into their respective monosaccharides and disaccharides (summarised in **Table 2**) (Ashida et al., 2009; Katayama et al., 2004; Kiyohara et al., 2011; Miwa et al., 2010). Lacto-*N*-biosidase acts upon LNT motifs to release LNB and lactose (Wada et al., 2008). LNB is then internalized by the GNB/ LNB transporter (Asakuma et al., 2011), where it is phosphorolysed by galacto-*N*-biose/lacto-*N*-biose I phosphorylase (GLNBP) into galactose-1-phosphate and GlcNAc (**Figure 4**) (Nishimoto & Kitaoka, 2007; Xiao et al., 2010). These monosaccharides then enter into the Bifid Shunt glycolytic pathway resulting in the production of significant quantities of acetate and lactate (O'Callaghan & van Sinderen, 2016; Palframan et al., 2003). Unlike *B. infantis*, *B. bifidum* strains do not possess the metabolic pathway required for the production of 1,2-PD (Centanni et al., 2019).

Comparative genomics of several *B. bifidum* isolates (JCM1254, JCM7004, TMC3108, and TMC3115) has revealed that the HMO-degrading enzymes are conserved across the species (Gotoh, Katoh, Sakanaka, Ling, Yamada, Asakuma, Urashima, Tomabechi, Katayama-Ikegami, & Kurihara, 2018). AfcA, AfcB, LnbB, BbgIII, BbhI, GNB/ LNB transporter, and GLNBP were found highly conserved in all strains, with amino acid sequence identity >97.4% for all strains (Gotoh, Katoh, Sakanaka, Ling, Yamada, Asakuma, Urashima, Tomabechi, Katayama-Ikegami, & Kurihara, 2018). These seven enzymes have recently been described as “the minimum set” necessary for complete assimilation of neutral HMOs (Gotoh, Katoh, Sakanaka, Ling, Yamada, Asakuma, Urashima, Tomabechi, Katayama-Ikegami, & Kurihara, 2018). A small number of *B. bifidum* strains demonstrate limited growth on HMO-supplemented medium, most notably strain JCM1255 (Locascio et al., 2009). A frameshift mutation in the GLNBP enzyme of this strain may account for its diminished HMO-metabolising capacity (Asakuma et al., 2011).

Tannock et al. (2013) examined the relationship between the presence of *B. bifidum* and the dominance of genus *Bifidobacterium* in the infant gut (Tannock et al., 2013). Interestingly, they found that, when *B. bifidum* occupies >10% of genus *Bifidobacterium*, the corresponding microbiota possesses a higher ratio of *Bifidobacterium* abundance than those containing *B. bifidum* of less than 10% population among the genus (Tannock et al., 2013). The predominance of genus *Bifidobacterium* in the presence of adequate amount of *B. bifidum* was observed only for breast-fed infant stools but not for cow-based formula milk-fed infant feces. Asakuma et al (2011) had also noticed that *B. bifidum* leaves HMO degradants unconsumed in the

spent media, even after prolonged incubation on HMOs (Asakuma et al., 2011). These results suggest that HMO degradants produced by *B. bifidum* extracellularly can be shared among other members of the bifidobacterial community (Asakuma et al., 2011). In 2015, Garrido et al. assessed the transcriptomic response of *B. bifidum* SC555 to pooled and individual HMOs, and observed significant upregulation of the LNB/ GNB gene cluster in the presence of HMO or LNT (Garrido et al., 2015). RNA-seq analysis identified that the transcriptome response of SC55 during growth on 2'FL, 3FL, or 6SL was almost identical to lactose (Garrido et al., 2015). This implies that, although *B. bifidum* encodes α -fucosidases and α -sialidases, neither fucose or sialic acid are used as carbon sources. Instead, these enzymes release these monosaccharides into the lumen, where they become available as nutrients for other gut microbes. An *in vitro* study by Gotoh et al (2018) provided reasoning for these observations, as they demonstrated *B. bifidum* mediated cross-feeding of HMO degradants within infant-borne bifidobacterial communities (Gotoh, Katoh, Sakanaka, Ling, Yamada, Asakuma, Urashima, Tomabechi, Katayama-Ikegami, & Kurihara, 2018). The release of HMO degradants by *B. bifidum* stimulated growth of other *Bifidobacterium* species. In particular, syntrophy between *B. bifidum* and *B. breve* have been frequently demonstrated in the *in vitro* setting (Cheng et al., 2020; Egan, Motherway, et al., 2014; Motherway et al., 2018; Turrone et al., 2015). Some studies suggest that the selfless behaviour of *B. bifidum* may also occur in the adult intestine as it liberates mucin degradants from the wall of the large intestine (Egan, Motherway, et al., 2014). Across the *Bifidobacterium* genera, *B. bifidum* is the only species which has demonstrated capacity to assimilate these gut mucins, signifying a potential role for this species in the adult gut microbiota

as well as the infant. The data mining review by Sakanaka et al. (2020) revealed that a high conservation of all genes required for extracellular HMO degradation (note that LnbB and LnbXY catalyze the same reaction) was evident in the 60 *B. bifidum* genomes examined (Sakanaka et al., 2020). The results are consistent not only with the shared high HMO assimilation ability, but also with the shared cross-feeding ability of this species.

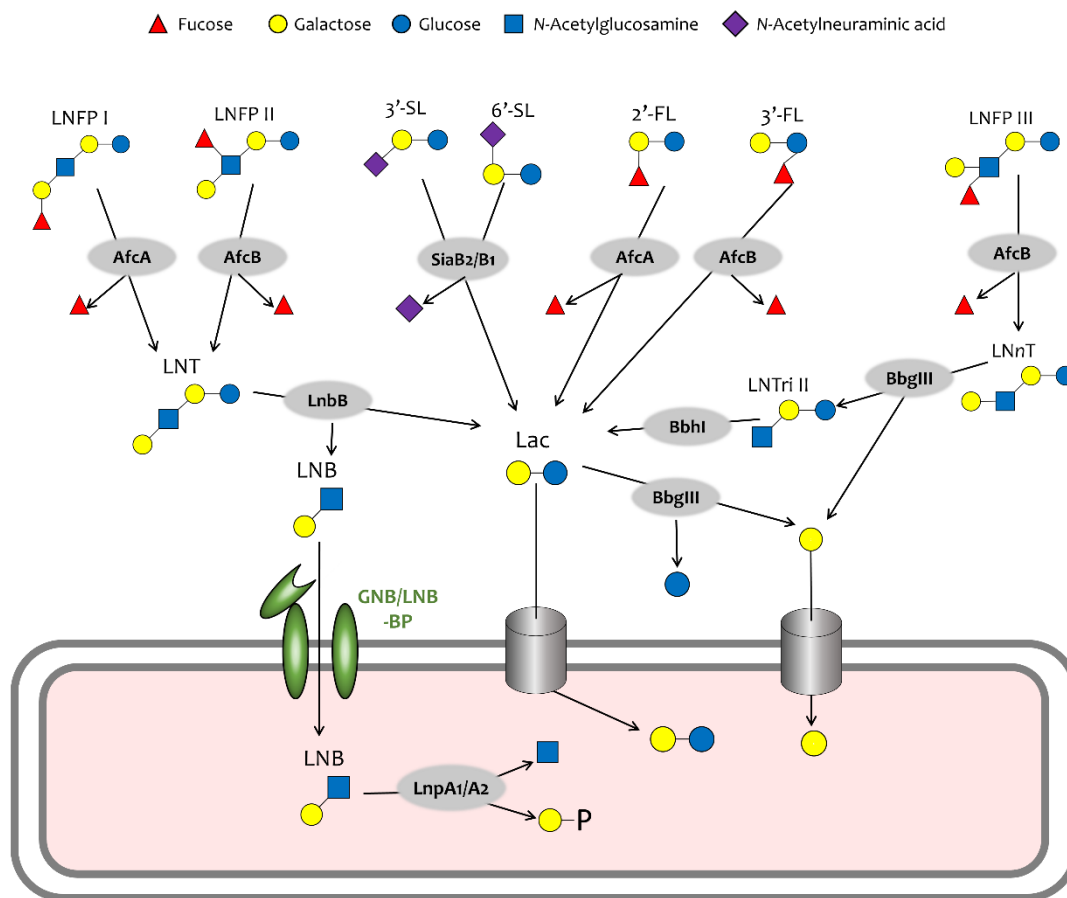


Figure 4: Schematic summary of HMO-assimilation pathways in *Bifidobacterium bifidum*. *B. bifidum* employs cell wall-anchored secretory glycosidases to hydrolyse HMOs extracellularly releasing mono- and di-saccharides. Lacto-*N*-biose (LNB) is internalised through ABC transporters and processed intra-cellularly. *B. bifidum* strains are capable of hydrolysing most HMO linkages and are considered a ‘prototypical HMO users’. GH enzyme abbreviations and locus tags according to Table 2.

2.3. *Bifidobacterium breve*

While *B. breve* is commonly found as one of the most dominant species in the gut, the precise reason for its predominance is poorly understood. Fewer studies exist on the HMO-utilizing strategy employed by this species when compared to *B. infantis* and *B. bifidum*. In comparison to the aforementioned HMO “super users”, the ability of *B. breve* to consume and grow directly on HMOs is limited. *B. breve* has been described as a ‘selective’ consumer, with high variability among strains in their capacity to consume HMO. It had been originally postulated that *B. breve* functions as a ‘scavenger’ species feeding on the HMO monosaccharide constituents produced by other members of the microbiota. Recent studies, however, have demonstrated that certain strains of *B. breve* are active HMO utilizers, capable of assimilating larger HMO structures such as fucosyllactose, LNT, sialyl-LNT or derived structures such as LNB (**Figure 5**).

In order to provide rationale for the high numbers of *B. breve* found in the intestinal microbiota of nursing infants, Ruiz-Moyano et al. (2013) characterised the growth of 24 *B. breve* isolates on HMO species and examined the molecular mechanisms employed for their utilization (Ruiz-Moyano et al., 2013). Although the authors found high variability in HMO assimilation phenotypes, consumption of LNT and LNnT was well conserved (Ruiz-Moyano et al., 2013). Growth on fucosylated HMOs, however, was strain dependent. While all isolates contained a GH95 α -fucosidase, only certain strains possessed a GH29 α -fucosidase (**Table 1, Figure 2**). In this study, only strains SC95, SC568, and SC154 demonstrate significant growth on 2’FL, with consumption of 2’-FL dictated by the presence of this GH29 α -fucosidase (Ruiz-Moyano et al., 2013). However, some isolates which encode

GH29 α -fucosidase did not display growth on 2'-FL, suggesting the absence of other enzymes or transporters necessary for the metabolism of 2'-FL. Researchers assessing digestion of HMO by *B. breve* in the premature infant verified that selective consumption and strain-to-strain variation is also prevalent in the physiological setting (Underwood et al., 2017). A mechanistic model for utilization of sialic acid has been characterised for *B. breve* strain UCC2003 including a putative sialic acid-ABC transporter, and an intracellular α -sialidase (Egan, O'Connell Motherway, et al., 2014). The authors suggested that hydrolysis of sialic acid residues occurs internally, wherein the monosaccharide is converted to fructose-6-P. A mechanistic pathway for digestion of LNT and LNnT has also been characterized for the UCC2003 strain (James et al., 2016) (**Figure 5**). James et al. (2016) concluded that consumption of these isomers occurs via overlapping, yet divergent metabolic pathways (James et al., 2016). Similar to the mechanistic model described in *B. infantis*, metabolism of LNnT requires internalisation of oligosaccharides via ABC transporters. Mutagenesis and transcriptomic analysis identified the *nah* locus-encoded ABC transporter as the solitary transporter implicated in the internalisation of LNnT (James et al., 2016). It has been suggested that this transporter may also be involved in LNT transport but its function is aided by the presence of one or more additional transport systems (James et al., 2016). Once inside the cell, molecules of LNnT are acted upon by a series of β -galactosidases. While the β -galactosidase encoded by *IntA* acts upon both Gal β -1,3GlcNAc linkages in LNT and Gal β -1,3GlcNAc linkages of LNnT, hydrolysis of LNnT is catalysed by other GHs such as LacZ6 and LacZ2 (**Table 2**). Enzymatic activity of these β -galactosidase releases lacto-*N*-triose and galactose. The

HMO degradants produced from LNT and LNnT are identical and enter into the same metabolic pathways for energy production feeding directly and indirectly into the Bifid Shunt (Desjardins et al., 1990). The review by Sakanaka et al. (2020) also validated the finding that almost all strains of *B. breve* are capable of utilizing LNB, LNT, and LNnT in the 91 genomes examined. GNB/LNB-BP (GltA), LNnT-BP (NahS), and the intracellular enzymes required for degrading them (GNB/LNB phosphorylase, LNT β -1,3-galactosidase, two β -1,4-galactosidases (LacZ2/6), and one β -*N*-acetylglucosaminidase (NahA) are highly conserved (Sakanaka et al., 2020).

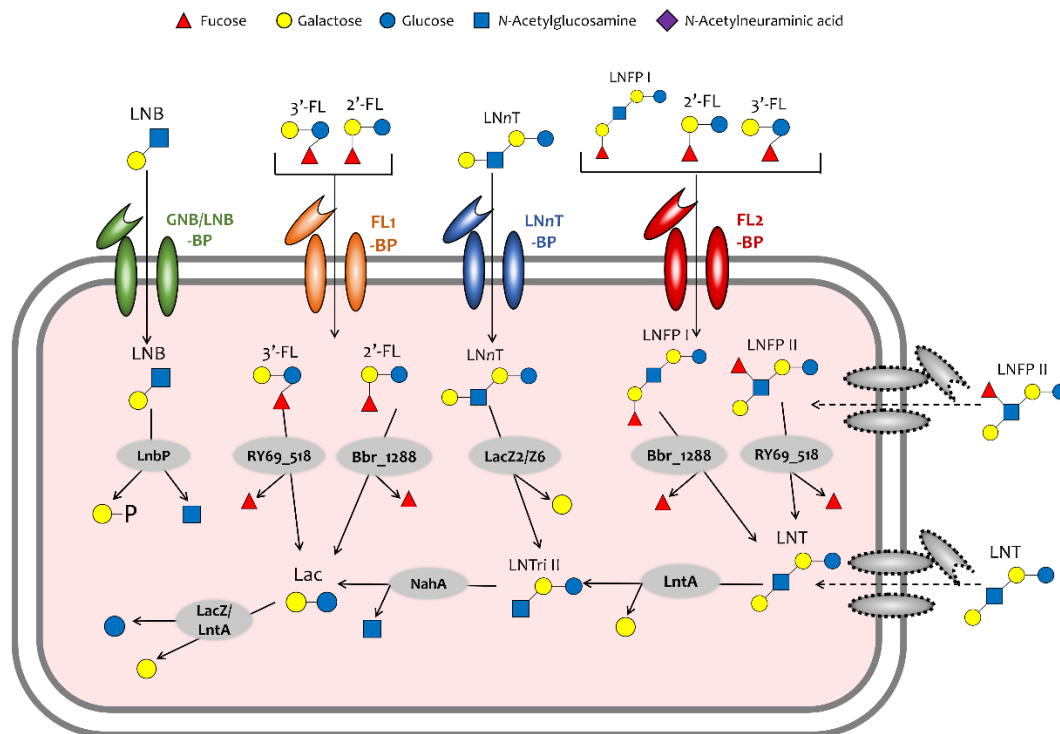


Figure 5: Schematic summary of HMO-assimilation pathways in *Bifidobacterium breve*. *B. breve* internalizes intact HMO through ATP-Binding cassette (ABC) wherein HMOs are assimilated by intracellular glycosyl hydrolases (GH). *B. breve* are often considered as a scavenger species consuming monosaccharide liberated by other infant gut bacteria. However, certain strains are capable of metabolising more complex HMO structures. Transporters not yet characterised are shown in grey with black dotted lines. GH enzyme abbreviations and locus tags according to Table 2.

2.4. *Bifidobacterium longum* subsp. *longum* (*B. longum*)

Despite the fact that *B. longum* has routinely been detected in high abundance in the infant gut microbiota, genomic and phenotypic studies of many *B. longum* strains indicate preference for plant-derived oligosaccharides (Chaplin et al., 2015; O’Callaghan et al., 2015). This suggests that *B. longum* is more adapted to the adult gut and provides no rationale for its predominance in the infant. Although *B. longum* and *B. infantis* are members of the same species, they vary considerably in their carbohydrate utilization capabilities. As described previously, *B. infantis* possess an array of genes related to assimilation of HMOs, demonstrating adaption to the HMO rich environment. This capacity is conserved across the subspecies. On the other hand, it appears strain to strain variation in HMO consumption is more pronounced in the *B. longum* subspecies than in any other bifidobacterial species (Sakanaka et al., 2020).

In order to understand the variability in HMO assimilation phenotypes, Garrido et al. (2016) characterised infant-borne *B. longum* isolates and examined the genetic loci responsible for enrichment of certain *B. longum* strains in the presence of HMO (Garrido et al., 2016). All tested strains were capable of growth on LNT, but only certain isolates demonstrated growth on LNnT and 2’FL. Glycoprofiling of strain *B. longum* SC596 demonstrated preferential consumption of fucosylated HMOs, a phenotype which had previously not been described in this subspecies (Garrido et al., 2016). Transcriptomic analysis revealed a novel gene cluster (termed FHMO cluster) responsible for assimilation of fucosylated HMO. This cluster contained 2 fucosidases, an ABC transporter, and a LacI-type regulator. The cluster was expressed as a single transcriptional unit, induced by fucosylated

structures and not neutral HMOs. Remarkably, many of the fucose metabolism genes were found to be homologous to genes found in the *B. infantis* ATCC 15697 HMO cluster I, highlighting genetic linkages between HMO assimilation mechanisms in these two strains (Garrido et al., 2016).

Recent identification of an important HMO-hydrolysing enzyme provides further rationale for the selective consumption of HMOs by *B. longum* strains. A novel lacto-*N*-biosidase (encoded by the gene *lnbX*) was first described in strain *B. longum* JCM1217 (Sakurama et al., 2013) (**Figure 6**). Further analysis demonstrated elevated levels of this enzyme in the feces of breast-fed infants, when compared to the feces of formula-fed neonates (Yamada et al., 2017). The mechanism of this lacto-*N*-biosidase is distinct from the lacto-*N*-biosidase (encoded by *lnbB*) described in *B. bifidum* previously. While LnbB cannot act on LNB units which have been substituted with additional monosaccharide residues, LnbX is much less specific regarding its substrates, and releases LNB units decorated with fucose and sialic acid residues. These findings indicate that the gene *lnbX* is a significant contributing factor to the persistence of specific *B. longum* strains in gut of breast-fed infants. In the Sakanaka et al. (2020) review, LnbX is detected for 38% of *B. longum* genomes of the 151 examined. LnbX-positive strains use GNB/LNB-BP (GltA) for LNB uptake and GNB/LNB phosphorylase (LnpA) for subsequent intracellular phosphorolysis, while LnbX-negative strains use LNT β -1,3-galactosidase for the intracellular hydrolysis of LNT (Sakanaka et al., 2020) (**Figure 6**). GltA, LnpA, GH42 LNT β -1,3-galactosidase, GH2 β -1,4-galactosidase (Bga2A homolog), and GH20 β -*N*-acetylglucosaminidase (Blon_0732 homolog) were found to be highly conserved in *B. longum* (**Table 2**).

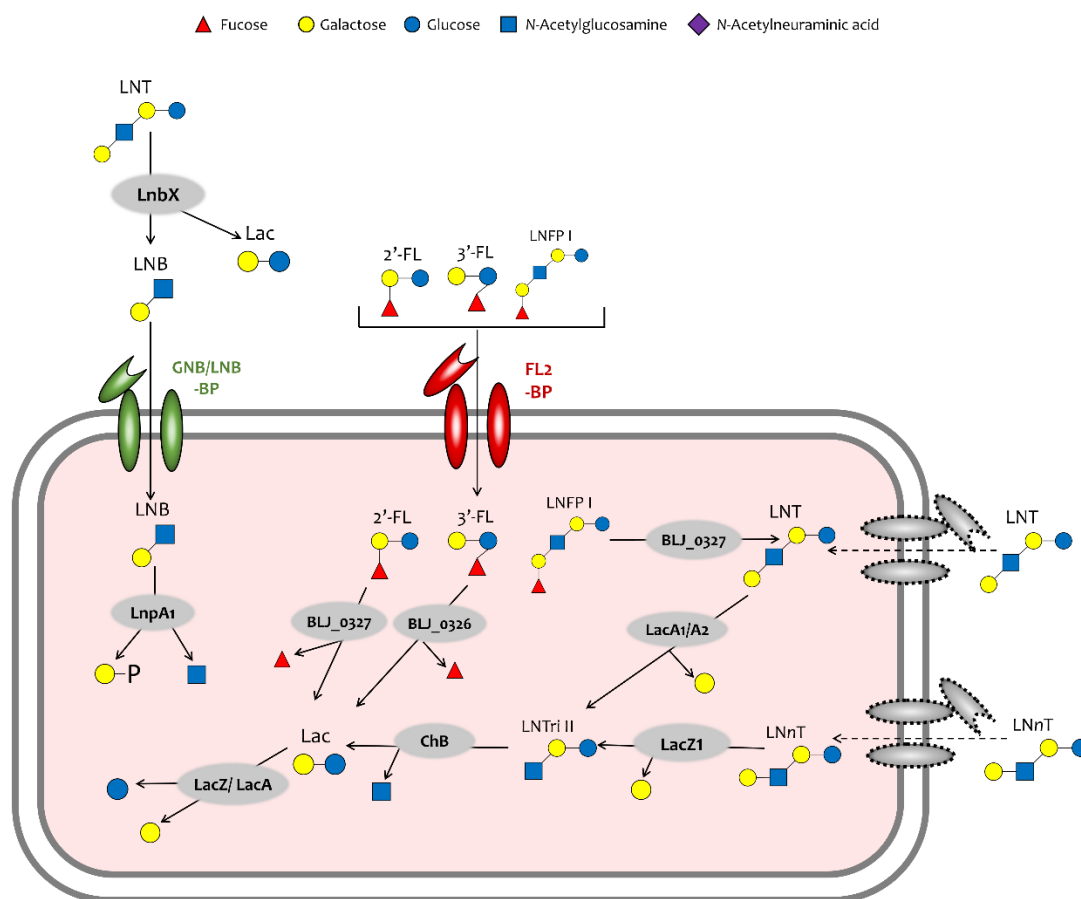


Figure 6: Schematic summary of HMO-assimilation pathways in *Bifidobacterium longum* subsp. *longum*. The strategy utilized by *B. longum* is dependent on the presence of the gene encoding lacto-N-biosidase (LnbX). LnbX-positive *B. longum* utilize extracellular GHs, while LnbX-negative strains employ oligosaccharide transporters to metabolise HMO internally. Transporters not yet characterised are shown in grey with black dotted lines. GH enzyme abbreviations and locus tags according to Table 2.

2.5. *Bifidobacterium catenulatum* subsp. *kashiwanohense* (*B. kashiwanohense*)

B. kashiwanohense is occasionally isolated from the faeces of neonates (Morita et al., 2011; Vazquez-Gutierrez et al., 2015), although it is not considered a common member of the neonatal gut microbiota. In addition, there is limited information on its metabolic capabilities. The ability of two *B. kashiwanohense* strains to grow on the HMO components 2'-FL and 3-FL

was recently shown, although these strains did not appear to metabolize the (fucosyllactose component) L-fucose (Bunesova et al., 2016). The authors suggest that the inability of *B. kashiwanohense* to grow on L-fucose is due to the lack of two enzymes, L-fucose mutarotase and fucose permease. James et al. (2019) recently sequenced the genome of *B. kashiwanohense* strain APCKJ1, isolated from faeces of a breast-fed infant using 2'-FL as a selective carbohydrate (James et al., 2019). Employing various approaches the researchers dissected fucosyllactose metabolism in APCKJ1. The fucosidase encoded by *fumA1* is thought to be specifically required for 2'-FL hydrolysis, and capable of hydrolysing 3-FL, while the solute binding protein and two permeases encoded by *fumS*, *fumT1* and *fumT2*, respectively, are responsible for the uptake and internalisation of 2'-FL and 3-FL. The ability of *B. kashiwanohense* to consume LDFT and LNFP I has also been recently suggested and might be a common characteristic of this species (Sakanaka et al., 2019). Sakanaka et al. (2020) analysed two deposited genomes of *B. kashiwanohense* and found they possessed the homologs of FL2-BP, GH95 α -L-fucosidase (AfcA), LNT β -1,3-galactosidase, and one GH2 β -1,4-galactosidase (Bga2A) (Sakanaka et al., 2020). The occurrence of GltA and GH29 α -L-fucosidase (AfcB) is strain-dependent, but the GH20 β -N-acetylglucosaminidase gene homolog was not detected. The ability to assimilate LNT has not been described in this species to date.

2.6. *Bifidobacterium pseudocatenulatum*

Although *Bifidobacterium pseudocatenulatum* is generally considered an adult-type bifidobacterial species, it has been isolated from the stools of breast fed infants (Benítez-Páez et al., 2016) and was shown to assimilate certain HMOs. Xiao et al. (2010) demonstrated that 33 out of 61 strains of *B.*

pseudocatenulatum examined were able to utilize LNB (Xiao et al., 2010). Matsuki et al. (2016) also reported LNT consumption by *B. pseudocatenulatum* (Matsuki et al., 2016). The authors also noted that some strains of this species assimilate 2'-FL, 3-FL, and LDFT. The Sakanaka et al. (2020) review examined 64 genomes of *B. pseudocatenulatum* which showed relatively high conservation of the GNB/LNB transporter (GltA) and GH2 β -1,4-galactosidase (Bga2A) homolog genes (Sakanaka et al., 2020). About half of the strains of those examined for this species were capable of assimilating LNB, as observed by Xiao et al. (2010), but lacked the GNB/LNB phosphorylase gene homolog. LNT β -1,3-galactosidase, which is moderately conserved in this species, may be involved in the disaccharide assimilation. The FL2-BP (13%) and GH95 α -L-fucosidase (13%) homologs were variably present, which is consistent with the results of Matsuki et al. (2016).

2.7. Other species and taxa

The genomes of other *Bifidobacterium* species, which reside in adult guts or mouths, and non-human animal intestines or dairy products (176 genomes examined), were found to lack HMO degradation genes with a few exceptions (Sakanaka et al., 2020). Instead, they appear to have gene sets for assimilating plant-derived glycans contained in adult or animal diet (Milani et al., 2016). Similarly, Lugli et al. (2020) employed 749 sequenced bifidobacterial genomes, encompassing *B. adolescentis*, *B. animalis*, *B. bifidum*, *B. breve*, *B. dentium*, *B. longum* and *B. pseudolongum* species to perform a screening of enzymes responsible for HMO degradation (Lugli et al., 2020). While putative β -galactosidase enzymes were identified among all analyzed genomes, each bifidobacterial strain was shown to contain a different composition of additional potential enzymes capable of breaking down HMO

molecules, *i.e.*, β -hexosaminidase, lacto-*N*-biase phosphorylase, α -L-fucosidase and sialidase (Lugli et al., 2020). Members of the *B. adolescentis* and *B. animalis* show limited presence of these additional enzymes (2 % and 15 % exhibited the presence of a predicted lacto-*N*-biase-encoding gene in their genomes, respectively), followed by *B. pseudolongum* strains of which 25 % and 32 % displayed the presence of genes encoding a putative lacto-*N*-biase and α -L-fucosidase in their genomes, respectively. To validate the glycobioime screening of the 749 sequenced bifidobacterial genomes, 86 bifidobacterial taxa belonging to the same species assessed were cultivated using HMOs as the unique carbon source. In this context, bifidobacteria were cultivated in media containing LNT, 2'FL and 3'SL. As expected, members of the *B. adolescentis* and *B. animalis* species did not grow in any of the HMOs media used, nor did members of the *B. dentium* species (Lugli et al., 2020).

Besides infant gut-associated bifidobacteria, it has been reported that a few other gut microbes belonging to other taxa have HMO consumption abilities. Several *Bacteroides* species were shown to assimilate 2'-FL, 3-FL, 3'-SL, 6'-SL, and LDFT (Yu et al., 2013). *Lactobacillus casei* utilizes the HMO degradation products lacto-*N*-triose II and LNB (Bidart et al., 2016; Bidart et al., 2014). Indeed, *Lactobacillus* has evolved a specific phosphotransferase system to import LNB (Bidart et al., 2014). HMO utilization capability of these gut microbes is generally not very high, but it is worth noting that minority species during the breast-feeding stage persist until weaning, which potentially influences the microbiota population in adulthood.

3. Milk Glycoconjugate Metabolism

While the high concentrations of oligosaccharides in human milk can in part explain the enrichment in bifidobacteria in breast-fed infant faeces, it should be noted that glycans conjugated to other molecules in milk, such as proteins, peptides or lipids, can also play a prebiotic role (Garrido et al., 2013). Protein glycosylation is a post-translational modification in which a glycan is covalently linked to predetermined amino acids in the protein structure. There are two major types of oligosaccharides attached to eukaryotic proteins: *N*-linked and *O*-linked glycans. *N*-Linked glycans are attached to the protein via specific asparagines in the sequence Asn-xxx-Ser/Thr (Stanley et al., 2017). *O*-Linked glycans differ from *N*-linked glycans by attachment to serine or threonine residues, with no clear surrounding amino acid consensus sequence (Brockhausen et al., 2009). In human milk, a large number of proteins are glycosylated, including immunoglobulins, lactoferrin and κ -casein among many others (Froehlich et al., 2010). Linked glycoproteins are classified into the following three groups based on composition: high-mannose, complex, and hybrid (combination of high-mannose and complex types) glycoproteins (Stanley, 2009). The vast array of combinations of linkages, compositions, and structures presented by complex glycan synthesis can create a diverse range of possible glycoprotein structures (Robinson et al., 2012). Some glycoproteins, such as lactoferrin and the κ -casein-derived glycomacropeptide, have previously been associated with a bifidogenic effect (Garrido, Nwosu, et al., 2012; Karav et al., 2016). The ability to deglycosylate or degrade glycoproteins has been demonstrated for select species of *Bifidobacterium* (Garrido, Nwosu, et al., 2012; Kiyohara et al., 2012; Ruas-Madiedo et al., 2008). Garrido et al (2012) have shown that *B. infantis* ATCC

15697 contains an endoglycosidase, EndoBI-1 (glycosyl hydrolase family 18), that has activity on major types of *N*-linked glycans found in glycoproteins and can release glycans from human lactoferrin and immunoglobulins (Garrido, Nwosu, et al., 2012) (Garrido et al. 2012).

Glycolipids are mostly associated with the milk fat globule membrane (Newburg & Chaturvedi, 1992) and are composed of a lipid chain of ceramide, a fatty acid linked to a sphingoid base, covalently attached to one or more monosaccharides. The most abundant gangliosides in human milk are GD3 and GM3 (NeuAc α 2-3Gal β 1-4Glc β 1-1Cer) (Lee et al., 2011). The ability of bifidobacteria to release sialic acid from predominant milk gangliosides such as GD3 and GM3 has been detected (Falk et al., 1990). Recently, Sugiyama et al. (2017) described a α -1,2-l-fucosidase AfcA isolated from *B. bifidum* which was capable of releasing fucose from fucosyl-GM1 ganglioside (Sugiyama et al., 2016). *B. infantis* and *B. bifidum* also have α -2,8-sialidase activity that released Neu5Ac from GD3 (Lee et al., 2014) (Lee et al., 2014). In general, however, when compared to the role of HMOs in shaping the distal gut microbiota of infants, the role of glycoconjugates has been less explored.

4. Conclusions

Microbiota composition during infancy plays an important role in processes that have life-long health consequences, such as educating the immune system, metabolic programming, and facilitating nutrient utilisation. Thus, it is important to gain better understanding of the biological function of the different HMOs in shaping microbiota development and function. Further longitudinal studies investigating the establishment of infant GI microbiota in relation to changes in breastmilk HMO composition are required.

Comparative genomic analysis reveals that bifidobacteria are adapted to their hosts. Altogether, bifidobacteria employ a variety of strategies to utilise milk oligosaccharides as described above. Many predicted transport systems from the bifidobacterial pangenome should be investigated to enhance our understanding of their capability to utilise HMO. Interestingly, encoding a large number of SBPs related genes in their genomes, may be linked to an ability to adapt to the wide variety of oligosaccharides present in the human gut. Therefore, special emphasis should be given to explore predicted/annotated HMO utilisation and cross-feeding mechanisms, which will provide new leads in novel gene/enzyme or metabolite discovery that may have important implications for host-microbe interactions and host health.

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CHAPTER IV

Tailored combinations of human milk oligosaccharides modulate the immune response in an in vitro model of intestinal inflammation

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Abstract

Infants rely on their developing immune system and the protective components of breast milk to defend against bacterial and viral pathogens, as well as immune disorders such as food allergies, prior to the introduction of solid foods. When breastfeeding is not feasible, fortified infant formula will most frequently be offered, usually based on a cow's milk-derived substitute. The current study aimed to explore the immunomodulatory effects of combinations of commercially available human milk oligosaccharides (HMOs). An *in vitro* co-culture model of Caco-2 intestinal epithelial cells and THP-1 macrophages was established to replicate the hallmarks of intestinal inflammation and to evaluate the direct effects of different synthetic HMO combinations. Notably, a blend of the most prevalent fucosylated and sialylated HMOs, 2'-fucosyllactose (2'-FL) and 6'-sialyllactose (6'-SL), respectively, resulted in decreased pro-inflammatory cytokine levels. These effects were dependent on the HMO concentration and on the HMO ratio resembling those in breastmilk. Interestingly, adding additional HMO structures did not enhance the anti-inflammatory effects. This research highlights the importance of carefully selecting HMO combinations in nutritional products, particularly for infant milk formulations, to effectively mimic the benefits associated with breastmilk.

1. Introduction

To protect against foreign invaders, humans have evolved intricate defence mechanisms, collectively known as the immune system. Immune system development begins before birth and continues postnatally as the body encounters new molecular signals. Shortly after birth, a newborn's immune system undergoes rapid changes, with various immune cells - including neutrophils, macrophages, monocytes, natural killer (NK) cells, T cells, and dendritic cells - actively participating in the defense against pathogenic invaders (Spellberg & Edwards, 2001; Zhao & Yang, 2022). At birth, there is an observable imbalance in T-helper (Th) cell types, specifically between Th1/17 and Th2 phenotypes. In neonates, the immune response leans towards the Th2 phenotype, which enhances humoral immunity and provides protection against extracellular pathogens (Basha et al., 2014; Debock & Flamand, 2014). Conversely, pathways associated with Th1/Th17, which target intracellular pathogens, are less prominent in early neonatal development (Adkins, 2000; Li et al., 2018). Disruption of this system by various factors such as stress, nutrient deficiencies, environmental factors or exposure to pathogens can lead to inflammation, a fundamental response to foreign materials. Inflammatory reactions involve the secretion of chemokines and cytokines attracting immune cells such as macrophages and T-cells, which act against the pathogen (Arango Duque & Descoteaux, 2014). However, dysregulated immune responses can lead to excessive inflammation, damaging healthy tissues and contributing to the emergence of inflammatory diseases, including necrotizing enterocolitis (NEC) (Cho et al., 2016; Denning et al., 2017). Breastfeeding has been shown to significantly decrease the risk of these conditions, implying that human milk has

components supporting the balanced maturation of the gut and immune system (Camacho-Morales et al., 2021; Nolan et al., 2020).

Human milk oligosaccharides (HMOs), which represent a diverse group of lactose-based sugars, are the third-largest solid component in breastmilk that have been shown to play a crucial role in early life development (Bode, 2012; Walsh et al., 2020). Although extensively studied for their prebiotic effects, through promoting growth of beneficial bacteria in the gut (Sela, 2011; Thomson et al., 2018), HMOs also offer a broader range of health benefits. These sugars can influence gut motility, exhibit anti-adhesive and antimicrobial properties, and regulate epithelial cell responses (Farhin et al., 2019; Figueroa-Lozano & de Vos, 2019; Moore et al., 2021). The advantages of HMOs may extend beyond infancy, potentially reducing allergy risk or supporting cognitive function (Fan et al., 2023; Seppo et al., 2017; Zuurveld et al., 2020), which has made HMOs the focus of intense current scientific research with an increasing number of studies unravelling their role in human physiology. Over the past decade, substantial clinical evidence has highlighted HMO benefits across various populations (as evaluated in a recent systemic review by Schönknecht *et al.* (Schönknecht et al., 2023) across varying study populations including healthy infants (Bosheva et al., 2022; Marriage et al., 2015; Parschat et al., 2021; Puccio et al., 2017), infants with specific health needs (Hascoët et al., 2022; Nowak-Wegrzyn et al., 2019; Vandenplas et al., 2022), children (Leung et al., 2020), and adults (Elison et al., 2016). While HMOs are believed to support immune system development by aiding the establishment of a healthy gut microbiome and its metabolic functions, recent studies also suggest that HMOs can directly impact on immune responses (Rosa et al., 2022).

HMOs have been found to exhibit anti-inflammatory activity by regulating the production of interleukins (ILs) and lymphocyte activation (Chleilat et al., 2020). He *et al.* utilized intestinal epithelial cells (T84, HCT8, FHs74) and HeLa cells to explore the immunomodulatory effects of human milk oligosaccharides (HMOs) derived from colostrum (He et al., 2016). Their research identified key networks involved in immune cell communication, differentiation of the intestinal mucosal immune system, and maintenance of homeostasis. HMO treatment was shown to result in a reduction in pro-inflammatory cytokine levels, including IL-8, IL-6, monocyte chemoattractant protein-1/2, and IL-1 β . Conversely, it elevated the levels of cytokines associated with tissue repair and homeostasis, highlighting the potential of HMOs to modulate immune responses and promote intestinal health (He et al., 2016). In a similar study, the transcriptional response of colonic epithelial cells treated with human milk oligosaccharides (HMOs) was examined using human colorectal adenocarcinoma HT-29 cells. The treatment with breastmilk-derived HMOs was shown to significantly affect expression of several cytokines, including IL-1 β , IL-8, IL-17C, and platelet factor 4 (PF4). Additionally, the levels of various chemokines, such as CXCL1, CXCL2, CXCL3, CXCL6, CCL5, CCL20, and CX3CL1, as well as the cell surface receptor interferon γ receptor 1 (IFNGR1), were also modulated by HMO administration (Lane et al., 2013). Newburg *et al.* revealed that HMOs isolated from breastmilk attenuated TNF- α and IL-1 β -induced expression of IL-8, monocyte chemoattractant protein 1 (MCP-1), and macrophage inflammatory protein-3 α (MIP-3 α) (Newburg et al., 2016). These findings underscore the role of breastmilk-derived HMOs in regulating immune signalling pathways in colonic epithelial cells. However, these anti-

inflammatory effects have also been demonstrated for individual HMO structures. Recent advancements in synthetic biology and metabolic engineering have enabled microbial systems to produce various HMOs, facilitating their use in both commercial and research settings. He *et al.* examined the influence of 2'-fucosyllactose (2'-FL), one of the most prominent short-chain oligosaccharides in milk, on *Escherichia coli*-induced interleukin (IL)-8 release by T84 and H4 intestinal epithelial cells (He *et al.*, 2016). The study demonstrated that 2'-FL quenches pro-inflammatory signalling through attenuation of CD14 induction in this *in vitro* model (He *et al.*, 2016). These HMO-induced anti-inflammatory effects were also observed in human epithelial cell responses related to allergic disease (Zehra *et al.*, 2018). For example, Zehra *et al.* investigated the therapeutic potential of specific HMOs in food allergy and demonstrated that the HMO, 6'-sialyllactose (6'-SL), inhibits chemokine (IL-8 and CCL20) release from lung T-84 and intestinal HT-29 cells stimulated with antigen-antibody complex (Zehra *et al.*, 2018). This effect was PPAR γ dependent while 2'-FL selectively inhibited CCL20 release in response to antigen antibody complex in a PPAR γ independent manner (Zehra *et al.*, 2018). In addition, sialylated HMOs suppressed inflammatory cytokines (IL-1 β , TNF- α and IL-6) production and reduced NEC incidence and pathological damages in inflamed ileum of NEC rats *in vivo* (Zhang *et al.*, 2021). It is clear, therefore, that HMOs have structure-specific impacts, with clear distinctions between neutral and acidic oligosaccharides, in modulating cytokine production and cell differentiation.

While previous *in vitro* studies indicate the potential of HMOs as anti-inflammatory agents in intestinal settings, research has largely focused on individual HMO structures. Blends of synthetic HMOs can mimic the diverse

mixture found in natural breast milk more closely, potentially offering a broader range of benefits. Synergy between specific HMO combinations may enhance immune modulation, with combined effects on reducing inflammation, improving gut barrier integrity, and supporting overall immune development. This study aims to explore whether specific combinations of commercial HMO ingredients can replicate the immunomodulatory effects of natural HMO pools. A co-culture model of Caco-2 intestinal epithelial cells and THP-1 macrophages was developed in order to mimic the hallmarks of intestinal inflammation and used to investigate the effects of different synthetic HMO combinations. Concentration and ratio-dependency assays were performed to elucidate the functional synergy between HMO structures in mitigating inflammation.

2. Materials and methods:

2.1. Materials

Cell culture media (DMEM, RPMI-1640, PBS), fetal bovine serum (FBS), trypsin/EDTA, antibiotics (penicillin/streptomycin solution), human interferon- γ (IFN- γ), MILLIPLEX[®] Human High Sensitivity T Cell Panel, and the Lactate Dehydrogenase Activity Assay Kit were supplied by Sigma-Aldrich (St Louis, MO, USA). Lipopolysaccharide (LPS) derived from *Escherichia coli* O55:B5 and obtained from Sigma-Aldrich. Phenol red-free DMEM was obtained from GIBCO (Carlsbad, CA, USA). CellTiter 96[®] Aqueous One Solution Reagent was purchased from Promega (Madison, WI, USA). Phorbol 12-Myristate 13-Acetate (PMA) was obtained from WVR (Radnor, PA, USA).

2.2. Ingredient generation and selection

2.2.1 Selection of commercial HMO ingredients:

Commercial HMO powders were received from Glycom A/S (Hørsholm, Denmark, purity > 90%). For use in experiments, HMO ingredients had to fulfill the following criteria: a) a positive response as a novel food ingredient from European Food Safety Authority (EFSA) as of 2022; b) endotoxin levels < 0.025 EU/mg; c) no cytotoxic effects at concentrations specified in **Table 1**. HMO or control ingredients were dissolved in glucose-free, phenol red-free media (Fisher, Waltham, MA, USA) and applied throughout experiments at a concentration relative to the regulatory concentration outlined in EFSA's Union list of novel foods (https://food.ec.europa.eu/food-safety/novel-food/authorisations/union-list-novel-foods_en accessed on 30/07/24)

Table 1: HMO concentrations applied during structure dependency assay

Sample	HMO concentration (g/L)					Total
	2'-FL	LNT	LNnT	6'-SL	LNFP I	
Glucose						3.8*
BM-HMO						3.8**
HMO5	1.2	0.8	0.6	0.4	0.8	3.8
HMO4	1.2	0.8	0.6		0.8	3.4
HMO3	1.2		0.6	0.4		2.2
2'-FL+LNFP I	1.2				0.8	2
2'-FL+6'-SL	1.2			0.4		1.6
2'-FL	1.2					1.2
LNT		0.8				0.8
LNnT			0.6			0.6
6'-SL				0.4		0.4

* refers to concentration of glucose present in glucose-supplemented DMEM control. ** refers to concentration of breastmilk-derived HMO in BM-HMO-supplemented DMEM control. 2'-FL (2'-fucosyllactose) and 6'-SL (6'-sialyllactose) were applied at low, standard, and high doses equivalent to 0.5×, 1×, and 2× the maximum regulated dose (according to the European Union List of authorized novel foods). The shading intensity corresponds to concentration levels, with darker green indicating higher concentrations of HMO.

2.2.2. Generation of HMO-enriched fraction:

Human milk samples of unknown secretor status were generously provided by the Irvinestown Human Milk Bank (Co. Fermanagh, Ireland) and were stored at -80 °C upon arrival. Briefly, lipids and proteins were removed from breastmilk as previously described (Quinn et al., 2018) after which lactose and HMO fractions were separated using size-exclusion chromatography (BioGel P2, 92 cm × 5 cm; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Fractions confirmed to be free of peptides (assessed with the Pierce colorimetric peptide assay) and containing minimal lactose (measured using high-pH anion exchange chromatography [HPAEC] with pulsed amperometric detection [Dionex Corporation, Sunnyvale, CA, USA]) were combined and freeze-dried. The resulting HMO-rich fraction was stored at 4 °C until use in experiments. Analysis by HPAEC revealed small amounts of 2'-fucosyllactose (2'-FL) in the HMO-enriched fraction, likely due to the co-fractionation of 2'-FL with lactose during purification. To restore the 2'-FL content that may have been lost in the size-exclusion process, commercial 2'-FL was reintroduced, following a previously described method (Walsh et al., 2022), to give a powder representative of secretor HMO (designated as breastmilk-derived HMO). The composition of the breastmilk-derived HMO is shown in Supplementary **Figure S1**.

2.3. Cell culture

2.3.1 Cell line maintenance

An *in vitro* model using co-cultures of Caco-2 and PMA-differentiated THP-1 cells was established and optimized to mimic the intestine in an inflammatory state based on methods previously outlined (Kämpfer et al., 2017). Caco-2 cells were obtained from the American Type Culture

Collection (ATCC) and cultured in DMEM-based cell culture medium (phenol red-free, Merck, Darmstadt, Germany) substituted with 10% heat-inactivated FBS (Merck) and 1% Penicillin/Streptomycin at 37 °C, 5% CO₂. For co-cultures, Caco-2 cells were seeded on transwell inserts (0.4 µm pore size, Corning) at a density of 6×10^4 cells/well and maintained for 21 days. On the apical (AP) side, the cells were cultured in DMEM, whereas the medium in the basolateral (BL) compartment was changed to RPMI-based medium (10% FBS). Caco-2 cells were used for experiments at three different passages (passage numbers 41 to 45) to provide three biological replicates. THP-1 cells were purchased from the European Collection of Authenticated Cell Cultures (ECACC) and cultured at 37 °C, 5% CO₂ using RPMI-1640 medium substituted with 10% heat-inactivated FBS and 0.1% mercaptoethanol. For co-culture experiments, THP-1 cells were seeded on transwell-suitable 12-well plates at a density of cells/well and differentiated with PMA (100 nM) for 24 h (37 °C, 5% CO₂). Following differentiation, the PMA was removed, and the THP-1 cells cultured for a further 24 h before use in experiments. THP-1 cells were used for experiments at three different passages (passage numbers 4 to 6) to provide three biological replicates.

2.3.2. Cytotoxic/ proliferative effects of HMO ingredients

To assess the cytotoxicity of HMO ingredients, the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) was employed. This colorimetric method quantifies the number of viable cells. Caco-2 cells were seeded at a concentration of 5×10^4 cells per well in a 96-well plate and incubated for 24 h with DMEM (10% FBS). Following cell attachment, the spent media were aspirated, and cells were rinsed twice with PBS. Subsequently, cells were treated with 500 µL HMO solutions for 24 h.

HMO solutions were prepared by dissolving ingredients in DMEM (without glucose or phenol red, GIBCO) at low (0.5× regulatory concentration), standard (1× regulatory concentration), and high (2× regulatory concentration) doses. A control group with DMEM medium without HMOs was included. After the 24 h treatment, cells were exposed to 20 µL/well of CellTiter 96 Aqueous One Solution Reagent, which contains MTS tetrazolium. The MTS tetrazolium compound is enzymatically converted by viable cells into a coloured formazan product. Following a 2 h incubation at 37 °C, absorbance was measured at 490 nm using a 96-well plate reader. Each experiment was conducted in duplicate, and the recorded values of the HMO solutions were adjusted based on the results obtained from the control well.

2.4. Induction of inflammation-like response

2.4.1. Optimization of inflammatory conditions:

An *in vitro* model using co-cultures of Caco-2 and PMA-differentiated THP-1 cells was established (see **Figure 1**) and optimised to mimic the intestine in an inflammatory state based on previously described methods (Kämpfer et al., 2017). THP-1 cells were pre-stimulated with LPS and IFN- γ (1, 10, or 100 ng/mL each) for 4 h. IEC Caco-2 transwells were then transferred into THP-1 wells and incubated with primed THP-1 cells for 24 h at 37 °C, 5% CO₂. Control wells containing (a) Caco-2 and THP-1 cells without stimulation and (b) Caco-2 cells in monoculture were included in experiments. Following this 24 h incubation period, supernatants from the Caco-2 apical compartment were collected in polypropylene tubes and stored at –20 °C for quantification of inflammatory cytokines (as outlined below). In addition, lactate dehydrogenase (LDH) activity in the cell culture medium was measured after 24 h co-culture using a Lactate Dehydrogenase Activity

Assay Kit (Sigma-Aldrich) according to manufacturer's instructions. The TEER was measured using an Ohm-meter (EVOM2) to assess the barrier development of the Caco-2 cell layer (every 6–7 days of culture), as well as the barrier integrity throughout the co-culture with THP-1 cells (after 4, 18, 24, and 48 h). The electrode was sterilized in 70% ethanol (15 min) and neutralized in PBS. Results were corrected for the blank and multiplied by the membrane filter area (1.12 cm^2) to obtain the final results in Ohm per cm^2 ($\Omega \cdot \text{cm}^2$).

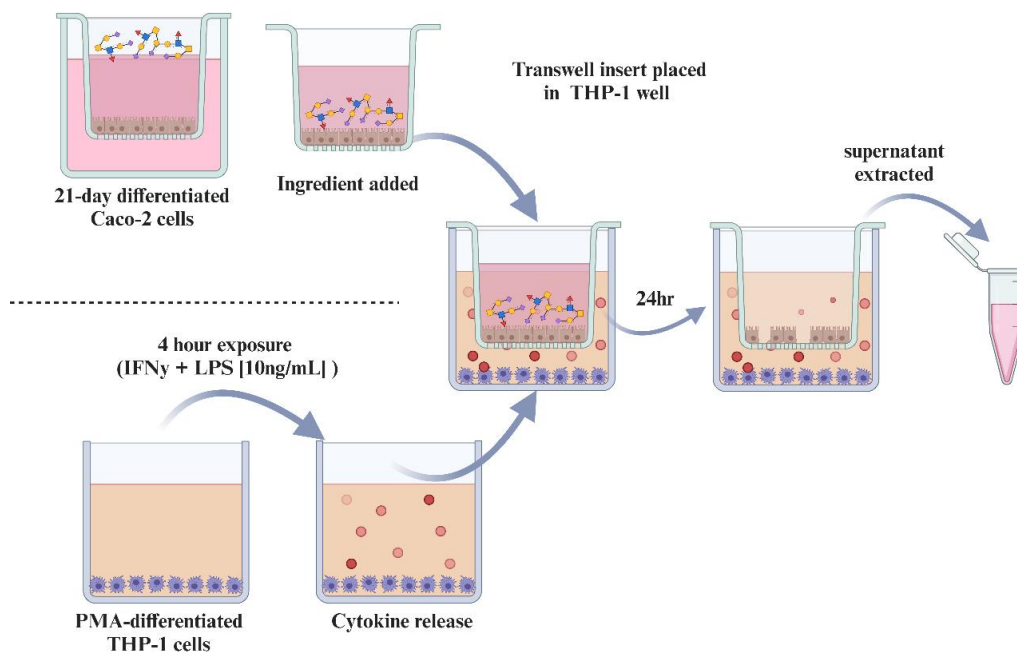


Figure 1: Schematic description of the *in vitro* model using co-cultures of Caco-2 and PMA-differentiated THP-1 cells to mimic the intestine in an inflammatory state.

The following criteria for the co-culture to be classified as ‘inflamed’ were defined:

1. A TEER reduction by at least 25% compared to the Caco-2 monoculture

2. After 24 h of co-culture, the levels of pro-inflammatory cytokines need to significantly exceed the concentrations recovered in the stable co-culture (Caco-2 and THP-1 cells without stimulation)
3. Re-establishment of TEER after 48 h of co-culture (>90% recovery to TEER values at initiation of co-culture)
4. Significantly lower levels of LDH activity (as measured by absorbance at 490nm)

2.4.2. Exposure of Caco-2 IECs to HMO ingredients:

Following selection of optimal stimulation (10ng/mL LPS and IFN- γ) HMO ingredients were introduced to the system and changes in cytokine expression assessed. HMO ingredients were prepared as outlined below (to assess structure-dependency, concentration-dependency, and ratio-dependency effects) and added to the Caco-2 monolayers, before transferring the Caco-2 transwells into the pre-stimulated THP-1 wells. Caco-2 cells were incubated with primed THP-1 cells and ingredients for 24 hours at 37 °C, 5% CO₂. Following this 24-hour incubation period, supernatants from the Caco-2 apical compartment were collected in polypropylene tubes and stored at -20°C.

Structure dependency assay:

Initially, the anti-inflammatory effects of HMO combinations were assessed and compared to the anti-inflammatory properties of individual HMOs. HMO ingredients were dissolved in DMEM media at maximum regulatory concentrations as outlined in **Table 1**. Media supplemented with glucose or breastmilk-derived HMOs (at equivalent concentrations to synthetic HMOs) were used as controls. After 24 h co-exposure of the Caco-2 transwells to the HMO ingredients and activated THP-1, supernatants were collected and the

release of IL-8 in the apical compartment was quantified using IL-8 ELISA (Biolegend). The absorbance was read spectrophotometrically at 450 nm.

Concentration dependency assay:

To further understand the immunomodulatory effects of these HMO ingredients, the co-culture experiments were repeated using various concentrations of 2'-FL (0.6–2.4 g/L) and 6'-SL (0.2–0.8 g/L). These were applied as singular doses, in combination with each other (2'-FL + 6'-SL), and in combination with LNFP-I, LNT, and LNnT (5-HMO) (**Table 2**). All HMOs were applied at low, standard, and high doses equivalent to 0.5×, 1×, and 2× the maximum regulated dose (according to the European Union List of authorized novel foods). The Caco-2 transwells were co-exposed to the activated THP-1 cells and the HMO ingredients for 24 h before collecting the supernatants from the apical compartment and measuring cytokine levels. The release of TNF- α , IL-8, IL-10, IL-6, and IL-1 β was quantified using a magnetic bead-based assay (Milliplex HSTCMAG-28SK high-sensitivity panel, Merck Millipore, Burlington, MA, USA) and analyzed with the Bio-Plex MAGPIX Multiplex Reader (Luminex). For the analysis, supernatants from the inflamed model were diluted 1:20 in cell culture media. Samples were added to appropriate wells and incubated overnight at 4 °C. Subsequently, the detection antibodies were added, and the plate incubated again for 1 h at RT. The wells were incubated with streptavidin-PE and incubated for 30 min at RT before the beads were re-suspended in assay buffer and the plate read using a Bio-Plex MAGPIX Multiplex Reader. Blanks and standard curves were included on each plate.

Table 2: HMO concentrations applied during concentration dependency assay

	Dosage	Relation to regulatory	HMO concentration (g/L)					
			2'-FL	LNT	LNnT	6'-SL	LNFP I	Total
Glucose	Low	NA						1.9*
	Standard	NA						3.8*
	High	NA						7.6*
BM-HMO	Low	NA						1.9**
	Standard	NA						3.8**
	High	NA						7.6**
HMO5	Low	0.5×	0.6	0.4	0.3	0.2	0.4	1.9
	Standard	1×	1.2	0.8	0.6	0.4	0.8	3.8
	High	2×	2.4	1.6	1.2	0.8	1.6	7.6
2'-FL & 6'-SL	Low	0.5×	0.6			0.2		0.8
	Standard	1×	1.2			0.4		1.6
	High	2×	2.4			0.8		3.2
2'-FL	Low	0.5×	0.6					0.6
	Standard	1×	1.2					1.2
	High	2×	2.4					2.4
6'-SL	Low	0.5×				0.2		0.2
	Standard	1×				0.4		0.4
	High	2×				0.8		0.8

* refers to concentration of glucose present in glucose-supplemented DMEM control. ** refers to concentration of breastmilk-derived HMO in BM-HMO-supplemented DMEM control. 2'-FL (2'-fucosyllactose) and 6'-SL (6'-sialyllactose) were applied at low, standard, and high doses equivalent to 0.5×, 1×, and 2× the maximum regulated dose (according to the European Union List of authorized novel foods). The shading intensity corresponds to concentration levels, with darker green indicating higher concentrations of HMO.

Ratio dependency assay

To assess whether the potency of HMOs could be modulated by adjusting the ratio of HMO components, the immune model was treated with various combinations of 2'-FL and 6'-SL provided in various ratios ranging from 1:2 to 3 to 6:1 according to **Table 3**. The equivalent concentrations of individual 2'-FL and 6'-SL were included for comparative purposes. The release of TNF-

α , IL-8, IL-10, IL-6, and IL-1 β were quantified using a magnetic bead-based assay (Milliplex HSTCMAG-28SK high-sensitivity panel). In addition, the effects of these combinations on barrier integrity were assessed using TEER as an indicative measurement. The TEER was measured using an Ohm-meter. Results were corrected for the blank and multiplied by the membrane filter area (1.12 cm²) to obtain the final results in Ohm per cm² ($\Omega \cdot \text{cm}^2$).

Table 3: HMO concentrations applied during ratio dependency assay

Treatment	HMO concentration (mg/mL)			Ratio
	2'-FL	6'-SL	Final	2'-FL: 6'-SL
Glucose			1.6*	
BM-HMO			1.6**	
2'-FL, 6'-SL combination	1.2	0.4	1.6	3:1
	1.2	0.3	1.5	4:1
	1.2	0.2	1.4	6:1
	0.6	0.3	0.9	2:1
	0.6	0.2	0.8	3:1
2'-FL	1.2		1.2	
	0.6		0.6	
6'-SL		0.4	0.4	
		0.3	0.3	
		0.2	0.2	

* refers to concentration of glucose present in glucose-supplemented DMEM control. ** refers to concentration of breastmilk-derived HMO in BM-HMO-supplemented DMEM control. 2'-FL (2'-fucosyllactose) and 6'-SL (6'-sialyllactose) were applied at low, standard, and high doses equivalent to 0.5 $\times$, 1 $\times$, and 2 $\times$ the maximum regulated dose (according to the European Union List of authorized novel foods). The shading intensity corresponds to concentration levels, with darker green indicating higher concentrations of HMO.

2.5. Cytokine quantification

2.5.1. Enzyme-Linked Immuno-Sorbent Assay (ELISA)

The release of IL-8 in the apical compartment was quantified after 24 h co-culture following pre- and co-exposure to ingredients. The ELISA was run

according to manufacturer's instructions (Biolegend, San Diego, CA, USA).

The absorbance was read spectrophotometrically at 450 nm.

2.5.2. Bio-Plex Magpix

Release of TNF- α , IL-8, IL-10, IL-6, or IL-1 β was quantified using a magnetic bead-based assay (Milliplex HSTCMAG-28SK high-sensitivity panel, Merck Millipore) and analyzed with the Bio-Plex MAGPIX Multiplex Reader (Luminex, Austin, TX, USA). For the analysis, supernatants from the inflamed model were diluted 1:20 in cell culture media. Samples were added to appropriate wells and incubated overnight at 4 °C. Subsequently, the detection antibodies were added and the plate incubated again for 1 h at RT. The wells were incubated with streptavidin-PE and incubated for 30 min at RT before the beads were re-suspended in assay buffer and the plate read using a Bio-Plex MAGPIX Multiplex Reader. Blanks and standard curves were included on each plate.

2.6. Statistical analysis

Experimental results are expressed as means $\pm$ the standard errors of the means. Statistical analyses were performed by means of one-way analysis of variance (ANOVA), followed by Tukey's test for comparing all pairs of groups. Significant differences between 2 groups were determined using the unpaired Student's *t*-test. A statistical software package (GraphPad PRISM™ version 5.0, GraphPad Software Inc, La Jolla, CA, U.S.A.) was used for the analysis. A *p*-value of <0.05 was considered statistically significant.

3. Results

3.1. Cytotoxic and proliferative effects of HMO ingredients

The effect of each treatment of various HMO combinations/concentrations (2'-FL, LNFP-I, LNT, LNnT, 3'-SL, and/or 6'-SL at various concentrations) on Caco-2 proliferation is summarized in **Figure 2**. All comparisons of proliferation rates were made relative to the control group (cell culture medium without HMO exposure, designated with a value of 100%). The results showed that the majority of HMO treatments did not affect the viability of Caco-2 cells (**Figure 1**). In fact, increased proliferation of Caco-2 cells was observed in the presence of LNT at 2× ($p = 0.0185$), LNnT at 1× ($p = 0.0023$), LNnT at 2× ($p = 0.0028$), LNFP-I at 1× ($p = 0.0013$), LNFP-I at 2× ($p = 0.0006$), 6'-SL at 0.5× ($p = 0.0342$), and 6'-SL at 1× ($p = 0.0010$). In contrast, when Caco-2 cells were incubated with 0.4 g/L (2×) of 3'-SL (**Figure 2**), the number of viable cells decreased to $76.9 \pm 2.55\%$, indicating potential cytotoxic effects of 3'-SL at higher concentrations. This HMO was thereby eliminated from further analyses.

3.2. Optimisation of inflamed co-culture

For the co-culture setup, transwell filters with Caco-2 cells were placed into well plates containing THP-1 cells that had been differentiated over 24 h and stimulated with varying concentrations of LPS and IFN γ (0 ng/mL for stable co-culture, 1 ng/mL, 10 ng/mL, and 100 ng/mL). To determine the most effective concentration of these immune stimulants, the functional characteristics, cellular integrity, and cytokine release in the inflamed co-culture models were compared with those in a Caco-2 monoculture.

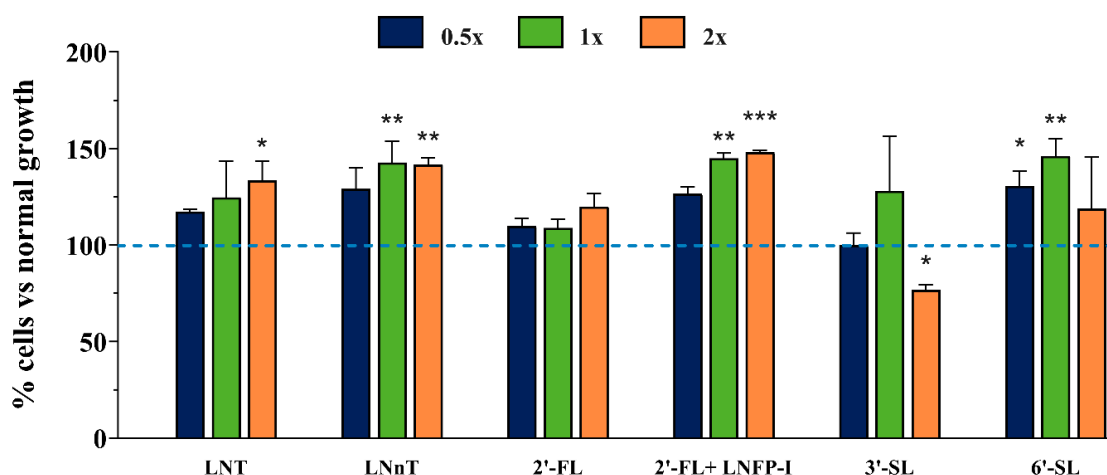


Figure 2: Effect of HMO treatments on Caco-2 proliferation and viability as measured by CellTiter 96 Aqueous One Solution Cell Proliferation Assay. HMOs were applied at low (0.5x regulatory concentration, navy), standard (1x regulatory concentration, green), and high (2x regulatory concentration, orange) doses. All comparisons of proliferation rates were made to the control group (cell culture medium without HMO, designated with a value of 100%, dotted blue line). LNT= Lacto-*N*-tetraose, LNnT= Lacto-*N*-neotetraose, 2'-FL= 2'-fucosyllactose, LNFP-I= Lacto-*N*-fucopentaose-I, 3'-SL= 3'-sialyllactose, 6'-SL= 6'-sialyllactose. Univariate analysis of variance (ANOVA) and post-hoc Tukey tests were performed to determine the significant differences between treatment groups and the control group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs control).

Initially, cytokine levels in the apical compartment from the Caco-2 monoculture and co-culture models were assessed using a MAGPIX magnetic bead-based assay. As summarized in **Figure 3A**, the levels of TNF- α , IL-1 β , IL-6, and IL-8 released after 24 h culturing varied significantly depending on the presence or absence of THP-1 cells and on the levels of immunostimulant present. In general, baseline cytokine levels in the Caco-2 monoculture were below the limit of detection, although IL-8 was expressed at low concentrations (13.9 ± 0.11 pg/mL). These levels of IL-8 increased to 159.8 ± 57.68 pg/mL when the Caco-2 cells were co-cultured with THP-1 cells under stable conditions, with low levels of IL-1 β also detected (5.7 ± 0.99

pg/mL). The inflamed co-culture models showed marked cytokine release compared to both the monoculture and stable co-culture, although responses to 1 ng/mL LPS + IFN- γ were relatively modest (12.5 pg/mL TNF- α , 11.0 pg/mL IL-1 β , 2.3 pg/mL IL-6, and 556.5 pg/mL IL-8). In contrast, stimulation of THP-1 cells with 10 ng/mL LPS + IFN- γ resulted in the release of 577.7 pg/mL of TNF- α , 205.3 pg/mL of IL-1 β , and 85.5 pg/mL of IL-6.

Most strikingly, the levels of IL-8 detected in the Caco-2 supernatants increased to 13,843.3 pg/mL in response to 10 ng/mL LPS + IFN- γ stimulation (**Figure 3A**). These concentrations of pro-inflammatory cytokines were therefore sufficient to meet the criteria defined for an inflamed model. Surprisingly, however, increasing the concentration of LPS + IFN- γ to 100 ng/mL did not result in significant increases in cytokine concentrations. As shown in **Figure 3A**, high doses of LPS + IFN- γ resulted in significantly lower levels of TNF- α , IL-1 β , and IL-8 ($p < 0.01$) when compared to stimulation with 10 ng/mL LPS + IFN- γ .

The barrier integrity of the Caco-2 cells was assessed by measuring TEER at 0, 2, and 24 h of co-culture. In addition, the ability of the Caco-2 IECs to re-establish was assessed by measuring TEER 48 h after the co-culture experiments (corresponding to 72 h after initiation of experiments). As summarized in **Figure 3B**, the stable co-culture of Caco-2 cells and THP-1 cells induced a slight reduction in TEER over a 24 h period. When compared to Caco-2 in monoculture, the TEER reduced from $2106.1 \pm 166.16 \Omega \cdot \text{cm}^2$ to $1728.1 \pm 102.76 \Omega \cdot \text{cm}^2$ in the stable co-culture (corresponding to 82.1% TEER of the monoculture control, $p = 0.0141$). Activation of inflammation in the THP-1 cells using 1 ng/mL LPS + IFN- γ did not induce a significant reduction in TEER when compared to the stable culture (1611.5 ± 151.18

$\Omega \cdot \text{cm}^2$, $p = 0.837$), whereas stimulation with 10 ng/mL and 100 ng/mL LPS + IFN- γ resulted in significant barrier disruption (**Figure 3B**). After 24 h, the TEER in the inflamed co-cultures using 10 ng/mL and 100 ng/mL LPS + IFN- γ was 57.4% ($p < 0.0001$) and 23.2% ($p < 0.0001$), respectively, versus the monoculture control (**Figure 3B**). The barrier disruption was therefore sufficient to meet the criteria defined for an inflamed model (>25%). Notably, however, the barrier integrity of the Caco-2 cells in the presence of THP-1 cells activated by 100 ng/mL LPS + IFN- γ did not re-establish to the levels of the Caco-2 monoculture after 48 h ($655.8 \pm 287.80 \Omega \cdot \text{cm}^2$, $p < 0.0001$), suggesting that excessive cell death had occurred. In contrast, the TEER in all other co-cultures (stable, 1 ng/mL, and 10 ng/mL) had re-established to >85% ($p > 0.05$) of the Caco-2 monoculture after 48 h (**Figure 3B**).

To further investigate cell damage, lactate dehydrogenase (LDH) release was measured, as high levels of THP-1 activation may induce cell death. LDH, a stable cytoplasmic enzyme released when cell membranes are damaged, was quantified by measuring the conversion of NAD to NADH, which absorbs at 490 nm. Low LDH activity ($0.5 \pm 0.09 \text{ A}_{450}$) was observed in the Caco-2 monoculture (**Figure 3C**). No significant increase in LDH was observed in the stable co-culture or the 1 ng/mL inflamed co-culture. Activation of inflammation in the THP-1 cells using 10 ng/mL LPS + IFN- γ resulted in large increases (192.6%) in LDH activity. This activity was further increased in the presence of 100 ng/mL LPS + IFN- γ -stimulated THP-1 cells. Such activation resulted in a 373.2% increase in LDH activity, indicating the occurrence of necrotic cell death (**Figure 3C**). These LDH readings confirm that excessive cell death occurred in Caco-2 intestinal cells during high activation of THP-1 macrophages. In contrast, low doses of LPS + IFN- γ (1

ng/mL) did not induce a sufficiently strong reaction to meet the criteria defined for an inflamed intestine. A concentration of 10 ng/mL LPS + IFN- γ was therefore selected as the optimal concentration to activate THP-1 cells and all subsequent experiments were carried out using this dose.

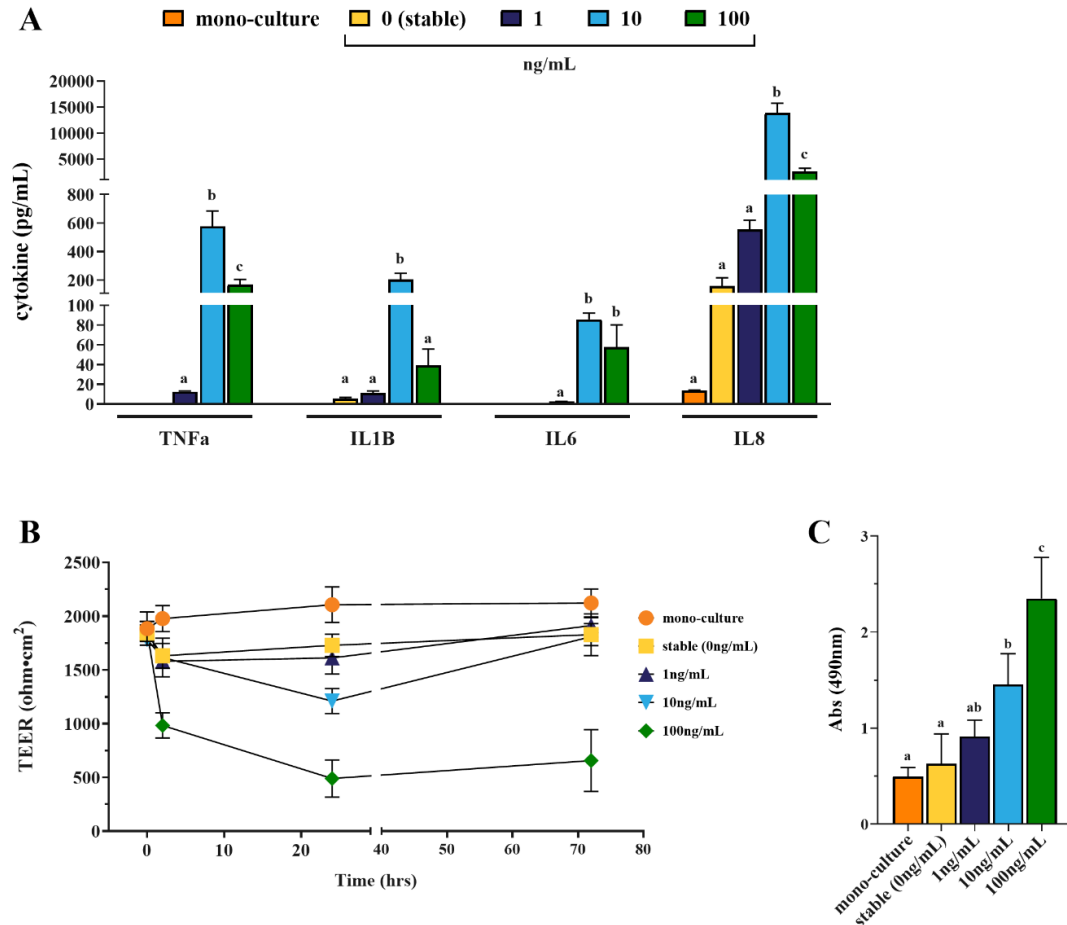


Figure 3: Optimisation of *in vitro* model A) Release of pro-inflammatory cytokines (TNF α , IL-1 β , IL-6, and IL-8) after 24h Caco-2 monoculture (orange), stable (yellow) or inflamed THP-1 co-culture following treatment with 1 (navy), 10 (blue), and 100 (green) ng/mL of both LPS and IFN γ , as measured by Milliplex HSTCMAG-28SK high sensitivity panel. Values for each cytokine with common letter= $p > 0.05$, calculated using Tukey's multiple comparisons test. B) Change in transepithelial electrical resistance after 24h inflammation followed by 48h recovery period (total 72 h) as measured using an EVOM2 Ohm-meter. Results were corrected for the blank and multiplied by the membrane filter area (1.12 cm²) to obtain the final results in Ohm per cm² ($\Omega \cdot \text{cm}^2$). C) LDH release after 24h Caco-2 monoculture, stable, and inflamed co-culture, using LDH detection kit and measuring spectrophotometrically at 490 nm. Values with common letter= $p > 0.05$, calculated using Tukey's multiple comparisons test.

3.3. Influence of human milk oligosaccharides on cytokine/ chemokine release

3.3.1 *Identifying the optimal combination of HMO for amelioration of inflammation*

Following optimization and establishment of the inflamed model (employing 10 ng/mL LPS + IFN- γ), the anti-inflammatory effects of various HMO mixes were assessed and compared to the anti-inflammatory properties of the same HMO applied individually. Breastmilk-derived HMOs (BM-HMO) were used as a positive control. As a negative control, DMEM supplemented with glucose at an equivalent concentration to HMO was used. HMOs were applied at the maximum approved concentration as per the European Union List of authorized novel foods, as outlined in **Table 1**. The quantification of IL-8 using Biolegend ELISA is summarized in **Figure 4** and shows that the levels of IL-8 released into Caco-2 supernatants varied significantly depending on the number and specific structure of the HMO(s) applied. Levels of IL-8 expression in the glucose control were $14,059.8 \pm 3615.99$ pg/mL, while in the BM-HMO-treated cells, levels were 6432.3 ± 555.68 pg/mL ($p = 0.0036$). The majority of the HMO treatments assessed demonstrated a capacity to modulate cytokine expression in the inflamed model. Following disruption of immune homeostasis, it was found that all HMO treatments, aside from LNnT (at 0.6 g/L) ($p < 0.0001$), resulted in similar IL-8 levels to HMOs isolated from breastmilk (BM-HMO at 3.8 g/L). Notably, the lowest concentrations of IL-8 were detected in Caco-2 supernatants where a 2-HMO combination of the most prevalent fucosylated (2'-FL at 1.2 g/L) and sialylated (6'-SL at 0.4 g/L) HMO had been applied to the Caco-2 cells. Treatment with this HMO combination reduced the levels

of pro-inflammatory IL-8 by $63.7 \pm 17.21\%$ when compared to the levels detected in the glucose control ($p = 0.0001$). Similar IL-8 levels were detected following application of a 2-HMO combination of 2'-FL and LNFP-1 (7488.5 pg/mL) as well as a treatment with a 3-HMO blend (8556.5 pg/mL). An increase in the number of HMO structures (4-HMO or 5-HMO) did not significantly reduce IL-8 levels when compared to those detected in the glucose control.

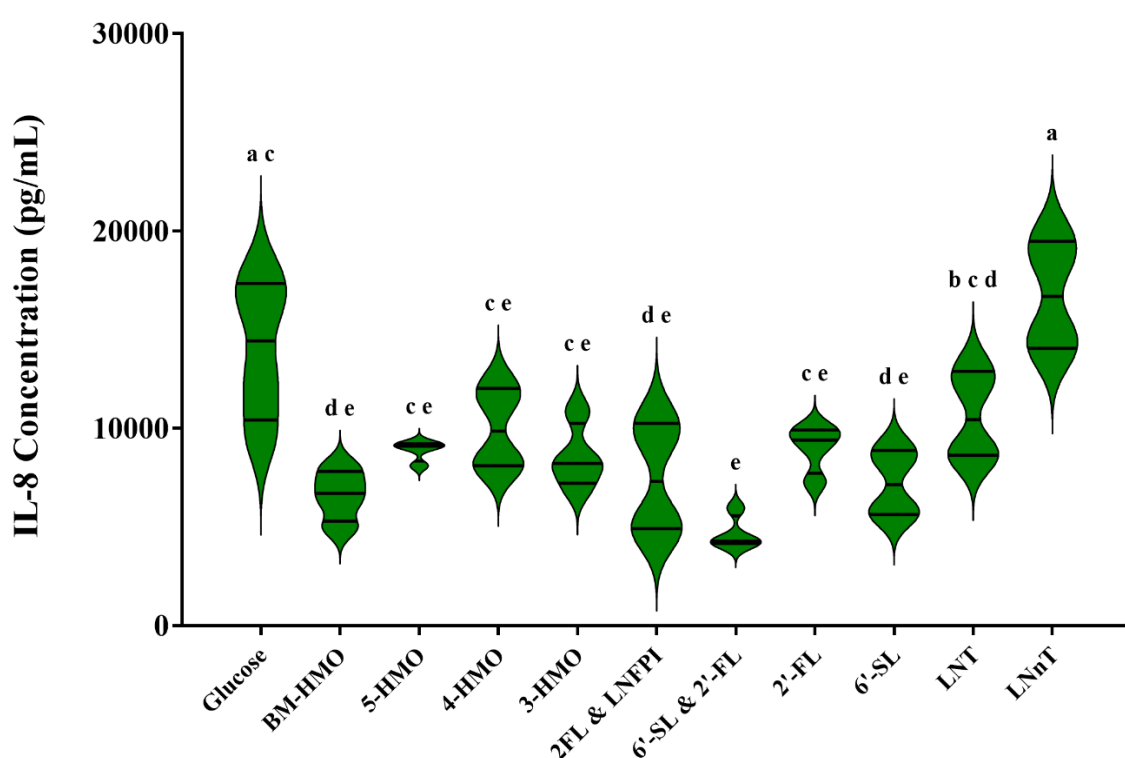


Figure 4: Release of pro-inflammatory IL-8 after 24h Caco-2 THP-1 inflamed co-culture induced using 10ng/mL of both LPS and IFN γ , following treatment with HMO. Concentrations of HMO used in treatments can be found in Table 1. BM-HMO= breastmilk-derived HMO, 2'-FL= 2'-fucosyllactose, LNFP-I= Lacto-*N*-fucopentaose-I, 6'-SL= 6'-sialyllactose, LNT= Lacto-*N*-tetraose, LNnT= Lacto-*N*-neotetraose. Values with common letter= $p > 0.05$, calculated using Tukey's multiple comparisons test.

3.3.2 Identifying the optimal HMO concentration for amelioration of inflammation:

To determine whether the immunomodulatory effects of these HMO ingredients were concentration-dependent, the co-culture experiments were repeated using various concentrations of 2'-FL (0.6–2.4 g/L) and 6'-SL (0.2–0.8 g/L). These were applied individually, in combination with each other (2'-FL + 6'-SL), and in combination with LNFP-I, LNT, and LNnT (5-HMO) (**Table 2**). The Caco-2 transwells were co-exposed to the activated THP-1 cells and the HMO ingredients for 24 h before collecting the supernatants from the apical compartment and measuring cytokine levels (IL-1 β , IL-6, IL-8, TNF- α , IFN γ , and IL-10) using Magpix ELISA. Anti-inflammatory IL-10 was only detected in Caco-2 supernatants where the inflamed intestinal cells had been exposed to standard and high doses of BM-HMO, glucose, 2'-FL + 6'-SL, or 6'-SL (**Figure 5**). Pro-inflammatory IL-1 β , IL-6, IL-8, TNF- α , and IFN γ were detected in all Caco-2 supernatants (**Figure 5**). Concentration dependency assays indicated that, in general, the protective effects of HMO treatments lessened at lower doses, but no added benefits were observed when higher doses were applied. This was noted in particular for expression of pro-inflammatory IL-8. Across all treatment groups, higher levels of IL-8 were detected following exposure of Caco-2 cells to low doses (0.5 $\times$ regulatory doses) of HMO when compared to standard and high doses (1 $\times$ and 2 $\times$ regulatory doses, respectively) ($p < 0.05$). No significant difference in IL-8 expression was observed when comparing standard and high doses of HMO ($p > 0.05$). Magpix analysis of inflammatory cytokines indicated that 2'-FL, individually and in combination with 6'-SL, reduced the expression of pro-inflammatory TNF α when compared to the glucose control ($p < 0.0001$)

across all doses. Similar findings were observed for expression of IL-8, IL-1 β , IL-6 and IFN γ where treatment with 2'-FL, 6'-SL, and combinations thereof resulted in reduced levels of inflammatory cytokines compared to treatment with glucose ($p < 0.05$). These HMO treatments (2'-FL, 6'-SL, and 2'-FL + 6'-SL) resulted in similar inflammatory phenotypes to breastmilk-derived HMO (BM-HMO) (Figure 5).

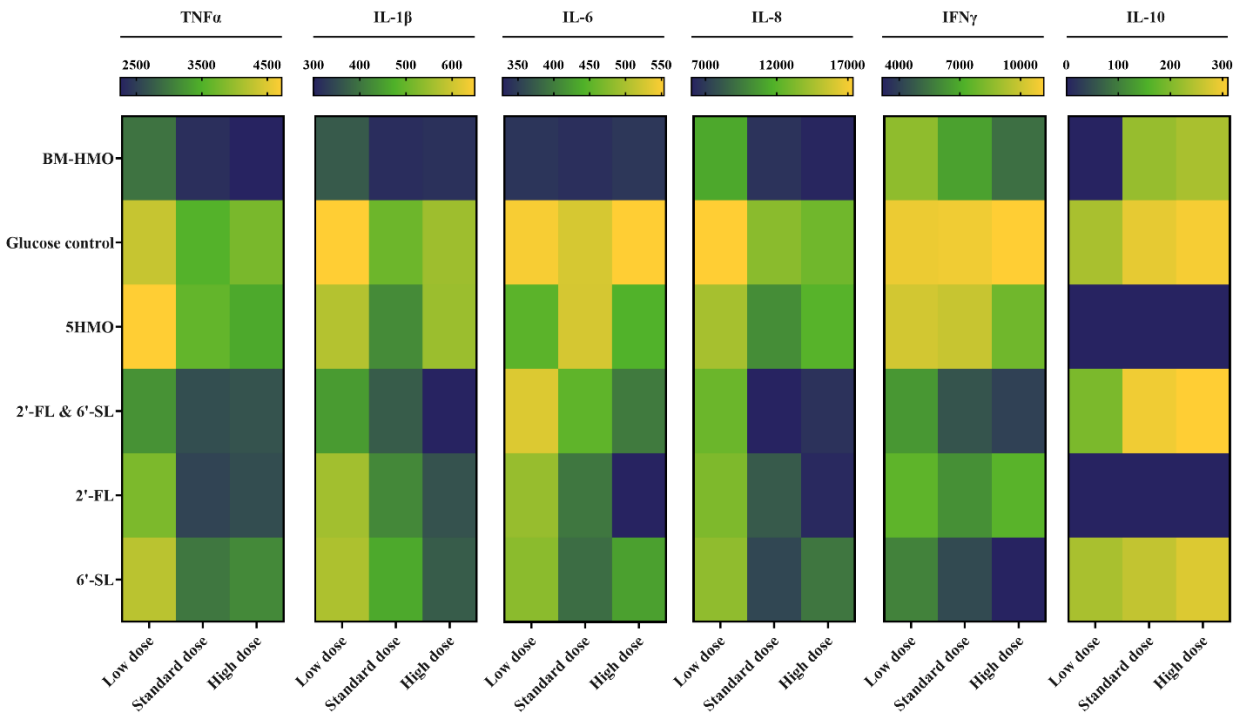


Figure 5: Heatmap summarising release of pro- and anti-inflammatory cytokines (TNF α , IL-1 β , IL-6, IL-8, IFN γ , and IL-10) following 24hr HMO treatment of inflamed Caco-2 THP-1 co-culture (induced using 10ng/mL of both LPS and IFN γ). Cytokine release was measured using Milliplex HSTCMAG-28SK high sensitivity panel. Lower concentrations of cytokines are shown in navy, while higher levels of cytokine release are depicted in yellow as per the scale indicated on top of graph. HMOs were applied at low (0.5x regulatory concentration, navy), standard (1x regulatory concentration, green), and high (2x regulatory concentration, orange) doses as per Table 3. BM-HMO= breastmilk-derived HMO, 2'-FL= 2'-fucosyllactose, 6'-SL= 6'-sialyllactose.

3.3.2 Assessing the optimal ratio of HMO for amelioration of inflammation:

To further understand the potential synergistic action between 2'-FL and 6'-SL in reducing intestinal inflammatory markers, these two HMOs were applied to the inflammatory model at various concentrations (0.2 g/L to 1.2 g/L) and various ratios (from 2:1 to 6:1) (**Table 3**). Levels of inflammatory cytokines, as measured by multiplex ELISA, are summarized in **Table 4**. Treatment with BM-HMO reduced expression of all cytokines measured (IL-1 β , IL-6, IL-8, and TNF- α) when compared to treatment with DMEM supplemented with glucose ($p < 0.05$). Although the ratio of 2'-FL to 6'-SL can fluctuate based on the mother's lactation stage and individual variation (Soyyilmaz et al., 2021), we found that application of these HMOs at ratios similar to those in colostrum and transitional milk (between 2:1 and 4:1) was superior to treatment with a 6:1 ratio of 2'-FL: 6'-SL (representative of mature and late-stage milk). Interestingly, application of 0.6 g/L 2'-FL and 0.3 g/L 6'-SL (2:1 ratio, total = 0.9 g/L) significantly reduced expression of IL-1 β and IL-8 when compared to the glucose control ($p < 0.05$), while application of 1.2 g/L 2'-FL and 0.2 g/L 6'-SL (6:1 ratio, total = 0.9 g/L) had no significant effect on the expression of these cytokines. Anti-inflammatory IL-10 was detected across all treatments of the 2'-FL and 6'-SL combination. The expression of IL-10 was not dependent on the ratio applied, with similar levels detected across all treatment groups ($p > 0.05$).

Table 4: Release of pro-inflammatory cytokines (TNF α , IL-1 β , IL-6, IL-8) and TEER (ohm·cm²) following 24hr treatment of HMO

Treatment	Concentration (mg/mL)			Ratio	Cytokine concentration (pg/mL)								TEER (ohm·cm²)	
	2'-FL	6'-SL	Total	2'-FL: 6'-SL	TNFα		IL-1B		IL-6		IL-8			
BM-HMO			1.6		2295	± 289.7 ^c	338.2	± 33.78 ^{de}	350.3	± 44.75 ^c	7207.0	± 1738.22 ^d	1002.7	± 151.48 ^a
Glucose			1.6		4165	± 716.3 ^a	528.9	± 76.28 ^a	515.4	± 37.65 ^a	17907.6	± 4091.36 ^{ab}	781.7	± 95.09 ^b
2'-FL, 6'-SL combination	1.2	0.4	1.6	3:1	2423	± 366.4 ^c	323.5	± 60.07 ^e	425.7	± 66.35 ^{ac}	7197.0	± 1198.30 ^d	957.7	± 125.59 ^{ab}
	1.2	0.3	1.5	4:1	2397	± 237.6 ^{bc}	336.7	± 29.48 ^{ce}	489.4	± 57.13 ^{ac}	8874.7	± 3159.06 ^{cd}	965.2	± 117.83 ^{ab}
	1.2	0.2	1.4	6:1	2401	± 601.89 ^{bc}	412.2	± 35.81 ^{ae}	492.1	± 43.63 ^{ac}	10353.0	± 3150.69 ^{bd}	855.5	± 131.16 ^{ab}
	0.6	0.3	0.9	2:1	2769	± 564.30 ^{bc}	321.1	± 88.95 ^{de}	491.1	± 92.87 ^{ab}	8404.4	± 1826.54 ^{cd}	920.5	± 89.39 ^{ab}
	0.6	0.2	0.8	3:1	2647	± 618.41 ^{bc}	378.8	± 68.18 ^{be}	552.1	± 21.88 ^a	13439.3	± 4449.65 ^{ad}	880.5	± 70.40 ^{ab}
2'-FL	1.2		1.2		2389	± 350.03 ^c	376	± 88.39 ^{abc}	464.7	± 113.93 ^{ab}	10791.7	± 3017.57 ^{bd}	955.7	± 94.31 ^{ab}
	0.6		0.6		2979	± 927.92 ^{bc}	487	± 91.04 ^{be}	481.3	± 47.50 ^{ab}	14515.3	± 3097.11 ^{abc}	886.5	± 64.35 ^{ab}
6'-SL		0.4	0.4		2741	± 464.5 ^{bc}	445.3	± 55.64 ^{abcd}	384.1	± 39.64 ^a	10542.2	± 2908.20 ^{bd}	911.5	± 101.89 ^{ab}
		0.3	0.3		2460	± 501.64 ^c	503.5	± 138.80 ^{ab}	496.7	± 26.59 ^b	16151.1	± 3327.28 ^{abc}	871.2	± 84.20 ^{ab}
		0.2	0.2		3564	± 993.39 ^{ab}	512.6	± 108.16 ^{ae}	461.1	± 53.15 ^{ab}	18472.2	± 8935.83 ^a	756.2	± 77.08 ^{ab}

Values within a column with common letter= $p > 0.05$, calculated using Tukey's multiple comparisons test
 Analysis was performed using technical triplicate data from biological triplicate experiments.

4. Discussion

In the realm of neonatal health, inflammatory disorders of the intestine present significant challenges, necessitating extensive research to understand their pathophysiology and develop prevention strategies (Blum et al., 2024). Such disorders often arise from chronic inflammation, which can lead to intestinal mucosal barrier dysfunction, marked by diminished gut mucus thickness and heightened intestinal permeability (Managlia et al., 2022). This inflammation can be triggered by various perinatal insults, including bacterial colonization, excessive expression of toll-like receptor 4 (TLR4), and hypoxic stress (Mihi & Good, 2019). It is widely recognized that human milk is the optimal nutrition source for the first six months of life, offering protection against various forms of neonatal intestinal inflammation (Jeurink et al., 2013). Preterm infants fed human milk have a substantially lower risk of developing severe intestinal issues than those fed formula (Altobelli et al., 2020). Research from *in vitro* tissue models, *in vivo* animal studies, and human mother-infant cohort studies highlights the protective role of HMOs, which are abundant in human milk but absent in formula (Bode, 2018). Our research utilizes an *in vitro* co-culture model of intestinal epithelial Caco-2 cells and THP-1 macrophages to explore whether synthetically-produced HMO blends can mimic the protective effects of breastmilk against inflammation-associated with intestinal disorders.

Following the approach of Kampfer *et al.*, we used *E. coli*-derived LPS as an inflammatory stimulus, directly applied to THP-1 cells to elicit an inflammation-like response (Kämpfer et al., 2017). Caco-2 cells express the LPS-binding receptor CD14 and Toll-like Receptor (TLR)-2 but lack TLR4, essential for LPS-induced signalling (Böcker et al., 2003). This co-culture

model mimics bacterial antigens crossing the intestinal epithelial cell barrier, activating gut-associated lymphoid tissue cells. Priming THP-1 cells with LPS and IFN- γ induced an inflammation-like response, replicating key mechanisms of inflammatory disorders. This Caco-2 inflammation model exhibits hallmarks of inflammatory disorders of the intestine, including a disrupted epithelial barrier, high pro-inflammatory cytokine levels, and cell death (Kandasamy et al., 2014).

Bacterial invasion and the presence of metabolites like LPS in the intestinal mucosa stimulate the production of inflammatory cytokines, including IL-8, IL-1 β , IL-6, IFN- γ , and TNF α . These cytokines further enhance intestinal permeability, perpetuating a cycle of inflammation (Andrews et al., 2018; Candelli et al., 2021). In intestinal inflammation scenarios, IL-8 attracts neutrophils, releasing enzymes and reactive oxygen species that damage tissue. IL-1 β and TNF α increase intestinal permeability, promoting inflammation and cellular apoptosis. Elevated IL-6 levels facilitate T and B cell differentiation and the production of acute-phase proteins, intensifying inflammation. IFN- γ exacerbates inflammation by activating macrophages. IL-10, however, modulates the immune response, balancing defense and tolerance, thereby suppressing excessive inflammation and protecting against tissue damage (Cho et al., 2020; Hunter & De Plaen, 2014; Maheshwari et al., 2014).

This current study was designed to target these pro-inflammatory cytokines to investigate the direct, non-prebiotic effect of blends of 2-HMO, 3-HMO, 4-HMO, and 5-HMO on intestinal epithelial cell responses associated with inflammatory disorders and compared to the anti-inflammatory effects of breastmilk-derived HMO. As expected, the

accumulation of LPS-IFN γ -induced pro-inflammatory cytokines were reduced most significantly by breastmilk-derived HMO. HMOs isolated directly from breast milk may provide superior protection against inflammation compared to a synthetic blend of HMOs because of the greater structural diversity, synergistic effects, and presence of minor HMOs which may provide a more comprehensive anti-inflammatory response than a limited synthetic HMO blend. The breastmilk derived-HMO included in this study may contain up to 200 distinct HMO structures, each with unique functions and interactions. This diversity of oligosaccharides allows for a broader range of biological activities. Synthetic blends, typically limited to a few HMOs cannot replicate this complexity, potentially reducing the protective effect. It has been assumed that blending multiple HMOs can provide a broader range of potential benefits by targeting different pathways involved in inflammation. Remarkably, we show that increasing the range and diversity of HMO did not improve inflammatory signatures in this Caco-2 model. Of the synthetic blends, the highest anti-inflammatory effects were observed in a combination of two HMO: 2'-FL and 6'-SL. These findings align with previous studies which has indicated that specific oligosaccharides such as 2'-FL and 6'-SL, when applied individually, can modulate multiple functions of intestinal epithelial cells, *in vitro*, *in vivo* and *ex vivo* (Holscher et al., 2014; Huang et al., 2021; Kim et al., 2024; Kuntz et al., 2008; Lv et al., 2023; Newburg et al., 2016; Sodhi et al., 2021; Yu et al., 2016). However, in comparison to these studies, we demonstrate that ratio and concentration specific combinations of these two HMO, which represent the most abundant fucosylated and sialylated HMO structures, can offer even greater anti-inflammatory effects by targeting the suppression of a greater multitude of

cytokines. All tested concentrations of 2'-FL and 6'-SL in combination reduced the expression of IL-1 β and TNF- α . While only the higher tested concentrations of 2'-FL (1.2 and 2.4g/L) and 6'-SL (0.4 and 0.8g/L) inhibited LPS induced IL-8 release which are well within the range of 2'-FL and 6'-SL levels found in human milk (2'-FL: 1.65g/L in mature milk to 3.18g/L in early term milk, 6'-SL: 0.3g/L in mature milk to 0.71 in early term milk) and can thus be considered physiologically relevant. Although the ratio of 2'-FL to 6'-SL can fluctuate based on the mother's lactation stage and individual variation (Soyyılmaz et al., 2021), we found that application of these HMO at ratios similar to those in colostrum and transitional milk (between 2:1 and 4:1) were superior to treatment with a 6:1 ratio of 2'-FL: 6'-SL (representative of mature and late-stage milk) (Soyyılmaz et al., 2021). In contrast to prior research, our findings indicate that LNT did not contribute to the reduction of inflammation. Previous studies have reported that LNT can suppress Th1-type responses, which produce cytokines like IFN- γ , IL-2, and TNF- β , all of which play roles in immune and inflammatory reactions (Terrazas et al., 2001). Additionally, research by Farhadihosseinabadi *et al.* demonstrated that LNT promoted a type 2 anti-inflammatory response in a mouse model, where the treated group exhibited higher levels of IL-10, IL-4, and IL-13 compared to the control group (administered PBS) (Farhadihosseinabadi et al., 2020). The discrepancies observed between this study and previous studies demonstrating anti-inflammatory effects, is likely due to the different models applied.

We propose that the increased anti-inflammatory effects observed with the combination of 2'-FL and 6'-SL might be due to their capacity to interact with epithelial cells through distinct but complementary pathways,

thus modulating the immune response. This synergistic blend could exert anti-inflammatory effects in the Caco-2 and THP-1 co-culture model through several mechanisms and receptors associated with immune modulation, primarily involving interactions with pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-containing protein 2 (NOD2), engagement with immune-associated lectins, and reinforcement of barrier integrity. This synergy may improve gut health, enhance immune resilience, and reduce inflammation more effectively than either HMO alone.

The first evidence of intact HMOs in the urine of breastfed infants was reported by Rudloff and colleagues in 1996 (Rudloff et al., 1996), and subsequent studies have shown absorption rates of 1–5% of HMOs from the gastrointestinal tract into the bloodstream (Goehring et al., 2014; Rudloff & Kunz, 2012; Ruhaak et al., 2014). HMOs are known to influence immune cell interactions, promoting balanced inflammatory responses (Rosa et al., 2022). Human lectins (glycan-binding proteins) are particularly relevant as they directly interact with HMOs and other milk glycans. To explore these interactions Noll *et al.* screened the glycan binding potential of C-type lectins and sialic acid-binding immunoglobulin-like lectins (Siglecs) with over 200 human milk glycans (Noll et al., 2016). Their findings showed that 2'-FL and 3-FL bind to dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), while LNT did not (Noll et al., 2016). On the other hand, sialyllactoses (including 6'-SL and 3'-SL) have been shown to bind to sialoadhesin (Siglec-1) and also to Siglec-3, Siglec-5, Siglec-7, Siglec-9, and Siglec-10 (Bhunia et al., 2004; Ha et al., 2020; Koliwer-Brandl et al., 2011; Soares et al., 2021). Studies have also demonstrated that galectins

bind selectively to siallylactoses, although with a significantly higher affinity for 3'-SL when compared with 6'-SL (de Kivit et al., 2013; Kitova et al., 2014).

In addition, the anti-inflammatory effect of the 2'-FL and 6'-SL combination used here could be mediated through interactions with toll-like receptors (TLRs). TLRs act as primary sensors of microbial products and activate signalling pathways through NF- κ B resulting in the modulation of cytokine release (Duan et al., 2022; Kawai & Akira, 2007). Several classes of TLRs with HMO binding specificity have been described in the literature. For example, a study using THP-1 macrophages demonstrated that 2'-FL and 6'-SL (as well as 3-FL and LNnT) suppressed TLR-5 and TLR-7 activation (Cheng et al., 2019). Asakuma *et al.* (2010) showed that 3'-SL, 6'-SL, and 6'-galactosyllactose (6'-GL) influenced both TLR-2 and TLR-4 expression (Asakuma et al., 2010). Anti-inflammatory effects via interactions with TLR4 have also been demonstrated for 2'-FL (He et al., 2016). This is relevant because Gram-negative bacteria initiate mucosal inflammation by binding LPS to TLR-4 on intestinal epithelial cells (IECs) (Vinderola et al., 2005). *In silico* modeling has predicted that 2'-FL and 6'-SL can dock onto TLR-4, thereby inhibiting its signaling (Sodhi et al., 2021). In mouse and piglet models of NEC, 2'-FL and 6'-SL have been shown to reduce inflammation and NEC symptoms, partly by inhibiting TLR-4 signaling, which is implicated in NEC onset (Sodhi et al., 2021). In contrast, pooled goat milk oligosaccharides, inulin, and synthetic fructo- and galacto-oligosaccharides were shown to stimulate immune responses and induce chemokine release through TLR-4 and NF κ B activation. Additionally, 2'-FL has been found to inhibit *E. coli*-induced IL-8 release by down-regulating the LPS receptor

complex component CD14 (He et al., 2016), a mechanism which has also been validated *in vivo*. As CD14 serves as a co-receptor for LPS and is involved in TLR-4 signaling, this may lead to decreased intestinal inflammation following LPS exposure.

Mechanistically, it is also plausible to hypothesise that the observed immunomodulatory effects imparted by this combination of 2'-FL and 6'-SL may be due to these HMOs supporting the maturation of the intestine by increasing barrier integrity and stimulating mucin expression. Although changes in TEER were not significant, the barrier enhancing effects were more pronounced for this HMO combination, especially at higher concentrations. The intestinal epithelium is crucial for gut homeostasis, as its cells and the overlying mucus layer form a barrier separating the lumen from the submucosa and systemic circulation (Takiishi et al., 2017). An impaired intestinal barrier allows luminal contents, including bacteria, to translocate into submucosal tissues, causing inflammation and potentially leading to gastrointestinal and systemic diseases (Nagpal & Yadav, 2017). Barrier impairment is especially concerning in preterm infants as it increases NEC risk. Breastfed infants show a quicker reduction in intestinal permeability compared to formula-fed infants, suggesting that components in breastmilk aid intestinal maturation (Chong et al., 2022; O'Sullivan et al., 2015). *In vitro* studies have demonstrated a capacity for 6'-SL, as well as 2'-FL, to support the development of a mature intestine (Holscher et al., 2017; Holscher et al., 2014). [58,87]. In an animal study, breastmilk-derived HMO fractions increased MUC2 expression and reduced intestinal permeability in a neonatal mouse model of NEC (Wu et al., 2019). HMOs were found to promote mucin expression by acting on chaperone proteins such as protein disulfide

isomerase (Wu et al., 2019). The results of this animal study are in line with findings from an *in vitro* study (Cheng et al., 2020). Here, the effects of HMOs on expression of goblet cell secretory genes in a colorectal cell model were assessed (Cheng et al., 2020). 2'-FL increased MUC2 expression under IL-13 exposure, while 3-FL upregulated both MUC2 and TFF3 expression under TNF α and IL-13 exposure (Cheng et al., 2020). Additionally, sialyllactose has been shown to promote the differentiation and growth of human intestinal epithelial cells, as evidenced by the upregulation of alkaline phosphatase expression (Perdijk et al., 2019). Alkaline phosphatase plays a role in gut barrier maintenance, potentially by deactivating LPS through phosphate removal (Bates et al., 2007).

Overall, these studies confirm that 2'-FL and 6'-SL have a variety of direct effects on epithelial cell functions, particularly concerning bacterial infection and intestinal inflammation. These diverse effects are likely dependent on improvements in barrier function, inhibition of TLR-4, down-regulation of CD14, and decreased activity of NF κ B pathways. Further studies assessing the regulation and activation of genes along these immune-associated pathways are required to fully elucidate the mechanisms through which the beneficial effects of HMO combinations are elicited.

5. Conclusion

This study presents a co-culture model of Caco-2 intestinal epithelial cells and THP-1 macrophages to investigate the anti-inflammatory effects of human milk oligosaccharides (HMOs) against inflammatory disorders of the intestine. Our findings reveal that specific combinations of synthetic HMOs, particularly 2'-fucosyllactose (2'-FL) and 6'-sialyllactose (6'-SL), effectively reduce pro-inflammatory cytokine levels. These results underscore the

potential of targeted HMO blends in infant nutrition to mimic breast milk benefits and prevent excessive inflammation. This study reinforces the concept that structurally different oligosaccharides have distinct biological activities and identifies, for the first time, that certain HMOs, i.e. 6'-SL, and 2'-FL, modulate human epithelial cell responses related to inflammatory pathologies. Taken together, our results indicate that HMOs including 2'-FL and 6'-SL could be applied as functional food ingredients for maintaining intestinal function and preventing excessive inflammation. It's important to note that the specific interactions and outcomes can depend on the individual HMO structures, concentrations, and the experimental conditions. Ongoing research is continually refining our understanding of how different HMOs work individually and in combination to influence inflammation and other aspects of immune function.

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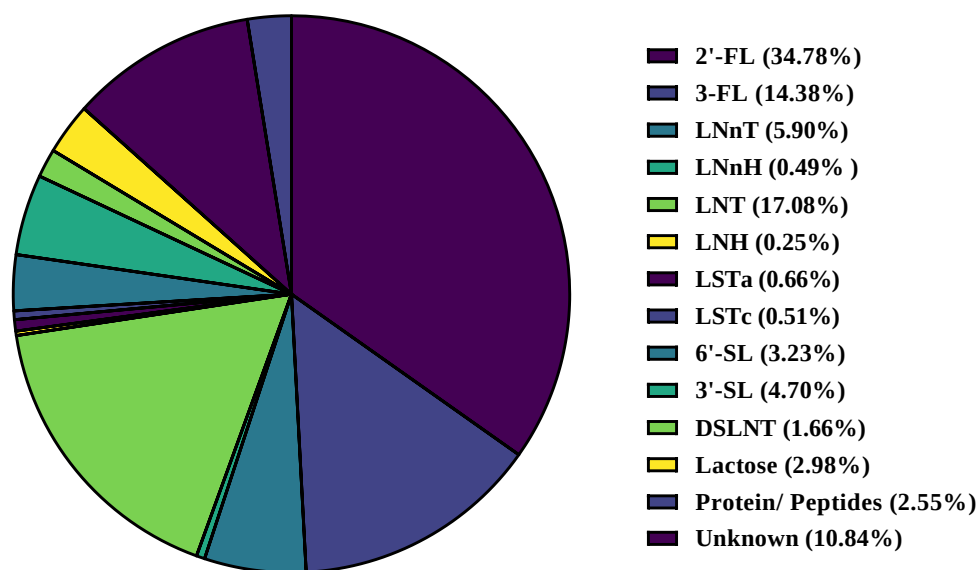
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Supplementary Materials



Supplementary Figure 1: Pie chart showing the relative abundance of the major oligosaccharides in breastmilk-derived HMO detected via HPAEC-PAD analysis by comparing to the following external HMO standards: lactose, 3-fucosyllactose (3-FL), 2'-fucosyllactose (2'-FL), lacto-*N*-neotetraose (LNnT), lacto-*N*-neohexaose (LNnH), lacto-*N*-tetraose (LNT), lacto-*N*-hexaose (LNH), sialyllacto-*N*-tetraose a (LSTa), sialyllacto-*N*-tetraose a (LSTc), 6'-sialyllactose (6'-SL), 3'-sialyllactose (3'-SL), disialyllacto-*N*-tetraose (DSLNT). Data represents average concentrations of technical duplicate data from biological triplicate experiments

CHAPTER V

Human Milk Oligosaccharide-Sharing by a Consortium of Infant Derived *Bifidobacterium* species

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Acknowledgements Clodagh Walsh designed the experimental model, performed growth assays and data analysis. Helen Slattery performed HPLC oligosaccharide analysis. Jonathan Lane, Douwe van Sinderen and Rita Hickey contributed to experimental design.

Abstract

Bifidobacteria are associated with a host of health benefits and are typically dominant in the gut microbiota of healthy, breast-fed infants. A key adaptation, facilitating the establishment of these species, is their ability to consume particular sugars, known as human milk oligosaccharides (HMOs), which are abundantly found in breastmilk. In the current study, we aimed to characterise the co-operative metabolism of four commercial infant-derived bifidobacteria (*Bifidobacterium bifidum* R0071, *Bifidobacterium breve* M-16V, *Bifidobacterium infantis* R0033, and *Bifidobacterium infantis* M-63) when grown on HMOs. Three different HMO substrates (2'-fucosyllactose alone and oligosaccharides isolated from human milk representing non-secretor and secretor status) were employed. The four-strain combination resulted in increased bifidobacterial numbers (>21%) in comparison to single strain cultivation. The relative abundance of *B. breve* increased by >30% during co-cultivation with the other strains despite demonstrating limited ability to assimilate HMO in monoculture. HPLC analysis revealed strain-level variations in HMO consumption. Metabolomics confirmed the production of formate, acetate, 1,2-propanediol, and lactate with an overall increase in such metabolites during co-cultivation. These results support the concept of positive co-operation between multiple bifidobacterial strains during HMO utilisation which may result in higher cell numbers and a potentially healthier balance of metabolites.

1. Introduction

Establishment of the infant gut microflora is a highly complex process, with feeding regime profoundly impacting on the development of neonate's microbial gut ecology (Ho et al., 2018; Timmerman et al., 2017; van den Elsen et al., 2019). Human milk contains a high concentration of indigestible carbohydrates, known as human milk oligosaccharides (HMOs), which can reach the large intestine in an intact form, where they can act as growth substrates for beneficial bacteria (Barile & Rastall, 2013). To date, it is estimated that over 200 different HMO structures exist, with overall levels in human milk varying from 5 to 23 g/L. All HMOs consist of a lactose backbone which is decorated (see below) and/or elongated into a variety of oligomeric structures by the addition of β 1-3- or β 1-6-linked lacto-*N*-biose (Gal β 1-3GlcNAc-, type 1 chain) or *N*-acetylglucosamine (Gal β 1-4GlcNAc-, type 2 chain) (Bode, 2012; Plaza-Díaz et al., 2018). HMO decoration is achieved by the addition of fucose residues (*e.g.*, 2'-fucosyllactose [2'-FL] and 3-fucosyllactose [3-FL]), or *N*-acetylneuraminic acid residues [NeuAc- also known as sialic acid] (*e.g.*, 3'-sialyllactose [3'-SL] and 6'-sialyllactose [6'-SL]) (Bode, 2012; Plaza-Díaz et al., 2018). Variability in the chemical and structural conformations of HMOs is biologically relevant (Smilowitz et al., 2014).

These glycans serve as selective regulators of infant gut microbiota composition, and facilitate the development of a *Bifidobacterium*-dominant microbiota in breast-fed infants (Rodriguez & Martiny, 2020; Thomson et al., 2018). A high level of bifidobacteria in the gut microbiota of infants is believed to contribute to enhanced development of the immature gut immune system, and is associated with reduced levels of intestinal infections and

diarrhea (Mitsuoka, 1990; Turrone et al., 2020). Due to their purported health-promoting effects, bifidobacteria have been incorporated into many functional foods and supplements as probiotic ingredients (Di Gioia et al., 2014; Sarkar & Mandal, 2016). Many members of the genus *Bifidobacterium* are known to dedicate a relatively large proportion of their genome to carbohydrate utilization, reflecting their adaptation to metabolism of host-produced and/or host-ingested/dietary carbohydrates. Some of these genomes, particularly infant bifidobacterial species, display a clear adaptation to the nursing period when the diet of their host may be solely composed of (HMO-rich) breast milk (Sela et al., 2008; Turrone et al., 2010; Underwood et al., 2015). One of the reasons as to why certain bifidobacterial species are such abundant and prevalent members of the (early) gut microbiota is due to their remarkable capacity to metabolize and consume (specific) HMOs. *In vitro* growth experiments have revealed species-dependent utilization of individual HMOs among *Bifidobacterium* (LoCascio et al., 2007; Locascio et al., 2009). These studies have enabled detailed profiling of (bifido)bacterial consumption of individual HMOs and demonstrate specific and preferential oligosaccharide consumption by particular bifidobacterial strains (Asakuma et al., 2011; Marcobal et al., 2010; Ruiz-Moyano et al., 2013). However, in order to fully appreciate the role that HMOs play in the creation of a *Bifidobacterium* dominant microbiota, consideration must be given to the cross-feeding relationships that exist between members of the infant microbiota (Milani et al., 2015). A key determinant of microbial dynamics in the gut microbiota is represented by nutrient competition and sharing. In the current study, we performed co-cultivation experiments involving four bifidobacterial strains when grown on HMO substrates to elucidate if, and to

what extent, trophic interactions prevail among bifidobacteria when present in a competitive environment. Parameters intrinsic to efficient HMO metabolism (ability to support microbial growth, utility as substrates for fermentation, and production of organic acid metabolites) were measured to determine the impact of this syntrophic/competitive relationship. Unravelling HMO assimilation phenotypes and understanding the oligosaccharide structural complexity required to enrich specific beneficial bacterial communities allows for the development of targeted and personalised HMO-containing probiotic products with well-defined microbial, metabolic, and infant health outcomes.

2. Results

2.1. *Bifidobacterium* strains display divergent and strain-specific growth phenotypes, with enhanced growth performance during co-cultivation

Growth profiles of *B. bifidum* Rosell[®]-71 (R0071), *B. infantis* Rosell[®]-33 (R0033), *B. breve* M-16V, and *B. infantis* M-63 cultivated in mono-culture on HMO-supplemented media were evaluated and compared to the growth profiles of the strains when grown in a four strain co-culture on the same carbohydrate sources (**Figure. 1**). The same experiments were performed on MRS without any carbohydrate source, revealing, as expected, an absence of appreciable growth (showing that carbohydrate free MRS [cfMRS] is unable to support bacterial growth above an OD_{600nm} of 0.1). HMO was successfully separated and purified from pooled breast milk by gel-filtration chromatography. Preliminary screenings indicated that, during size exclusion on Bio-Gel polyacrylamide beads, lactose and 2'-FL tended to fractionate together in the same eluates. Here, this apparent hindrance was exploited in order to generate multiple HMO powders which mimicked physiologically

relevant oligosaccharide blends. All fractions with high levels of lactose and 2'-FL were eliminated from the final HMO-enriched blend to give a powder more representative of non-secretor HMO (designated NS-HMO). Although the most abundant α 1,2-fucosylated HMO was eliminated from the powder, the preparation contained other H-antigen containing HMOs such as difucosyllactose (DFL), lacto-*N*-fucopentaose I (LNFP I), and lacto-*N*-difucohexaose I (LNDFH I) (**Supplementary Figure S1**). The 2'-FL which had been eliminated during the size exclusion process was then resubstituted to give a powder representative of secretor HMOs (designated S-HMO) (**Figure S1**). In total, therefore, three HMO substrates were assessed in this study: 2'-FL (at 1.2 g/L), NS-HMO (at 3.8 g/L), and S-HMO (at 5 g/L) (compositions of the HMO powders are outlined in **Figure S1**).

Most notably, significant growth differences were observed between the two *B. infantis* strains (**Figure 1**) despite these strains showing similar growth in MRS media with glucose and lactose as sole carbohydrate sources (data not shown). When grown on 2'-FL, strain M-63 demonstrated a significantly higher maximum OD₆₀₀ ($p = 0.0004$) and higher growth rate during the exponential phase ($p = <0.0001$) of growth, when compared to strain R0033 (**Figure 1**). Similar findings were noted during growth on NS-HMO (**Figure 1**). Although R0033 and M-63 both grew vigorously on S-HMO (reaching final OD₆₀₀ values of 0.81 and 0.99, respectively), the observed growth rate of M-63 during the exponential phase was significantly higher than that of R0033 ($p = 0.0023$) (**Figure 1A**). M-63, however, did display comparable growth profiles to R0071. *B. bifidum* R0071 reached a maximum OD₆₀₀ of 0.81 and 0.98 on NS-HMO and S-HMO, respectively (**Figure 1**).

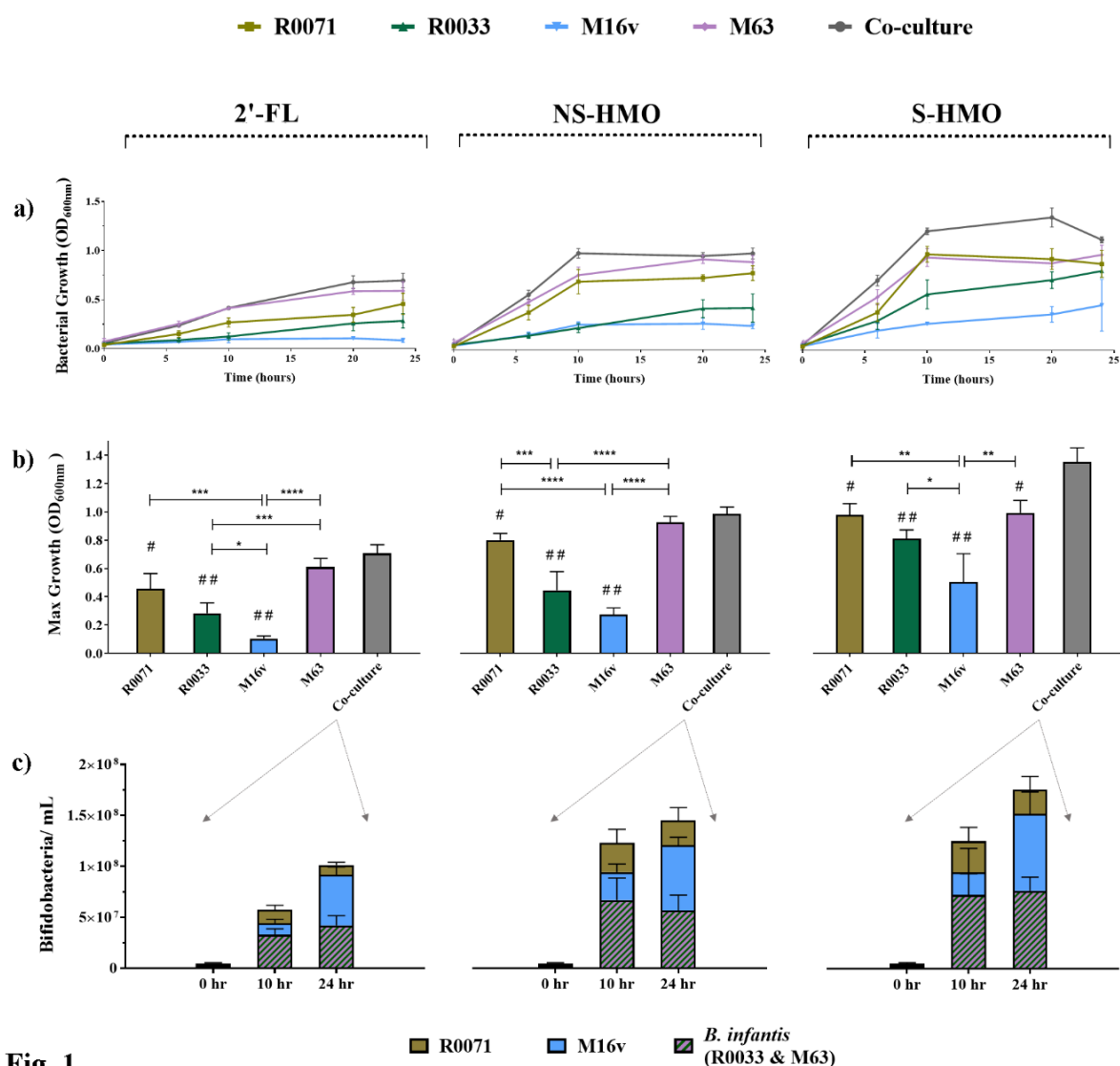


Fig. 1

Figure 1: Growth profiles of bifidobacterial strains as mono-cultures and as a four strain co-culture with the following HMO as the sole carbon source: 2'-FL [1.2g/L], model of NS-HMO [3.8g/L] and model of S-HMO [5g/L]. **a)** Growth of strains in mono- and co-culture at 0 hr, 6 hr, 10 hr, 20 hr and 24hr. **b)** Maximum levels of growth reached by the strains in mono- and co-culture **c)** Growth (bifidobacteria/mL) of subspecies within the four strain co-culture at 0 hr, 10 hr and 24 hr. Strains within co-culture were quantified using qPCR with primers targeting the 16s gene region of each subspecies. OD600nm= Optical density at 600nm. R0071= *Bifidobacterium bifidum* R0071, R0033= *Bifidobacterium infantis* R0033, M-16V= *Bifidobacterium breve* M-16V, M-63= *Bifidobacterium infantis* M-63. Analysis was calculated using technical triplicate data from biological triplicate experiments and data are means +/- SD. Univariate analysis of variance (ANOVA) and post-hoc Tukey tests were performed to determine the significant differences between the groups (# = $p < 0.05$, ## = $p < 0.001$ versus co-culture) (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ between other strain combinations).

B. breve M-16V did not exhibit any significant growth on 2'-FL (Figure 1) yet reached an OD₆₀₀ of 0.27 and 0.51 when grown on NS-HMO and S-HMO, respectively (**Figure 1B**). Co-cultivation of this strain with R0071, R0033, and M-63 on 2'-FL ($p < 0.0001$), NS-HMO ($p = 0.0033$) or S-HMO ($p = 0.0002$) resulted in significant increases of M-16V cell numbers when compared to its growth in mono-culture (**Figure 1C**). Overall, co-cultivation of all four *Bifidobacterium* strains together on S-HMO resulted in significantly higher cell numbers when compared to single strain cultivations (Figure 1B). During growth on S-HMO co-cultivation resulted in a 27.7% increase in cell numbers when compared to the highest mono-culture fermentation of R0071 ($p = 0.0096$) (**Figure 1B**). Quantitative PCR analysis targeting the 16S rRNA region allowed for quantification of cell numbers of each species within the co-culture, but did not distinguish between the two *B. infantis* strains. Nonetheless, qPCR analysis provided insights into the microbial dynamics in the co-culture system and revealed significant shifts in microbial distribution across the fermentation period. Despite significant increases in bacterial cell numbers, no change in the relative abundance of species within the co-culture occurred from 0 to 10 hours. At the initiation of fermentation on S-HMO the co-culture contained $1.23 (\pm 0.41) \times 10^6$ bifidobacteria/ mL *B. bifidum*, $1.38 (\pm 0.39) \times 10^6$ bifidobacteria/ mL *B. breve* and $2.61 (\pm 0.46) \times 10^6$ bifidobacteria/ mL *B. infantis*. No significant change had occurred in this population distribution by late exponential phase (10 hours) with each species within the co-culture displaying similar growth rates (μ) and doubling times (T_d). During this period (0 to 10 hours) *B. bifidum*, *B. breve*, and *B. infantis* grew at a rate of 0.32 ± 0.01 , 0.3 ± 0.02 , and 0.33 ± 0.04 hour⁻¹, respectively. However, between exponential (10 hours) and stationary

phase (24 hours), significant shifts in microbial distribution were observed. While bacterial cell numbers of *B. bifidum* ($p= 0.8476$) and *B. infantis* ($p= 0.9419$) remained constant between 10 and 24 hours fermentation, growth of *B. breve* ($p= 0.0010$) persisted. Therefore, after 24 hours growth on S-HMO, the co-culture contained $2.63 (\pm 0.95) \times 10^7$ bifidobacteria/ mL *B. bifidum*, $7.57 (\pm 2.14) \times 10^7$ bifidobacteria/ mL *B. breve* and $7.63 (\pm 1.36) \times 10^7$ bifidobacteria/ mL *B. infantis*. qPCR analysis showed an overrepresentation of *B. breve* at 24 hours when compared to bifidobacterial numbers of *B. bifidum* and *B. infantis*.

Further insights into growth parameters of strains during co-cultivation were provided by performing co-culture growth of *B. bifidum* R0071 with a) *B. infantis* R0033 on DFL (**Supplementary Figure S2**) and b) *B. breve* M-16V on 3'-SL (**Supplementary Figure S3**). In both instances, co-cultivation resulted in significantly higher cell numbers in comparison to mono-culture growth of strains on the same carbohydrates. During growth on DFL, single-strain cultures of R0071 and R0033 achieved maximum growth of $2.02 (\pm 0.36) \times 10^8$ CFU/ mL and $1.98 (\pm 0.57) \times 10^8$ CFU/ mL, respectively (**Figure S2**). When these strains were grown in co-culture on the same carbohydrate, the culture contained $3.47 (\pm 0.35) \times 10^8$ CFU/ mL ($p < 0.0001$ versus both mono-culture situations) with *B. bifidum* R0071 representing $22.08 \pm 4.16\%$ of total cell numbers (**Figure S2**).

B. bifidum R0071 was shown to grow well on 3'-SL in mono-culture reaching a viable count of $1.89 (\pm 0.51) \times 10^8$ CFU/ mL after 24 hours incubation. As per its growth on more complex HMO blends, *B. breve* M-16V demonstrated limited ability to grow on 3'-SL in mono-culture (**Figure S3**). However, significant growth of this strain in 3'-SL was observed when

cultured in combination with *B. bifidum* R0071 (**Figure S3**). The co-culture of R0071 and M-16V contained $3.11 (\pm 0.86) \times 10^8$ CFU/ mL after 24 hours growth on 3'-SL ($p < 0.0001$ versus either of the mono-culture situations) with *B. breve* M-16V accounting for $86.89 \pm 4.84\%$ of the total cell number proportion (**Figure S3**).

2.2. Detailed analysis of bifidobacterial degradation of oligosaccharides confirms strain-specific and structurally selective consumption of HMO

Glycoprofiling of bifidobacterial strains using HPLC methods revealed significant variations in oligosaccharide utilization (**Supplementary Figure S4**). Typically, higher levels of HMO consumption were observed in cultures with greater cell numbers. For instance, the numerical dominance of *B. infantis* M-63 over its subspecies counterpart *B. infantis* R0033 was similarly reflected in these strains' glycoprofile patterns. In comparison to M-63, significantly higher oligosaccharide amounts remained in the culture after growth of R0033 in 2'-FL ($p = 0.0093$), NS-HMO ($p < 0.0001$), or S-HMO ($p = 0.0091$) (**Figure S4**). During growth on S-HMO, M-63 consumed 78.8% of the total HMO, while R0033 only assimilated 45.5% of available HMO (**Figure 2A**). HPLC analysis of the culture supernatant showed that the majority of 2'-FL (79.2%), 3-FL (57.4%), 3'-SL (74.1%), 6'-SL (51.9%), LNT (100%) and LNnT (94.2%) was degraded after 10 hours fermentation with M-63 (**Figure 2**). Overall, glycoprofiling of M-63 culture supernatants indicated that LNT and LNnT are preferred substrates for this *B. infantis* strain (**Figure 2C**). On the other hand, R0033 exhibited limited consumption of LNT (30.4%) and LNnT (3.7%), as well as 3'-SL (16.7%) and 6'-SL (15.1%). Instead, the fucosyllactoses served as preferred nutrients for R0033, with 58.1% of 2'-FL and 80.6% of 3-FL hydrolysed by late exponential phase

(**Figure 2D**). Notably, during fermentation on S-HMO, R0033 preferentially consumed 3-FL rather than its predominant structural isomer 2'-FL ($p < 0.0001$). Again, this diverges from the saccharolytic activity observed in *B. infantis* M-63, with this strain preferentially consuming 2'-FL over 3-FL ($p = 0.0058$) (**Figure 2D**). Further structure-specific consumption of HMO isomers was observed for M-63, with significantly higher preference for 3'-SL over 6'-SL ($p < 0.0001$) (**Figure 2B**).

In comparison to M-63, *B. breve* M-16V demonstrated limited utilization of most HMO structures. During growth on S-HMO, M-16V consumed 24.4% of 3'-SL, 18.6% of 6'-SL, 8.9% of 2'-FL, 30.1% of 3-FL, and 8.1% of LNnT (**Figure 2**). LNnT represents the core structure of type 2 HMO, whereas LNT represents the core structure of type 1 HMO. M-16V demonstrated significant consumption of the type 1 HMO LNT (**Figure 2C**). One hundred percent of available LNT was metabolised by M-16V after 10 hours fermentation (**Figure 2C**).

B. bifidum R0071 readily assimilated most HMO structures (**Figure 2 & Figure S4**). As per M-16V consumption of the tetrasaccharides, R0071 preferentially consumed type 1 LNT rather than type 2 LNnT (99.3% consumption vs 80.9%, $p = 0.0023$) (**Figure 2C**). Similarly, R0071 favoured consumption of lacto-*N*-hexaose (LNH) (which harbours the type 1 moiety LNB) over lacto-*N*-neo-hexaose (LNnH) (which harbours the type 2 moiety *N*-acetyllactosamine) ($p < 0.0001$). Moreover, R0071 demonstrated preferential consumption of 3'-SL over 6'-SL (88.1 % consumption vs 42.6 % consumption, $p < 0.0001$) (**Figure 2B**). One of the most notable observations made during oligosaccharide glycoprofiling of *B. bifidum* R0071 was the significant increase in the levels of 3-FL following

fermentation on NS-HMO (increase of 36.76% of 3-FL) and S-HMO (increase of 46.09% of 3-FL) (**Figure 2D**). No other strain combination resulted in increased levels of 3-FL. Increased levels of 3-FL were also observed during growth of *B. bifidum* R0071 with DFL as sole carbohydrate source (**Figure S2**). After 10 hours growth of *B. bifidum* R0071, 68.03% DFL had been consumed which corresponded to the release of 232.1 ± 26.1 mg/L of 3-FL. HPAEC-PAD analysis indicated that this increased level of 3-FL did not persist throughout fermentation, with only 17.3 mg/mL 3-FL remaining after 24 hours growth of *B. bifidum* R0071 (decrease of 92.47% versus 10 hours) (**Figure S2**). During co-cultivation of R0071 with *B. infantis* R0033 on the same DFL-containing media, detectable levels of DFL and 3-FL were found in the culture after 10 hours, but significantly less when compared to mono-culture growth of R0071 (**Figure S2**). After 10 hours co-culture of R0071 and R0033 the concentration of DFL had decreased by 92.01% ($p < 0.0001$ versus R0071 mono-culture) with 21.9 mg/mL of 3-FL detected in the culture ($p < 0.0001$ versus R0071 mono-culture). After 24 hours co-cultivation of R0071 and R0033, neither DFL nor 3-FL were detected in the culture supernatant (**Figure S2**).

Monosaccharide analysis using HPAEC-PAD revealed that large quantities of sialic acid are released during growth of *B. bifidum* strain R0071 on NS-HMO (118.8 mg/L) and S-HMO (134.6 mg/L) (**Supplementary Figure S5**). Significant quantities of sialic acid (24 mg/L in NS-HMO and 30 mg/L in S-HMO) were also detected in the M-63 cell-free supernatant after 10 hours growth (**Figure S5**). The levels of sialic acid produced following fermentation with *B. bifidum* R0071 were in stoichiometric accordance with levels of depleted sialylated HMO. In S-HMO, R0071 assimilated 0.46 ± 0.04

mM sialylated HMO, and released 0.44 ± 0.03 mM sialic acid (molar ratio of depleted sialylated HMO to sialic acid produced = 1.07 ± 0.13). This molar balance of depleted sialylated HMO and production of sialic acid was not observed during M-63 fermentation. In S-HMO, *B. infantis* M63 consumed 0.46 ± 0.04 mM sialylated HMOs but produced only 0.1 ± 0.02 mM sialic acid (molar ratio of depleted sialylated HMOs to sialic acid produced = 4.82 ± 1.03).

Glycoprofiling of *B. bifidum* R0071 and *B. breve* M-16V cultures with 3'-SL as sole carbohydrate source were performed to assess whether the sialic acid released by *B. bifidum* R0071 is utilised by other strains in a co-culture system. In accordance with its growth on more complex HMO blends, M-16V was incapable of utilising 3'-SL (**Figure S3**). Mono-culture growth of R0071 resulted in the consumption of 60.51% 3'-SL at 10 hours and 100% 3'-SL at 24 hours which corresponded to a release of 159.3 mg/mL (10 hours) and 234.6 mg/mL (24 hours) of sialic acid (**Figure S3**). Co-cultivation of the two strains resulted in similar depletions of 3'-SL (69.91% at 10 hours, 100% at 24 hours) but significantly lower amounts of sialic acid (41.7 mg/mL at 10 hours, 0 mg/mL at 24 hours) (**Figure S3**).

Across all strains and carbohydrate combinations assessed in this study, levels of lactose after 10 hours growth were below the limit of detection. All residual lactose found in the HMO powders at initiation of fermentation (approximately 130 mg/L) and any lactose released from the breakdown of HMO into its constituent building blocks was consumed by the strain(s) by 10 hours.

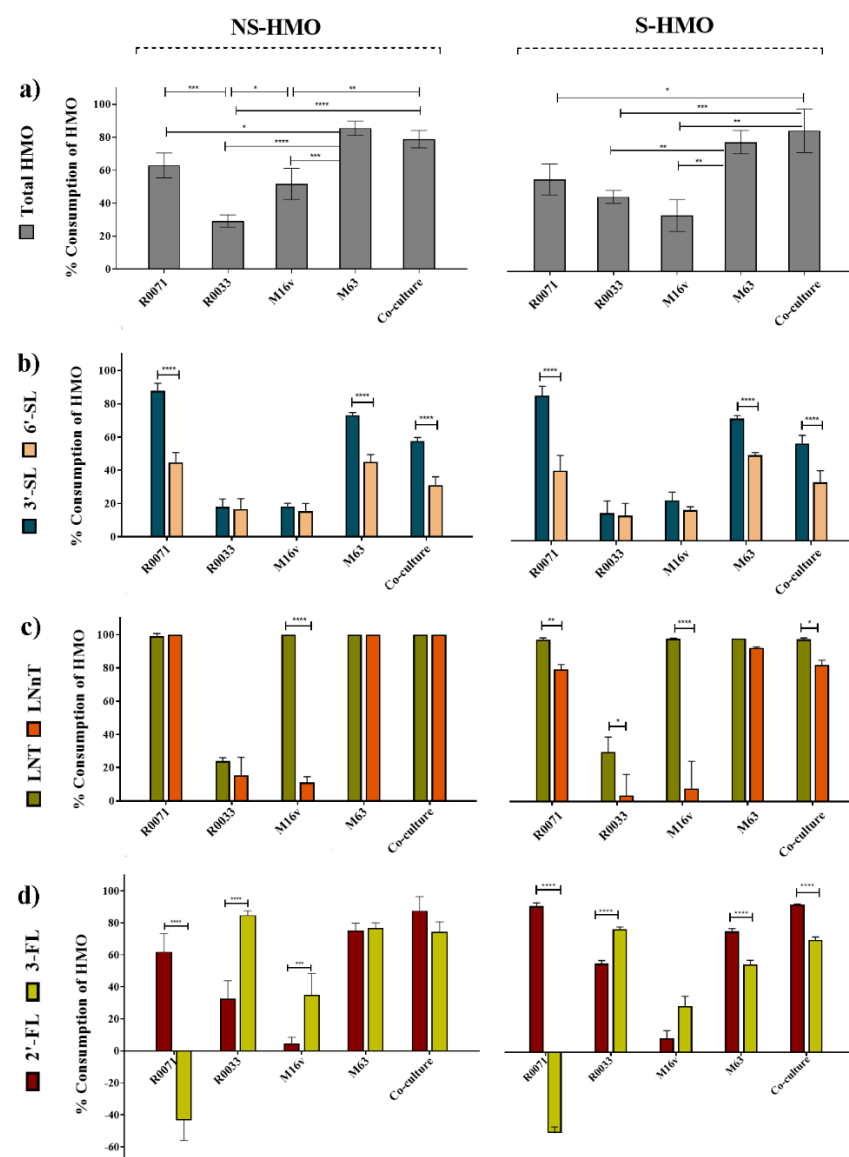


Fig. 2

Figure 2: Percentage consumption of oligosaccharides after 10h of bifidobacterial fermentation on a model of NS-HMO and S-HMO versus the uninoculated control. The concentrations of oligosaccharide in culture were determined by HPAEC-PAD. The eluted sugars were identified from their elution times relative to those of external standards (lactose, 3-fucosyllactose [3-FL], 2'-fucosyllactose [2'-FL], lacto-*N*-neotetraose [LNnT], lacto-*N*-neohexaose [LNnH], lacto-*N*-tetraose [LNT], lacto-*N*-hexaose [LNH], sialic acid, sialyllacto-*N*-tetraose a [LSTa], sialyllacto-*N*-tetraose a [LSTc], 6'-sialyllactose [6'-SL], 3'-sialyllactose [3'-SL], disialyllacto-*N*-tetraose [DSLNT]) **a)** Consumption of total HMO, **b)** Consumption of the sialyllactoses, 3'-SL and 6'-SL, **c)** Consumption of the tetraoses, LNT and LNnT and **d)** Consumption of the fucosyllactoses, 2'-FL and 3-FL. R0071= *Bifidobacterium bifidum* R0071, R0033= *Bifidobacterium infantis* R0033, M-16V= *Bifidobacterium breve* M-16V, M-63= *Bifidobacterium infantis* M-63. Analysis was calculated using technical duplicate data from biological triplicate experiments and data are means $\pm$ SD. Univariate analysis of variance (ANOVA) and post-hoc Tukey tests were performed to determine the significant differences between the groups (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$).

2.3. Production of metabolic end-products is dependent on strain combination and HMO substrate

To assess the metabolic consequences of bifidobacterial growth on HMO, levels of the possible fermentative end-products acetate, lactate, formate, and 1,2-propanediol (1,2-PD) were evaluated. The absolute concentrations of these metabolites following 10 hours fermentation are presented in **Figure 3** and **Supplementary Table S1**. The central metabolic pathways employed in *Bifidobacterium* for the production of these organic acids is shown in **Supplementary Figure S6**. Measurable levels of acetate, lactate, and formate were detected across all strain/HMO combinations, albeit with significant variations (**Figure 3**). Consistent with increased bifidobacterial numbers detected during growth analysis, co-culture fermentation of the four bifidobacterial strains resulted in significantly higher total metabolites compared to mono-culture fermentations (**Figure 3, Table S1**). In comparison to single-strain cultivation with M63, metabolite production increased by 45.4%, 13.6%, and 15.9% during co-cultivation on 2'-FL, NS-HMO, and S-HMO, respectively. On the whole, acetate, and lactate levels were lowest during metabolism of 2'-FL and highest in cultures grown in S-HMO. Production of acetate ranged from 2.36 mM during M-16V growth on 2'-FL to 25.6 mM during co-culture metabolism of S-HMO. Likewise, lowest concentrations of lactate were detected following M-16V growth on 2'-FL (0.21mM) and highest during co-culture growth on S-HMO (17.29mM). Notable variances were observed between metabolic profiles of the two *B. infantis* strains. *B. infantis* M-63 produced significantly higher amounts of acetate than R0033 during metabolism of 2'-FL (7.76mM vs 3.94mM, $p=0.0233$), NS-HMO (19.87mM vs 3.08mM, $p<0.0001$), and S-HMO (23.11mM vs 11.55mM, $p<0.000$).

Conversely, variability in formate production between strains was less pronounced, ranging from 2.68 mM to 9.1 mM (**Figure 3, Table S1**). Overall, it is presumed that the concentrations of organic acids produced during bifidobacterial fermentation of carbohydrates are positively correlated with bacterial cell numbers. Pearson correlation and linear regression analysis was performed to assess the relationship between bacterial biomass and the subsequent yields of acetate, lactate, and formate (data not shown). Findings indicate that, across all three HMO substrates, acetate and lactate concentrations are positively correlated with bacterial cell numbers. However, no correlation between bacterial cell numbers and levels of organic acids were noted in the case of formic acid production.

Quantitative analysis of metabolite 1,2-PD using GC-MS revealed that production of 1,2-PD only occurred in the presence of the *B. infantis* strains and not during either *B. bifidum* or *B. breve* mono-culture fermentations, irrespective of the carbohydrate source (**Figure 3, Table S1**). During growth on 2'-FL as sole carbon source, 1,2-PD production was only observed after fermentation with the four strain *Bifidobacterium* co-culture, with 1.7 mM detected after 10 hours growth (**Figure 3**). Co-culture fermentation of NS-HMO and S-HMO also resulted in significant quantities of 1,2-PD being produced (2.4 mM and 3 mM, respectively) (**Figure 3**). 1,2-PD was also detected in *B. infantis* R0033 culture supernatants on S-HMO (2.4 mM), and in M-63 culture supernatants when grown on NS-HMO (2.1 mM) and S-HMO (2.1 mM) (**Figure 3**).

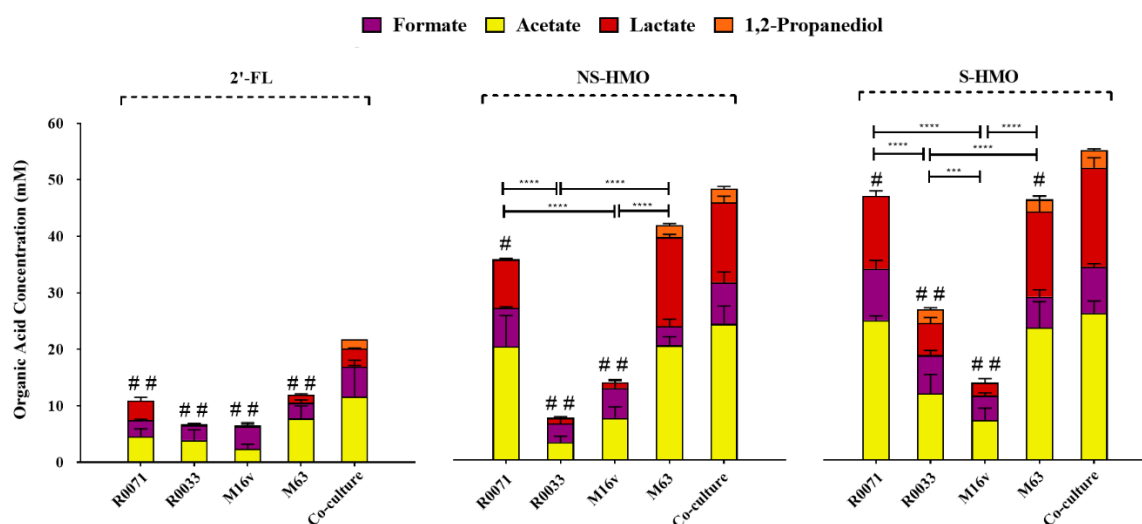


Fig. 3

Figure 3: Levels of organic acids and 1,2-propanediol produced after 10h of bifidobacterial growth as mono-cultures and as a four strain co-culture. The following HMO were used as the sole carbon source: 2'-FL [1.2g/L], model of NS-HMO [3.8g/L] and a model of S-HMO [5g/L]. Levels of acetic acid, formic acid, and lactic acid were quantified using HPLC coupled with an RI detector. Levels of 1,2-PD were quantified using GC-MS. R0071= *Bifidobacterium bifidum* R0071, R0033= *Bifidobacterium infantis* R0033, M-16V= *Bifidobacterium breve* M-16V, M-63= *Bifidobacterium infantis* M-63. Analysis was calculated using technical duplicate data from biological triplicate experiments and data are means +/- SD. Univariate analysis of variance (ANOVA) and post-hoc Tukey tests were performed to determine the significant differences between the groups (# = $p < 0.05$, ## = $p < 0.001$ versus co-culture) (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ between other strain combinations).

Molar ratios of organic acids were determined to provide further insights into the metabolic trajectories during bifidobacterial HMO fermentation. These ratios are given in **Table 1**. For every 2 moles of hexose that enter into the *Bifidobacteriaceae*-specific bifid shunt metabolic pathway, 3 moles of acetic acid and 2 moles of lactic acid are produced (**Figure S6**). The theoretical ratio of 1.5 acetate: lactate was observed during *B. bifidum* R0071, *B. infantis* M-63, and co-culture fermentation across all HMO substrates (**Table 1**). In contrast, growth of *B. infantis* R0033 and *B. breve* M-16V on 2'-FL as sole carbon source significantly increased the acetate to

lactate ratio to 7.4 ± 1.8 and 9.9 ± 3.4 (**Table 1**). Findings show that *B. infantis* R0033 and *B. breve* M-16V metabolism was characterized by an increase in formic acid production (relative to acetic and lactic acid production) (**Table 1**). A significant increase in the ratios of formate: acetate and formate: lactate was observed for R0033 and M-16V when compared to all other strain combinations (**Table 1**).

Table 1: Ratios of organic acids after 10 hours fermentation

		<i>B. bifidum</i> R0071	<i>B. infantis</i> R0033	<i>B. breve</i> M-16V	<i>B. infantis</i> M-63	4Bif co-culture
acetate: lactate	2'-FL	1.1 ± 0.2	$7.4 \pm 1.8 *$	$9.9 \pm 3.4 *$	4.9 ± 2	3.6 ± 1.7
	NS-HMO	2.3 ± 0.6	3.4 ± 1.7	$8 \pm 3.2 *$	1.3 ± 0.1	1.7 ± 0.1
	S-HMO	1.9 ± 0.2	2.1 ± 0.4	$3.2 \pm 0.8 *$	1.6 ± 0.6	1.5 ± 0.1
formate: acetate	2'-FL	0.6 ± 0.1	$0.9 \pm 0.2 *$	$1.9 \pm 0.4 *$	0.4 ± 0.1	0.5 ± 0.3
	NS-HMO	0.4 ± 0.1	$1.1 \pm 0.2 *$	0.8 ± 0.2	0.2 ± 0.1	0.3 ± 0.1
	S-HMO	0.4 ± 0.1	$0.7 \pm 0.1 *$	$0.7 \pm 0.1 *$	0.2 ± 0.2	0.3 ± 0.1
formate: lactate	2'-FL	0.9 ± 0.2	$17.9 \pm 6 *$	$26.1 \pm 9.4 *$	2.1 ± 0.7	$1.6 \pm 0.3 *$
	NS-HMO	0.8 ± 0.3	$3.6 \pm 1.9 *$	$6.2 \pm 2.1 *$	0.2 ± 0.1	0.5 ± 0.1
	S-HMO	0.7 ± 0.1	$1.2 \pm 0.2 *$	$2 \pm 0.6 *$	0.4 ± 0.2	0.5 ± 0.1

*= designates significant difference (p value < 0.05) versus theoretical ratio (acetate: lactate = 1.5, formate: acetate = 0.4, formate: lactate = 0.6)

3. Discussion

This study aimed to characterise the metabolic capability and co-operative metabolism of a community of four infant *Bifidobacterium* strains when grown on HMOs. Since formula-fed infants lack exposure to these potentially health-promoting oligosaccharides and microbes, delivery of HMOs and probiotic bifidobacteria in infant formula or supplements remains a priority (Walsh et al., 2020). The four strains included in this study were selected based on their proven efficacy in clinical studies (Giannetti et al., 2017; Wong et al., 2019; Xiao et al., 2019). For instance, formula-fed infants receiving a probiotic supplement containing *B. bifidum* R0071 and *B. infantis* R0033 in addition to a *Lactobacillus* strain, maintained higher faecal SIgA levels at the end of the four-week treatment period (Xiao et al., 2019). *B. breve* M-16V is also a commonly used probiotic strain in infants and has been shown to offer potential in protecting infants from developing the necrotising enterocolitis (NEC) and allergic diseases (reviewed by Wong et al. 2019 (Wong et al., 2019)). A mixture containing *B. infantis* M63 and other two probiotic strains (one being M-16V) was associated with better control of functional abdominal pain and improved quality of life when compared to the placebo in children with IBS (Giannetti et al., 2017).

A key factor in determining microbial dynamics in the microbiota is the sharing and/or competition for nutrients (Gould et al., 2018). In this context, microbe–microbe interactions can either positively or negatively influence the fitness of affected organisms. The current study demonstrates that, during growth on HMOs, cross-feeding interactions between multiple bifidobacterial strains increases overall cell numbers. This increase was hallmarked by notable shifts in microbial distribution from exponential to

stationary phase. We found that under certain conditions (i.e., when fermentable carbohydrates were readily available) these *Bifidobacterium* strains stably coexist and no (sub)species appeared to have a considerable competitive advantage. However, over time, consumption of limiting nutrients and release of acidic metabolites appeared to shape the course of competition, thereby altering the relative abundance of species in the culture. No increase in bacterial cell numbers was observed for *B. bifidum* or *B. infantis* from exponential to stationary phases of growth during co-cultivation. Any increase in growth was due to continued proliferation of *B. breve*. This indicates that, despite demonstrating limited capacity to metabolize HMOs in pure culture, the ability of M-16V to act as a ‘scavenger species’ and potential higher resistance to acidic conditions allows the strain to persist at the lowest resource level and become the competitive dominant in a shared environment. These findings appear to reflect the distribution of bifidobacterial species in the gut of breast-fed infants. Prototypical HMO-consumers *B. bifidum* and *B. infantis*, are often detected in low numbers in the feces of breast-fed infants, while *B. longum* and *B. breve* are regularly found as the dominant species in infant stools, even though they demonstrate minimal growth on HMOs *in vitro* (Turroni et al., 2012).

Generally, it is assumed that *B. breve* has limited ability to metabolise complex HMO structures, but can grow on mono- and di-saccharide HMO degradants (Ward et al., 2007). More recent studies have identified *B. breve* strains capable of metabolising a greater repertoire of HMO structures, including LNT and LNnT (James et al., 2016), and those decorated with fucose and sialic residues (Ruiz-Moyano et al., 2013). Thus, the ability of *B. breve* to assimilate particular HMOs is strain-dependent. In mono-culture *B.*

breve M-16V was shown to exhibit rather poor growth on all assessed HMO substrates. Glycoprofiling revealed that M-16V consumed minimal amounts of 3-FL, 2'-FL, 3'-SL, 6'-SL, and LNnT, corroborating findings from previous *in-vitro* growth studies (Thongaram et al., 2017). We demonstrate here that M-16V is an efficient consumer of lacto-*N*-tetraose (LNT), the central moiety of Type I HMO. Similar selective consumption patterns have also been observed *in vivo* during probiotic administration of *B. breve* M-16V to premature breast-fed infants (Underwood et al., 2017).

Growth and sugar analysis presented here revealed notable strain-level differences in the ability of the two *B. infantis* strains to utilize HMO. *B. infantis* R0033 exhibited minimal growth on HMOs and only demonstrated significant consumption of the fucosyllactoses. In contrast, the probiotic strain *B. infantis* M-63 showed high levels of growth on 2'-FL, NS-HMO, and S-HMO. HPLC analysis of the resultant supernatant showed that the majority of HMO structures are degraded after 10 hours fermentation with M-63, with preferential consumption of LNT and LNnT. Sakanaka et al. (2020), via genome data mining, found conservation of HMO-related genes (including GHs) across *B. infantis* to be very high (Sakanaka et al., 2020). It is assumed, therefore, that all *B. infantis* strains behave in the same manner when utilizing HMOs. However, a recent study has reported genetic differences in HMO utilization clusters among *B. infantis* strains which has significant implications on fitness and performance of *B. infantis* strains in culture and in the infant gut (Duar, Casaburi, et al., 2020). Comparative genome analysis of commercial *B. infantis* probiotics revealed two main variants; one which harboured the full repertoire of HMO utilization genes and another variant which lacks an ABC-transporter needed for binding of

core HMO structures (such as LNT and LNnT) (Duar, Casaburi, et al., 2020). We hypothesise that similar genotypic variations exist between *B. infantis* strains M-63 and R0033 which may have implications for their fitness and survival in the infant gut.

In contrast to the intracellular consumption strategies used by *B. infantis*, *B. bifidum* employs cell wall-anchored secretory glycosidases which hydrolyse the HMO extra-cellularly releasing mono- and di-saccharides, which are then internalized for metabolism and growth (Asakuma et al., 2011; Sakanaka et al., 2020). Here, we report that *B. bifidum* R0071 grows well on HMO and readily assimilates most HMO structures including type I structures, type II structures and sialylated structures. R0071 demonstrated preference for consumption of 3'-SL over 6'-SL. Both these structures are trisaccharide HMO that have the same molecular composition and only differ in the attachment of sialic acid residues around the lactose core. These findings highlight that the specific structure of the HMO isomers is biologically relevant. *B. bifidum* R0071 also demonstrated substantial consumption of 2'-FL, with the majority of the substrate degraded by late exponential phase. Remarkably, dramatic increases in the levels of 3-FL were observed during R0071 fermentation of HMOs. To the best of our knowledge this is the first time that an increase in the level of 3-FL during bifidobacterial growth on HMOs has been reported. We speculate that this increase in 3-FL can be accounted for by differences in the catalytic activity of 1,2- α -L-fucosidase (AfcA) and 1,3-1,4- α -L-fucosidase (AfcB) as previously described (Gotoh et al., 2018). It is plausible to hypothesise that the rapid action of AfcA cleaves the α -1,2 linked fucose from DFL to release 3-FL into the supernatant, which subsequently remains in the culture due to the low

catalytic activity of AfcB. To test this hypothesis, growth experiments on DFL alone were performed. Glycoprofiling of culture supernatants confirmed that 3-FL was released during growth of R0071. However, this increase in 3-FL levels was found to be transient, suggesting that the AfcB harboured by R0071 begins catalysing α 1,3-fucosylated HMO once other preferred substrates have been assimilated. Interestingly, when R0071 was co-cultured on the same media with *B. infantis* R0033, notable increases in bacterial cell numbers were observed. This suggests that, despite its transient nature, 3-FL released by R0071, becomes available as a metabolic cross-feeding substrate for other gut commensals, particularly *B. infantis* strains which demonstrate adaptation for uptake and assimilation of the major fucosylated HMO(Sakanaka et al., 2019).

Quantification of HMO degradants also revealed that *B. bifidum* R0071 produced sialic acid in stoichiometric accordance with consumption of sialylated HMO. No increase in the levels of lactose was observed after 10 hours growth, which demonstrates that *B. bifidum* R0071 hydrolyses HMO in order to access the disaccharide moieties (such as lactose and lacto-*N*-biose) which it then internalises and utilizes for growth. Findings from previous studies suggest that extracellular breakdown of HMO and subsequent liberation of monosaccharides by *B. bifidum* strains can facilitate cross-feeding within microbial communities (Egan et al., 2014; Motherway et al., 2018; Turroni et al., 2015). In particular, studies have highlighted the existence of syntrophic relationships between *B. bifidum* and *B. breve* strains(Egan, O'ConnellMotherway, et al., 2014). We hypothesise that release of HMO degradants by *B. bifidum* R0071 facilitated growth of *B. breve* M-16V during co-culture growth, with syntrophy between the strains prevailing

in the presence of other infant-derived bifidobacteria. When M-16V was co-cultured with *B. bifidum* R0071 on 3'-SL (without the *B. infantis* strains present) its growth was considerably enhanced. These findings highlight that *B. bifidum*, which is generally known to be the minority species in the early gut microbiota, can have a major influence on the prevalence of other species within the *Bifidobacterium* genus. The significance of *B. bifidum* altruistic behaviour in shared environments has been highlighted previously, with studies showing that *B. bifidum* supplementation results in enhanced HMO consumption and increased dominance of bifidobacteria (Gotoh et al., 2018; Tannock et al., 2013).

Here we demonstrate that all four *Bifidobacterium*, individually, have the metabolic machinery required to capture and metabolize HMO into complex health-promoting metabolites, with enhanced production during co-fermentation. We found detectable levels of lactate, acetate, and formate in all cultures. This has potential implications for infant nutritional and health outcomes since production of these key metabolites is critical to the delivery of fundamental ecosystem services (Duar, Henrick, et al., 2020; Fukuda et al., 2011; Fukuda et al., 2012; Mächler et al., 2016; Smith et al., 2013; Zabel et al., 2019). In general, higher bacterial biomass resulted in increased secretion of acetate and lactate and correlated with superior rates of HMO consumption. It has been proposed that a theoretical molar ratio of 1.5 acetate to 1 lactate is produced during efficient metabolism of HMOs, although other studies have shown that this theoretical ratio is not always obtained (Pokusaeva et al., 2011). The variation in the theoretical ratio is explained by the production of other sugar metabolites, such as formate and ethanol, which limit the production of lactate. This theoretical ratio was only achieved during *B.*

infantis M-63 and co-culture metabolism of S-HMO. In contrast, inefficient hydrolysis of 2'-FL by *B. infantis* R0033 and *B. breve* M-16V shifted metabolism towards greater acetate production resulting in higher average ratios. Studies have demonstrated that increased levels of acetate and formate allow *Bifidobacterium* with atypical HMO consumption phenotypes to balance their reduction-oxidation levels and produce enough energy to continue growing (Özcan & Sela, 2018). Formate is produced when pyruvate is shunted away from lactate production, and towards the production of acetyl-CoA (Özcan & Sela, 2018). When more than 50% acetyl-CoA is converted to acetate by *B. longum*, the theoretical formate: acetate ratio is 0.4 (Van der Meulen et al., 2006). This ratio was achieved throughout fermentation with the co-culture, irrespective of carbohydrate source. Here, *B. infantis* R0033 and *B. breve* M-16V surpassed that threshold, suggesting that there is a shift to produce more acetyl-CoA than lactate from pyruvate. It is hypothesised that this metabolic shift to favour formate secretion is to prioritize ATP production through a branch in the bifid shunt. We speculate that by shifting metabolic pathways towards formate production, M-16V and R0033 can generate NADH from NAD⁺ without having to use NADH to reduce pyruvate to lactate, resulting in a net positive NADH.

Unlike glucose, galactose, sialic acid, and *N*-acetylglucosamine derivatives, fucose is not predicted to enter the bifid shunt central metabolic pathway. Instead, it is hypothesized that fucose derivatives enter in a separate fermentative pathway, which results in the production of an equimolar ratio of pyruvate and intermediary metabolite 1,2-PD per mole of fucose consumed (Dedon et al., 2020). Here it was found that co-fermentation enhanced production of 1,2-PD. During growth on 2'-FL, the co-culture and *B. infantis*

M-63 exhibited similar levels of growth but 1,2-PD was only detected in the co-culture system. Therefore, factors other than biomass accumulation may play a role in production of this compound. It is possible that fucose transport is repressed in the presence of high concentrations of efficient substrates. James et al. (2019) putatively linked a LacI-family regulator in *Bifidobacterium catenulatum* subsp. *kashowanohense* (*fumR*) to fucose metabolism (James et al., 2019). Subsequently, Dedon et al. (2020) determined that *B. infantis* ATCC 15697 possesses a homolog of this *fumR* and hypothesised that this putative repressor may restrict fucose metabolism in the presence of other efficiently utilized sugars. Therefore, it is possible that increased competition and demand for lactose, glucose, and galactose during co-culture drives metabolism of fucose and subsequent production of 1,2-PD. Enhanced production of 1,2-PD may also occur as a consequence of the trophic chain from the release of fucose during extracellular metabolism of HMO by *B. bifidum* R0071 (Cheng et al., 2020). Cross-feeding based on the metabolite 1,2-PD has been proposed to have an important role in the establishment of trophic interactions among gut symbionts (Bunesova et al., 2018; Schwab et al., 2017). Accordingly, it has been reported that the infant-associated *Eubacterium hallii* metabolizes 1,2-PD to produce short chain fatty acids butyrate, formate, and propionate (Engels et al., 2016; Morrison & Preston, 2016). The shift to prioritize 1,2-PD production may therefore facilitate trophic relationships within the infant gut microbiome.

4. Conclusion

Deciphering relationships between early colonizing bacterial species and specific HMO types allows for the development of health-promoting products that more closely resemble natural infant microbiota interactions. Findings

presented here contribute to our knowledge of HMO-microbe interactions and demonstrate the potential for synbiotic combinations of pre- and pro- biotics. The work demonstrates that small differences in the molecular structure of HMOs can have considerable impacts on their biological efficacy and on the catalytic ability of bifidobacterial strains. The variances in HMO metabolism observed in this study highlight the importance of probiotic strain specificity for efficient metabolism of HMOs. Genomic and proteomic analyses are required to further understand the functional implications of genotypic variations in HMO-utilization clusters. Development of “HMObiotic” or HMO-containing probiotic products should account for these variations and combine HMOs with their preferred pairing of strains. Overall, we conclude that genotype, but also phenotype, of strains should be considered when selecting a strain for probiotic use, as variations in HMO utilization may determine fitness and performance in the gut. We demonstrate the potential benefits for the use of multi-strain bifidobacterial probiotics since co-operation between strains may expand HMO acquisition capabilities and help shift bacterial communities closer towards those observed in breast-fed infants.

5. Materials & Methods:

5.1. Bacterial strains and propagation

Bifidobacterium strains used in this study were obtained from Lallemand Health Solutions, France (*B. bifidum* [R0071] and *B. infantis* [R0033]) and Morinaga Milk, Japan (*B. breve* [M-16V] and *B. infantis* [M-63]). All strains were stored at -80 °C in de Man-Rogosa-Sharpe (MRS) broth (Difco, BD, Ireland), supplemented with 50% (v/v) glycerol as a cryoprotectant. For routine experiments, strains were grown at 37 °C under anaerobic conditions

using MRS medium supplemented with 0.05% (wt/v) L-cysteine hydrochloride (Sigma, St. Louis, MO) and 0.01% (wt/v) mupirocin (Merck, Darmstadt, Germany). Bacterial strains were routinely validated using bifidobacterial-specific PCR targeting the 16S region using primers g-Bifid-F (5'-CTCCTGGAAACGGGTGG-3') and g-Bifid-R (5'-CTCCTGGAAACGGGTGG-3') (Matsuki et al., 2004).

5.2. Carbohydrate substrates

5.2.1 Generation of human milk oligosaccharide enriched fraction:

Oligosaccharide fractions were isolated from human milk as previously described (Quinn et al., 2018). In short, pooled human milk samples (of unknown secretor status) were kindly donated by Irvinestown Human Milk Bank (Co. Fermanagh, Ireland) and stored at -80 °C on arrival. Lipids and proteins were removed before applying samples to a BioGel P2 size exclusion column (92 × 5 cm; Bio-Rad Laboratories, Inc., Hercules, CA, USA) to separate lactose and HMO components. High pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was used, as described below, to quantify lactose in the eluted fractions while a Pierce colorimetric peptide assay (Thermo Fisher Scientific, Reinach, Switzerland) was used to measure levels of peptides. Peptide-free and low-trace lactose (<80 mg/L) fractions were pooled and freeze-dried and the resultant HMO-enriched fraction was stored at 4 °C prior to use in experiments.

5.2.2. Preparation of HMO blends

Three HMO powders were assessed in this study; a HMO-enriched fraction with and without 2'-FL (Friesland Campina, Amersfoort, Netherlands) was

used to mimic non-secretor (NS-HMO) and secretor (S-HMO) human milk oligosaccharide fractions, respectively. 2'-FL alone was used as a control (**Table 2**). Stock suspensions (20% wt/v) of 2'-FL, NS-HMO, and S-HMO were prepared in MQ-water and sterile filtered (0.2 μ m, Chromafil, Macherey-Nagel, Düren, Germany) Oligosaccharide substrates were used in fermentation studies at concentrations outlined in **Table 2**. The maximum levels of 2'-FL authorised according to the Official Journal of the European Union (EFSA Panel on Dietetic Products & Allergies, 2015) is 1.2 g/L while preliminary studies indicated that 3.8 g/L was the lowest HMO concentration that resulted in excess oligosaccharide in culture system after 24 hours bacterial fermentation (data not shown).

Table 1: Concentrations of HMO used in fermentation experiments

	2'-FL Control	NS-HMO	S-HMO
2'-fucosyllactose (g/L)	1.2	-	1.2
Isolated HMO (g/L)	-	3.8	3.8

5.3. Pure culture and co-culture fermentations

5.3.1. Growth Medium

Modifications were made to Man-Rogosa-Sharpe (MRS) (De Man et al., 1960) medium to generate a basal growth medium which contained no carbohydrate sources as previously described (O'Donnell et al., 2011). Media was sterilized by autoclaving at 121°C for 15 minutes. For use in fermentation studies, cfMRS medium was supplemented with sterile oligosaccharide solutions at concentrations outlined in Table 1.

5.3.2. Preparation of inoculum and initiation of fermentation process

For experiments, bacteria derived from -80 °C stock cultures were streaked on MRS agar supplemented with 0.05 % (wt/v) L-cysteine HCl and 0.01 % (wt/v) mupirocin and incubated for 48 hrs at 37 °C under anaerobic conditions. Single colonies of each strain were subsequently inoculated in supplemented MRS broth, and subcultured twice before use in experiments. To obtain working cultures, supernatants of overnight media was removed, the bacterial cell pellet washed with Phosphate Buffer Saline (PBS) (Merck), and resuspended in cfMRS such that the OD₆₀₀ of each bacterial culture was ~2. The following bacterial combinations were assessed: single cultures of *B. bifidum* (R0071), *B. infantis* (R0033), *B. breve* (M-16V), *B. infantis* (M-63) and a co-culture of all four *Bifidobacterium* (4Bif). Blanks of uninoculated media were also included. Ten milli-litres of oligosaccharide supplemented media (2'-FL, NS-HMO, S-HMO, or cfMRS) was inoculated with 1 % (v/v) of overnight cultures to initiate fermentations. All cultivations were carried out under anaerobic conditions at 37 °C.

More in-depth analysis of potential cross-feeding relationships was provided by performing mono- and co-culture fermentations of a) *B. bifidum* R0071 and *B. infantis* R0033 on 5mg/mL DFL and b) *B. bifidum* R0071 and *B. breve* M-16V on 5mg/mL 3'-SL. Cultures were prepared as described above. At 0, 10, and 24 hours bacterial cultures were serially diluted in Maximum Recovery Diluent (Oxoid) and enumerated by plating on MRS agar. Bacteria was quantified according to the formula:

$$cfu/mL = \frac{\text{no. of colonies} \times \text{dilution factor}}{\text{volume of culture plated}}$$

Definitive differences in colony characteristics between R0071 & R0033, and R0071 & M-16V allowed for strain-level quantification during co-cultivation of strains.

5.4. Growth analysis

5.4.1. Optical Density Readings

Variability in bacterial biomass was assessed throughout fermentation by measuring optical density at 600 nm (OD₆₀₀). Growth analysis was performed on cultures at 0 hr, 6hr (early exponential), 10 hr (late exponential), 20 hr (early stationary), and 24 hr (late stationary). At each time point, 200 µl of culture was transferred to the wells of 96 well microtiter plates in an anaerobic chamber and the OD values determined using a Synergy 2 plate reader (BioTek Instruments, Inc., Vermont, US). Turbidity of cultures was assumed to correlate with numbers of bacterial cells within solution.

5.4.2. Quantitative PCR

Possible enhancement or reduction of bifidobacterial growth as a consequence of co-cultivation with other strains was evaluated through absolute quantification of bacterial numbers by quantitative PCR (qPCR). One ml samples were harvested from cultures at 0 hours, 10 hours, and 24 hours. Bacterial cells were pelleted at $12,000 \times g$ for 2 min prior to the addition of the lysis buffer. Total bacterial DNA was isolated using GenElute Bacterial Genomic DNA Kit (Merck) according to manufacturer's instructions. The resultant DNA was quantified by a Nanodrop 2000 Spectrophotometer. Total bifidobacterial numbers were quantified on a 480 Lightcycler platform (Roche Applied Science, Penzberg, Germany) using the following programme: 95 °C for 5 min followed by 40 cycles of 95 °C for 10

s, 58 °C for 20 s and 72 °C for 30 s. The fluorescent product was detected at the last step of each cycle. Following amplification, melting temperature analysis of PCR products was performed to determine the specificity of the PCR. The melting curves were obtained by slow heating at 0.2°C/s increments from 60 to 99°C, with continuous fluorescence collection. Reactions took place in a 25 µl volume made up of 5 µl PCR grade water, 1.25 µl forward primer (0.10 µM), 1.25 µl reverse primer (0.10 µM), 5 µl DNA template and 12.5 µl SYBR green (Roche Diagnostics, West Sussex, United Kingdom). Experiments were based on previously designed strain-specific primers targeting the 16s gene region. For quantification of all *Bifidobacterium* strains present in samples, the primer pairs Bifid-F (CTCCTGGAAACGGGTGG) and g-Bifid-R (GGTGTCTTCCCGATATCTACA) were used. To determine *B. bifidum* cell numbers, primer pairs BiBIF-1 (5'-CCACATGATCGCATGTGATTG-3') and BiBIF-2 (5'-CCGGATGCTCCATCACAC-3') were used. *B. breve* cell numbers were quantified using primer pairs BiBRE-1 (5'-CCACATGATCGCATGTGATTG-3') and BiBRE-2 (5'-ACAAAGTGCCTTGCTCCCT-3'), while *B. infantis* cell numbers were determined using primer pairs BiINF-1 (5'-TTCCAGTTGATCGCATGGTC-3') and BiINF-2 (5'-GGAAACCCCATCTCTGGGA -3').

The copy-number of a given strain in co-culture was evaluated by comparing the cycle threshold (Ct) values with those from a standard curve of known copy number. Standard curves (10^9 to 10^2 copy number/ µL) were established by performing 1 in 10 serial dilutions of 16S rDNA of the specified bifidobacterial strain (in the case of *B. infantis* strains, M-63 was

used as the reference rDNA). The cell numbers of strains within co-culture (denoted by bifidobacteria/mL) were then deduced by comparing these copy number values with copy numbers of standards with known cell numbers (as determined by viable count assessment). Cell numbers were calculated according to the formula (Quigley et al., 2013):

$$\frac{(C/\mu\text{l})(TV)}{TCN} \times T \text{ cfu/ml} = \text{bifidobacteria/ml (S)}$$

Where; C/ μl = Copy number/ μl , TV = template volume, TCN = total copy number of the standard used, T cfu/ml = total cell number of standard used, and bifidobacteria/mL(S) = cell number of test sample. Samples were run in triplicate, while standards and negative controls (where template DNA was replaced with PCR grade water) were run in duplicate.

Growth rate (μ) of each subspecies within cultures was calculated using the following equation:

$$\mu = \frac{\ln X_2 - \ln X_1}{t_2 - t_1}$$

where X_2 and X_1 are the cell densities at t_2 and t_1 . Mean doubling time (T_d) was calculated as:

$$T_d = \ln 2 / \mu$$

5.5. Chemical analysis of culture supernatants

For chromatographic analysis of cultures, supernatants were extracted at late exponential phase (10 hours) and filtered using 0.2 μm nylon syringe filters (Chromafil, Macherey-Nagel, Düren, Germany) to remove bacterial cell debris.

5.5.1 Quantification of HMO utilization

The concentrations of oligosaccharide in culture after 10 hours microbial fermentation were determined by HPAEC-PAD using a Dionex ICS-3000 Series system (Dionex Corporation, Sunnyvale, CA, USA). Samples were diluted 100-fold with MQ-Water, and 20 µl injected onto a CarboPac PA100 column (250 × 4 mm) (Thermo Fisher Scientific) equipped with a guard column. 100 mM NaOH (A) and 100mM NaOH plus 500mM Na-acetate (B) were used as eluents at a flow rate of 1.0 mL/min. The column temperature and detector were maintained at 32 °C. HMO were separated for 22 min using an eluent gradient as follows: 95 % A and 5 % B for 3 min, 88 % A and 12 % B for 10 min, 84 % A and 16 % B for 4 min and hold time of 3 min, followed by 82 % A and 18 % B for 2 min.

At 22.1 min the gradient immediately changes to 50 % A and 50 % B for 8 min to regenerate the column and then back to 95 % A and 5 % B for a further 15 min to re-equilibrate the column. The eluent was monitored by pulsed amperometric detection (PAD). The eluted sugars were identified from their elution times relative to those of external standards: Lactose, 3-FL, 2'-FL, LNnT, LNnH, LNT, LNH, sialic acid, sialyllacto-*N*-tetraose a (LSTa), LSTc, 6'-SL, 3'-SL, disialyllacto-*N*-tetraose (DSLNT). Utilization of HMO was calculated as % consumption versus the uninoculated control.

5.5.2. Quantification of organic acid metabolites

To examine organic acid production, supernatants were extracted after 10 hours fermentation and filtered as above. Levels of organic acid metabolites were then quantified by HPLC using a Waters Alliance Separations module e2695 coupled to a Waters 2414 refractive index (RI) detector (Waters, Milford MA, USA). Samples or standards at a volume of 20 µl were injected

on to an Aminex HPX 87C fixed ion resin column (300 X 7.8mm) operated at 60°C. The samples were eluted with H₂SO₄ (0.005 N) at a flow rate of 0.6 mL/ min. Sample detection was performed by comparing retention times of standards. Analytical grade acetic acid, lactic acid, and formic acid supplied by Merck were used as standards.

5.5.3. Quantification of 1,2-Propanediol

To assess whether co-culture fermentation of HMO increased 1,2-PD production when compared to single culture fermentation, samples were sent to MS-Omics ApS, Frederiksberg, Denmark for analysis using GC-MS (Ahmed et al., 2019). A mixed pooled sample (QC sample) was created by taking an aliquot from each sample. This sample was analyzed with regular intervals throughout the sequence. For 1,2-PD analysis, samples were acidified using hydrochloric acid, and deuterium-labeled internal standards were added. Analysis was performed using a high-polarity column (ZebronTM ZB-FFAP, GC Cap. Column 30 m × 0.25 mm × 0.25 μm) installed in a GC (7890B, Agilent) coupled with a quadrupole detector (59977B, Agilent). Raw data was converted to netCDF format using Chemstation (Agilent), before the data was imported and processed in Matlab R2014b (Mathworks, Inc.) using the PARADISE software.

5.6. Statistical analysis:

Statistical analysis was calculated using technical triplicate data from biological triplicate experiments. Graphs were created using in PRISM v8 (GraphPad, La Jolla, CA). Univariate analysis of variance (ANOVA) and post-hoc Tukey tests were performed to determine the significant differences between the groups for growth, oligosaccharide consumption and metabolite production. Statistical significance was accepted as $P \leq 0.05$.

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Supplementary Materials

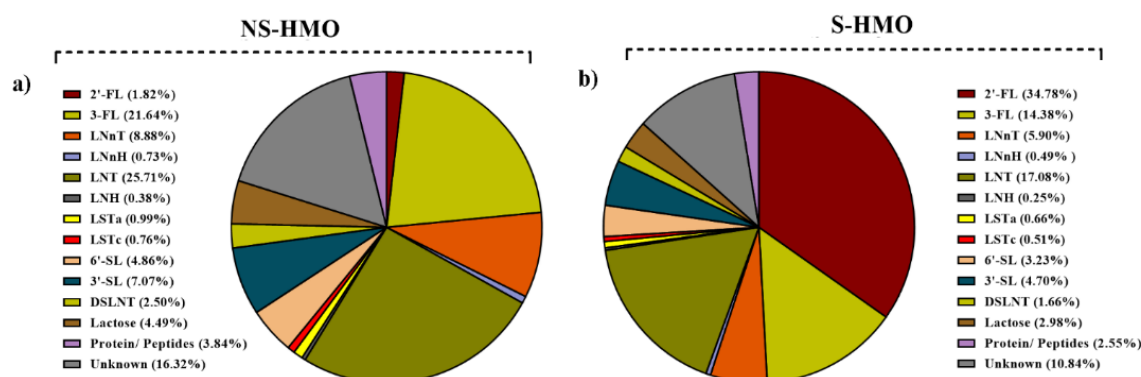


Fig. S1

Supplementary Figure 1: Pie chart showing the relative abundance of the major oligosaccharides found in a) NS-HMO and b) S-HMO detected via HPAEC-PAD analysis by comparing to the following external HMO standards: lactose, 3-fucosyllactose (3-FL), 2'-fucosyllactose (2'-FL), lacto-*N*-neotetraose (LNNt), lacto-*N*-neohexaose (LNNH), lacto-*N*-tetraose (LNT), lacto-*N*-hexaose (LNH), sialyllacto-*N*-tetraose a (LSTa), sialyllacto-*N*-tetraose a (LSTc), 6'-sialyllactose (6'-SL), 3'-sialyllactose (3'-SL), disialyllacto-*N*-tetraose (DSLNT). Data represents average concentrations of technical duplicate data from biological triplicate experiments.

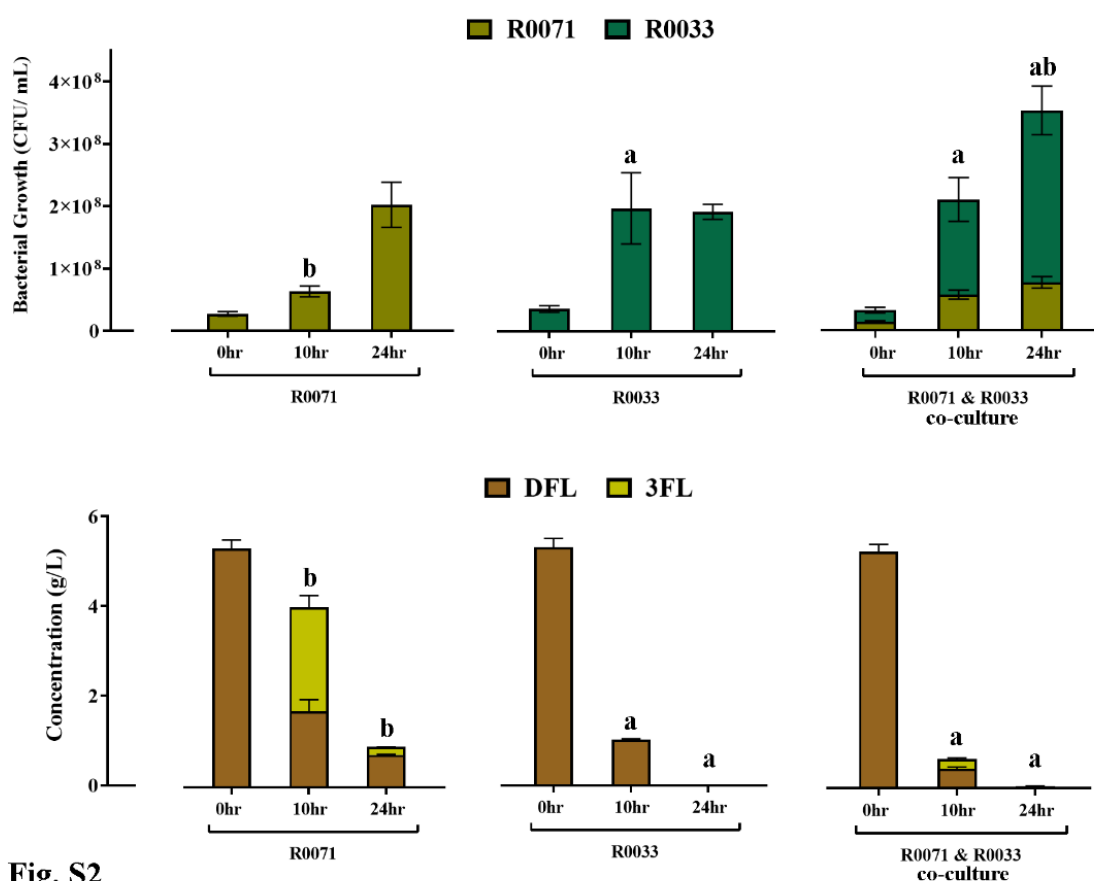


Fig. S2

Supplementary Figure 2: Growth profiles in CFU/mL (top) and glycoprofiles in g/L HMO (bottom) of *Bifidobacterium bifidum* R0071 and *Bifidobacterium infantis* R0033 as mono-cultures and as a two strain co-culture with 5g/L difucosyllactose (DFL) as the sole carbon source. Bacterial cell numbers were assessed using colony counts at 0 hr, 10 hr, and 24 hr. Definitive differences in colony characteristics between R0071 & R0033 allowed for strain-level quantification during co-cultivation of strains. The concentrations of oligosaccharides in the culture supernatants was determined by HPAEC-PAD. The sugars were identified from their elution times relative to those of external standards. Analysis was calculated using technical triplicate data from biological triplicate experiments and data are means $\pm$ SD. Univariate analysis of variance (ANOVA) and post-hoc Tukey tests were performed to determine the significant differences between the groups (a = $p < 0.05$ versus R0071 mono-culture, b = $p < 0.05$ versus R0033 mono-culture).

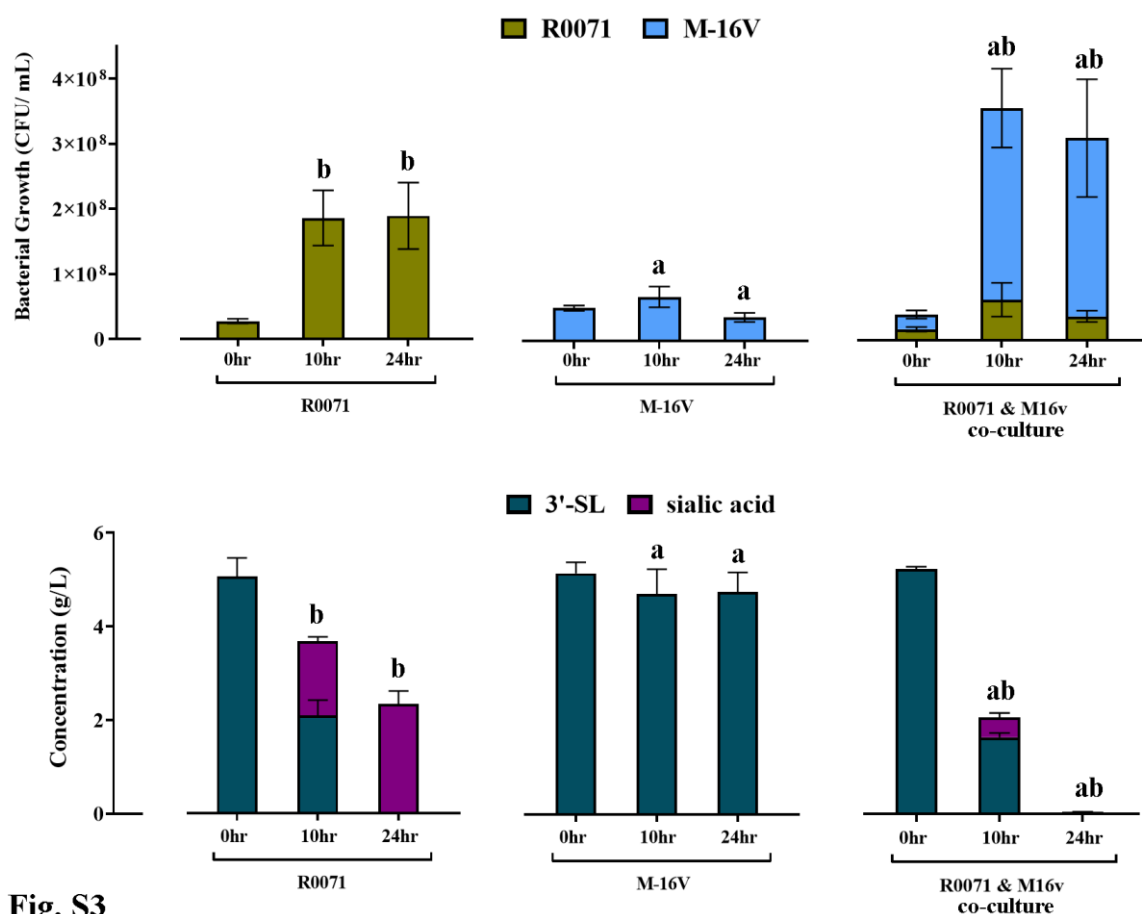


Fig. S3

Supplementary Figure 3: Growth profiles in CFU/mL (top) and glycoprofiles in g/L HMO (bottom) of *Bifidobacterium bifidum* R0071 and *Bifidobacterium breve* M-16V as mono-cultures and as a two strain co-culture with 5g/L 3'-sialylactose (3'-SL) as the sole carbon source. Bacterial cell numbers were assessed using colony counts at 0 hr, 10 hr, and 24 hr. Definitive differences in colony characteristics between R0071 & M-16V allowed for strain-level quantification during co-cultivation of strains. The concentrations of oligosaccharides in the culture supernatants was determined by HPAEC-PAD. The sugars were identified from their elution times relative to those of external standards. Analysis was calculated using technical triplicate data from biological triplicate experiments and data are means $\pm$ SD. Univariate analysis of variance (ANOVA) and post-hoc Tukey tests were performed to determine the significant differences between the groups (a = $p < 0.05$ versus R0071 mono-culture, b = $p < 0.05$ versus M-16V mono-culture).

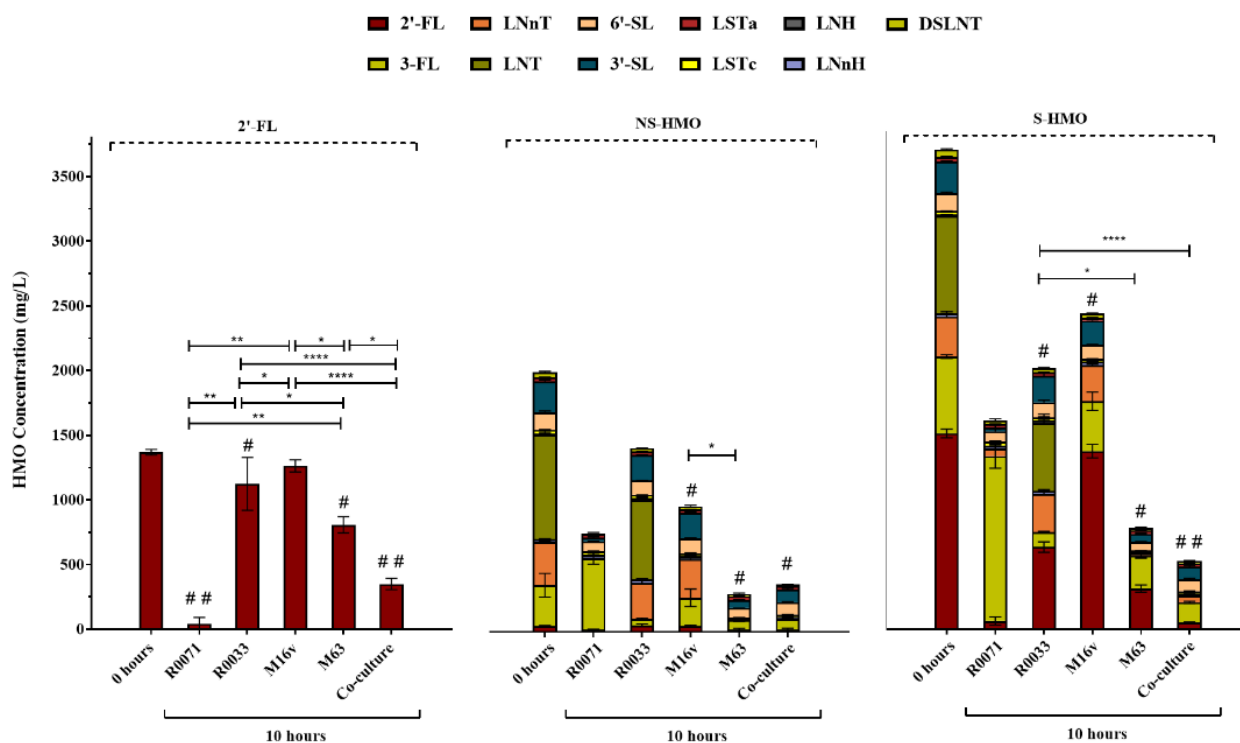


Fig. S4

Supplementary Figure 4: Concentration of HMO (2'-FL, NS-HMO and S-HMO) before and after 10h of bifidobacterial fermentation detected via HPAEC-PAD analysis by comparing to the following external HMO standards: 3-fucosyllactose (3-FL), 2'-fucosyllactose (2'-FL), lacto-*N*-neotetraose (LNnT), lacto-*N*-neohexaose (LNnH), lacto-*N*-tetraose (LNT), lacto-*N*-hexaose (LNH), sialyllacto-*N*-tetraose a (LSTa), sialyllacto-*N*-tetraose a (LSTc), 6'-sialyllactose (6'-SL), 3'-sialyllactose (3'-SL), disialyllacto-*N*-tetraose (DSLNT). R0071= *Bifidobacterium bifidum* R0071, R0033= *Bifidobacterium infantis* R0033, M-16V= *Bifidobacterium breve* M-16V, M-63= *Bifidobacterium infantis* M-63. Analysis was calculated using technical duplicate data from biological triplicate experiments and data are means $\pm$ SD. Univariate analysis of variance (ANOVA) and post-hoc Tukey tests were performed to determine the significant differences between the groups (# = $p < 0.05$, ## = $p < 0.001$ versus Time 0) (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ between strain combinations).

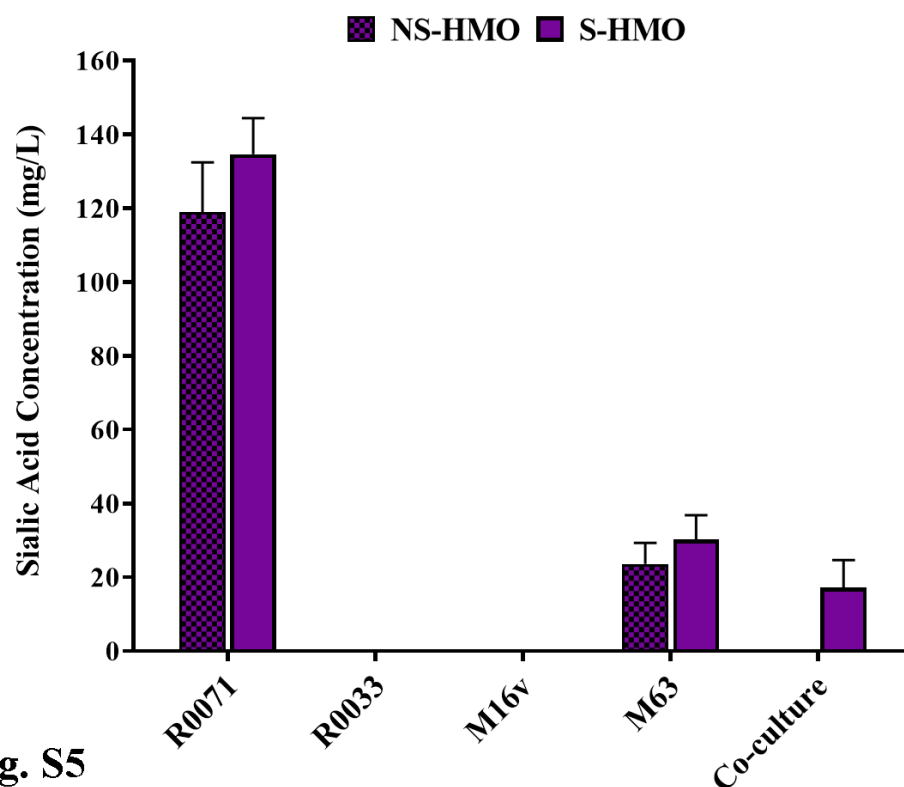


Fig. S5

Supplementary Figure 5: Quantification of free sialic acid following 10h of bifidobacterial fermentation on NS-HMO and S-HMO detected via HPAEC-PAD analysis by comparing to an external sialic acid standard. R0071= *Bifidobacterium bifidum* R0071, R0033= *Bifidobacterium infantis* R0033, M-16V= *Bifidobacterium breve* M-16V, M-63= *Bifidobacterium infantis* M-63. Analysis was calculated using technical duplicate data from biological triplicate experiments and data are means +/-SD.

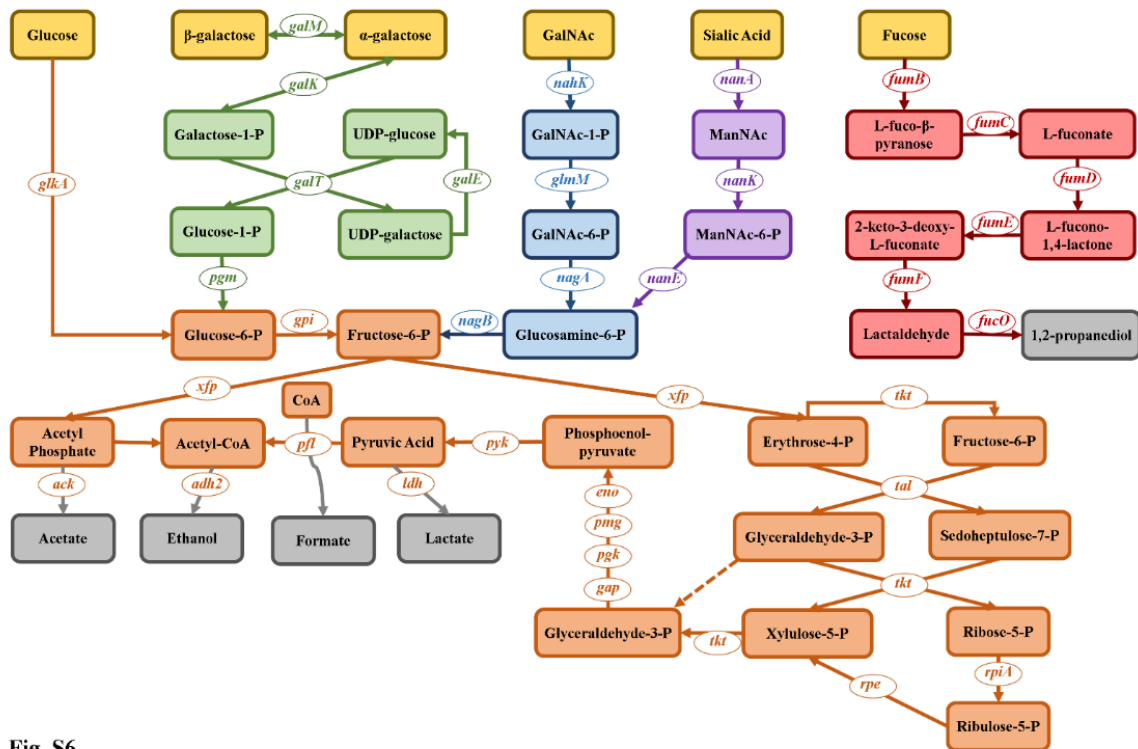


Fig. S6

Supplementary Figure 6: A schematic representation of carbohydrate degradation through the “bifid shunt” and related pathways in bifidobacteria.

Gene names: Leloir genes (green): *galM* galactose mutarotase; *galK* galactokinase; *galT* galactose-1-phosphate uridylyltransferase; *pgm* phosphoglucomutase. Central fermentative pathway genes (orange): *glkA* glucokinase; *gpi* glucose-6-phosphate isomerase; *xfp* fructose-6-phosphate phosphoketolase/ xylulose-5-phosphate phosphoketolase; *tkt* transketolase; *tal* transaldolase; *rpiA* ribose 5-phosphate isomerase; *rpe* ribulose 5-phosphate epimerase; *gap* glyceraldehyde-3-phosphate dehydrogenase; *pgk* phosphoglycerate kinase; *pmg* phosphoglycerate mutase; *eno* enolase; *pyk* pyruvate kinase; *ack* acetate kinase; *adh2* aldehyde-alcohol dehydrogenase; *pfl* formate acetyltransferase; *ldh* lactate dehydrogenase. N-acetylglucosamine genes (blue): *nahK* N-acetylhexosamine 1-kinase; *GlmM* phosphoglucosamine mutase; *nagA* N-acetylglucosamine-6-phosphate deacetylase; *nagB* glucosamine-6-phosphate isomerase. Sialic Acid genes (purple): *nana* N-acetylneuraminase lyase; *nanK* N-acetylmannosamine kinase; *nanE* N-acetylmannosamine-6-phosphate 2-epimerase. Fucose genes (red) (as proposed by Dedon et al. 2020): *fumB* L-fucose mutarotase; *fumC* L-fucose dehydrogenase; *fumD* L-fuconolactone hydrolase; *fumE* L-fuconate dehydratase; *fumF* aldolase; *fumO* lactaldehyde reductase. Monosaccharide HMO derivatives depicted in yellow. End metabolites depicted in grey

Supplementary Table 1: Organic Acid Concentrations

	Acetate (mM)			Lactate (mM)			Formate (mM)			1,2-propanediol (mM)		
	2'-FL	NS-HMO	S-HMO	2'-FL	NS-HMO	S-HMO	2'-FL	NS-HMO	S-HMO	2'-FL	NS-HMO	S-HMO
<i>Bbif</i> R0071	4.6 ± 1.4	19.7 ± 5.4	24.4 ± 0.8	3.5 ± 0.6	8.5 ± 0.2	12.7 ± 0.9	2.9 ± 0.2	6.7 ± 0.2	9.0 ± 1.4	<LOD	<LOD	<LOD
<i>BInf</i> R0033	3.9 ± 1.8	3.1 ± 1.0	11.6 ± 3.3	0.2 ± 0.1	1.0 ± 0.2	5.6 ± 1.0	2.7 ± 0.2	3.2 ± 0.9	6.7 ± 0.8	<LOD	<LOD	2.4 ± 0.3
<i>Bbrev</i> M-16V	2.4 ± 0.8	7.2 ± 2.0	6.9 ± 2.1	0.2 ± 0.2	1.0 ± 0.3	2.3 ± 0.7	4.0 ± 0.7	5.3 ± 1.5	4.3 ± 0.5	<LOD	<LOD	<LOD
<i>Binf</i> M-63	7.8 ± 2.2	19.9 ± 1.5	23.1 ± 4.5	1.4 ± 0.2	15.5 ± 0.5	14.9 ± 12.7	2.8 ± 0.5	3.3 ± 1.2	5.4 ± 1.2	<LOD	2.1 ± 0.3	2.1 ± 0.6
4Bif co-culture	11.6 ± 5.5	23.6 ± 3.1	25.6 ± 2.1	3.2 ± 0.1	13.9 ± 1.1	17.3 ± 1.7	5.3 ± 1.2	7.2 ± 1.8	8.1 ± 0.5	1.7 ± 0.0	2.4 ± 0.4	3.0 ± 0.3

CHAPTER VI

**Integrative Genomic and Proteomic Analysis of
Bifidobacterial Interactions During Human Milk
Oligosaccharide Utilization**

Acknowledgements Clodagh Walsh designed the experimental model, performed growth assays, created library of targeted genomic features, contributed to genomic and bioinformatic analysis, performed the data analysis. Helen Slattery performed HPLC oligosaccharide analysis. Rebecca Owens contributed to proteomic analysis. Francesca Bottacini contributed to sequence assembly and annotation. Jonathan Lane, Douwe van Sinderen and Rita Hickey contributed to experimental design.

Abstract

Bifidobacteria are prevalent and abundant components of the infant gut microbiome, at least in part due to their ability to metabolize human milk oligosaccharides (HMOs). However, the mechanisms governing HMO metabolism among bifidobacterial communities are not completely understood. The current study aimed to define bifidobacterial strain/species-specific differences in HMO utilization and unravel potential metabolic interactions between strains during co-cultivation. We performed whole-genome sequencing and subsystem-based genomic analysis to identify carbohydrate metabolism gene profiles across four *Bifidobacterium* strains (*B. bifidum* R0071, *B. breve* M-16V, *B. longum* subsp. *infantis* R0033, and *B. longum* subsp. *infantis* M-63) to characterize HMO utilization pathways. Label-free proteomic analysis was used to quantify protein expression during growth on secretor HMO-containing media, compared to a lactose control. These combinatorial genomic-proteomic analyses revealed distinct HMO metabolic strategies across *Bifidobacterium* species and highlight the cooperative resource-sharing networks that enable efficient HMO utilization. These findings provide critical insights into HMO-mediated metabolic interactions among bifidobacterial communities and their potential applications in probiotic formulations designed to mimic the infant gut microbiome.

1. Introduction

Bifidobacteria represent an important component of the human gut microbiome, particularly during early life, and their high prevalence and abundance in the infant gut has been associated with a myriad of health benefits (Saturio et al., 2021). Particular, human-derived members of the genus *Bifidobacterium*, such as *Bifidobacterium longum* subsp. *infantis*, *Bifidobacterium longum* subsp. *longum*, *Bifidobacterium breve*, and *Bifidobacterium bifidum*, are thought to contribute to immune development, pathogen resistance, and gut maturation in neonates and infants (Bottacini et al., 2014; Di Gioia et al., 2014; O'Callaghan & van Sinderen, 2016). One of the mechanisms by which bifidobacteria obtain their high abundance and prevalence in the neonatal gut, allowing them to exert their beneficial effects is through the metabolism of human milk oligosaccharides (HMOs). HMOs are a complex group of glycans that are the third most abundant solid component of human milk after lactose and lipids (Bode, 2012; Garrido et al., 2013; Walsh et al., 2020). HMOs resist digestion by the host and reach the colon intact, where they serve as substrates for particular bifidobacterial species/strains (Katayama, 2016; Lordan et al., 2024). HMO catabolism by bifidobacteria leads to the production of acetic acid and lactic acid, which have beneficial effects such as preventing pathogen infection by lowering the pH of the infant gut environment (Jiang & Savaiano, 1997; Morrison & Preston, 2016; Pokusaeva et al., 2011).

HMOs are highly diverse in structure, composed of varying combinations of glucose, galactose, fucose, *N*-acetylglucosamine, and sialic acid (Chen, 2015). Over 200 distinct HMO structures have been identified, categorized into neutral (fucosylated and non-fucosylated) and acidic

(sialylated) forms (Bode & Jantscher-Krenn, 2012; Walsh et al., 2020). Only a limited number of gut microbes possess the enzymatic machinery required to degrade these complex molecules. In general, carbohydrate metabolism by human gut microbiota is facilitated by carbohydrate-active enzymes (CAZymes) (Xiao et al., 2024). Bifidobacterial CAZymes include a plethora of glycosyl hydrolases (GHs), which, together with various sugar-specific transport systems, in particular the carbohydrate-specific ATP-binding cassette (ABC) transport systems, enable internalization, degradation, and metabolism of a wide range of dietary or host-derived glycans (Fushinobu & Abou Hachem, 2021; Wardman et al., 2022).

Studies by Sakanaka *et al.* (2020) and Hermes *et al.* (2024) have reported significant variation in the conservation of species-specific gene sets for HMO degradation (Hermes et al., 2024; Sakanaka et al., 2020). In *B. longum* subsp. *infantis* some strains exhibit broader HMO utilization profiles than others, largely due to differences in their genomic content. Duar *et al.* (2020) revealed significant variation in the gene sets related to HMO degradation among *B. infantis* strains, suggesting that not all strains are equally capable of metabolizing the particular spectrum of HMO structures (Duar, Casaburi, et al., 2020). This genomic heterogeneity holds implications for the selection of strains in probiotic formulations, where HMO-degrading capabilities may affect the strain's adaptability, resilience, and ability to positively influence host health (Duar, Casaburi, et al., 2020). Members of the *B. bifidum* species show the highest degree of conservation in terms of their HMO metabolism, whereas *B. breve* exhibit rather limited HMO utilization capabilities, relying more heavily on cross-feeding from other species (Hermes et al., 2024; Sakanaka et al., 2020). The cross-feeding

phenomenon, where extracellular HMO-degradation products are shared between bifidobacteria, has been well-documented in recent literature (Egan et al., 2014; Luo et al., 2021; Turrone et al., 2018; Xiao et al., 2024). Gotoh et al. (2018) demonstrated that *B. bifidum* plays a pivotal role in extracellularly releasing HMO degradation products such as fucose and sialic acid, which are then utilized by other species (Gotoh et al., 2018). This indicates that bifidobacterial communities often include "primary degraders" such as *B. bifidum*, while other species act as "secondary utilizers," benefiting from shared metabolic byproducts. The metabolic interactions that occur between bifidobacterial species in a community context are recognized as critical to the overall functioning of the infant gut microbiota. Previously, we have shown that *B. infantis*, *B. bifidum* and *B. breve* can collaborate to enhance overall HMO degradation efficiency, underscoring the importance of community-level interactions in shaping the infant gut microbiome (Walsh et al., 2022).

Despite the growing body of research on bifidobacterial HMO metabolism, several knowledge gaps persist. Most studies have focused on individual species or strains, with fewer addressing community-level interactions that occur within bifidobacterial consortia. Moreover, genomic studies, while insightful, do not fully capture the dynamic protein expression patterns that occur during growth on HMOs. Proteomic approaches, which quantify protein expression during HMO degradation, are therefore essential to validate predicted metabolic pathways and to uncover novel enzymes and transport systems involved in HMO assimilation. The present study aims to characterize the gene and protein sets involved in carbohydrate metabolism in four commercial infant bifidobacterial strains: two *B. longum* subsp.

infantis strains, one *B. breve* strain, and one *B. bifidum* strain. Whole-genome sequence and predictive genomic analysis of the bifidobacterial strains facilitated the identification of key enzymes, transporters, and regulatory elements involved in HMO degradation. These predicted HMO metabolic capabilities (or lack thereof) were validated *in vitro*. Additionally, we explored how these strains metabolize HMOs in co-culture, revealing important insights into the metabolic interactions and cross-feeding relationships within bifidobacterial communities. These findings offer valuable information for optimizing probiotic and synbiotic formulations tailored to support infant gut health by promoting *in situ* growth and activity of beneficial bifidobacteria.

2. Results and discussion

2.1. Identification and validation of HMO utilisation pathways in commercial bifidobacterial strains:

Bifidobacterial gut colonization and persistence is, at least partly, driven by their ability to efficiently utilize specific nutrient resources, particularly carbohydrates, the availability of which varies depending on diet, life stage and gut environment (Laforest-Lapointe & Arrieta, 2017; Milani et al., 2016; Sánchez et al., 2013). Understanding glycan utilization and cross-feeding mechanisms can help identify novel genes, enzymes, and metabolites that contribute to bifidobacteria-microbe associations and host health. The current study aimed to characterize the metabolic and cooperative behaviour of four commercial infant *Bifidobacterium* strains when grown on HMOs. As formula-fed infants may lack exposure to these beneficial oligosaccharides and microbes, delivering HMOs and bifidobacterial strains through formula

or supplements is a key goal (Hill et al., 2021; Schönknecht et al., 2023). The strains included in this study were selected based on their commercial relevance and their previously reported clinical efficacy. *B. bifidum* R0071 and *B. longum* subsp. *infantis* R0033, when administered in combination with a *Lactobacillus* strain, were shown to lead to higher fecal secretory immunoglobulin A (Sigal) levels in formula-fed infants after a four-week treatment (Xiao et al., 2019). *B. breve* M-16V has previously been demonstrated to show promise in protecting against necrotizing enterocolitis (NEC) and allergic diseases (Wong et al., 2019), while *B. longum* subsp. *infantis* M63, in combination with *B. breve* M-16V and *B. longum* subsp. *longum* BB536, improved outcomes in children with irritable bowel syndrome (IBS) (Giannetti et al., 2017). More recently, the efficacy and safety of *B. longum* subsp. *infantis* M63 were investigated in healthy infants in a double-blind, randomized, placebo-controlled trial (Hiraku et al., 2023). Supplementation with *B. infantis* M-63 significantly increased the relative abundance of *Bifidobacterium*, decreased stool pH and increased levels of acetic acid and IgA in the stool at 1 month of age, compared with the placebo group (Hiraku et al., 2023).

In order to identify key HMO-utilizing genes in these commercial bifidobacterial strains, a comprehensive literature search for studies assessing HMO metabolism (including fucosylated HMOs, sialylated HMOs, LNT, LNnT, HMO-derived mono and di-saccharides, and subsequent organic acid production) identified 207 genes with a potential role in HMO utilization in bifidobacterial strains (**Figure 1, 2, 3, Supplementary Table S1, S2, S3**). This literature search revealed variations among species of bifidobacteria in their capacity to utilize different HMO. Genes responsible for metabolism of

fucosylated HMOs (FHMOs), which constitutes around 70% of the total HMOs present in secretor milk by weight (Soyyılmaz et al., 2021), were found to be widespread in *B. longum* subsp. *infantis*, *B. bifidum*, and *Bifidobacterium catenulatum* subsp. *kashiwanohense*, yet appear to be much less common in strains of *B. longum*, *B. breve*, and *B. pseudocatenulatum* (Hermes et al., 2024). The key fucosidases enzymes for metabolism of FHMOs are generally classified under glycosyl hydrolase family 29 (GH29, 1,3/4- α -l-fucosidase) and glycosyl hydrolase family 95 (GH95, 1,2- α -l-fucosidase). HMOs are also decorated with sialic acid to form sialylated HMOs (sialylHMOs), including compounds such as 3'-sialyllactose (3'-SL), 6'-sialyllactose (6'-SL), and various sialyllacto-*N*-tetraoses (LSTa, LSTb, LSTc) (Lis-Kuberka & Orczyk-Pawłowicz, 2019). Sialylated glycoproteins, such as lactoferrin and β -casein, and glycolipids such as gangliosides are also abundant in human milk (Wang et al., 2021). Sialidases (neuraminidases), which cleave terminal sialic acids from these glycans, typically exhibit broad substrate specificity and hydrolyze α 2,3-, α 2,6-, and α 2,8-linked sialic acids (Muñoz-Provencio & Yebra, 2023). Studies have shown that several *Bifidobacterium* species possess sialidase activity. In bifidobacteria, both extracellular (SiaBb1 and SiaBb2) and intracellular (NanH1 and NanH2) exo- α -sialidases have been identified, with bifidobacterial sialidases classified under glycosyl hydrolase family 33 (GH33) in the CAZy database (Muñoz-Provencio & Yebra, 2023). Following cleavage of fucose and sialic acid residues, core HMO structures, such as type 1 lacto-*N*-tetraose (LNT) and its type 2 isomer lacto-*N*-neotetraose (LNnT), are assimilated using a repertoire of glycosyl hydrolases including GH2 β -1,4-galactosidases, GH42 β -1,3-galactosidases, GH20 β -1,3-*N*-acetylglucosaminidases, GH20/ GH136 lacto-

N-biosidases, and GH112 GNB/LNB phosphorylases. During this literature search for HMO-utilizing genes, pathways for metabolism of LNT (formed through the β 1-3 addition of lacto-*N*-biose (LNB) disaccharide units to the lactose core) and LNnT (formed through β 1-4 addition of *N*-acetyllactosamine disaccharide units to lactose) were identified across many infant-derived bifidobacterial species (James et al., 2016; Smilowitz et al., 2014).

For this study, sequencing, assembly, and annotation was performed on the commercial bifidobacterial strains (see section 4.3 for further details). Thereafter, the derived proteomes of *B. bifidum* R0071, *B. breve* M-16 V, *B. longum* subsp. *infantis* R0033, and *B. longum* subsp. *infantis* M-63 were searched for homologs of these HMO-utilising genes (e-value cutoff of $1e^{-5}$, query cover cutoff of 50%). Strains with well-characterized genetic and phenotypic profiles (namely *B. bifidum* JCM1255, *B. breve* UCC2003, and *B. longum* subsp. *infantis* ATCC15697) were included as benchmark references. Based on percent identity, these genes were classified into three categories: high identity (80-100%), moderate identity (60-80%), and low identity (40-60%). The genomic analysis suggested variations among the strains in their capacity to utilize different HMOs (**Figure 1, 2, 3, Table S1, S2, S3**). Growth experiments on HMO substrates - including 2'-fucosyllactose (2'-FL), 3-fucosyllactose (3-FL), 3'-sialyllactose (3'-SL), 6'-sialyllactose (6'-SL), LNT, LNnT, lactose, fucose, sialic acid, glucose, and galactose - linked genomic potential with actual metabolic function, thereby confirming or refining predictions regarding HMO-utilizing capabilities.

2.1.1 *Bifidobacterium longum* subsp. *infantis*

Previous research has shown that *B. longum* subsp. *infantis* ATCC 15697 efficiently utilizes small neutral HMOs, many of which are fucosylated (Sela et al., 2012). To utilize FHMOs, the *B. longum* subsp. *infantis* type strain ATCC 15697 encodes five predicted intracellular fucosidases; BLON_0248, BLON_0426, BLON_0346, BLON_2335, and BLON_2336 (Sela et al., 2012). BLON_2335, from glycoside hydrolase family 95 (GH95), is specific for α 1,2-L-fucosidic linkages, while BLON_2336, part of the GH29 subfamily B (GH29_B), shows preference for α 1,3/4-L-fucosidic linkages (Ashida et al., 2020). BLON_0346 was originally classified as a GH29 enzyme but was later reassigned to a new family, GH151, and shown to hydrolyze p-nitrophenyl- α -L-fucoside (pNP- α -Fuc) and Fuc α 1-2Gal, though it does not act on 2'-FL (Ashida et al., 2020). BLON_0248 and BLON_0426, being members of GH29_A, do not effectively hydrolyze disaccharides or trisaccharides such as 2'-FL and 3-FL, but have been found to slowly cleave the α 1,2-L-fucosidic linkage in lacto-*N*-fucopentaose I (LNFP I) and the α 1,3-L-fucosidic linkage in LNFP III (Ashida et al., 2020). Here, we found that *B. longum* subsp. *infantis* M-63 and *B. longum* subsp. *infantis* R0033 possess clear homologues of BLON_0248, BLON_0426, BLON_0346, BLON_2335, and BLON_2336 with 100% identity across all strains, indicating robust capabilities for breaking down FHMOs. Two of these fucosidases, GH95 1,2- α -l-fucosidase (BLON_2335) and GH29 1,3/4- α -l-fucosidase (BLON_2336), are found in a gene cluster (HMO cluster H1, **Figure 1**) dedicated to HMO metabolism, as previously described for ATCC 15697 (Sela et al., 2008).

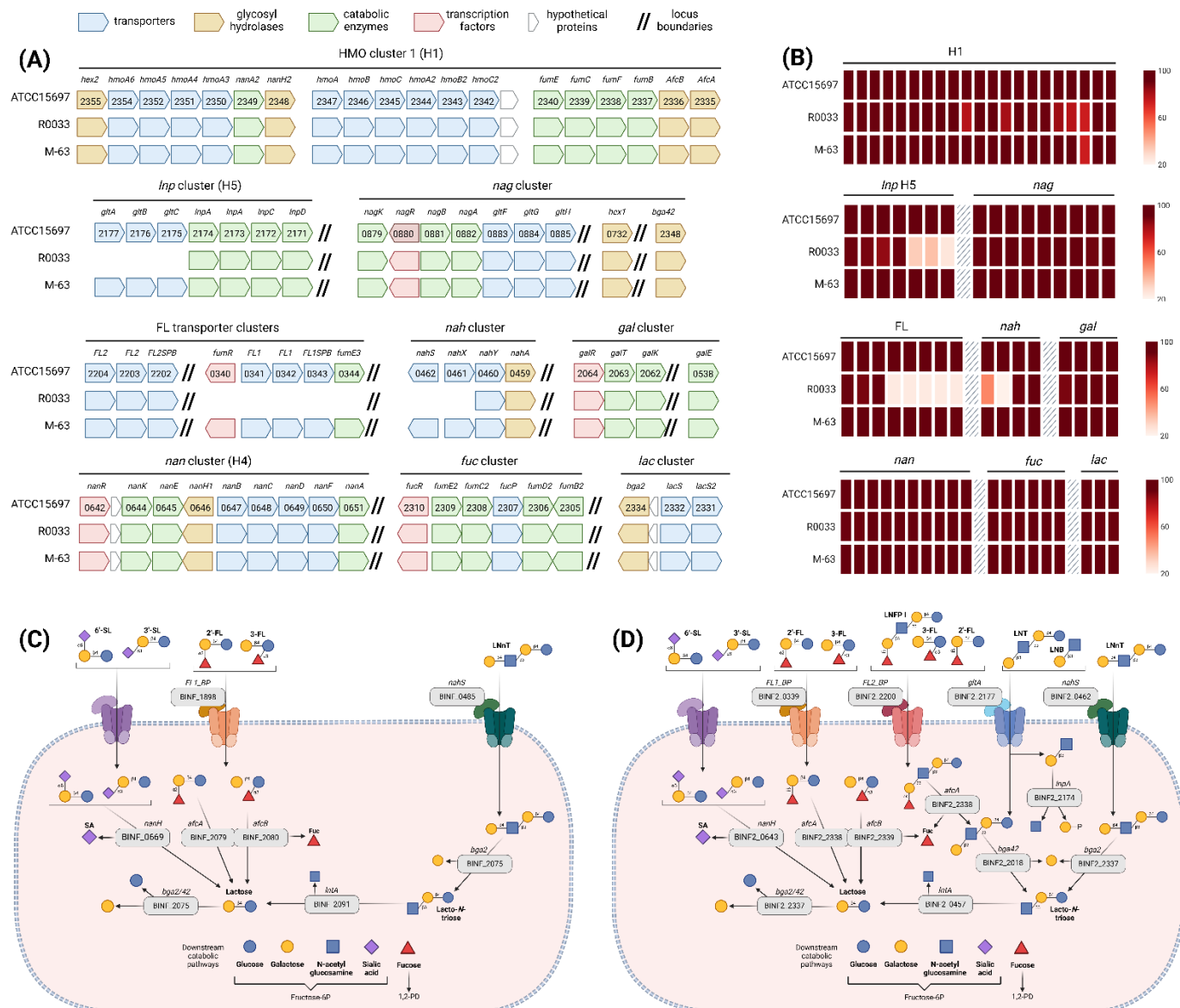


Figure 1: (A) Gene clusters involved in HMO utilization in *Bifidobacterium infantis* ATCC15697, R0033 and M-63 (based on *B. infantis* HMO clusters defined initially by Sela 2008 and more recently by Arzamasov et al 2022). Genes are colored according to the function of encoded proteins with locus position indicated by numbers within arrows. (B) Heat maps summarising the percent identity of *B. infantis* strains against glycosyl hydrolases, transporters, transcription factors, and catabolic enzymes found within the HMO-utilising gene clusters based on BLAST searches (using e-value cutoff of 1e-5 and query cover cutoff of 50%). Degree of conservation indicated by varying intensities of red (lighter shades represent lower conservation, while darker shades represent higher conservation). Schematic representation of R0033 (C) and M-63 (D) HMO utilization pathways based on presence/ absence of HMO utilisation features and HMO growth data. Locus regions shown along reaction arrows with designated gene names shown above in italics. Schematic shows pathways for utilisation of 2'-fucosyllactose (2'-FL), 3-fucosyllactose (3-FL), 3'-sialyllactose (3'-SL), 6'-sialyllactose (6'-SL), lacto-N-tetraose (LNT), lacto-N-neotetraose (LNNt), lacto-N-fucopentaose (LNFP), lactose (Lac), fucose (fuc), sialic acid (SA), glucose, and galactose.

This gene cluster (**Figure 1**), corresponding to locus tags BLON_2331-2361 in ATCC15697, is equivalent to BINF_2073-2097 in strain R0033, and BINF2_2334-2363 in strain M-63, and is predicted to encode five intracellular glycosyl hydrolases, including one GH33 sialidase, one GH95 fucosidase, one GH29 fucosidase, one GH2 β -galactosidase, and one GH20 *N*-acetyl- β -hexosaminidase. These enzymes are predicted to break down a variety of HMOs into their constituent monosaccharides. Seven extracellular solute-binding proteins, which are predicted to facilitate oligosaccharide transport by interacting with proximal permeases, are also predicted to be encoded within this cluster (**Figure 1**). It appears, therefore, that the investigated *B. longum* subsp. *infantis* strains are not only equipped with intracellular glycosidases but also possess a range of transporters to import various HMOs. Regarding FHMO transport, FL transporter gene homologs are distributed in only a few strains of *Bifidobacterium*, which are categorized into four clusters based on phylogenetic analysis: cluster 1-IV (FL1-BP) and clusters 2-I, 2-II, and 2-III (FL2-BP) (Sakanaka, 2022). In the current study, notable differences in FL transport systems were observed between *B. longum* subsp. *infantis* strains R0033 and M-63 (**Figure 1, Table S1**). While both strains harbour the FL2-BP associated gene cluster (BLON_2202-2204), which encodes a versatile ABC transporter system (importing 2'-FL, 3-FL, and lactodifucotetraose [LDFT]), R0033 lacks the FL1-II BP system (BLON_0341-0343) present in ATCC15697 (**Figure 1**). Strain M-63, on the other hand, encompasses homologues for both transport systems. Despite the variance in FL transporters, all three *B. longum* subsp. *infantis* strains (R0033, M-63, and ATCC15697) demonstrated substantial growth on 2'-FL and 3-FL (>8.5 logCFU) suggesting, and as outlined in

previous studies, that 2'-FL and 3-FL metabolism is an important phenotypic trait for *B. infantis* (Hermes et al., 2024). At the time of writing, and based on publicly available studies, all *B. longum* subsp. *infantis* strain examined have been shown to contain either FL1-BP and FL2-BP (Hermes et al., 2024). As suggested by Hermes *et al.* it seems that the competitive advantage of FHMO utilization is indispensable for *B. infantis* and that strains within the subspecies may be broadly divided into generalists (such as M-63 or ATCC 15697) or those focused more on the utilization of smaller (fucosylated) HMOs (such as R0033).

Regarding the sialidases characterized in bifidobacteria, two *B. longum* subsp. *infantis* sialidases have been identified which are located intracellularly and release α -2,3- and α -2,6-linked sialosides, with preference for α -2,6-glycosyl linkage (Sela et al., 2011). Among these, the NanH2 sialidase, encoded within the H1 HMO catabolic gene cluster (**Figure 1A**) of *B. longum* subsp. *infantis* ATCC 15697, plays a key role in breaking down sialylated HMOs and is upregulated during HMO fermentation. This sialidase gene (BLON_2348) is highly conserved across the *B. longum* subsp. *infantis* strains studied here, with homologs present at BINF_2086 in strain R0033 and BINF2_2351 in strain M-63. All three strains—R0033, M-63, and ATCC15697—were shown to exhibit notable growth on sialylated HMOs (3'-SL: 7.7-8.2 logCFU; 6'-SL: 8.1-8.3 logCFU), confirming their ability to utilize sialylHMO. Homologous genes to a sialic acid metabolism pathway previously described for *B. breve* UCC2003 (Egan et al., 2014) were also identified in *B. longum* subsp. *infantis* R0033 (BINF_0666-0674) and M-63 (BINF2_0640-0648) (**Figure 1, Figure 5**). Robust growth on free sialic acid

was observed across all *B. infantis* strains, with logCFU values of 8.1 ± 0.04 (R0033), 8.1 ± 0.09 (M-63), and 7.8 ± 0.07 (ATCC15697).

Previous studies have shown that *B. longum* subsp. *infantis* ATCC 15697 uses two different β -galactosidases to selectively degrade type-1 and type-2 HMOs (Yoshida et al., 2012). Yoshida et al (2012) revealed that the locus tag BLON_2016 gene, which is distantly located from the HMO cluster-1 (**Figure 1A**), encodes a β -galactosidase (Bga42A) with a significantly higher specificity for LNT than for LNB, lactose and type-2 HMOs (Yoshida et al., 2012). The BLON_2334 gene located within the HMO cluster-1 encodes a GH2 β -galactosidase specific for Lac and type-2 HMOs (Yoshida et al., 2012). Both the β -1,3-galactosidase Bga42A/BLON_2016 and GH2 Bga2A/BLON_2334, located in the H1 gene cluster, were shown to be conserved in the genomes of strains R0033 and M-63 (**Figure 1, Table S1**). The presence of such β -galactosidases would suggest high capacity for degradation of both type 1 and type 2 HMO structures. However, while both strains demonstrated robust capacity for growth on LNT (R0033: 7.9 ± 0.13 logCFU, M-63: 8.8 ± 0.08 logCFU), only M-63 demonstrated a capacity to utilise LNT (**Figure 1**). R0033 reached 6.5 ± 0.32 logCFU in comparison to the 8.9 ± 0.07 logCFU achieved by M-63. The reduced growth of R0033 on LNT may correlate with the absence of the H5 cluster BLON_2175-2177 transporter system (**Figure 1, Table S1**), which recognizes LNT/GNB/LNB and a wide range of larger type 1 HMOs (Duar, Casaburi, et al., 2020; LoCascio Riccardo et al., 2010). Loss of such transporter systems within the HMO cluster-5 has been shown for numerous strains across the *B. longum* subsp. *infantis* subspecies. Duar et al. (2020) showed that *in vitro*, H5-positive strains (i.e. strains that possessed the full repertoire of HMO

utilization genes) had a significant fitness advantage over H5-negative strains (i.e. those lacking BLON_2175-2177 transporter system), which was also observed *in vivo* in breastfed infants (Duar, Casaburi, et al., 2020). Authors from this latter study suggested that the absence of the BLON_2175-2177 ABC transporter is the result of gene reduction in the production of probiotics. However, the absence of this ABC transporter has been identified in a recently isolated native strain (Barratt et al., 2022), suggesting that this feature is also prevalent in ‘natural’ *B. longum* subsp. *infantis* populations.

Overall, our genomic and growth analysis of these commercial *B. longum* subsp. *infantis* suggests that 1) FHMO utilization is indispensable for *B. infantis* despite variability in FL transport systems, 2) the absence of ABC transporters in the H5 gene region in *B. infantis* R0033 limits its metabolism of core HMO structures such as LNT and 3) the conservation of sialidase and sialic acid transport genes across *B. longum* subsp. *infantis* strains underscores their specialization for sialylated HMOs and sialic acid metabolism.

2.1.2 *Bifidobacterium bifidum*

B. bifidum has been shown to encode several secreted glycosyl hydrolases which enable it to extracellularly degrade nearly all types of FHMOs (Ashida et al., 2009; Katayama et al., 2004; Katoh et al., 2020). These diverse extracellular glycosidase activities represent a common feature across different *B. bifidum* strains, with homologs of the relevant genes present in most members of the species (Sakanaka et al., 2020). Ashida et al. (2009) identified two critical α -L-fucosidases encoded by *B. bifidum* strain JCM 1254, designated *afcA* and *afcB*, which are responsible for fucose removal

from fucosylated milk oligosaccharides and glycoconjugates (Ashida et al., 2009). Clear homologues of *afcA* and *afcB*, which encode a GH95 1,2- α -l-fucosidase and a GH29 1,3/4- α -l-fucosidase, are present in the genome of *B. bifidum* R0071 (showing over 97% identity) (**Figure 2, Supplementary Table S2**). As described by Turrone *et al.* (2010) for *B. bifidum* PRL2010, both fucosidases contain a signal peptide, but only BBIF_0227 (1,2- α -l-fucosidase) has an LPXTG motif, indicating that it is secreted and anchored to the cell wall (Turrone et al., 2010). In contrast, the 1,3/4- α -l-fucosidase encoded by BBIF1_1367 is predicted (based on TMHMM analysis) to contain two transmembrane domains (one at the C-terminus [position 13-35] and one at the N-terminus [position 1463-1485]). This suggests, and as outlined previously by Turrone *et al.* that for PRL2010 fucosidase, the enzyme stays membrane-bound following secretion (Turrone et al., 2010). These enzymes are believed to cleave fucose residues from various FHMOs including 2'-FL and 3-FL (Turrone et al., 2010). Our growth assays corroborated this predicted ability of *B. bifidum* R0071, revealing substantial growth on FHMOs, with final counts of 8.6 ± 0.14 logCFU on 2'-FL and 8.7 ± 0.13 logCFU on 3-FL (**Figure 2B**). However, *B. bifidum* R0071 displayed very little growth on free fucose (6.4 ± 0.27 logCFU, **Figure 2B**), consistent with findings by Bunesova *et al.* (2016), where the researchers reported that while *B. bifidum* strains are efficient at releasing fucose from HMOs, they are incapable of metabolizing free fucose (Bunesova et al., 2018). Previous studies suggest that *B. bifidum* acquires mucin-associated galacto-*N*-biose (GNB) or HMO-associated lacto-*N*-biose (LNB) via extracellular enzymes that defucosylate these glycans, which are subsequently broken down by lacto-*N*-biosidase and other enzymes (Bunesova et al., 2016; Wada et al., 2008). The liberated extracellular fucose

and other non-preferred mono- and disaccharides are then available to other (bifido)bacterial species in the infant gut that cannot directly utilize FHMOs. This cross-feeding mechanism promoted by *B. bifidum* supports the formation of a bifidobacteria-rich microbiota, indicating that *B. bifidum* plays a crucial role in shaping the infant gut ecosystem (Gotoh et al., 2018).

B. bifidum possesses two extracellular sialidases (SiaBb1 and SiaBb2) that release free sialic acid from mucin sialoglycans and sialylHMO with both preferentially hydrolyzing α -2,3-linked sialic acid over α -2,6-linked sialic acid substrates (Nishiyama et al., 2018). Genomic analysis confirmed the presence of the sialidase genes *siaBb1* and *siaBb2* in *B. bifidum* R0071 (Table S2). Growth assays corroborated the strain's proficiency in degrading sialylated HMOs, with final growth counts of 8.7 ± 0.08 logCFU on 3'SL and 8.5 ± 0.19 logCFU on 6'SL (**Figure 2B**). This aligns with previous work by Nishiyama et al. (2017), which demonstrated that *B. bifidum* strains enhance carbohydrate assimilation through extracellular sialidases, contributing to their competitive advantage in the infant gut (Nishiyama et al., 2018). *B. bifidum* R0071 exhibited negligible growth on free sialic acid (6.2 ± 0.27 logCFU, **Figure 2B**) and lacked key genes for sialic acid metabolism (< 40% identity, **Figure 2A**) indicating a limited capacity to metabolize sialic acid beyond releasing it from sialylated HMOs. This supports the theory that *B. bifidum*'s primary role in sialic acid metabolism is the production of extracellular sialidases, which remove sialic acid from sialylated glycans to access carbohydrates such as LNB and lactose. Consequently, *B. bifidum* also plays a secondary role in sialic acid metabolism by releasing sialic acid for other microbes to utilize.

In contrast to the reliance on galactosidases described for *B. longum* subsp. *infantis*, isolates of *B. bifidum* rely on β -1,4-galactosidase (BbgIII, GH2), β -1,3-*N*-acetylglucosaminidase (BbhI, GH20), lacto-*N*-biosidase (LnbB, GH20), GNB/LNB phosphorylase (LnpA1, GH112) and GNB/LNB transporter (GltA) activities for assimilation of LNT and LNnT (Garrido et al., 2012; Gotoh et al., 2018; Kitaoka et al., 2005; Miwa et al., 2010; Wada et al., 2008). LnbB acts upon LNT motifs to release LNB and lactose, which is then internalized by the GNB/ LNB transporter, where it is phosphorolysed by LnpA1 into galactose-1-phosphate and GlcNAc. LNnT, on the other hand, is acted upon by BbgIII to release galactose and lacto-*N*-triose, which is further broken down to lactose and glucose by the action of BbhI. *B. bifidum* R0071 was expected to utilize both LNT and LNnT, as its genome contains clear homologues encoding BbgIII, BbhI, LnbB, LnpA1, and the GNB/LNB transporter positioned at BBIF1_0505, BBIF1_1522, BBIF1_1436, BBIF1_1018, and BBIF1_1021, respectively (**Figure 2, Table S2**). Growth assays indeed revealed robust growth on both LNT and LNnT, with *B. bifidum* R0071 reaching >8.8 logCFU (**Figure 2B**).

2.1.3 *Bifidobacterium breve*

Compared to *B. bifidum* and *B. longum* subsp. *infantis* strains, *B. breve* M-16V and UCC2003 exhibit a very much reduced genomic capacity to directly metabolize FHMOs (**Figure 3**), likely due to the absence of key fucosidase genes (**Figure 3A**), as noted by James *et al.* (2018) (James et al., 2018). However, both *B. breve* strains demonstrate modest growth on free fucose, with final counts of 7.8 ± 0.09 logCFU in M-16V and 7.7 ± 0.11 logCFU in UCC2003 (**Figure 3B**). A proposed fucose utilization pathway, initially described by Bunesova *et al.* (2016) and supported by Dedon *et al.* (2020),

mirrors rhamnose metabolism in *Campylobacter* and *Xanthomonas campestris* (Bunesova et al., 2016; Dedon et al., 2020). This pathway, detailed by Dedon *et al.* (2020) for *B. infantis* ATCC15697, involves *fucP* (BLON_2305, L-fucose permease), which transports fucose into the cell (Dedon et al., 2020). Fucose is then converted into fuconolactone by L-fucose mutarose (*fumB* BLON_2306) and fucose dehydrogenase (*fumC*, BLON_2309). The resulting fuconolactone is hydrolyzed into fuconate by fuconolactone hydrolase (*fumD*, BLON_2307) and further transformed by fuconate dehydratase (*fumE*, BLON_2340) into 2-keto-3-deoxy-fuconate, which is subsequently dehydrogenated to 2,4-diketo-3-deoxy-fuconate. In some species, this compound is metabolized into lactate and pyruvate, or lactaldehyde and pyruvate, depending on the enzymatic reaction. Homologous genes supporting this pathway are found in *B. breve* M-16V, as highlighted by the genome alignment in **Figure 3**.

B. breve M-16V and UCC2003 both demonstrated substantial growth on free sialic acid, with M-16V reaching 8.5 ± 0.09 logCFU. Neither strain could directly utilize sialylated HMOs, likely due to the absence of sialylHMO degrading sialidases. The *nan* cluster, as previously described for *B. breve* UCC2003 (Egan et al., 2014), was highly conserved in *B. breve* M-16V, supporting efficient sialic acid utilization (**Figure 3, Table S3**).

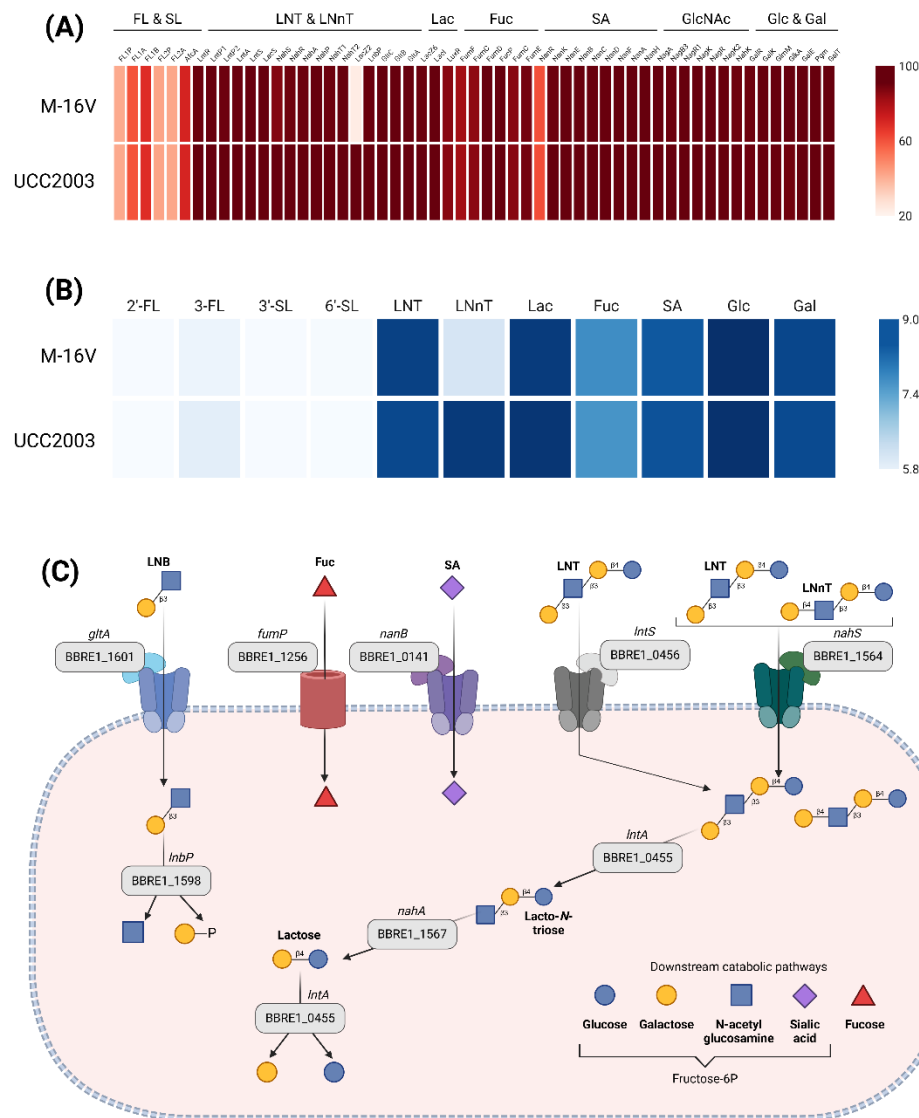


Figure 3: **(A)** Heat map summarising the percent identity of *Bifidobacterium breve* M-16V and UCC2003 strains against genes predicted to be involved in degradation of fucosylated HMO (fHMO), sialylated HMO (sialHMO), lacto-*N*-tetraose (LNT), lacto-*N*-neotetraose (LNT), lactose (Lac), fucose (fuc), sialic acid (SA), *N*-Acetylglucosamine (GlcNAc), glucose (Glc), and galactose (Gal) based on BLAST searches using e-value cutoff of 1e-5 and query cover cutoff of 50%. Degree of conservation indicated by varying intensities of red (lighter shades represent lower conservation, while darker shades represent higher conservation). **(B)** Growth (given as logCFU) of *B. breve* M-16V and UCC2003 on various HMO-associated oligo-, di-, and monosaccharides. **(C)** Schematic representation of M-16V HMO utilization pathways based on presence/ absence of HMO utilisation features and HMO growth data. Locus regions shown along reaction arrows with designated gene names shown above in italics.

B. breve M-16V and UCC2003 also showed strong genomic capacity for LNT metabolism, with the β -1,3-galactosidase gene *lntA* showing >99% identity in both strains (**Figure 1A, Table S3**). M-16V exhibited high growth on LNT (8.8 ± 0.06 logCFU), consistent with its genomic profile. However, M16V was not predicted to encode a LacZ2 homologue, essential for LNT degradation (**Figure 1, Table S3**). LNT metabolism was therefore impaired in M-16V, reaching a final logCFU value of 6.3 ± 0.28 during growth on LNT. In comparison, UCC2003 displayed strong growth on both LNT and LNT (8.8 $\pm$ 0.08 logCFU) (**Figure 1B**), highlighting the variability in HMO-degrading capabilities within *B. breve* strains (James et al., 2016; James et al., 2018).

2.2 Proteomic analysis of HMO-utilisation pathways during co-cultivation of commercial bifidobacterial strains

Insights from genome analysis and mono-culture growth studies highlight the adaptation of certain *Bifidobacterium* species to the nursing period, during which the host's diet primarily consists of HMO-rich breast milk. While these studies are informative, mono-culture experiments fail to capture the community-level interactions that are essential for understanding the cooperative metabolism of HMOs among bifidobacterial species and may overestimate the survival capabilities of individual strains on HMOs in competitive environments (Walsh et al., 2022). Furthermore, genomic analyses reveal potential metabolic capacities but do not necessarily reflect the functional activities expressed under specific conditions. In contrast, proteomic studies of multi-species co-cultures grown on HMOs uncover dynamic interactions, active metabolic processes, and adaptive responses that are not observable through genomic or mono-culture approaches alone. This

method offers a more comprehensive and accurate perspective on how bifidobacteria metabolize HMOs in complex, competitive microbial ecosystems.

Therefore, in order to fully appreciate the role that HMO play in the creation of a *Bifidobacterium* dominant microbiota, label-free proteomic analysis was applied assessing the response of *B. bifidum* R0071, *B. longum* subsp. *infantis* M-63, and *B. breve* M-16V when grown together in a medium containing lactose, or a mimic of secretor HMOs (designated here as S-HMO; see Materials and Methods for further information on how these were obtained) as the sole carbon source (**Supplementary Figure S2**). After 10 hours, bacterial cells were harvested, processed for protein extraction and digestion, and analyzed by LC-MS/MS on a Thermo Q-Exactive mass spectrometer. Across all treatment groups, 2199 distinct proteins were identified, representing 33.2% of the combined deduced proteome of these strains (**Supplementary Figure S3**). Protein abundance patterns varied based on the carbohydrate(s) used in the growth experiment, revealing distinct shifts in expression when cultivated on HMOs in comparison to growth on lactose (**Figure S2**). A total of 883 identified proteins were attributed to *B. bifidum*, 532 to *B. breve*, and 500 to *B. longum* subsp. *infantis* (**Figure S3**). Notably, growth on S-HMO as the sole carbon source of the medium was associated with a significant increase in abundance of proteins related to carbohydrate metabolism and transport, particularly those involved in sialic acid, fucose, and GlcNAc metabolism, all components of HMOs (**Figure S4**). In contrast, lactose-mediated growth caused a comparative increase in proteins associated with DNA replication and oxidative stress.

2.2.1 *Bifidobacterium longum* subsp. *infantis*

Proteomic analysis of *B. longum* subsp. *infantis* M-63 and R0033 during co-culture with *B. bifidum* R0071 and *B. breve* M-16V revealed significant shifts in HMO-utilizing genes including fucosidases and sialidases, as well as proteins involved in fucose/ sialic acid metabolism, and subsequent central metabolism pathways. Specifically, GH95 1,2- α -l-fucosidase (BINF2_2336) was detected only in the HMO conditions, while GH29 1,3/4- α -l-fucosidase (BINF2_2337) increased 4.36-fold when grown on S-HMO compared to lactose. The proteomic analysis also revealed protein abundance shifts in *B. infantis* fucose metabolism enzymes. FumP and FumC were detected exclusively when grown in HMO conditions (and not in lactose growth conditions) (**Table 1**), while FucO and FumE showed substantial increases (>6.5-fold and >3.5-fold, respectively) compared to when grown on lactose. Unlike glucose, galactose, and sialic acid, fucose does not enter the bifid shunt pathway but is instead hypothesized to undergo a separate fermentative pathway, leading to pyruvate and 1,2-propanediol (1,2-PD) production in an equimolar ratio. *B. longum* subsp. *infantis* catabolizes both free fucose and fucosyl oligosaccharide residues to produce 1,2-PD along this fucose metabolism pathway (Dedon et al., 2020). Notably, we have previously shown that 1,2-PD production is enhanced during co-culture of *B. longum* subsp. *infantis* M-63 and *B. longum* subsp. *infantis* R0033 with *B. bifidum* R0071 and *B. breve* M-16V, potentially as a result of the trophic chain initiated by extracellular fucose release from HMO metabolism by *B. bifidum* (Walsh et al., 2022). Cross-feeding on 1,2-PD plays a vital role in establishing symbiotic interactions in the gut microbiome, with the infant-associated *Eubacterium hallii* using 1,2-PD to produce short-chain fatty acids such as

butyrate, formate, and propionate (Schwab et al., 2017). 1,2-PD has also been shown to enhance the ecological performance of *Limosilactobacillus reuteri* in gnotobiotic mice (Cheng et al., 2020). While the ecological and physiological roles of 1,2-PD in the infant gut remain unclear, further research may elucidate why only a select group of *Bifidobacterium* strains have evolved to metabolize FHMOs.

Proteomic analysis of the sialidase enzymes in *B. infantis* during co-culture growth on HMO suggests distinct roles for NanH1 and NanH2 during metabolism of sialylated glycans. NanH2 was exclusively expressed during growth on HMO and not in lactose growth conditions. On the other hand, the NanH1 sialidase (identified at BINF2_0643) was below the limit of detection during analysis of the bifidobacterial proteome following growth on HMOs (**Table 1**). Limited evidence exists corroborating a direct link between NanH1 and sialylHMO utilization, suggesting that NanH1 may act on other sialylated glycans found in the infant gut, such as milk glycopeptides, monosialogangliosides, or sialylated mucins (Sela et al., 2011). The *nanH1* gene (BLON_0646) is part of a gene cluster dedicated to sialic acid catabolism, including *nanA1* (*N*-acetyl neuraminic acid lyase), a putative kinase, and an epimerase (*nanE*). Proximal to this cluster, a sialic acid-specific ABC transporter (*nanB*, *nanC*, *nanD*, *nanF*) shares sequence similarity with the *satABCD* sialic acid transport system as described in *Haemophilus ducreyi* (Post et al., 2005). Proteomic analysis revealed that several of these sialic acid metabolism enzymes and transporters, such as *N*-acetyl neuraminate lyase NanA (BINF2_0648) and ABC transport binding protein NanD (BINF2_0646), were either exclusively detected or increased in abundance during growth on S-HMOs compared to lactose (**Table 1**).

Table 1: Proteins matched to *B. infantis* found at significantly higher abundance/ uniquely detected after co-culture growth in S-HMO

Locus	Function	Fold change v lactose*		
BINF2_0513	Transcriptional regulator, LacI family	2.08	±	0.34
BINF2_0535	Lactaldehyde reductase	2.32	±	0.35
BINF2_0640	Uncharacterized protein	exclusive		
BINF2_0642	<i>N</i> -acetylmannosamine-6-phosphate 2-epimerase	<lod		
BINF2_0646	Oligopeptide/dipeptide ABC transporter,	exclusive		
BINF2_0648	Dihydrodipicolinate synthetase	exclusive		
BINF2_0731	β- <i>N</i> -acetylhexosaminidase	1.76	±	0.09
BINF2_0876	Sugar kinase, ROK family	exclusive		
BINF2_0878	glucosamine-6-phosphate isomerase	7.94	±	1.13
BINF2_0879	<i>N</i> -acetylglucosamine-6-phosphate deacetylase	7.02	±	0.11
BINF2_0880	Extracellular solute-binding protein, family 1	29.40	±	17.97
BINF2_1768	Formate acetyltransferase	3.07	±	0.19
BINF2_2062	Galactokinase	2.29	±	1.14
BINF2_2167	Hypothetical protein	exclusive		
BINF2_2171	Galactose-1-phosphate uridylyltransferase	8.47	±	2.15
BINF2_2173	GNB/LNB phosphorylase	4.68	±	1.70
BINF2_2199	Extracellular solute-binding protein, family 1	exclusive		
BINF2_2238	Aldehyde-alcohol dehydrogenase	1.79	±	0.31
BINF2_2305	Fucose isomerase (fucose transport protein)	exclusive		
BINF2_2308	Short-chain dehydrogenase/reductase SDR	exclusive		
BINF2_2335	β-1,4-Galactosidase	4.95	±	0.64
BINF2_2336	1,2-α-l-Fucosidase	exclusive		
BINF2_2337	1,3/4-α-l-Fucosidase	4.36	±	0.48
BINF2_2339	Dihydrodipicolinate synthase family protein	4.07	±	0.30
BINF2_2340	SDR family oxidoreductase	6.50	±	2.61
BINF2_2341	Fuconate dehydratase	3.51	±	0.36
BINF2_2343	Binding-protein-dependent transport system	exclusive		
BINF2_2345	Extracellular solute-binding protein, family 1	exclusive		
BINF2_2346	Binding-protein-dependent transport system	exclusive		
BINF2_2348	Extracellular solute-binding protein, family 1	exclusive		
BINF2_2350	dihydrodipicolinate synthase family protein	exclusive		
BINF2_2355	β- <i>N</i> -acetylhexosaminidase	exclusive		

*Protein abundance evaluated using label-free quantitative proteomic analysis
Significant changes in protein abundance determined using Persus (v 1.6.2.2)

Notably, these findings suggest that *B. infantis* may benefit from the extracellular release of sialic acid by strains of *B. bifidum* during co-cultivation. This sialic acid metabolism pathway produces *N*-acetylglucosamine-6-P, which is further processed by NagA1 glucosamine-6-phosphate isomerase (BLON_0881) and NagB2 *N*-acetylglucosamine-6-phosphate deacetylase (BLON_0882), encoded within the *nag* cluster, into fructose-6-P, entering central metabolism (Egan et al., 2014; O’Connell Motherway et al., 2018). When compared to growth of *B. longum* subsp. *infantis* on lactose, we observed significant upregulation of BINF2_0878 NagA1 (>7.86-fold change) and BINF2_0879 NagB2 (>7.02-fold change), as well as a >26.77-fold change in the Nag cluster transport protein during growth on HMO substrates (**Table 1**). The potential trophic interactions between *B. infantis* and *B. bifidum* during growth on sialylHMO and cross-feeding on sialic acid residues may therefore impact bifid shunt-mediated production of acetate and lactate, which have beneficial effects such as preventing pathogen infection by lowering the pH of the infant gut environment.

2.2.2 *Bifidobacterium bifidum*

For *B. bifidum* R0071, proteomic analysis validated *B. bifidum* adaptation for FHMO degradation, with 1,3/4- α -l-fucosidase (BBIF1_1367) displaying a 4.82-fold increase and 1,2- α -l-fucosidase (BBIF_0227) exhibiting a 2.16-fold increase respectively when grown on S-HMOs (**Table 2**). This analysis also revealed significant increases in the abundance of key *B. bifidum* enzymes involved in LNT metabolism including BbgIII (>2.6-fold increase on S-HMOs when compared to lactose) and BbhI (> 4.4-fold increase on S-HMOs when compared to lactose) (**Table 2**). Notably, we also observed significant

increases in protein abundance of LnpA1 GNB/LNB phosphorylase and the GNB/LNB transporter GltA (**Table 2**), corroborating our previously outlined hypothesis that *B. bifidum* acts to defucosylate and desialylate in order to access mucin- or HMO-associated LNB. Proteomic data also revealed substantial upregulation of α -2,3-sialidase (BBIF1_1779), which displayed a fold increase of 73.03 on S-HMOs when compared to co-culture growth on lactose (**Table 2**).

Table 2: Proteins matched to *B. bifidum* found at significantly higher abundance/ uniquely detected after co-culture growth in S-HMO

Locus	Function	Fold change v lactose*		
BBIF1_1779	α -2,3-sialidase	73.03	±	21.39
BBIF1_1367	1,3/4- α -l-Fucosidase	4.82	±	0.36
BBIF1_1522	β -N-acetylhexosaminidase	4.37	±	0.33
BBIF1_1014	Galactose-1-phosphate uridylyltransferase	3.94	±	0.04
BBIF1_1015	N-acetylhexosamine 1-kinase	3.87	±	0.14
BBIF1_0582	Sugar kinase, pfkB family	2.76	±	0.12
BBIF1_0505	β -1,4-Galactosidase	2.66	±	0.17
BBIF1_1436	Lacto-N-biosidase	2.63	±	0.18
BBIF1_0483	Lacto-N-biosidase	2.59	±	0.17
BBIF1_0588	N-acetylglucosamine-6-phosphate deacetylase	2.58	±	0.04
BBIF1_1021	GNB/LNB transporter SBP	2.27	±	0.04
BBIF1_0227	Alpha-Fucosidase	2.16	±	0.11
BBIF1_1780	2,3/6- α -Sialidase	2.06	±	0.16
BBIF1_0587	Glucosamine-6-phosphate isomerase	1.97	±	0.07
BBIF1_1017	Sugar kinase, ROK family	1.92	±	0.17

*Protein abundance evaluated using label-free quantitative proteomic analysis
Significant changes in protein abundance determined using Persus (v 1.6.2.2)

Moderate increases in the sialidase at BBIF1_1780 were also observed, with fold increases of 2.1 ± 0.16 when grown on the S-HMOs when compared to the lactose control. Interestingly, we have previously shown that this sortase-dependent exo- α -sialidase in R0071 is significantly upregulated during exposure to intestinal epithelial cells and promotes *B. bifidum* adhesion to

mucins (Walsh et al., 2023, Chapter VII). We hypothesise, therefore, that the extracellular BBIF1_1780 sialidase from *B. bifidum* acts as a bifunctional extracellular glycosidase that not only modulates *Bifidobacterium*-mucosal surface interactions but also serves to assimilate HMOs and intestinal glycoconjugates. The role of these extracellular sialidases in mucin cross-feeding between sialidase-carrying *B. bifidum* and sialic acid-utilizing *B. breve* has been previously investigated (Egan et al., 2014; Nishiyama et al., 2017). Egan et al. demonstrated that *B. breve* UCC2003 has the ability to cross-feed on sugars released by the mucin-degrading activity of *B. bifidum* PRL2010 (Egan et al., 2014). We hypothesize that similar trophic relationships exist between *B. bifidum* R0071 and *B. breve* M-16V during co-cultivation on HMOs.

2.2.3 *Bifidobacterium breve*

Proteomic analysis of *B. breve* M-16V in mono- and co-culture conditions highlights the importance of interspecies cooperation for effective fucose (**Figure 4**) and sialic acid (**Figure 5**) metabolism. Key fucose metabolism enzymes such as FumP (L-fucose permease), FumB (L-fucose mutarose), FumC (fucose dehydrogenase), FumD (fuconolactone hydrolase), and FumE (L-fuconate dehydratase) were detected during co-culture on S-HMOs yet were absent or at low levels in mono-culture (**Table 3, Figure 4**). This suggests that *B. breve* relies on other species such as *B. bifidum* to provide necessary fucose substrates, supporting earlier findings by Lawson et al. (2020) that cooperative interactions among bifidobacteria, driven by breast milk-derived HMOs, promote niche specialization and growth of species such as *B. breve* through scavenging of degradation products released by more specialized strains such as *B. bifidum* (Lawson et al., 2019).

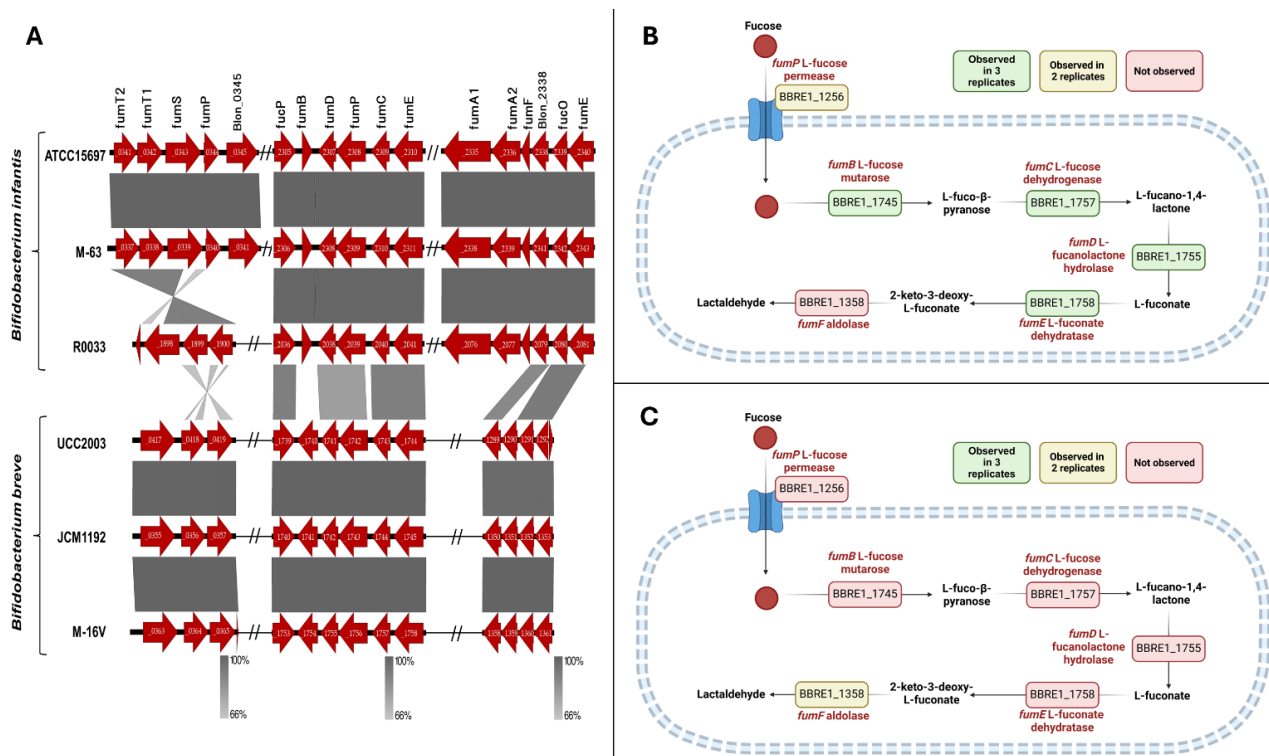


Figure 4: Fucose gene clusters; alignment and M-16V proteome shift **(A)** Homology of the fucose utilisation gene clusters (BLON_0341-345, BLON_2305-2310, BLON_2335-2340) as described for *B. infantis* ATCC15697 by Dedon et al. (2020) in bifidobacterial strains *B. infantis* M-63, *B. infantis* R0033, *B. breve* UCC2003, *B. breve* JCM1192, and *B. breve* M-16V. Linear comparison figures of genomic loci were created using Easyfig (v2.2.5). Homologous regions between strains are shaded in grey, with varying intensities to indicate the degree of conservation (lighter shades represent lower conservation, while darker shades represent higher conservation). Figure also presents proposed fucose pathways for M-16V response based on proteome during growth on HMO in **(B)** co-culture with *B. bifidum* R0071 and *B. infantis* strains and **(C)** mono-culture conditions. Locus regions along reaction arrows are designated in red (not observed), yellow (observed in two replicates), and green (observed in all replicates). Gene symbols and corresponding protein names are provided in red above the reaction arrows.

Proteomic analysis of the M-16V proteome following co-cultivation with *B. bifidum* and *B. longum* subsp. *infantis* also lead to the detection of key proteins involved in sialic acid metabolism, which were either absent or present at lower levels in mono-culture (**Table 3, Figure 5**). These include substrate binding protein NanE (BBRE1_0141), ABC transport ATP-binding proteins NanD and NanF (BBRE1_0143-0144), and sugar kinase NanK (BBRE1_0138) (**Figure 5**). These findings are consistent with our previous research demonstrating that *B. breve* benefits from cross-feeding interactions on sialic acid within bifidobacterial communities (Walsh et al., 2022, Chapter V), highlighting the ecological importance of sialic acid sharing in the infant gut. Interestingly, the proteome of M-16V demonstrated distinct upregulation of the GNB/LNB transporter GltA during co-cultivation on HMOs, when compared to co-cultivation on lactose. GltA in M-16V, which demonstrated 78.8% sequence identity to the GNB/ LNB transporter in *B. bifidum*, increased 3.3-fold on S-HMOs, providing further evidence of the cross-feeding (and perhaps competitive) relationship between *B. bifidum* and *B. breve* during growth on HMOs (**Table 3**).

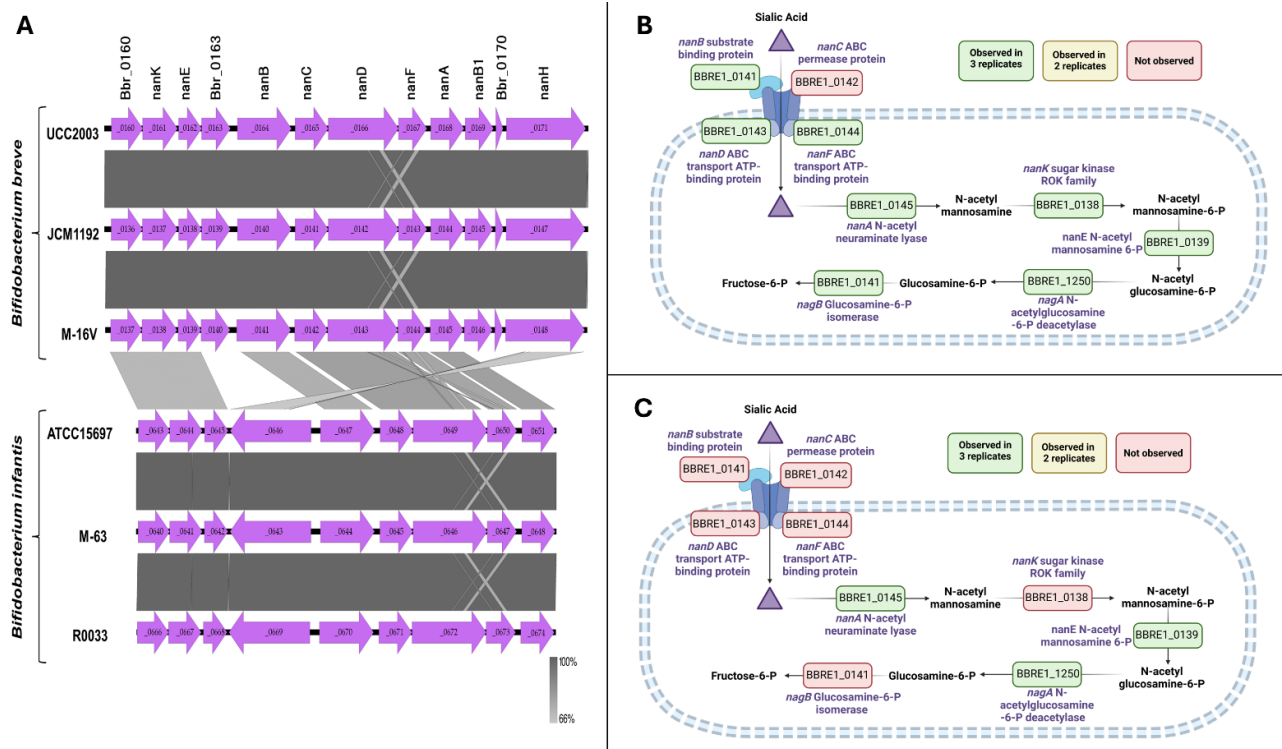


Figure 5: Sialic acid gene clusters: alignment and M-16V proteomic shift **(A)** Homology of the sialic acid utilisation gene clusters (Bbr_0160-0171) as described for *B. breve* UCC2003 by Egan et al. (2014) in bifidobacterial strains *B. breve* JCM1192, *B. breve* M-16V, *B. infantis* ATCC15697, *B. infantis* M-63, and *B. infantis* R0033. Linear comparison figures of genomic loci were created using Easyfig (v2.2.5). Homologous regions between strains are shaded in grey, with varying intensities to indicate the degree of conservation (lighter shades represent lower conservation, while darker shades represent higher conservation). Figure also presents proposed sialic acid pathways for M-16V response based on proteome during growth on HMO in **(B)** co-culture with *B. bifidum* R0071 and *B. infantis* strains and **(C)** mono-culture conditions. Locus regions along reaction arrows are designated in red (not observed), yellow (observed in two replicates), and green (observed in all replicates). Gene symbols and corresponding protein names are provided in purple above the reaction arrows.

Table 3: Proteins matched to *B. breve* found at significantly higher abundance/ uniquely detected after co-culture growth in S-HMO

Locus	Function	Fold change v lactose		
BBRE1_0010	β -1,4-Galactosidase	1.90	$\pm$	0.17
BBRE1_0137	Uncharacterized protein	exclusive		
BBRE1_0138	<i>N</i> -acetylmannosamine kinase/ ROK family	<LOD		
BBRE1_0139	<i>N</i> -Acetylmannosamine-6-phosphate 2-epimerase	exclusive		
BBRE1_0141	Substrate-binding protein	exclusive		
BBRE1_0143	ABC transport system ATP-binding protein	exclusive		
BBRE1_0144	ABC transport system ATP-binding protein	exclusive		
BBRE1_0145	<i>N</i> -Acetylneuraminate lyase	exclusive		
BBRE1_0148	<i>N</i> -acetylneuraminate lyase	exclusive		
BBRE1_0150	GntR domain protein	exclusive		
BBRE1_0364	Binding-protein-dependent permease	<LOD		
BBRE1_0418	Galactose-1-phosphate uridylyltransferase	exclusive		
BBRE1_0419	Galactokinase	1.91	$\pm$	0.14
BBRE1_0453	ABC transport system permease protein	<LOD		
BBRE1_0456	Solute-binding protein of ABC transporter	3.85	$\pm$	0.19
BBRE1_0484	Extracellular solute-binding protein, family 1	exclusive		
BBRE1_0748	Formate acetyltransferase	2.97	$\pm$	0.14
BBRE1_0802	NagB2 Glucosamine-6-phosphate isomerase	1.90	$\pm$	0.15
BBRE1_0807	Sulfatase family protein	2.90	$\pm$	0.57
BBRE1_1250	<i>N</i> -acetylglucosamine-6-phosphate deacetylase	exclusive		
BBRE1_1251	Glucosamine-6-phosphate isomerase	2.21	$\pm$	0.22
BBRE1_1360	SDR family oxidoreductase	7.09	$\pm$	2.52
BBRE1_1361		3.36	$\pm$	0.20
BBRE1_1374	Alpha-L-fucosidase	exclusive		
BBRE1_1556	Lactaldehyde reductase	1.96	$\pm$	0.24
BBRE1_1561	β -1,4-Galactosidase	3.56	$\pm$	0.17
BBRE1_1564	Solute-binding protein of ABC transporter	3.91	$\pm$	0.13
BBRE1_1597	<i>N</i> -acetylhexosamine 1-kinase	exclusive		
BBRE1_1598	GNB/LNB phosphorylase	exclusive		
BBRE1_1601	Solute-binding protein of the ABC transporter	3.35	$\pm$	0.08
BBRE1_1656	aldehyde-alcohol dehydrogenase	2.30	$\pm$	0.15
BBRE1_1754	Dihydrodipicolinate synthase	exclusive		
BBRE1_1755	amidohydrolase family protein	exclusive		
BBRE1_1757	Short-chain dehydrogenase/reductase SDR	exclusive		
BBRE1_1758	fuconate dehydratase	exclusive		
BBRE1_1893	galactose-1-phosphate uridylyltransferase	exclusive		
BBRE1_1904	ABC transporter related	2.87	$\pm$	0.24

3. Conclusions

The results from our study significantly contribute to understanding the metabolic interactions within bifidobacterial communities, particularly their capacity to degrade and utilize various human milk oligosaccharides (HMOs). Key findings demonstrate the specific abilities of each assessed *Bifidobacterium* strain to metabolize fucosylated, sialylated, and/or neutral HMOs, which are believed to play a crucial role in bifidobacterial colonization of the neonatal gut. Our genomic and proteomic analyses reveal how *B. bifidum*, *B. infantis*, and *B. breve* differ in their ability to metabolize these complex glycans, with *B. bifidum* showing a specialization in extracellular degradation of sialylated and fucosylated HMOs, while *B. longum* subsp. *infantis* is particularly efficient at utilizing a broad spectrum of HMOs due to its extensive glycosyl hydrolase repertoire. Our study also highlights the importance of cross-feeding interactions among bifidobacterial species. The ability of *B. bifidum* to release HMO degradation products such as fucose and sialic acid, which are then utilized by other species like *B. breve*, illustrates the cooperative nature of certain bifidobacterial species. This trophic relationship may enhance growth and survival of bifidobacteria in the infant gut and emphasizes the value of developing multispecies probiotic formulations that mimic natural microbial interactions.

A critical practical implication of these findings is their relevance to the formulation of probiotic supplements and infant formulas. By selecting specific bifidobacterial strains that can complement each other's metabolic capabilities, particularly through cross-feeding, it is possible to design more effective probiotic formulations that support the healthy establishment of the infant gut microbiota. These insights can guide the selection of strains that

are best suited to promote gut health in formula-fed infants, who are typically deprived of the HMOs present in breast milk. In conclusion, this integrative genomic and proteomic analysis has provided valuable insights into the metabolic strategies of bifidobacteria and their roles in HMO utilization. These findings underscore the importance of interspecies cooperation in the gut microbiota and provide a foundation for optimizing probiotic interventions aimed at supporting infant health.

4. Materials and methods

4.1. Carbohydrate substrates

Commercial HMO powders (2'-FL, 3-FL, 6'-SL, 3'-SL, LNT, LNnT) were received from Glycom A/S (Hørsholm, Denmark, purity > 90%). The di- and mono-saccharides used in this study were lactose, fucose, galactose, glucose (Sigma-Aldrich, Steinheim, Germany), and sialic acid (Carbosynth, Compton, Berkshire, United Kingdom).

4.1.1. Generation of HMO-enriched fraction:

Oligosaccharide fractions were isolated from human milk as previously described (Walsh et al., 2022). In short, pooled human milk samples (of unknown secretor status) were kindly donated by Irvinestown Human Milk Bank (Co. Fermanagh, Ireland) and stored at -80 C on arrival. Lipids and proteins were removed before applying samples to a BioGel P2 size exclusion column (92 × 5 cm; Bio-Rad Laboratories, Inc., Hercules, CA, USA) to separate lactose and HMO components. High pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was used, as described below, to quantify lactose in the eluted fractions while a Pierce colorimetric peptide assay (Thermo Fisher Scientific, Reinach,

Switzerland) was used to measure levels of peptides. Peptide-free and low-trace lactose (<80 mg/L) fractions were pooled and freeze-dried and the resultant HMO-enriched fraction was stored at 4°C prior to use in experiments. HPAEC analysis indicated that low levels of 2'-fucosyllactose (2'-FL) were found in the HMO-enriched fraction, likely due to the purification process where 2'-FL and lactose fractionated together in the same eluates. The 2'-FL which had been eliminated during the size exclusion process was then resubstituted using commercial 2'-FL (Glycom) to give a powder representative of secretor HMOs (designated S-HMOs, **Supplementary Figure S1**) as previously described (Walsh et al., 2022).

4.2. Bacterial strains and culture conditions

Commercial *Bifidobacterium* strains used in this study were obtained from Lallemand Health Solutions, France (*B. bifidum* R0071 and *B. longum* subsp. *infantis* R0033), and Morinaga Milk, Japan (*B. breve* M-16V and *B. longum* subsp. *infantis* M-63). Strains were stored at -80 °C in de Man-Rogosa-Sharpe (MRS) broth (Difco, BD, Ireland), supplemented with 50% (v/v) glycerol as a cryoprotectant. For routine experiments, strains were grown at 37 °C under anaerobic conditions using MRS medium supplemented with 0.05% (wt/v) L-cysteine hydrochloride (Merck) and 0.01% (wt/v) mupirocin (Merck)). Bacterial stocks were regularly checked for contamination by closely monitoring colony morphology during culturing. In addition, the bacterial stocks were routinely validated as being specific to their genera using bifidobacterial-specific PCR targeting the 16S region using primers g-Bifid-F (5'-CTCCTGGAAACGGGTGG-3') and g-Bifid-R (5'-CTCCTGGAAACGGGTGG-3') (Matsuki et al., 2004). Resultant amplicons were sequenced using Sanger sequencing.

4.3. Whole Genome Sequencing Assembly and Annotation

Following growth of R0071, R0033, M-16V and M-63 as described in section 2.3 above, bacterial cells were harvested, lysed, and total bacterial DNA was isolated. Genome sequences of the bifidobacterial strains were generated by MacroGen Inc. (Seoul, South Korea) using Pacific Biosciences SMRT RSII technology. The raw sequencing reads were *de novo* assembled using the Hierarchical Genome Assembly Process (HGAP) protocol RS_Assembly.2 implemented in the SMRT Analysis software v.2.3 with default parameters (<https://github.com/PacificBiosciences/SMRT-Analysis>) as described previously (Bottacini et al., 2017).

Open Reading Frame (ORF) prediction and automatic annotation was performed using Prodigal v2.0 (<http://prodigal.ornl.gov>) for gene predictions. BLASTP v2.2.26 (cut-off E-value of 0.0001) was used for sequence alignments against a combined bifidobacterial genome-based database, and MySQL relational database to assign annotations. Predicted functional assignments were manually revised and edited using similarity searches against the non-redundant protein database curated by the National Centre for Biotechnology Information (<ftp://ftp.ncbi.nih.gov/blast/db/>) and PFAM database (<http://pfam.sanger.ac.uk>), which allowed for *in silico* characterization of hypothetical proteins. GenBank editing and manual inspection was performed using Artemis v18 (<http://www.sanger.ac.uk/resources/soft-ware/artemis/>). Transfer RNA genes were identified employing tRNAscan-SE v1.4 and ribosomal RNA genes were detected based on the software package Rnammer v1.2 (38) supported by BLASTN v2.2.26.

4.4. Genome-Wide Comparisons and Phylogenetic Analysis

The evolutionary relationships of *Bifidobacterium* strains were analyzed by comparing the genomes of the four commercial strains (*B. bifidum* R0071, *B. breve* M-16V, *B. longum* subsp. *infantis* M-63, and *B. longum* subsp. *infantis* R0033) to publicly available genomes from the NCBI database. Phylogenetic trees were constructed using multiple sequence alignment of 49 core genes via the FastTree plugin on KBase. Linear comparison figures of genomic loci (*B. longum* subsp. *infantis* HMO Cluster 1 (Sela 2008), *B. breve* UCC2003 sialic acid utilization cluster (Egan et al., 2014) and *B. infantis* fucose utilization cluster (Dedon et al., 2020) were created using Easyfig (v2.2.5). Average nucleotide identity was calculated using the ANIm algorithm from FastANI v1.33.

4.5. Carbohydrate Utilisation Prediction

The carbohydrate utilization capabilities of the commercial strains and prototype strains were assessed using two different approaches. Firstly, BlastKoala was used to assign KEGG orthology numbers to the bifidobacterial sequences (<https://www.kegg.jp/blastkoala/>) and group genes into KEGG modules describing metabolic pathways. This allowed for identification of complete and incomplete pathways associated with carbohydrate utilisation pathways. Secondly, Carbohydrate Active Enzymes (CAZymes) were identified by annotating the genomes on the dbCAN metaserver (v10.0) against the CAZY database using HMMER (E-Value < $1e^{-15}$, coverage > 0.35) tools. The CAZY database separates enzymes into one of six classes: glycoside hydrolases, glycosyl transferases, polysaccharide

lyases, carbohydrate esterases, auxiliary activities, and carbohydrate-binding modules.

4.6. Prediction of HMO utilisation capabilities:

Scientific literature databases including PubMed, Web of Science, UCC library, and Google Scholar were searched for studies providing mechanistic insight into HMO metabolism (including HMOs, HMO-derivates, and subsequent organic acid production). A list of HMO utilisation features (**Supplementary Tables S1, S2, and S3**) was then compiled from these studies which included gene-trait matching, transcriptomic, proteomic, and knock-out studies (Arzamasov & Osterman, 2022; Asakuma et al., 2011; Ashida et al., 2020; Ashida et al., 2009; Bunesova et al., 2016; Dedon et al., 2020; Duar, Henrick, et al., 2020; M. Egan et al., 2014; Muireann Egan, Mary O’Connell Motherway, et al., 2014; Garrido et al., 2013; Garrido et al., 2011; Garrido et al., 2015; Garrido et al., 2012; González-Rodríguez et al., 2012; González et al., 2008; James et al., 2016; James et al., 2018; Katayama et al., 2004; Kim et al., 2013; Kitaoka, 2012; Kitaoka et al., 2005; Kiyohara et al., 2011; Lanigan et al., 2019; Lawson et al., 2019; LoCascio Riccardo et al., 2010; Miwa et al., 2010; Nishiyama et al., 2018; O’Connell Motherway et al., 2018; Pokusaeva et al., 2011; Ruiz-Moyano et al., 2013; Sakanaka et al., 2020; Sakanaka et al., 2019; Sakurama et al., 2013; Schwab et al., 2017; Sela et al., 2008; Sela et al., 2012; Sela et al., 2011; Thomson et al., 2018; Turroni et al., 2010; Turroni et al., 2012; Xiao et al., 2010; Yamada et al., 2017; Yoshida et al., 2012; Zabel et al., 2019). A Blast search was then performed (e-value cutoff of $1e^{-5}$, query cover cutoff of 50%) of the database against the bifidobacterial genomes of interest. Percent identity was recorded for each

bifidobacterial strain of interest and classified as “low identity” = 40-60%, “moderate identity” = 60-80%, and “high identity” = 80-100%.

4.7. Validation of HMO genotype/phenotype associations

Carbohydrate-dependent growth profile analysis of bifidobacterial strains on individual HMO and HMO-derivatives was performed to validate the predicted HMO utilisation pathways. For growth analysis, a 5% (w/v) stock solution of a given substrate (2'-FL, 3-FL, 3'-SL, 6'-SL, LNT, LNnT, lactose, fucose, sialic acid, glucose, and galactose) was prepared using distilled water. The obtained solution was filter sterilized using a 0.45 µm membrane filter. To test the growth potential of each strain, carbohydrate-free de Man Rogosa and Sharpe (cfMRS) medium was freshly prepared from first principles as previously described (O'Donnell et al., 2011) and sterilised by autoclaving. For use in experiments, the medium was supplemented with 0.5% (v/v) of an individual growth substrate solution and 0.05% (v/v) of L-cysteine HCl as essential nitrogen source for bifidobacterial growth. To this medium, 1% (v/v) of an overnight culture previously cultivated in 2% glucose was added. Medium without any supplemented carbohydrate was used as a negative control for each experiment. Cultures were grown anaerobically at 37 °C and bacterial numbers quantified at regular intervals for 24 hours. Growth profiles were obtained by recording optical density measurements at OD_{600nm} and by serial diluting and plating on MRS agar to obtain colony counts (cfu/mL).

4.8. Proteomic analysis of HMO utilisation-related factors

4.8.1. Sample preparation:

Overnight cultures of *Bifidobacterium* were prepared as previously described (Walsh et al., 2022). Co-cultures containing equal amounts of *B. bifidum*

R0071, *B. longum* subsp. *infantis* R0033, *B. breve* M-16V, & *B. longum* subsp. *infantis* M-63 were prepared and cultured under anaerobic conditions at 37 °C for 10 hours in oligosaccharide supplemented media (Lactose, 2'-FL, HMO, or cfMRS). For comparative purposes and in order to understand the impact of bifidobacterial co-culturing on M-16V proteome, a mono-culture of M-16V was similarly prepared on the same carbohydrate sources. At the end of fermentation, bacterial cells were pelleted by centrifugation and washed three times in PBS to remove any residual media. Bacterial cell pellets were immediately frozen in liquid nitrogen and stored at -70 °C for proteomic analysis.

4.8.2. Protein extraction and digestion:

Cold lysis buffer (0.1 M Tris-HCl, 10 % glycerol, 20 mM EDTA, 50 mM NaCl, 1 mM PMSF, 1 µg/ml, pH 7.5) was added to each cell pellet and sonicated using a MS72 probe (3 x 10 sec, cycle 6, 20 % power), and placed on ice between each sonication round. An aliquot of each lysate was clarified by centrifugation and a Bradford protein assay (Bio-Rad) was used to determine the protein concentration. Total lysates (without clarification) were adjusted to 0.25 mg/ml protein concentration with 50 mM ammonium bicarbonate and heated at 95 °C, 5 min. Samples were reduced using dithiothreitol (DTT; 5mM final, 15 min), alkylated using iodoacetamide (IAA; 15mM final, 20 min, dark) and subjected to overnight trypsin digestion at 37°C (trypsin: 8 µg/ml final) in the presence of ProteaseMax (0.01 % (w/v)). Digestion was stopped by addition of trifluoroacetic acid (1% (v/v) final) and samples were dried under vacuum. Digests were resuspended in 0.5% (v/v) TFA prior to clean-up using C18 ZipTips (Millipore) according to

manufacturer's instructions. Samples were dried under vacuum and stored at -70 °C until LC-MS/MS analysis.

4.8.3. Label-free quantitative (LFQ) proteomic analysis:

Peptide extracts (0.75 µg) were analysed by LC-MS/MS using the Thermo Q-Exactive mass spectrometer coupled to a Dionex Ultimate 3000 RSLCnano, using a 2.25 hour method for separation on an Easy-Spray PepMap™ column (50 cm × 75 µm, 2 µm particles). Samples were searched against a composite database containing the proteomes of R0071, M-16V, and M-63, appended with the *Homo sapiens* proteome (downloaded from Uniprot 15/02/2019) using MaxQuant (v 1.6.2.10) with MaxLFQ algorithm included for protein ratio characterisation (Cox et al., 2014) and 1% FDR applied. Perseus (v 1.6.2.2) was used for data analysis (Tyanova et al., 2016). Proteins were filtered to exclude single peptide identifications and proteins matching a reverse or a contaminants database. Proteins were retained for analysis only if detected in at least two biological replicates from either comparator group. Qualitative results were based on unique protein detection in at least two biological replicates in a condition, with absence of detection in all replicates of the comparator set. Quantitative and qualitative results were combined prior to functional characterization.

Proteins with quantitative or qualitative changes were collated. Functional analysis was carried out on the entire set of proteins identified in the study using BLAST2GO. BLAST analysis was carried out using CloudBlast with the blastp-fast program against the non-redundant NCBI Bacteria database. Mapping and annotation were then carried out to assign gene ontology (GO) annotations based on best BLAST hits. InterProScan analysis was carried out in tandem to identify protein families, domains, sites

and repeats associated with the protein sequences identified. Associated GO annotations from InterProScan analysis were then merged to the existing results. To determine which functional groups/annotations were enriched among the proteins with altered abundance, enrichment analysis was performed using Fisher's Exact test, by GO or InterProScan term. Due to redundancy in GO terms and presence of very general terms, further enrichment was carried out to reduce the results to the lowest level term in a family (i.e. most specific description) which was still significantly enriched ($p < 0.05$).

4.9 Statistical analysis

Statistical analysis for adhesion assays were performed using technical triplicate data (three wells from each passage of cells) from biological triplicate experiments (across three successive passages of HT29-MTX cells). Graphs were created using PRISM v8 (GraphPad, La Jolla, CA) and were plotted as mean $\pm$ sd. Univariate analysis of variance (ANOVA) and post-hoc Tukey tests were performed to determine the significant differences between strains and carbohydrates for levels of bifidobacterial adhesion. Statistical significance was accepted as $p \leq 0.05$. For proteomic analysis Perseus was used for statistical analysis, with quantitative results composed of proteins with a significant change in abundance ($p < 0.05$ (Student's t-test), fold change ≥ 2).

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Supplementary Materials

Supplementary Table 1: Genome of *Bifidobacterium infantis* R0033, M-63, and ATCC15697 matched against genes[†] associated with metabolism of HMO and HMO-derivatives

Designation	Protein name	Strain*	Accession number	Cluster	GH no.	Percent identity			Locus	
						ATCC15697	R0033	M-63	R0033	M-63
<i>blon_0341</i>	Binding-protein-dependent permease	<i>B.infantis</i> ATCC 15697	ACJ51465.1			100		100		BINF2_0337
<i>blon_0342</i>	Binding-protein-dependent permease	<i>B.infantis</i> ATCC 15697	ACJ51466.1			100		100		BINF2_0338
<i>blon_0343</i>	Extracellular solute-binding protein, family 1	<i>B.infantis</i> ATCC 15697	ACJ51467.1			100		100		BINF2_0339
<i>blon_2202</i>	Extracellular solute-binding protein, family 1	<i>B.infantis</i> ATCC 15697	ACJ53262.1			100	97.23	100	BINF_1898	BINF2_2200
<i>blon_2203</i>	Binding-protein-dependent permease	<i>B.infantis</i> ATCC 15697	ACJ53263.1			100	97.23	100	BINF_1899	BINF2_2201
<i>blon_2204</i>	Binding-protein-dependent permease	<i>B.infantis</i> ATCC 15697	ACJ53264.1			100	99.67	100	BINF_1900	BINF2_2202
<i>blon_2335</i>	1,2- α -l-Fucosidase	<i>B.infantis</i> ATCC 15697	ACJ53393.1	H-1	GH95	100	98.85	100	BINF_2076	BINF2_2338
<i>blon_2336</i>	1,3/4- α -l-Fucosidase	<i>B.infantis</i> ATCC 15697	ACJ53394.1	H-1	GH29	100	99.58	100	BINF_2077	BINF2_2339
<i>blon_2348</i>	2,3/6- α -Sialidase	<i>B.infantis</i> ATCC 15697	ACJ53406.1	H-1	GH33	100	100	100	BINF_2086	BINF2_2351
<i>bbr_0235</i>	β -1,4-Galactosidase	<i>B.breve</i> UCC2003	ABE94991.1		GH2	100		100		BINF2_0264
<i>bbr_0530</i>	Solute-binding protein of ABC transporter system	<i>B.breve</i> UCC2003	ABE95227.1			48.54	49.37	48.54	BINF_1706	BINF2_2016
<i>bbr_1554</i>	Solute-binding protein of ABC transporter system	<i>B.breve</i> UCC2003	ABE96225.1			93.89	41.83	93.71	BINF_1706	BINF2_0460
<i>bbr_1558</i>	Permease protein of the ABC transporter system	<i>B.breve</i> UCC2003	ABE96229.1			99.02	98.77	99.02	BINF_1838	BINF2_2135
<i>bbr_1559</i>	ATP-binding protein of ABC transporter system	<i>B.breve</i> UCC2003	ABE96230.1			98.58	98.1	98.58	BINF_1839	BINF2_2136
<i>bbr_1560</i>	ATP-binding protein of ABC transporter system	<i>B.breve</i> UCC2003	ABE96231.1			95.35	95.35	95.35	BINF_1840	BINF2_2137
<i>blon_0346</i>	Beta-galactosidase	<i>B.infantis</i> ATCC 15697	ACJ51470.1		GH42	100	97.28	100	BINF_0275	BINF2_0342
<i>blon_0459</i>	β -N-acetylhexosaminidase	<i>B.infantis</i> ATCC 15697	ACJ51575.1		GH20	100	53.06	100	BINF_2091	BINF2_0457
<i>blon_0732</i>	β -N-acetylhexosaminidase	<i>B.infantis</i> ATCC 15697	ACJ51836.1		GH20	100	98.31	99.85	BINF_0766	BINF2_0731
<i>blon_0883</i>	Extracellular solute-binding protein, family 1	<i>B.infantis</i> ATCC 15697	ACJ51983.1			100	99.55	100	BINF_0908	BINF2_0881
<i>blon_2016</i>	β -1,3-Galactosidase	<i>B.infantis</i> ATCC 15697	ACJ53083.1		GH42	100	99.86	100	BINF_1708	BINF2_2018
<i>blon_2174</i>	GNB/LNB phosphorylase	<i>B.infantis</i> ATCC 15697	ACJ53235.1	H-5	GH112	100	99.07	100	BINF_1877	BINF2_2174
<i>blon_2175</i>	Binding-protein-dependent transport system	<i>B.infantis</i> ATCC 15697	ACJ53236.1	H-5		100		100		BINF2_2175

Supplementary Table 1: Genome of *Bifidobacterium infantis* R0033, M-63, and ATCC15697 matched against genes[†] associated with metabolism of HMO and HMO-derivatives

Designation	Protein name	Strain*	Accession number	Cluster	GH no.	Percent identity			Locus	
						ATCC15697	R0033	M-63	R0033	M-63
<i>blon_2176</i>	Binding-protein-dependent transport system	<i>B.infantis</i> ATCC 15697	ACJ53237.1	H-5		100		100		BINF2_2176
<i>blon_2177</i>	Extracellular solute-binding protein, family 1	<i>B.infantis</i> ATCC 15697	ACJ53238.1	H-5		100		100		BINF2_2177
<i>blon_2331</i>	sugar transporter	<i>B.infantis</i> ATCC 15697	ACJ53389.1	H-1		100	100	100	BINF_2073	BINF2_2334
<i>blon_2332</i>	sugar transporter	<i>B.infantis</i> ATCC 15697	ACJ53390.1	H-1		100	99.22	100	BINF_2074	BINF2_2335
<i>blon_2334</i>	β -1,4-Galactosidase	<i>B.infantis</i> ATCC 15697	ACJ53392.1	H-1	GH2	100	99.41	100	BINF_2075	BINF2_2337
<i>blon_2341</i>	Protein of unknown function DUF624	<i>B.infantis</i> ATCC 15697	ACJ53399.1	H-1		100	100	100	BINF_2082	BINF2_2344
<i>blon_2342</i>	Binding-protein-dependent transport system	<i>B.infantis</i> ATCC 15697	ACJ53400.1	H-1		100	100	100	BINF_2083	BINF2_2345
<i>blon_2343</i>	Binding-protein-dependent transport system	<i>B.infantis</i> ATCC 15697	ACJ53401.1	H-1		100	100	100	BINF_2084	BINF2_2346
<i>blon_2344</i>	Extracellular solute-binding protein, family 1	<i>B.infantis</i> ATCC 15697	ACJ53402.1	H-1		100	85.26	100	BINF_2085	BINF2_2347
<i>blon_2345</i>	Binding-protein-dependent transport system	<i>B.infantis</i> ATCC 15697	ACJ53403.1	H-1		100	100	100	BINF_2083	BINF2_2348
<i>blon_2346</i>	Binding-protein-dependent transport system	<i>B.infantis</i> ATCC 15697	ACJ53404.1	H-1		100	100	100	BINF_2084	BINF2_2349
<i>blon_2347</i>	Extracellular solute-binding protein, family 1	<i>B.infantis</i> ATCC 15697	ACJ53405.1	H-1		100	88.89	100	BINF_2085	BINF2_2350
<i>blon_2350</i>	Extracellular solute-binding protein, family 1	<i>B.infantis</i> ATCC 15697	ACJ53408.1	H-1		100	99.05	100	BINF_2088	BINF2_2353
<i>blon_2351</i>	Extracellular solute-binding protein, family 1	<i>B.infantis</i> ATCC 15697	ACJ53409.1	H-1		100	90.65	100	BINF_2089	BINF2_2354
<i>blon_2352</i>	Extracellular solute-binding protein, family 1	<i>B.infantis</i> ATCC 15697	ACJ53410.1	H-1		100	84.8	100	BINF_2090	BINF2_2355
<i>blon_2353</i>	Hypothetical protein	<i>B.infantis</i> ATCC 15697	ACJ53411.1	H-1	GH20	100	80	80	BINF_2091	BINF2_2356
<i>blon_2354</i>	Extracellular solute-binding protein, family 1	<i>B.infantis</i> ATCC 15697	ACJ53412.1	H-1		100	100	100	BINF_2090	BINF2_2357
<i>blon_2355</i>	β -N-acetylhexosaminidase	<i>B.infantis</i> ATCC 15697	ACJ53413.1	H-1	GH20	100	99.39	100	BINF_2091	BINF2_2358
<i>blon_0625</i>	Beta-glucosidase (L-arabinopyranosidase)	<i>B.infantis</i> ATCC 15697	ACJ51732.1	H-3	GH33	100	98.28	100	BINF_0645	BINF2_0622
<i>blon_2357</i>	Extracellular solute-binding protein, family 1	<i>B.infantis</i> ATCC 15697	ACJ53415.1	H-1		100	98.84	100	BINF_2093	BINF2_2359
<i>blon_2359</i>	Binding-protein-dependent transport system	<i>B.infantis</i> ATCC 15697	ACJ53417.1	H-1		100	99.26	99.63	BINF_2095	BINF2_2361
<i>blon_2360</i>	Binding-protein-dependent transport system	<i>B.infantis</i> ATCC 15697	ACJ53418.1	H-1		100	99.37	100	BINF_2096	BINF2_2362
<i>blon_2361</i>	Trehalose import ATP-binding protein SugC	<i>B.infantis</i> ATCC 15697	ACJ53419.1	H-1		100	99.73	100	BINF_2097	BINF2_2363
<i>blon_0243</i>	Two component transcriptional regulator, LuxR family	<i>B.infantis</i> ATCC 15697	ACJ51372.1	H-2		100	100	100	BINF_0238	BINF2_0241
<i>blon_0244</i>	Signal transduction histidine kinase-like protein	<i>B.infantis</i> ATCC 15697	ACJ51373.1	H-2		100	100	100	BINF_0239	BINF2_0242
<i>blon_0245</i>	MFS transporter	<i>B.infantis</i> ATCC 15697	ACJ51374.1	H-2		100	100	100	BINF_0240	BINF2_0243
<i>blon_0247</i>	major facilitator superfamily MFS_1	<i>B.infantis</i> ATCC 15697	ACJ51375.1	H-2		100	99.51	100	BINF_0241	BINF2_0244

Supplementary Table 1: Genome of *Bifidobacterium infantis* R0033, M-63, and ATCC15697 matched against genes[†] associated with metabolism of HMO and HMO-derivatives

Designation	Protein name	Strain*	Accession number	Cluster	GH no.	Percent identity			Locus	
						ATCC15697	R0033	M-63	R0033	M-63
<i>blon_0248</i>	Alpha-L-fucosidase	<i>B.infantis</i> ATCC 15697	ACJ51376.1	H-2	GH29	100	100	100	BINF_0242	BINF2_0245
<i>blon_0344</i>	Mandelate racemase/muconate lactonizing	<i>B.infantis</i> ATCC 15697	ACJ51468.1	H-2		100	98.96	99.52	BINF_2081	BINF2_0340
<i>blon_0345</i>	major facilitator superfamily MFS_1	<i>B.infantis</i> ATCC 15697	ACJ51469.1			100	58.56	100	BINF_0241	BINF2_0341
<i>blon_0423</i>	LacI type regulator	<i>B.infantis</i> ATCC 15697	ACJ51544.1	H-3		100	98.92	100	BINF_0406	BINF2_0420
<i>blon_0425</i>	MFS transporter	<i>B.infantis</i> ATCC 15697	ACJ51545.1	H-3		100	99.26	100	BINF_0407	BINF2_0421
<i>blon_0426</i>	Alpha-L-fucosidase	<i>B.infantis</i> ATCC 15697	ACJ51546.1	H-3	GH29	100	100	100	BINF_0408	BINF2_0422
<i>blon_0540</i>	Lactaldehyde reductase	<i>B.infantis</i> ATCC 15697	ACJ51653.1			100	99.74	100	BINF_0540	BINF2_0535
<i>blon_2305</i>	fucose isomerase (fucose transport protein)	<i>B.infantis</i> ATCC 15697	ACJ53363.1			100	100	100	BINF_2037	BINF2_2307
<i>blon_2306</i>	amidohydrolase family protein	<i>B.infantis</i> ATCC 15697	ACJ53364.1			100	98.43	100	BINF_2038	BINF2_2308
<i>blon_2307</i>	fucose transport protein	<i>B.infantis</i> ATCC 15697	ACJ53365.1			100	99.53	100	BINF_2039	BINF2_2309
<i>blon_2309</i>	fuconate dehydratase	<i>B.infantis</i> ATCC 15697	ACJ53367.1			100	100	100	BINF_2041	BINF2_2311
<i>blon_2337</i>	L-fucose mutarotase (fucose operon protein)	<i>B.infantis</i> ATCC 15697	ACJ53395.1	H-1		100	99.31	100	BINF_2078	BINF2_2340
<i>blon_2338</i>	dihydrodipicolinate synthase family protein	<i>B.infantis</i> ATCC 15697	ACJ53396.1	H-1		100	99.6	100	BINF_1595	BINF2_2341
<i>blon_2339</i>	SDR family oxidoreductase	<i>B.infantis</i> ATCC 15697	ACJ53397.1	H-1		100	100	100	BINF_2080	BINF2_2342
<i>blon_2340</i>	fuconate dehydratase	<i>B.infantis</i> ATCC 15697	ACJ53398.1	H-1		100	99.53	99.76	BINF_2081	BINF2_2343
<i>blon_2349</i>	dihydrodipicolinate synthase family protein	<i>B.infantis</i> ATCC 15697	ACJ53407.1	H-1		100	98.74	100	BINF_2087	BINF2_2352
<i>blon_2475</i>	ABC transporter related	<i>B.infantis</i> ATCC 15697	ACJ53529.1			100	100	100	BINF_2222	BINF2_2477
<i>blon_rs11960</i>	Short-chain dehydrogenase/reductase SDR	<i>B.infantis</i> ATCC 15697	ACJ53366.1			100	100	100	BINF_2040	BINF2_2310
<i>blon_0641</i>	LacI regulator	<i>B.infantis</i> ATCC 15697	ACJ51748.1	H-4		100	99.4	100	BINF_0664	BINF2_0638
<i>blon_0642</i>	GntR domain protein	<i>B.infantis</i> ATCC 15697	ACJ51749.1	H-4		100	99.18	100	BINF_0665	BINF2_0639
<i>blon_0643</i>	Uncharacterized protein	<i>B.infantis</i> ATCC 15697	ACJ51750.1	H-4		100	99.01	100	BINF_0666	BINF2_0640
<i>blon_0644</i>	ROK family protein	<i>B.infantis</i> ATCC 15697	ACJ51751.1	H-4		100	100	100	BINF_0667	BINF2_0641
<i>blon_0645</i>	N-acetylmannosamine-6-phosphate 2-epimerase	<i>B.infantis</i> ATCC 15697	ACJ51752.1	H-4		100	99.56	100	BINF_0668	BINF2_0642
<i>blon_0646</i>	α -2,3-sialidase	<i>B.infantis</i> ATCC 15697	ACJ51753.1	H-4	GH33	100	99.61	100	BINF_0669	BINF2_0643
<i>blon_0647</i>	Extracellular solute-binding protein	<i>B.infantis</i> ATCC 15697	ACJ51754.1	H-4		100	99.81	100	BINF_0670	BINF2_0644
<i>blon_0648</i>	Binding-protein-dependent transport system	<i>B.infantis</i> ATCC 15697	ACJ51755.1	H-4		100	99.69	100	BINF_0671	BINF2_0645
<i>blon_0649</i>	Oligopeptide/dipeptide ABC transporter,	<i>B.infantis</i> ATCC 15697	ACJ51756.1	H-4		100	99.72	100	BINF_0672	BINF2_0646

Supplementary Table 1: Genome of *Bifidobacterium infantis* R0033, M-63, and ATCC15697 matched against genes[†] associated with metabolism of HMO and HMO-derivatives

Designation	Protein name	Strain*	Accession number	Cluster	GH no.	Percent identity			Locus	
						ATCC15697	R0033	M-63	R0033	M-63
<i>blon_0650</i>	ABC transporter related	<i>B.infantis</i> ATCC 15697	ACJ51757.1	H-4		100	100	100	BINF_0673	BINF2_0647
<i>blon_0651</i>	Dihydrodipicolinate synthetase	<i>B.infantis</i> ATCC 15697	ACJ51758.1	H-4		100	99.69	100	BINF_0674	BINF2_0648
<i>blon_2402</i>	N-acetylmannosamine-6-phosphate 2-epimerase	<i>B.infantis</i> ATCC 15697	ACJ53457.1			100	99.13	100	BINF_2144	BINF2_2403
<i>bbr_1250</i>	Sugar kinase, ROK family	<i>B.breve</i> UCC2003	ABE95927.1			88.49	88.49	88.49	BINF_0904	BINF2_0877
<i>bbr_1251</i>	Transcriptional regulator, N-acetylglucosamine	<i>B.breve</i> UCC2003	ABE95928.1			83.17	83.42	83.17	BINF_0903	BINF2_0876
<i>bbr_1252</i>	Sugar kinase, pfkB family	<i>B.breve</i> UCC2003	ABE95929.1			93.62	93.96	93.62	BINF_0902	BINF2_0875
<i>blon_0880</i>	ROK family protein	<i>B.infantis</i> ATCC 15697	ACJ51980.1			100	99.73	100	BINF_0905	BINF2_0878
<i>blon_0881</i>	glucosamine-6-phosphate isomerase	<i>B.infantis</i> ATCC 15697	ACJ51981.1			100	100	100	BINF_0906	BINF2_0879
<i>blon_0882</i>	N-acetylglucosamine-6-phosphate deacetylase	<i>B.infantis</i> ATCC 15697	ACJ51982.1			100	98.83	100	BINF_0907	BINF2_0880
<i>blon_0538</i>	UDP-glucose 4-epimerase	<i>B.infantis</i> ATCC 15697	ACJ51651.1			100	99.7	100	BINF_0538	BINF2_0533
<i>blon_0565</i>	glucokinase	<i>B.infantis</i> ATCC 15697	ACJ51677.1			100	99.37	100	BINF_0569	BINF2_0561
<i>blon_2031</i>	phosphoglucosamine mutase	<i>B.infantis</i> ATCC 15697	ACJ53098.1			100	98.92	100	BINF_1723	BINF2_2033
<i>blon_2062</i>	galactokinase	<i>B.infantis</i> ATCC 15697	ACJ53126.1			100	96.88	100	BINF_1763	BINF2_2063
<i>blon_2063</i>	galactose-1-phosphate uridylyltransferase	<i>B.infantis</i> ATCC 15697	ACJ53127.1			100	99.52	100	BINF_1764	BINF2_2064
<i>blon_2171</i>	UDP-glucose-4-epimerase	<i>B.infantis</i> ATCC 15697	ACJ53232.1	H-5		100	98.82	100	BINF_1874	BINF2_2171
<i>blon_2172</i>	galactose-1-phosphate uridylyltransferase	<i>B.infantis</i> ATCC 15697	ACJ53233.1	H-5		100	97.28	100	BINF_1875	BINF2_2172
<i>blon_2173</i>	N-acetylhexosamine 1-kinase	<i>B.infantis</i> ATCC 15697	ACJ53234.1	H-5		100	92.76	100	BINF_1876	BINF2_2173
<i>blon_2184</i>	phosphoglucomutase	<i>B.infantis</i> ATCC 15697	ACJ53245.1			100	99.28	100	BINF_1882	BINF2_2184

[†]Gene database generated through extensive literature search as outlined in Materials and Methods (section 4.6)

*Strain column refers to strains in which colonization feature was identified

**Percent identity based on BLAST search using e-value cutoff of 1e-5 and query cover cutoff of 50%

Table formatted with graded colour scale based on percent identity in accordance with Figure 2 (higher % identities correspond to darker green colours)

Supplementary Table 2: Genome of *Bifidobacterium bifidum* R0071 and type strain JCM1255 matched against genes[†] associated with metabolism of HMO and HMO-derivatives

Designation	Function	Strain*	Accession number	GH family	Percent identity**		R0071 locus
					JCM1255	R0071	
<i>afcA</i>	1,2- α -l-Fucosidase	<i>B.bifidum</i> JCM 1254	AAQ72464.1	GH29	98.88	100	BBIF1_0227
<i>afcB</i>	1,3/4- α -l-Fucosidase	<i>B.bifidum</i> PRL2010	ADP36408.1	GH29	98.45	97.79	BBIF1_1367
<i>siaB2</i>	2,3/6- α -Sialidase	<i>B.bifidum</i> JCM 1254	BAK26854.1	GH33	97.85	100	BBIF1_1780
<i>blon_2353</i>	Hypothetical protein	<i>B.infantis</i> ATCC 15697	ACJ53411.1	GH20	64.71	64.71	BBIF1_0021
<i>lnpA2</i>	GNB/LNB phosphorylase	<i>B.bifidum</i> JCM 1254	BAE95374.1	GH112	99.33	100	BBIF1_0268
<i>lnbY</i>	Chaperon for LnbX	<i>B. longum</i> JCM 1217	KFI62468.1		44.65	43.91	BBIF1_0481
<i>lnbX</i>	Lacto- <i>N</i> -biosidase	<i>B. longum</i> JCM 1217	BAJ67172.1	GH136	59.88	59.79	BBIF1_0483
<i>bbgII</i>	β -1,4-Galactosidase	<i>B.bifidum</i> JCM 1254	BAI94821.1	GH2	98.81	100	BBIF1_0505
<i>lnpA1</i>	GNB/LNB phosphorylase	<i>B.bifidum</i> JCM 1254	BAD80752.1	GH112	99.47	100	BBIF1_1018
<i>gltC</i>	Permease protein of the ABC transporter system	<i>B.breve</i> UCC2003	ABE96257.1		91.77	91.77	BBIF1_1019
<i>blon_2176</i>	Binding-protein-dependent transport system	<i>B.infantis</i> ATCC 15697	ACJ53237.1		90.09	90.4	BBIF1_1020
<i>gltA</i>	GNB/LNB transporter SBP	<i>B.bifidum</i> JCM 1254	AEP83683.1		99.19	100	BBIF1_1021
<i>IntS</i>	Solute-binding protein of ABC transporter system	<i>B.breve</i> UCC2003	ABE95227.1		47.97	48.4	BBIF1_1360
<i>IntA</i>	β -1,3-Galactosidase	<i>B.breve</i> UCC2003	ABE95226.1	GH42	76.23	76.67	BBIF1_1362
<i>lnbB</i>	Lacto- <i>N</i> -biosidase	<i>B.bifidum</i> JCM 1254	ABZ78855.1	GH20	99.1	100	BBIF1_1436
<i>nahP</i>	Permease protein of the ABC transporter system	<i>B.breve</i> UCC2003	ABE96229.1		99.51	98.28	BBIF1_1462
<i>nahT1</i>	ATP-binding protein of ABC transporter system	<i>B.breve</i> UCC2003	ABE96230.1		98.57	98.57	BBIF1_1463
<i>nahT2</i>	ATP-binding protein of ABC transporter system	<i>B.breve</i> UCC2003	ABE96231.1		77.46	76.88	BBIF1_1466
<i>bbhI</i>	β - <i>N</i> -acetylhexosaminidase	<i>B.bifidum</i> JCM 1254	BAI94822.1	GH20	99.25	100	BBIF1_1522
<i>lacI</i>	LacI family transcriptional regulator	<i>B.bifidum</i> PRL2010	ADP36407.1		100	100	BBIF1_1365
<i>lacS</i>	lactose/raffinose/galactose permease	<i>B.bifidum</i> PRL2010	ADP36492.1		99.59	100	BBIF1_1456
<i>lacZ6</i>	β -1,4-Galactosidase	<i>B.breve</i> UCC2003	ABE96223.1	GH2	90.41	90.22	BBIF1_1457
<i>lacI</i>	LacI family transcriptional regulator	<i>B.bifidum</i> PRL2010	ADP36495.1		100	100	BBIF1_1459
<i>blon_0344</i>	Mandelate racemase/muconate lactonizing enzyme	<i>B.infantis</i> ATCC 15697	ACJ51468.1		71.88	71.88	BBIF1_0263
<i>fumE</i>	fuconate dehydratase	<i>B.infantis</i> ATCC 15697	ACJ53367.1		81.25	68.85	BBIF1_0265

Supplementary Table 2: Genome of *Bifidobacterium bifidum* R0071 and type strain JCM1255 matched against genes[†] associated with metabolism of HMO and HMO-derivatives

Designation	Function	Strain*	Accession number	GH family	Percent identity**		R0071 locus
					JCM1255	R0071	
<i>blon_2339</i>	SDR family oxidoreductase	<i>B.infantis</i> ATCC 15697	ACJ53397.1		70.7	70.7	BBIF1_0266
<i>blon_0423</i>	LacI type regulator	<i>B.infantis</i> ATCC 15697	ACJ51544.1		71.31	70.57	BBIF1_0397
<i>blon_2475</i>	ABC transporter related	<i>B.infantis</i> ATCC 15697	ACJ53529.1		95.2	95.47	BBIF1_1809
<i>blon_0649</i>	Oligopeptide/dipeptide ABC transporter,	<i>B.infantis</i> ATCC 15697	ACJ51756.1		44.83	44.48	BBIF1_0632
<i>nanE</i>	<i>N</i> -acetylmannosamine-6-phosphate 2-epimerase	<i>B.infantis</i> ATCC 15697	ACJ53457.1		57.58	57.58	BBIF1_1759
<i>nagK2</i>	Sugar kinase, pfkB family	<i>B.breve</i> UCC2003	ABE95929.1		81.54	81.21	BBIF1_0582
<i>nagR2</i>	Transcriptional regulator, <i>N</i> -acetylglucosamine	<i>B.breve</i> UCC2003	ABE95928.1		65.59	70.7	BBIF1_0584
<i>blon_0880</i>	ROK family protein	<i>B.infantis</i> ATCC 15697	ACJ51980.1		60.06	60.06	BBIF1_0586
<i>nagB</i>	glucosamine-6-phosphate isomerase	<i>B.bifidum</i> PRL2010	ADP35664.1		100	98.89	BBIF1_0587
<i>nagA</i>	<i>N</i> -acetylglucosamine-6-phosphate deacetylase	<i>B.bifidum</i> PRL2010	ADP35665.1		100	99.76	BBIF1_0588
<i>nahK</i>	<i>N</i> -acetylhexosamine 1-kinase	<i>B.bifidum</i> PRL2010	ADP36119.1		99.72	100	BBIF1_1015
<i>nagK</i>	Sugar kinase, ROK family	<i>B.breve</i> UCC2003	ABE95927.1		61.2	61.2	BBIF1_1017
<i>galT</i>	galactose-1-phosphate uridylyltransferase	<i>B.bifidum</i> PRL2010	ADP35518.1		100	100	BBIF1_0431
<i>galK</i>	galactokinase	<i>B.bifidum</i> PRL2010	ADP35519.1		100	100	BBIF1_0432
<i>glmM</i>	phosphoglucosamine mutase	<i>B.bifidum</i> PRL2010	ADP35549.1		100	99.78	BBIF1_0463
<i>galE1</i>	UDP-glucose-4-epimerase	<i>B.bifidum</i> PRL2010	ADP36117.1		99.41	99.71	BBIF1_1013
<i>galT</i>	galactose-1-phosphate uridylyltransferase	<i>B.bifidum</i> PRL2010	ADP36118.1		100	100	BBIF1_1014
<i>glkA</i>	glucokinase	<i>B.bifidum</i> PRL2010	ADP36455.1		100	100	BBIF1_1425
<i>galE1</i>	UDP-glucose-4-epimerase	<i>B.bifidum</i> PRL2010	ADP36489.1		100	100	BBIF1_1453
<i>pgm</i>	phosphoglucomutase	<i>B.bifidum</i> PRL2010	ADP36541.1		100	99.82	BBIF1_1504
<i>afcA</i>	1,2- α -l-Fucosidase	<i>B.bifidum</i> JCM 1254	AAQ72464.1	GH29	98.88	100	BBIF1_0227
<i>afcB</i>	1,3/4- α -l-Fucosidase	<i>B.bifidum</i> PRL2010	ADP36408.1	GH29	98.45	97.79	BBIF1_1367

[†]Gene database generated through extensive literature search as outlined in Materials and Methods (section 4.6)

*Strain column refers to strains in which colonization feature was identified

**Percent identity based on BLAST search using e-value cutoff of 1e-5 and query cover cutoff of 50%

Table formatted with graded colour scale based on percent identity in accordance with Figure 2 (higher % identities correspond to darker green colours)

Supplementary Table 3: Genome of *Bifidobacterium breve* M-16V and UCC2003 matched against genes[†] associated with metabolism of HMO and HMO-derivatives

Designation	Protein name	Strain*	Accession number	GH family	Percent identity**		M-16V locus
					UCC2003	M-16V	
<i>fl1-BP</i>	Extracellular solute-binding protein, family 1	<i>B.infantis</i> ATCC15697	ACJ51467.1		44.34	44.1	BBRE1_0291
<i>blon_0341</i>	Binding-protein-dependent permease	<i>B.infantis</i> ATCC15697	ACJ51465.1		65.02	65.02	BBRE1_0292
<i>blon_0342</i>	Binding-protein-dependent permease	<i>B.infantis</i> ATCC15697	ACJ51466.1		74.59	74.59	BBRE1_0293
<i>fl2-BP</i>	Binding-protein-dependent permease	<i>B.infantis</i> ATCC15697	ACJ51466.1		45.59	45.59	BBRE1_0365
<i>fl2-BP</i>	Binding-protein-dependent permease	<i>B.infantis</i> ATCC15697	ACJ51465.1		44.86	44.86	BBRE1_0364
<i>afcA</i>	1,2- α -l-Fucosidase	<i>B.infantis</i> ATCC15697	ACJ53393.1	GH95	77.14	77.52	BBRE1_1357
<i>bga2A</i>	β -1,4-Galactosidase	<i>B.infantis</i> ATCC15697	ACJ53392.1	GH2	57.65	57.84	BBRE1_0010
<i>lacZ2</i>	β -1,4-Galactosidase	<i>B.breve</i> UCC2003	ABE94991.1	GH2	100	23.99	
<i>lntR</i>	Transcriptional regulator, LacI family	<i>B.breve</i> UCC2003	ABE95223.1		100	99.4	BBRE1_0452
<i>lntp1</i>	ABC transport system permease protein	<i>B.breve</i> UCC2003	ABE95224.1		100	99.68	BBRE1_0453
<i>lntp2</i>	ABC transport system permease protein	<i>B.breve</i> UCC2003	ABE95225.1		100	99.1	BBRE1_0454
<i>lntA</i>	β -1,3-Galactosidase	<i>B.breve</i> UCC2003	ABE95226.1	GH42	100	99.28	BBRE1_0455
<i>lntA</i>	Solute-binding protein of ABC transporter system	<i>B.breve</i> UCC2003	ABE95227.1		100	99.78	BBRE1_0456
<i>lacA</i>	Galactoside symporter	<i>B.breve</i> UCC2003	ABE96222.1		100	100	BBRE1_1560
<i>nahA</i>	Solute-binding protein of ABC transporter system	<i>B.breve</i> UCC2003	ABE96225.1		100	95.46	BBRE1_1564
<i>nagC</i>	NagC/XylR-type transcriptional regulator	<i>B.breve</i> UCC2003	ABE96226.1		100	98.72	BBRE1_1565
<i>nahA</i>	β -N-acetylhexosaminidase	<i>B.breve</i> UCC2003	ABE96227.1	GH20	100	99.26	BBRE1_1567
<i>nahP</i>	Permease protein of the ABC transporter system	<i>B.breve</i> UCC2003	ABE96229.1		100	100	BBRE1_1570
<i>nahT1</i>	ATP-binding protein of ABC transporter system	<i>B.breve</i> UCC2003	ABE96230.1		100	99.05	BBRE1_1571
<i>nahT2</i>	ATP-binding protein of ABC transporter system	<i>B.breve</i> UCC2003	ABE96231.1		100	100	BBRE1_1573
<i>lnbP</i>	GNB/LNB phosphorylase	<i>B.breve</i> UCC2003	ABE96256.1	GH112	100	99.73	BBRE1_1598
<i>gltC</i>	Permease protein of the ABC transporter system	<i>B.breve</i> UCC2003	ABE96257.1		100	100	BBRE1_1599
<i>gltB</i>	Permease protein of the ABC transporter system	<i>B.breve</i> UCC2003	ABE96258.1		100	100	BBRE1_1600
<i>gltA</i>	Solute-binding protein of the ABC transporter system	<i>B.breve</i> UCC2003	ABE96259.1		100	100	BBRE1_1601
<i>lacI</i>	LacI regulator	<i>B.infantis</i> ATCC15697	ACJ51748.1		58.93	58.33	BBRE1_0008

Supplementary Table 3: Genome of *Bifidobacterium breve* M-16V and UCC2003 matched against genes[†] associated with metabolism of HMO and HMO-derivatives

Designation	Protein name	Strain*	Accession number	GH family	Percent identity**		M-16V locus
					UCC2003	M-16V	
<i>blon_0625</i>	Beta-glucosidase (L-arabinopyranosidase)	<i>B.infantis</i> ATCC15697	ACJ51732.1	GH33	93.05	92.64	BBRE1_1487
<i>lacZ6</i>	β -1,4-Galactosidase	<i>B.breve</i> UCC2003	ABE96223.1	GH2	100	99.33	BBRE1_1561
<i>lacI</i>	Transcriptional regulator, LacI family	<i>B.breve</i> UCC2003	ABE96224.1		100	100	BBRE1_1562
<i>luxR</i>	Two component transcriptional regulator, LuxR family	<i>B.infantis</i> ATCC15697	ACJ51372.1		94.5	94.5	BBRE1_0219
<i>blon_0244</i>	Signal transduction histidine kinase-like protein	<i>B.infantis</i> ATCC15697	ACJ51373.1		89.95	89.95	BBRE1_0220
<i>blon_0245</i>	MFS transporter	<i>B.infantis</i> ATCC15697	ACJ51374.1		97.76	98.75	BBRE1_0221
<i>blon_0423</i>	LacI type regulator	<i>B.infantis</i> ATCC15697	ACJ51544.1		91.11	91.11	BBRE1_0356
<i>fumF</i>	dihydrodipicolinate synthase family protein	<i>B.infantis</i> ATCC15697	ACJ53396.1		89.53	89.53	BBRE1_1358
<i>blon_2339</i>	SDR family oxidoreductase	<i>B.infantis</i> ATCC15697	ACJ53397.1		94.27	93.51	BBRE1_1360
<i>dapA4</i>	Dihydrodipicolinate synthase	<i>B.breve</i> UCC2003	EW41831.1		100	100	BBRE1_1754
<i>bbr_1741</i>	amidohydrolase family protein	<i>B.breve</i> UCC2003	EW41830.1		100	100	BBRE1_1755
<i>fumP</i>	L-fucose permease	<i>B.breve</i> UCC2003	EW41829.1		100	100	BBRE1_1756
<i>fumc</i>	Short-chain dehydrogenase/reductase SDR	<i>B.infantis</i> ATCC15697	ACJ53366.1		94.68	94.68	BBRE1_1757
<i>fume</i>	fuconate dehydratase	<i>B.infantis</i> ATCC15697	ACJ53367.1		97.89	97.89	BBRE1_1758
<i>blon_2475</i>	ABC transporter related	<i>B.infantis</i> ATCC15697	ACJ53529.1		98.13	98.13	BBRE1_1904
<i>blon_0643</i>	Uncharacterized protein	<i>B.infantis</i> ATCC15697	ACJ51750.1		66.22	65.89	BBRE1_0137
<i>nanK</i>	N-acetylmannosamine kinase/ ROK family	<i>B.breve</i> UCC2003	ABE94871.1		100	99.11	BBRE1_0138
<i>nanE</i>	N-acetylmannosamine-6-phosphate 2-epimerase	<i>B.breve</i> UCC2003	AHJ18490.1		100	100	BBRE1_0139
<i>nanB</i>	Substrate-binding protein	<i>B.breve</i> UCC2003	ABE94874.1		100	99.81	BBRE1_0141
<i>nanC</i>	ABC transport system permease protein	<i>B.breve</i> UCC2003	ABE94875.1		100	99.69	BBRE1_0142
<i>nanD</i>	ABC transport system ATP-binding protein	<i>B.breve</i> UCC2003	ABE94876.1		100	99.7	BBRE1_0143
<i>nanF</i>	ABC transport system ATP-binding protein	<i>B.breve</i> UCC2003	ABE94877.1		100	100	BBRE1_0144
<i>nanA</i>	N-Acetylneuraminase lyase	<i>B.breve</i> UCC2003	ABE94878.1		100	100	BBRE1_0145
<i>nanH</i>	N-acetylneuraminase lyase	<i>B.breve</i> UCC2003	EFE89784.1	GH33	100	100	BBRE1_0148
<i>blon_0642</i>	GntR domain protein	<i>B.infantis</i> ATCC15697	ACJ51749.1		71.37	71.37	BBRE1_0150

Supplementary Table 3: Genome of *Bifidobacterium breve* M-16V and UCC2003 matched against genes[†] associated with metabolism of HMO and HMO-derivatives

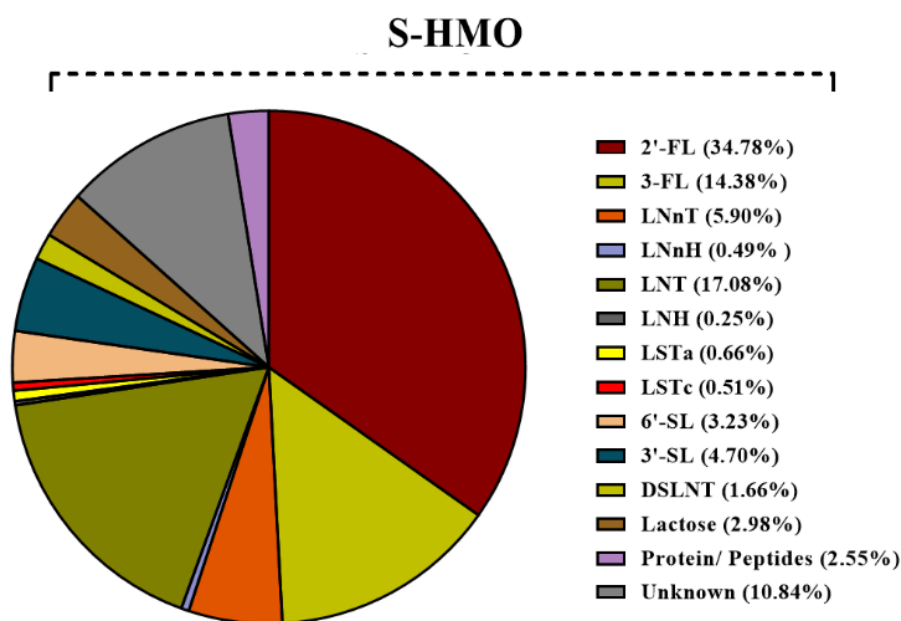
Designation	Protein name	Strain*	Accession number	GH family	Percent identity**		M-16V locus
					UCC2003	M-16V	
<i>nagA</i>	N-acetylglucosamine-6-phosphate deacetylase	<i>B.breve</i> JCM1192	EFE88218.1		99.76	100	BBRE1_1250
<i>nagB3</i>	Glucosamine-6-phosphate isomerase	<i>B.breve</i> UCC2003	ABE95925.1		100	99.26	BBRE1_1251
<i>nagR1</i>	Transcriptional regulator, ROK family	<i>B.breve</i> UCC2003	ABE95926.1		100	100	BBRE1_1252
<i>nagK</i>	Sugar kinase, ROK family	<i>B.breve</i> UCC2003	ABE95927.1		100	100	BBRE1_1253
<i>nagR2</i>	Transcriptional regulator, N-acetylglucosamine	<i>B.breve</i> UCC2003	ABE95928.1		100	99.75	BBRE1_1254
<i>nagK2</i>	Sugar kinase, pfkB family	<i>B.breve</i> UCC2003	ABE95929.1		100	99.66	BBRE1_1255
<i>nahK</i>	N-acetylhexosamine 1-kinase	<i>B.breve</i> UCC2003	ABE96255.1		100	98.88	BBRE1_1597
<i>blon_2063</i>	galactose-1-phosphate uridylyltransferase	<i>B.infantis</i> ATCC15697	ACJ53127.1		99.04	99.04	BBRE1_0418
<i>galK</i>	galactokinase	<i>B.breve</i> JCM1192	EFE88977.1		99.76	100	BBRE1_0419
<i>glmM</i>	phosphoglucosamine mutase	<i>B.breve</i> JCM1192	EFE89003.1		99.57	100	BBRE1_0438
<i>glkA</i>	glucokinase	<i>B.breve</i> JCM1192	EFE88681.1		98.42	100	BBRE1_1539
<i>galE</i>	UDP-glucose-4-epimerase	<i>B.breve</i> JCM1192	EFE88615.1		100	100	BBRE1_1596
<i>pgm</i>	phosphoglucomutase	<i>B.breve</i> JCM1192	EFE88604.1		100	100	BBRE1_1606
<i>galt</i>	galactose-1-phosphate uridylyltransferase	<i>B.breve</i> JCM1192	EFE90085.1		100	100	BBRE1_1893

[†]Gene database generated through extensive literature search as outlined in Materials and Methods (section 4.6)

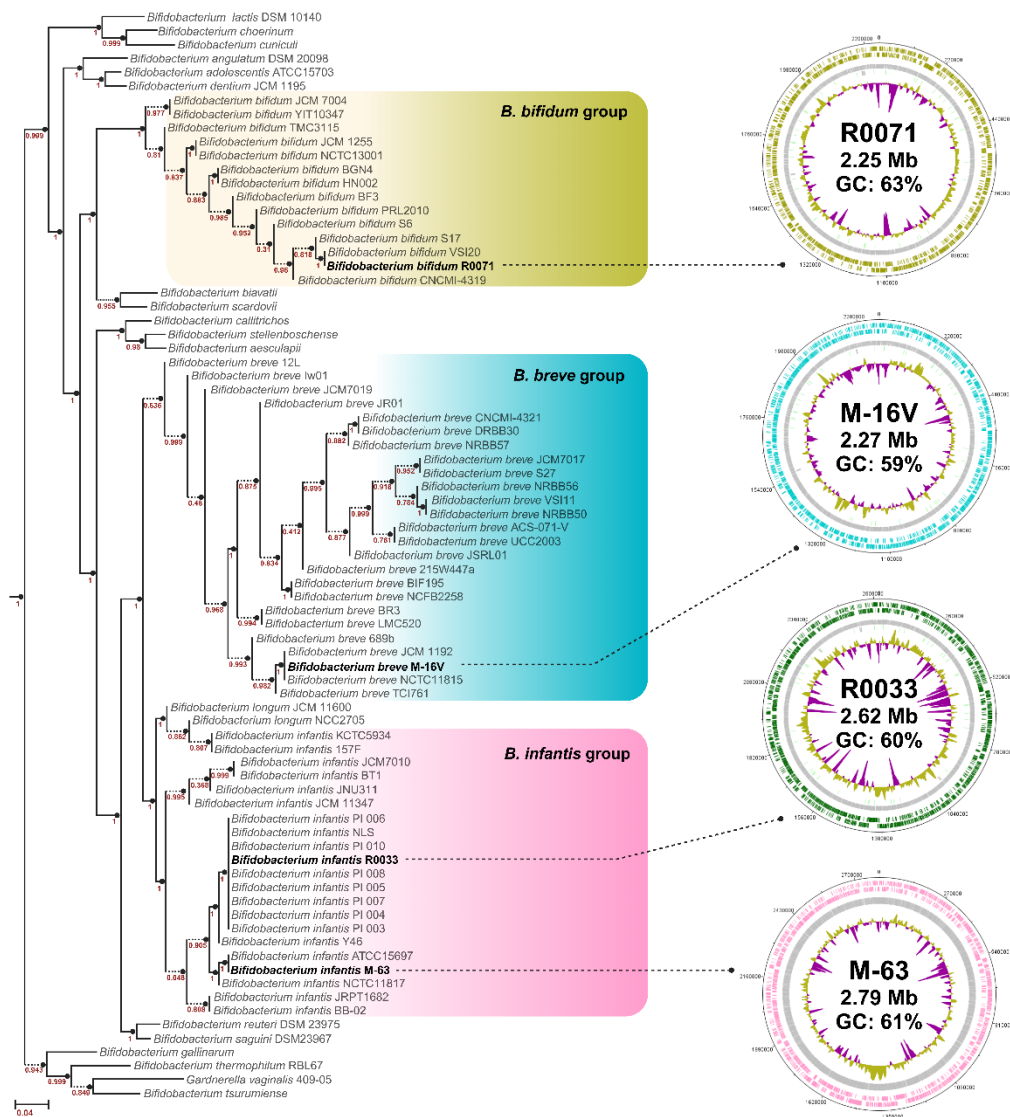
*Strain column refers to strains in which colonization feature was identified

**Percent identity based on BLAST search using e-value cutoff of 1e-5 and query cover cutoff of 50%

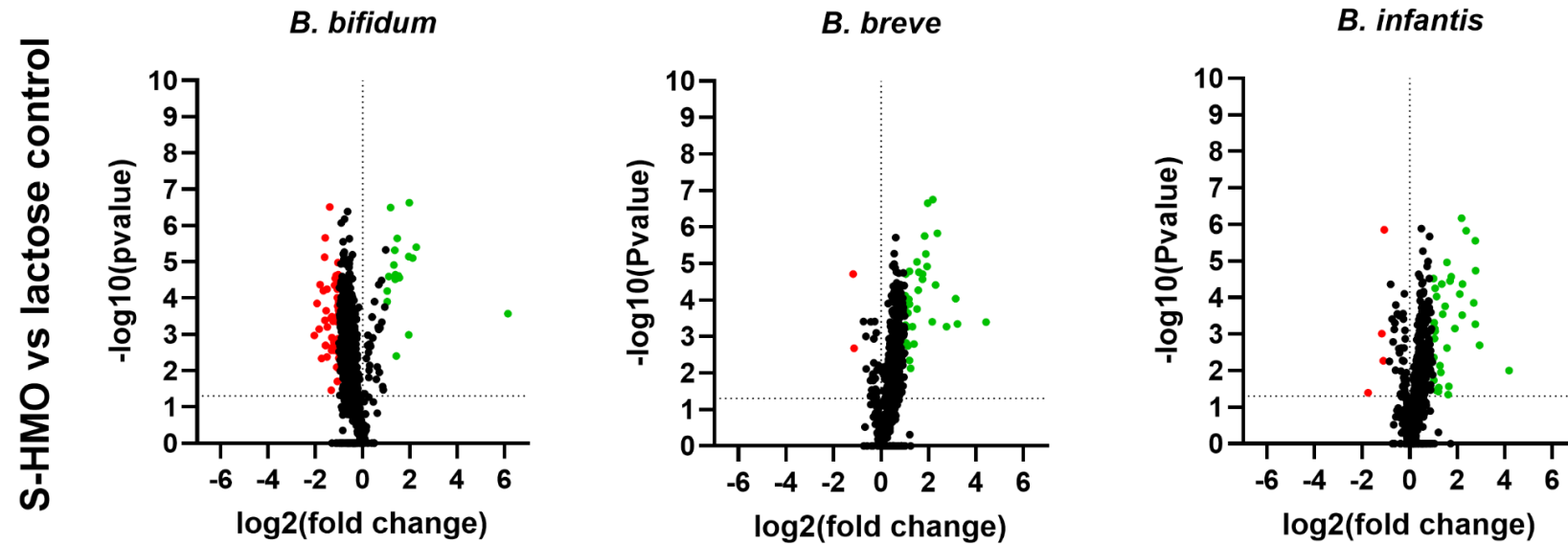
Table formatted with graded colour scale based on percent identity in accordance with Figure 2 (higher % identities correspond to darker green colours)



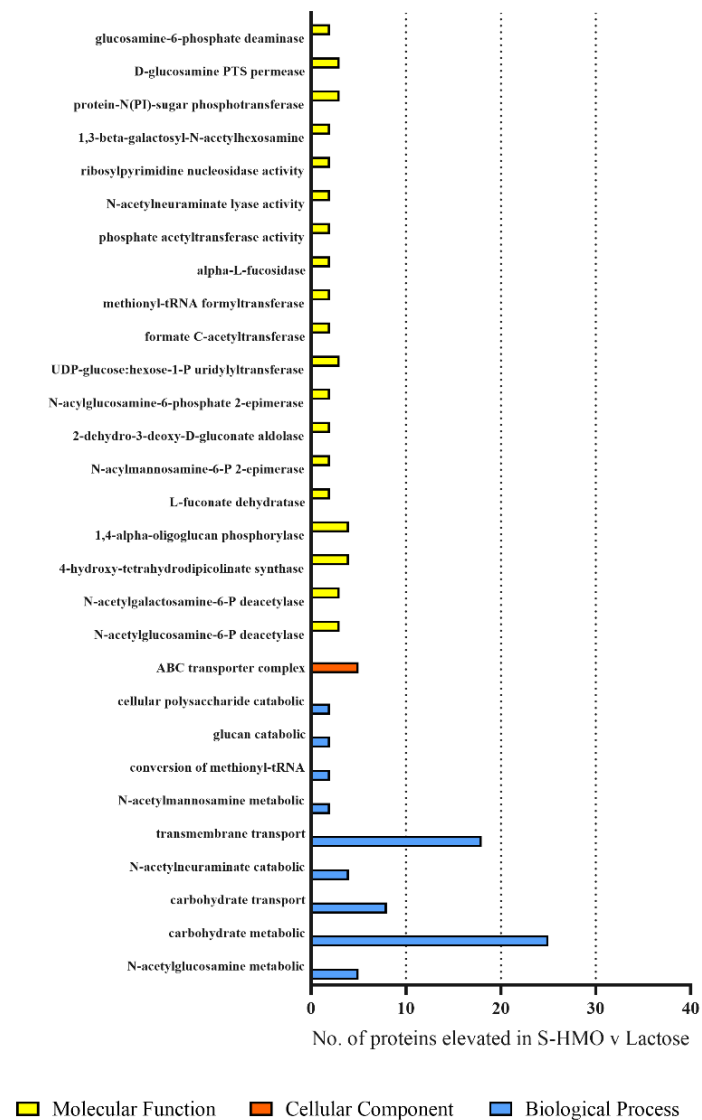
Supplementary Figure 1: Relative abundance of the major oligosaccharides found in S-HMO Detected via HPAEC-PAD analysis by comparing to the following external HMO standards: lactose, 3-fucosyllactose (3-FL), 2'-fucosyllactose (2'-FL), lacto-*N*-neotetraose (LNnT), lacto-*N*-neohexaose (LNnH), lacto-*N*-tetraose (LNT), lacto-*N*-hexaose (LNH), sialyllacto-*N*-tetraose a (LSTa), sialyllacto-*N*-tetraose a (LSTc), 6'-siallylactose (6'-SL), 3'-siallylactose (3'-SL), disialyllacto-*N*-tetraose (DSLNT). Data represents average concentrations of technical duplicate data from biological triplicate experiments.



Supplementary Figure 2: Circular genome plots and genome details, including GC content and base pair numbers and comparison of the evolutionary relationships of *B. bifidum* R0071, *B. breve* M-16V, *B. infantis* R0033, and *B. infantis* M-63 with publicly available genomes from the NCBI database. Phylogenetic trees (color-coded by species group: gold for *B. bifidum*, blue for *B. breve*, pink for *B. infantis*) are based on 49 core, universal genes defined by COG (Clusters of Orthologous Groups) gene families using the species tree function of KBase. The bar represents the genetic distance



Supplementary Figure 3: Volcano plots displaying quantitative changes in abundance of *Bifidobacterium bifidum*, *Bifidobacterium infantis*, and *Bifidobacterium breve* proteins following co-culture growth in A) non-secretor HMO (NS-HMO) and B) secretor HMO (S-HMO) The horizontal line represents a value of p of 0.05. Proteins with a significant change in abundance when compared to growth in lactose [$p < 0.05$ (Student's t -test), fold change ≥ 2] are highlighted in red to indicate decreased abundance and green to indicate increased abundance.



Supplementary Figure 4: Functional analysis of proteins with significant changes in abundance in bifidobacterial cultures Grown on human milk oligosaccharides (HMOs) versus lactose. The number of proteins with elevated expression in secretor HMO (S-HMO) compared to lactose is categorized by their gene ontology (GO) functional classifications. The yellow bars represent molecular functions, blue bars indicate biological processes, and the single red bar denotes cellular components. Proteins with fold changes ≥ 2 and p-value < 0.05 were considered significantly altered. Functional analysis was performed using BLAST2GO (OmicsBox) and InterProScan, followed by GO enrichment analysis via Fisher's Exact Test.

CHAPTER VII

HMO-primed bifidobacteria exhibit enhanced ability to adhere to intestinal epithelial cells

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Abstract

The ability of gut commensals to adhere to the intestinal epithelium can play a key role in influencing the composition of the gut microbiota. Bifidobacteria are associated with a multitude of health benefits and are one of the most widely used probiotics for humans. Enhanced bifidobacterial adhesion may prolong gut transit time and increase host-microbe, microbe-nutrient, and/or microbe-microbe interactions, thereby enabling consolidated health benefits to the host. The objective of this study was to determine the ability of human milk oligosaccharides (HMOs) to enhance bifidobacterial intestinal adhesion *in vitro*. The current study assessed the colonisation-promoting effects of HMOs on four commercial infant-associated *Bifidobacterium* strains (two *B. longum* subsp. *infantis* strains, *B. breve* and *B. bifidum*). HT29-MTX cells were used as *in vitro* intestinal models for bacterial adhesion. Short-term exposure of four commercial infant-associated *Bifidobacterium* strains to HMOs derived from breastmilk substantially increased the adherence (up to 47 %) of these probiotic strains. Interestingly, when strains were incubated with HMOs as a four-strain combination, the number of viable bacteria adhering to intestinal cells increased by > 90%. Proteomic analysis of this multi-strain bifidobacterial mixture revealed that the increased adherence resulting from exposure to HMOs was associated with notable increases in the abundance of sortase-dependent pili and glycosyl hydrolases matched to *Bifidobacterium bifidum*. This study suggests that HMOs may prime infant gut-associated *Bifidobacterium* for colonisation to intestinal epithelial cells by influencing the expression of various colonization factors.

1. Introduction

Bifidobacterial strains are the most abundant colonizers in the gut of breast-fed infants, accounting for 50-90% of the total bacterial population detected in their faeces (Milani et al., 2017). The successful adaptation of bifidobacteria to the infant gut has been largely attributed to their large arsenal of carbohydrate-active enzymes, which allows specific bifidobacterial strains to utilise breast-milk derived glycans such as human milk oligosaccharides (HMOs). HMOs act as prebiotics (“a substrate that is selectively utilized by host microorganisms conferring a health benefit”) by promoting growth of beneficial intestinal bacteria thereby generating short-chain fatty acids which are critical for gut health (Pokusaeva et al., 2011). Due to their historical use and health-promoting properties, bifidobacterial strains are some of the most widely used and clinically documented probiotics. As probiotics, bifidobacteria contribute to host health through modulation of the intestinal microbiota, promotion of immune system development, maintenance of mucosal barrier integrity, and resistance to pathogenic colonization (Gareau et al., 2010). However, in order for bifidobacterial probiotics to impart their beneficial role, they not only need to compete with other gut commensals for nutrient acquisition, but also need to survive gastrointestinal transit and stably colonize the gastrointestinal tract (Morrin & Hickey, 2020). Adhesion to intestinal cells and/or mucus should therefore act as a key selection criterion when developing probiotic-containing products.

Recent studies have identified a plethora of bacterial features that have been shown to be involved in host-microbe interactions (Chang & Kao, 2019; Viljoen et al., 2020; Xiao et al., 2021). In this regard, cell surface components, termed adhesins, play an important role in allowing bifidobacteria to adhere

to and interact with intestinal epithelial cells (Westermann et al., 2016). Many microbial adhesins display lectin-like properties and bind corresponding glycan receptors on the surface of the host cells via carbohydrate-recognition domains (CRDs). These interactions can be an important determinant of host and tissue tropism. Bacterial adhesion to human intestinal cells often involves the assembly of elongated, submicroscopic structures called pili (resembling "threads") or fimbriae (also known as short attachment pili or "hairs") (Nizet et al., 2017). Mounting evidence suggests that numerous bifidobacterial strains encode and express pilus-like structures (Foroni et al., 2011; O'Connell Motherway et al., 2019; O'Connell Motherway et al., 2011; Westermann et al., 2016). In recent years, several studies have indicated that certain milk components could enhance the adhesion ability of bifidobacterial strains by influencing the expression of these bacterial adhesins. For instance, González and colleagues found that the FimA fimbrial subunit and sortase-like fimbria-associated protein (involved in the assemblage of fimbriae) were highly upregulated during growth of *Bifidobacterium longum* in human milk and formula milk (González et al., 2008). Researchers have hypothesized that one such milk component contributing to this enhanced adhesion is the prebiotic fraction in human milk, i.e. HMOs. An *in vitro* study by Chichlowski et al. demonstrated that exposure of *B. infantis* ATCC 15697 to breastmilk-derived HMO significantly increased adherence of the strain to HT-29 intestinal cells (Chichlowski et al., 2012). More recently, findings from Zhang et al. indicate that 2'-fucosyllactose (2'-FL), typically the most abundant HMO in breastmilk, promotes *B. bifidum* DNG6 adhesion to Caco-2 cells through increased expression of genes encoding adhesion proteins (Zhang et al., 2020). Likewise, incubation of *B. infantis* ATCC 15697 in the

presence of 3'-siallylactose (3'-SL) and 6'-siallylactose (6'-SL), two predominant acidic oligosaccharides found in the milk of most mammals, was shown to substantially increase adherence of this strain to HT-29 cells (Kavanaugh et al., 2013). Subsequent transcriptomic analysis revealed that this increased adherence was associated with heightened levels of tight-adherence protein TadE expression. This protein plays a pivotal role in orchestrating the assembly of type IVb pili surface appendages (Kavanaugh et al., 2013).

The objective of the current study was to assess the ability of HMOs to enhance adhesion of a community of bifidobacterial strains to mucus-secreting intestinal cells. As outlined by Xiao et al. the majority of current studies assessing colonization capabilities of probiotic strains have been performed using allochthonous strains (i.e. those isolated from fermented foods or plants and then studied in humans) and therefore do not perform well in their new ecosystem (Xiao et al., 2021). It is suggested that, if the goal is stable colonization, autochthonous strains (i.e. those of human origin) should instead be examined (Xiao et al., 2021). The present study focused on four autochthonous bifidobacterial strains of human origin that are of commercial interest due to their historical employment as ingredients in functional foods and their health benefits are clinically documented (Giannetti et al., 2017; Wong et al., 2019; Xiao et al., 2019). Here, we investigated the adhesive properties of *Bifidobacterium bifidum* R0071, *Bifidobacterium infantis* R0033, *Bifidobacterium breve* M-16V, and *Bifidobacterium longum* subsp. *infantis* (*B. infantis*) M-63, which are all shown to elicit potent immunomodulatory and protective effects *in vivo* (Giannetti et al., 2017; Wong et al., 2019; Xiao et al., 2019). Genomic mining

and proteomic analyses were subsequently performed to understand the mechanisms underlying bifidobacterial adherence to intestinal cells. Previous work by our group has shown that positive co-operation between these bifidobacterial strains in utilising HMOs as a carbohydrate source resulted in higher cell numbers (Walsh et al., 2022). Given the evolution of these strains in a HMO-rich environment, we hypothesised that HMOs would not only influence the selective growth of these strains in a community setting, but also their specific adhesive ability. This study provides further insights into the role that HMOs play in selectively promoting bifidobacterial colonization in the infant gut and corroborates the notion that oligosaccharides acting as prebiotics also have the potential to enhance bifidobacterial adhesion.

2. Materials and Methods

2.1. Generation of HMO-enriched fraction

Pooled human milk samples (of unknown secretor status) were kindly donated by Irvinestown Human Milk Bank (Co. Fermanagh, Ireland) and stored at -80 °C on arrival. Briefly, lipids and proteins were removed from breastmilk as previously described (Quinn et al., 2018) before separation of lactose and HMO components using size-exclusion chromatography (BioGel P2, 92 × 5 cm; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Peptide-free (quantified using Pierce colorimetric peptide assay) and low-trace lactose (quantified using high pH anion exchange chromatography [HPAEC] with pulsed amperometric detection [Dionex Corporation, Sunnyvale, CA, USA]) fractions were pooled and freeze-dried. The resultant HMO-enriched fraction was stored at 4°C prior to use in experiments. HPAEC analysis indicated that low levels of 2'-fucosyllactose (2'-FL) were found in the HMO-enriched

fraction, likely due to the purification process where 2'-FL and lactose fractionated together in the same eluates. Therefore, the 2'-FL which had been eliminated during the size exclusion process was resubstituted using commercial 2'-FL (Friesland Campina), as previously described (Walsh et al., 2022), to give a powder representative of secretor HMO (designated S-HMO). S-HMO was used in adhesion studies at concentrations outlined in previous studies from our group (Walsh et al., 2022): final concentration of 5 g/L (3.8 g/L isolated HMO + 1.2 g/L 2'-FL). The composition of the S-HMO blend is shown in **Supplementary Figure S1**.

2.2. Epithelial cell line conditions

The human colonic adenocarcinoma cell line HT29-MTX (European Collection of Authenticated Cell Cultures [ECACC]) was used as a model of human intestinal epithelia. HT29-MTX cells were used for experiments at three different passages (passage numbers 38 to 41) to provide three biological replicates. Cells were routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM) (10% Fetal Bovine Serum [FBS], 1% non-essential amino acids) (Merck, Darmstadt, Germany) in 75 cm² tissue culture flasks at 37°C with humidified atmosphere (5% CO₂). The cultures were passaged by detaching with trypsin when cells had reached approximately 90% confluence. Cells were seeded at a density of 1x10⁵ cells/mL into 12-well plates (Cellbind; Corning, New York, USA). HT29-MTX cells were used once 100% confluency was reached (approximately 5 × 10⁶ cells/well at day 12-14). Growth medium was changed every other day and supplemented with 2% FBS 24 h prior to use.

2.3. Bacterial strains and culture conditions

Commercial *Bifidobacterium* strains used in this study were obtained from Lallemand Health Solutions, France (*Bifidobacterium bifidum* R0071 and *Bifidobacterium longum* subsp. *infantis* R0033), and Morinaga Milk, Japan (*Bifidobacterium breve* M-16V and *Bifidobacterium infantis* M-63). Strains were stored at -80 °C in de Man-Rogosa-Sharpe (MRS) broth (Difco, BD, Ireland), supplemented with 50% (v/v) glycerol as a cryoprotectant. For routine experiments, strains were grown at 37 °C under anaerobic conditions using MRS medium supplemented with 0.05% (wt/v) L-cysteine hydrochloride (Merck) and 0.01% (wt/v) mupirocin (Merck)). Bacterial stocks were regularly checked for contamination by closely monitoring colony morphology during culturing. In addition, the bacterial stocks were routinely validated as being specific to their genera using bifidobacterial-specific PCR targeting the 16S region using primers g-Bifid-F (5'-CTCCTGGAAACGGGTGG-3') and g-Bifid-R (5'-CTCCTGGAAACGGGTGG-3') (Matsuki et al., 2004). Resultant amplicons were sequenced using Sanger sequencing.

2.4. Colonisation Assays

2.4.1. Priming of bifidobacterial strain:

For experiments, bifidobacterial strains derived from -80 °C stock cultures were streaked on MRS agar supplemented with 0.05 % (wt/v) L-cysteine HCl and incubated for 48 hrs at 37 °C under anaerobic conditions. Single colonies of each strain were subsequently inoculated in supplemented MRS broth and sub-cultured twice before use in subsequent experiments. To obtain working cultures, supernatant of overnight culture was removed by centrifugation,

after which the bacterial cell pellet was washed with phosphate buffer saline (PBS) (Merck) and resuspended in tissue culture media (2% FBS) such that the OD₆₀₀ of each bacterial culture was ~0.4 (corresponding to ~5x10⁷ CFU/mL, as determined by plate counts). The following bacterial combinations were assessed: single cultures of *B. bifidum* (R0071), *B. infantis* (R0033), *B. breve* (M-16V), *B. infantis* (M-63) and a mixture of all four bifidobacterial strains (4Bif) containing equal volumes of each ~5x10⁷ CFU/mL bacterial culture prepared as described above. Bacterial suspension was added at a 1:1 ratio to DMEM tissue culture media (2% FBS) supplemented with 10 mg/mL of S-HMO or 2'-FL. Therefore, the final concentration of bacteria and carbohydrate at initiation of incubation was 2.5x10⁷ CFU/mL and 5 mg/mL, respectively. Bacteria suspended in non-supplemented DMEM tissue culture media (without S-HMO or 2'-FL) was used as a control. Bacteria were exposed to oligosaccharides at 37°C under anaerobic conditions for 1hr before removal of residual oligosaccharides by centrifugation. The bacterial pellet was washed three times using PBS and centrifugation before resuspending in non-supplemented DMEM media prior to use in the assays.

2.4.2. Bacterial exposure to eukaryotic cells:

Eukaryotic cells were washed twice with PBS, and 0.5 mL of bacterium:medium suspensions were added to the appropriate wells (corresponding to approximately 10–20 bacterial cells per eukaryotic cell). Bacteria were exposed to eukaryotic cells for 2 hours at 37°C under anaerobic conditions, after which wells were washed three times with PBS to remove non-adherent bacteria, and then lysed with 0.1% Triton X100 (Merck) for 30 min. Bacteria within extracted lysates were quantified using two methods.

Firstly, viable bacteria adhering to cells were enumerated by serially diluting lysates and spread-plating (in triplicate) on MRS plates. Aliquots of the experimental inocula were retained, diluted, and plated to determine original CFU/mL. Results were expressed as adherent bacteria as a percentage of the original inoculum, thereby accounting for variations in the original inocula between treatment groups. Percentage adherent = [CFU/mL of recovered adherent bacteria/ CFU/mL of inoculum] x 100. Comparisons between strains and treatments groups were calculated as fold change. Fold change in adherence= Percentage adherent treatment group/ percentage adherent control group.

Secondly, bacterial numbers were determined using quantitative PCR (qPCR) as previously described (Walsh et al., 2022). Bacterial DNA was extracted using GenElute Bacterial Genomic DNA Kit (Merck) and quantified using a 480 Lightcycler platform (Roche Applied Science, Penzberg, Germany) using the following program: 95 °C for 5 min followed by 40 cycles of 95 °C for 10 s, 58 °C for 20 s and 72 °C for 30s. Species within the co-culture were enumerated using primers targeting the 16S rDNA gene region. For quantification of all *Bifidobacterium* strains present in samples, the primer pairs Bifid-F and g-Bifid-R were used. To determine *B. bifidum* cell numbers, primer pairs BiBIF-1 (5'-CCACATGATCGCATGTGATTG-3') and BiBIF-2 (5'-CCGGATGCTCCATCACAC-3') were employed. *B. breve* cells were quantified using primer pair BiBRE-1 (5'-CCACATGATCGCATGTGATTG-3') and BiBRE-2 (5'-ACAAAGTGCCTTGCTCCCT-3'), while *B. infantis* cell numbers were determined using primer pair BiINF-1 (5'-TTCCAGTTGATCGCATGGTC-

3') and BiINF-2 (5'- GGAAACCCCATCTCTGGGA -3'). The copy-number of a given species in co-culture was evaluated by comparing the cycle threshold (Ct) values with those from a standard curve of known copy number. Standard curves (10^9 to 10^2 copy number/ μ L) were established by performing 1 in 10 serial dilutions of 16S rDNA of the specified bifidobacterial species (in the case of *B. infantis* strains, M-63 was used as the reference rDNA). The cell numbers of species within co-culture (denoted by bifidobacteria/mL) were then deduced by comparing these copy number values with copy numbers of standards with known cell numbers (as determined by viable count assessment). Cell numbers were calculated according to the formula (Quigley et al., 2013):

$$\frac{(C/\mu\text{l})(TV)}{TCN} \times T \text{ cfu/ml} = \text{bifidobacteria/ml (S)}$$

Where; C/ μ l = Copy number/ μ l, TV = template volume (μ l), TCN = total copy number of the standard used, T cfu/ml = total cell number of standard used, and bifidobacteria/mL(S) = cell number of test sample. Samples were run in triplicate, while standards and negative controls (where template DNA was replaced with PCR grade water) were run in duplicate.

2.5. Whole Genome Sequencing Assembly and Annotation:

Following growth of R0071, R0033, M-16V and M-63 as described in section 2.3 above, bacterial cells were harvested, lysed, and total bacterial DNA was isolated. Genome sequences of the bifidobacterial strains were generated by Macrogen Inc. (Seoul, South Korea) using Pacific Biosciences SMRT RSII technology. The raw sequencing reads were *de novo* assembled using the Hierarchical Genome Assembly Process (HGAP) protocol RS_Assembly.2 implemented in the SMRT Analysis software v.2.3 with default parameters

(<https://github.com/PacificBiosciences/SMRT-Analysis>) as previously described (Bottacini et al., 2017).

Open Reading Frame (ORF) prediction and automatic annotation was performed using Prodigal v2.0 (<http://prodigal.ornl.gov>) for gene predictions. BLASTP v2.2.26 (cut-off E-value of 0.0001) was used for sequence alignments against a combined bifidobacterial genome-based database, and MySQL relational database to assign annotations. Predicted functional assignments were manually revised and edited using similarity searches against the non-redundant protein database curated by the National Centre for Biotechnology Information (<ftp://ftp.ncbi.nih.gov/blast/db/>) and PFAM database (<http://pfam.sanger.ac.uk>), which allowed for *in silico* characterization of hypothetical proteins. GenBank editing and manual inspection was performed using Artemis v18 (<http://www.sanger.ac.uk/resources/soft-ware/artemis/>). Transfer RNA genes were identified employing tRNAscan-SE v1.4 and ribosomal RNA genes were detected based on the software package Rnammer v1.2 (38) supported by BLASTN v2.2.26.

2.6. Prediction of colonisation capabilities

Scientific literature databases including PubMed, Web of Science, UCC library, and Google Scholar were searched for studies providing mechanistic insight into probiotic colonization and survival in the gastrointestinal tract. A list of probiotic (*Lactobacillus* and *Bifidobacterium* genera) colonisation-related features (**Supplementary Table S3**) was then compiled from these studies which included gene-trait matching, transcriptomic, proteomic, and knock-out studies (Bron et al., 2007; Call et al., 2015; Christiaen et al., 2014;

Fanning et al., 2012; Foroni et al., 2011; Frese et al., 2011; Frese et al., 2013; Ganesh et al., 2018; Goh & Klaenhammer, 2014; González-Rodríguez et al., 2012; Gueimonde et al., 2009; Guglielmetti et al., 2008; Ishikawa et al., 2021; Kankainen et al., 2009; Liu et al., 2014; O'Connell Motherway et al., 2011; Shkoporov et al., 2008; Sims et al., 2011; Tanaka et al., 2000; Turrone et al., 2013; Walter et al., 2005; Walter et al., 2007; Walter et al., 2008; Wilson et al., 2014). A Blast search was then performed (e-value cutoff of 1e-5, query cover cutoff of 50%) of the database against the bifidobacterial genomes of interest. Percent identity was recorded for each bifidobacterial strain of interest and classified as “low identity” = 30-50%, “moderate identity” = 50-70%, and “high identity” = 70-100%.

The bifidobacterial sequences were also searched for sortase-dependent proteins since these proteins can play a pivotal role in adhesion and mucin-degradation. The bifidobacterial strains were surveyed for features containing the following sequence:

1. a signal peptide at the N-terminus
2. a pentaglycine recognition motif
 - LPXTG in the case of sortase A- and sortase C-dependent proteins
 - [L/I/V][S/A]XTG in the case of sortase E-dependent proteins
3. followed by a hydrophobic membrane-spanning region (verified using ProtScale <https://web.expasy.org/protscale/> and DeepTMHMM v1.0.13 <https://dtu.biolib.com/DeepTMHMM>)
4. positively charged residues at the C-terminus.

2.7. Proteomic analysis of colonisation-related factors

2.7.1 Sample preparation:

Label-free quantitative proteomic analysis was carried out on the four-strain bifidobacterial mixture with/without S-HMO pre-treatment and after exposure to the HT29-MTX cell line. Sample groups included HMO-treated (HMO⁺ Cells⁺), and untreated bifidobacteria (HMO⁻ Cells⁺), following incubation with cells, and untreated bifidobacterial control (HMO⁻ Cells⁻) without incubation with HT29-MTX cells. Biological replicates of treated and control cells were prepared ($n = 3$ per condition).

HT29-MTX cells were seeded in 6-well plates and cultured until 100% confluence was reached. A bifidobacterial mixture containing equal amounts of *B. bifidum* R0071, *B. infantis* R0033, *B. breve* M-16V, & *B. infantis* M-63 was prepared as outlined in section 2.4. This bacterial mixture was then incubated for 2 hours in non-supplemented serum-free phenol-red free DMEM with and without HMO. For experiments, HT29-MTX cells were washed with PBS and treated with 3mL bacterial solutions. The plates were then incubated at 37 °C for 2 h in a humidified atmosphere (5% CO₂). As a control, bacteria were incubated under the same conditions in DMEM without HT29-MTX cells. After 2 hours incubation, medium containing the treated bacteria was aspirated (Wei et al., 2014). Thereafter, bacteria were pelleted, washed three times in PBS and immediately frozen in liquid nitrogen. Adherent bacteria were also extracted from the system by detaching HT29-MTX cells with ice-cold PBS. Harvested cells were immediately frozen in liquid nitrogen. All samples were stored at -70 °C until protein extraction was performed.

2.7.2 Protein extraction and digestion:

Cold lysis buffer (0.1 M TrisHCl, 10 % glycerol, 20 mM EDTA, 50 mM NaCl, 1 mM PMSF, 1 µg/ml, pH 7.5) was added to each cell pellet and sonicated using a MS72 probe (3 x 10 sec, cycle 6, 20 % power), and placed on ice between each sonication round. An aliquot of each lysate was clarified by centrifugation and a Bradford protein assay (Bio-Rad) was used to determine the protein concentration. Total lysates (without clarification) were adjusted to 0.25 mg/ml protein concentration with 50 mM ammonium bicarbonate and heated at 95 °C, 5 min. Samples were reduced using dithiothreitol (DTT; 5mM final, 15 min), alkylated using iodoacetamide (IAA; 15mM final, 20 min, dark) and subjected to overnight trypsin digestion at 37°C (trypsin: 8 µg/ml final) in the presence of ProteaseMax (0.01 % (w/v)). Digestion was stopped by addition of trifluoroacetic acid (1% (v/v) final) and samples were dried under vacuum. Digests were resuspended in 0.5% (v/v) TFA prior to clean-up using C18 ZipTips (Millipore) according to manufacturer's instructions. Samples were dried under vacuum and stored at -70 °C until LC-MS/MS analysis.

2.7.3 Label-free quantitative (LFQ) proteomic analysis:

Peptide extracts (0.75 µg) were analysed by LC-MS/MS using the Thermo Q-Exactive mass spectrometer coupled to a Dionex Ultimate 3000 RSLCnano, using a 2.25 hour method for separation on an Easy-Spray PepMap™ column (50 cm × 75 µm, 2 µm particles). Samples were searched against a composite database containing the proteomes of R0071, M-16V, and M-63, appended with the *Homo sapiens* proteome (downloaded from Uniprot 15/02/2019) using MaxQuant (v 1.6.2.10) with MaxLFQ algorithm included for protein ratio characterisation (Cox et al., 2014) and 1% FDR applied. Perseus (v

1.6.2.2) was used for data analysis (Tyanova et al., 2016). Proteins were filtered to exclude single peptide identifications and proteins matching a reverse or a contaminants database. Proteins were retained for analysis only if detected in at least two biological replicates from either comparator group. Qualitative results were based on unique protein detection in at least two biological replicates in a condition, with absence of detection in all replicates of the comparator set. Quantitative and qualitative results were combined prior to functional characterization.

2.8. Statistical analysis

Statistical analysis for adhesion assays were performed using technical triplicate data (three wells from each passage of cells) from biological triplicate experiments (across three successive passages of HT29-MTX cells). Graphs were created using PRISM v8 (GraphPad, La Jolla, CA) and were plotted as mean $\pm$ sd. Univariate analysis of variance (ANOVA) and post-hoc Tukey tests were performed to determine the significant differences between strains and carbohydrates for levels of bifidobacterial adhesion. Statistical significance was accepted as $p \leq 0.05$. For proteomic analysis Perseus was used for statistical analysis, with quantitative results composed of proteins with a significant change in abundance ($p < 0.05$ (Student's t-test), fold change ≥ 2).

3. Results

3.1. Single strain and multi-strain adherence to HT29-MTX

Adherence of probiotic bacteria was evaluated using HT29-MTX human tumorigenic cell lines to mimic bifidobacterial adhesion to intestinal epithelial cells (IECs). Bifidobacterial strains were incubated with various carbohydrates (glucose, 2'-FL, or S-HMOs) after which their ability to adhere to HT29-MTX cells was determined. As shown in **Figure 1** and **Supplementary Table S1**, adhesion of bifidobacterial strains varied significantly depending on strain and carbon source. The lowest levels of adherence were observed with *B. breve* M-16V in control group where glucose was the sole carbon source ($7.2 \pm 0.6\%$ of original inoculum), while highest levels of adherence were observed with strain *B. bifidum* R0071 after exposure to S-HMOs ($13.8 \pm 1.4\%$ of original inoculum). Across all treatment groups, *B. bifidum* R0071 demonstrated significantly higher adherence when compared to *B. breve* M-16V (**Table S1**).

Addition of 2'-FL resulted in a marked increase in the adhesive ability of the *B. infantis* strains, as represented by a 1.26 fold increase in adhesion of M-63 ($p = 0.0007$) and a 1.15 fold increase in adhesion of R0033 ($p = 0.0456$) when compared to treatment of these strains in control media (**Figure 1**). Exposure of *B. bifidum* R0071 and *B. breve* M-16V to 2'-FL had no significant impact on the adhesion of these strains to the cell line. However, exposure of each of the four *Bifidobacterium* strains to S-HMOs significantly increased adherence to HT29-MTX cells by an average of 1.31 fold (vs control). The HMO-induced promotion of adhesion was observed, in particular, for *B. bifidum* R0071 which demonstrated significantly higher

adhesion following S-HMO treatment when compared to the other strains (vs R0033 $p=0.0170$, vs M-16V and M-63 $p<0.0001$, **Table S1**). The adhesive ability of R0071 increased 1.37 fold after S-HMO treatment when compared to adhesion of the strain in control conditions ($p<0.0001$). Interestingly, we also observed higher adhesion levels of *B. infantis* R0033 when compared to its species counterpart *B. infantis* M-63 following exposure of the strains to S-HMO ($p=0.0118$).

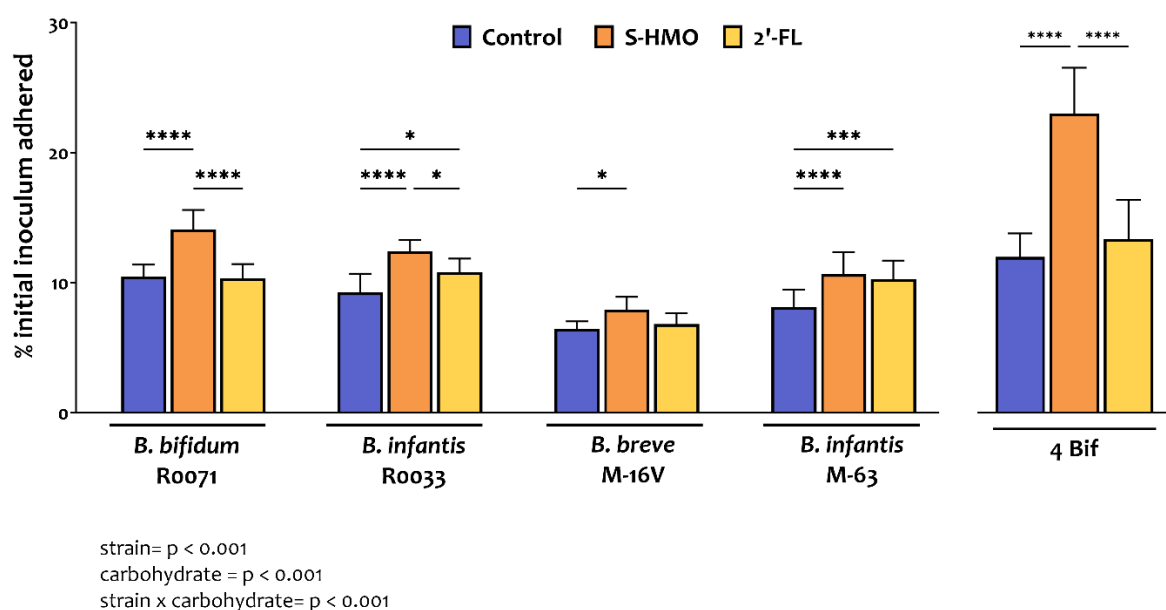


Figure 1: Levels of bifidobacterial adherence to HT29-MTX cells following pre-exposure to glucose control, breastmilk-derived human milk oligosaccharides (S-HMO), and 2'-fucosyllactose (2'-FL). Adherent bacteria plotted as a percentage of the original inoculum. Percentage adherent = [CFU/mL of recovered adherent bacteria/ CFU/mL of inoculum] x 100. R0071= *Bifidobacterium bifidum* R0071, R0033= *Bifidobacterium infantis* R0033, M-16V= *Bifidobacterium breve* M-16V, M-63= *Bifidobacterium infantis* M-63, 4Bif= 4 *Bifidobacterium* mixture. Analysis was calculated using technical triplicate data from biological triplicate experiments and data are means +/- SD. Univariate analysis of variance (ANOVA) and post-hoc Tukey tests were performed to determine the significant differences between the groups (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$). Results of ANOVA are as outlined in figure where row factor= bifidobacterial strain(s), column factor= carbohydrate source, and interaction= bifidobacterial strain x carbohydrate source.

To assess whether a probiotic combination represents better colonists than single strains, the adhesive capacity of a bifidobacterial mixture containing equal amounts of all four strains was assessed using HT29-MTX cells. Significant levels of bacterial adhesion occurred with this bifidobacterial consortium (**Figure 1, Figure 2**). Small increases in adhesion were observed in the control group when compared to colonization by single strains ($11.9 \pm 1.8\%$ in co-culture versus $10.5 \pm 0.9\%$ in *B. bifidum* R0071 alone). Most notably, dramatic increases in adherence levels of the four-strain mixture were observed following S-HMO-exposure when compared treatment with control media (**Figure 1**). A large proportion of the original inoculum ($23.0 \pm 3.5\%$) was found to have adhered to intestinal cells following treatment with HMOs, which corresponds to a 1.92 fold increase when compared to the non-supplemented control ($p < 0.0001$).

Quantitative PCR analysis targeting the 16S rRNA-encoding region allowed for quantification of cell numbers of each species within the four-strain combination but did not distinguish between the two *B. infantis* strains. Nonetheless, qPCR analysis provided insights into the microbial composition of adhered bacteria (**Figure 2**). Across all treatments, *B. bifidum* occupied the largest proportion of adhered bacteria accounting for 53.0%, 57.8%, and 52.3% in control, HMO, and 2'-FL treatment groups, respectively (**Figure 2**). As shown in **Supplementary Table S2**, significantly higher levels of adhered *B. bifidum* were detected following S-HMO exposure, when compared to the two other species present within the bifidobacterial combination (vs *B. breve* $p < 0.0001$, vs R0033 $p = 0.0157$).

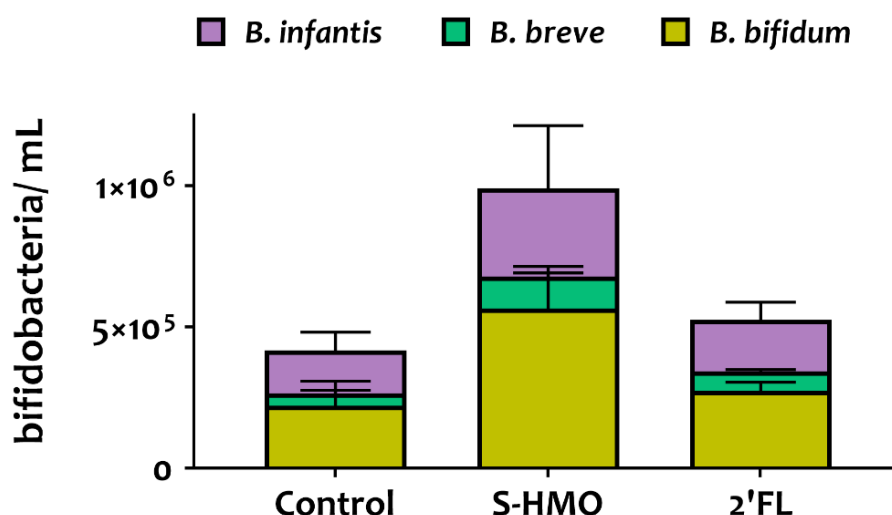


Figure 2: qPCR analysis of adhered *Bifidobacterium* mixture (equal parts *Bifidobacterium bifidum* R0071, *Bifidobacterium infantis* R0033, *Bifidobacterium breve* M-16V, *Bifidobacterium infantis* M-63) following pre-exposure to glucose control, breastmilk-derived human milk oligosaccharides (S-HMO), and 2'-fucosyllactose (2'-FL). Species within bifidobacterial mixture quantified using primers targeting the 16s gene region of each species. Analysis was calculated using technical triplicate data from biological triplicate experiments and data are means +/- SD.

3.2. Mining of bifidobacterial sequences for colonization-related features

To elucidate the key factors that are responsible for the observed differences in bifidobacterial adherence to intestinal cells, the bifidobacterial genomes were surveyed for colonisation-related factors. A literature search for studies assessing probiotic colonisation was performed and resulted in identification of 45 genes with a potential role in *Lactobacillus* and *Bifidobacterium* survival and persistence in the gut (**Supplementary Table S3**). Thereafter, the genomes of *B. bifidum* R0071, *B. breve* M-16V, *B. infantis* R0033, and *B. infantis* M-63 were searched for homologs of these genes. All bifidobacterial strains studied here harboured genes associated with microbe-host interactions (>50% coverage, e-value <1 e-5). Genes with high similarity

(>70% identity) to bile salt hydrolase (*bsh*, shown to improve adhesion ability of *Lactobacillus plantarum*), transaldolase (*tal*, demonstrated to bind mucin in strains of *B. bifidum*), S-ribosylhomocysteine lyase (*luxS*, shown to be involved in biofilm production in *B. breve* UCC2003), early secretory antigen target 6-like protein (*esat-6* [BBPR_0651], found to be highly induced in *B. bifidum* PRL2010 following HT29 exposure), and hemolysin-like protein (*tlyC1*, shown to be involved in bile tolerance in *B. longum* BBMN68) were identified across all four genomes as well as genes involved in exopolysaccharide production such as undecaprenyl-phosphate galactosephosphotransferase *rfbP* (**Figure 3, Table S3**).

The *tad* locus (as first described for the bifidobacterial genera in UCC2003 (O'Connell Motherway et al., 2011)), which encodes for site-determining protein TadZ, ATPase TadA, integral membrane proteins TadB and TadC, pre-pilin precursor Flp, and the pseudopilins TadE and TadF was found to be moderately conserved across the infant bifidobacterial strains studied here (as highlighted in **Figure 3**). Genes with homology (>50% coverage, e-value <1 e-5) to the UCC2003 *tad* locus were identified in all four genomes (*B. breve* M-16V [BBREV_0119- BBREV_0125], *B. infantis* R0033 [BINF_0150-BINF_0156], *B. infantis* M-63 [BINF2_0148-BINF2_0154], and *B. bifidum* R0071 [BBIF_1744-BBIF_1750]), although the level of genomic similarity was low (30-50% identity) in *B. bifidum* R0071 (**Table S3, Figure 3**). Notably, we identified clear homologs (>70% identity) of the UCC2003 *tad* locus in *B. breve* M-16V (**Table S3, Figure 3**).

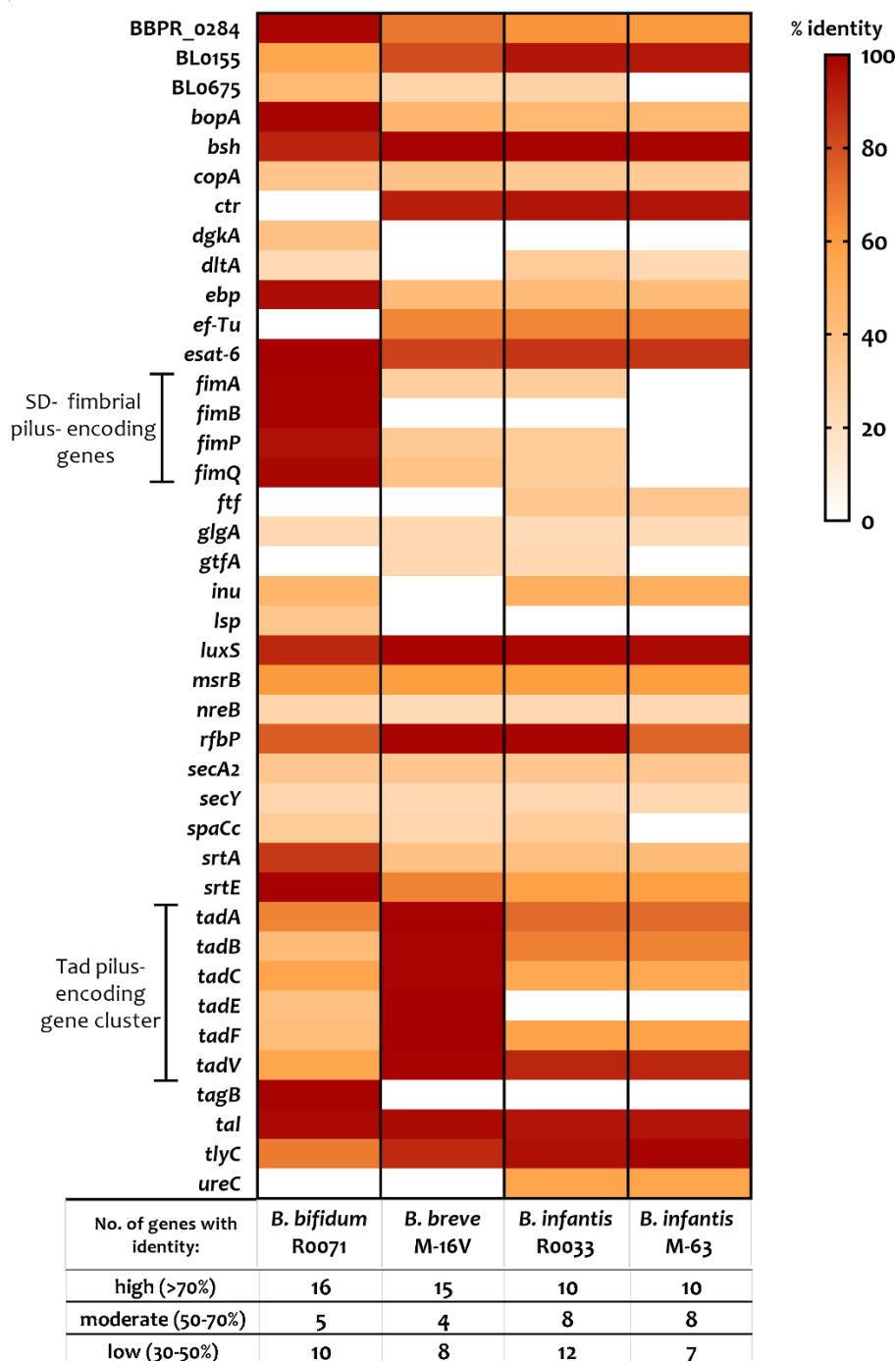


Figure 3: Percentage identity of genes predicted to be involved in probiotic survival & persistence in the gut. Genes, including those associated with assemblage of sortase-dependent (SD-) fimbriae or Tad pili (as highlighted), were identified using a variety of sources including gene-trait matching, transcriptomic, proteomic, and knock-out studies (see Supplementary Table S3 for further information). Percent identity (classified as “low identity” = 30-50%, “moderate identity” = 50-70%, and “high identity” = 70-100%) is based on BLAST searches using e-value cutoff of 1e-5 and query cover cutoff of 50%.

In contrast to the *tad* pili, genes encoding for fimbrial proteins such as FimA, FimB, FimP, and FimQ (as described in *B. bifidum* PRL2010 (Turrone et al., 2013)) were not found in all the bifidobacterial strains studied here (**Table S3, Figure 3**). Genes with low homology (30-50%) to *fimA*, *fimP*, and *fimQ* were identified in *B. breve* M-16V and *B. infantis* R0033, but not in *B. infantis* M-63 (**Table S3, Figure 3**). On the other hand, clear homologs (>70% identity) of these fimbrial-associated genes were identified in *B. bifidum* R0071. The pilus shaft components encoded by *fimA* and *fimP* are polymerized and assembled with pilus tip components (encoded by *fimB* and *fimQ*) by an adjacent class C sortase (srtC). A further class of sortase enzymes (sortase E [srtE]) has also recently been found to be critical for bifidobacterial adhesion (Ishikawa et al., 2021). It is clear, therefore, that specific sortase enzymes may contribute to the adherence phenotypes observed in this study. These sortase-enzymes work by covalently attaching specific sortase-dependent proteins (SDPs) to the cell-wall of Gram-positive organisms by recognizing a conserved C-terminal motif. Therefore, to understand if variances in SDPs could account for variances in adherence of the strains studied here, the bifidobacterial proteomes were searched for proteins with a signal peptide at the N-terminus, the srtC (LPXTG) or srtE ([L/I/V][S/A]XTG) recognition motif, followed by a stretch of hydrophobic amino acids and a positively charged C-terminus as previously described (Call & Klaenhammer, 2013; Ishikawa et al., 2021). We found nine such proteins in *B. breve* M-16V (**Figure S2, Supplementary Table S4**), 13 in *B. infantis* R0033 (**Figure S2, Supplementary Table S5**), and 10 in *B. infantis* M-63 (**Figure S2, Supplementary Table S6**). Comparative analysis between *B. infantis* strains revealed the presence of fimbrial proteins in R0033 (at

BINF_1976 and BINF_1977), but not M-63, which contained the SrtC-recognition motif LPXTG (Table S5) and Von Willebrand Factor-type A (VWA) domain.

In comparison to the lower numbers of SDPs identified in the other bifidobacterial strains, we identified 36 putative sortase-dependent proteins in *B. bifidum* R0071, including seven proteins containing the srtC-recognition motif and 29 proteins containing the srtE-recognition motif (**Figure S2, Supplementary Table S7**). Of these putative srtE-dependent proteins identified in *B. bifidum* R0071, 15 were found to represent glycosyl hydrolases (GHs) associated with degradation of HMOs and mucins such as those expressed on the epithelial cell surface, while the predicted srtC-dependent proteins (genetically) corresponded to the fimbrial proteins FimA/ FimP major pilin subunits or FimB/ FimQ minor pilin subunits. In *B. bifidum* R0071 we identified six putative sortase-dependent pilus proteins arranged in three pilin clusters at *BBIF*_1805-1807 (*pil1*: *BBPR*_1820-1822 in PRL2010), *BBIF*_1693-1695 (*pil2*: *BBPR*_1707-1709) and *BBIF*_0313-0315 (*pil3*: *BBPR*_0282-0284). A recent study has established that the presence of a stretch of polyG influences the on-off switch in the expression of these pili genes (Penno et al., 2022). Therefore, the identified pilin clusters in *B. bifidum* R0071 were searched for the presence of a G-tract up to 500bp upstream and 200bp downstream of the predicted gene start in the first gene of the operon. In comparison to PRL2010, which harbours only one G-tract containing pilus gene cluster (Penno et al., 2022), polyG sequences were found to be present in two pilus-specifying gene clusters in *B. bifidum* R0071. A G-tract was identified in the *pil3* gene cluster (*BBIF*_0313- 0315) at the

position 363939-36394911 (11 base pairs) and in the *pil1* gene cluster (*BBIF_1805-1807*) at the position 2216145 to 2216157 (13 base pairs).

3.3. Quantitative proteomic analysis of colonization-related factors

Label-free quantitative proteomic analysis was carried out on the four-strain bifidobacterial mixture after cell exposure and compared to control bacteria that had not been in contact with HT29-MTX cells. Furthermore, the impact of HMO pre-treatment on colonization-associated pathways was also assessed by comparing HMO-treated and untreated bifidobacteria following exposure to a human cell line. Preliminary screenings of the adhered cell lysate (which contained both lysed HT29-MTX cells and adhered bacteria) showed substantial masking of bacterial proteins. A total of 2908 proteins were detected with only 12 proteins matched to bacterial proteins (2 x *B. infantis*, 2 x *B. breve*, and 8 x *B. bifidum*). The remaining 2896 proteins were matched to human proteins. Since analysis of the adhered cell lysate did not allow for in-depth understanding of the impact of HMO and cell treatments on the bifidobacterial mixture, these samples were eliminated from subsequent analyses. Analysis of bifidobacteria aspirated from the cell apical compartment resulted in the identification of 2191 bacterial proteins (which covered 37.4% of the proteome of the strains studied here) and 1385 human proteins. **Supplementary Figure S3** shows details of bacterial protein numbers divided by species (**Figure S3A**) and by treatment (**Figure S3B**).

To assess the influence of epithelial cell exposure on bifidobacterial proteome expression, a 2-fold increase or decrease in the level of protein abundance was used as a cut-off for statistically significant differentially abundant (SSDA) proteins ($p < 0.05$). Interestingly, exposure of the

bifidobacterial mixture to HT29-MTX cells (HMO⁻cells⁺) appeared to reduce abundance of many *B. infantis* and *B. breve* proteins (**Figure 4A**).

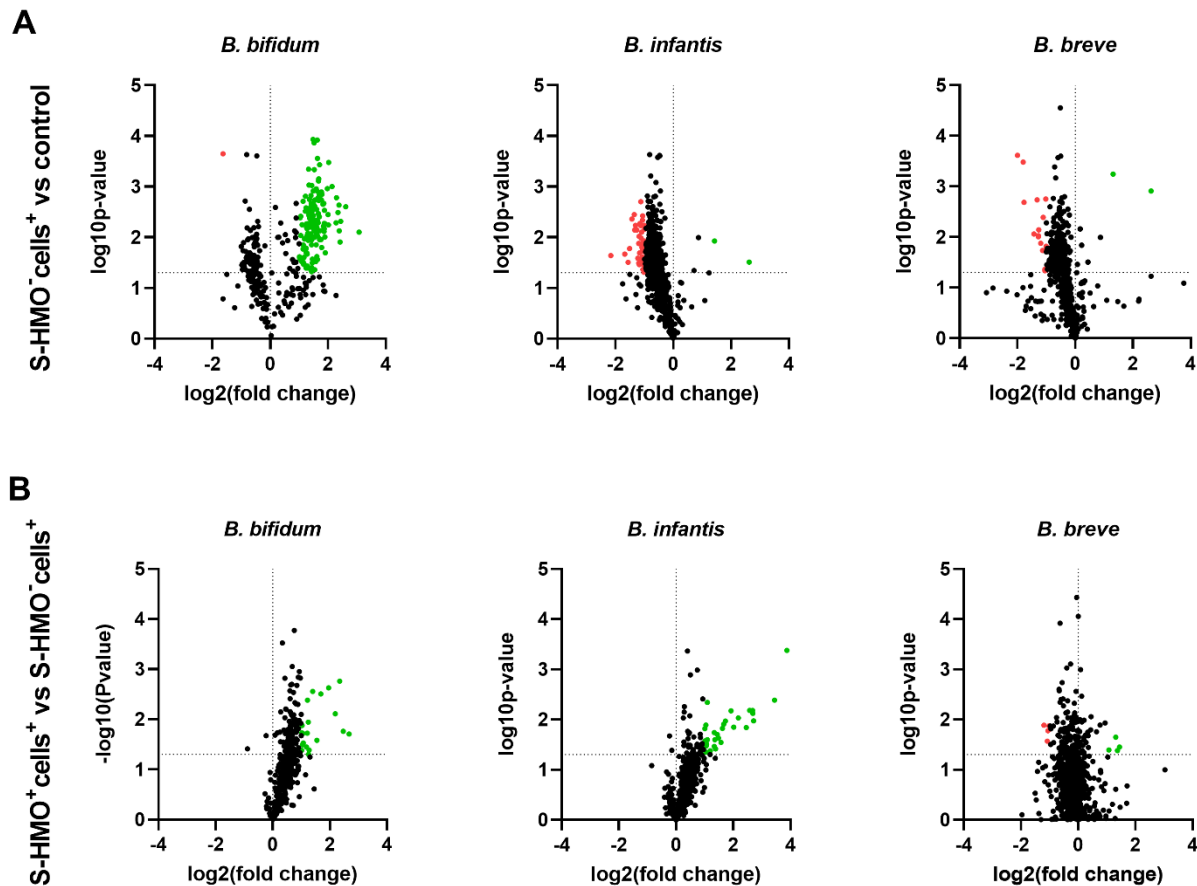


Figure 4: Volcano plots displaying quantitative changes in abundance of *Bifidobacterium bifidum*, *Bifidobacterium infantis*, and *Bifidobacterium breve* proteins following treatment A) with and without mucus-secreting HT29-MTX cells (S-HMO⁻cells⁺ vs S-HMO⁻cells⁻) B) with mucus-secreting HT29-MTX cells with and without pre-exposure to S-HMO (S-HMO⁺cells⁺ vs S-HMO⁻cells⁺) as detected using label-free quantitative proteomics. The horizontal line represents a p-value of 0.05. Proteins with a significant change in abundance ($p < 0.05$ [Student's t-test], fold change ≥ 2) are highlighted in red to indicate decreased abundance and green to indicate increased abundance.

Following treatment with cells (HMO⁻cells⁺), only two *B. infantis* proteins were found in higher abundance when compared to the control group; 30S ribosomal protein S17 (fold increase of 6.37) and sugar kinase (fold increase of 2.74) (**Supplementary Table S9**). A further two *B. infantis*

proteins were uniquely detected after treatment with cells; DNA polymerase III delta subunit and uracil-xanthine transport protein (**Table S9**). Similarly, treatment with cells resulted in increased abundances of only four *B. breve* proteins (prolipoprotein diacylglycerol transferase, transport protein, 30S ribosomal protein S17, and undecaprenyl-phosphate α -N-acetylglucosaminophosphotransferase) (**Supplementary Table S10**). In contrast, 42 *B. infantis* and 14 *B. breve* proteins decreased in abundance following incubation with HT29-MTX cells (**Figure 4A**). Qualitative analysis revealed that a further 49 *B. infantis* and 60 *B. breve* proteins were no longer detected after cell-treatment (HMO⁻cells⁺).

In contrast to *B. infantis* and *B. breve*, incubation of the bifidobacterial mixture with HT29-MTX cells (HMO⁻cells⁺) drastically enhanced the expression of *B. bifidum* proteins compared to the control (HMO⁻cells⁻) (**Figure 4A, Table S8**). Only nine *B. bifidum* proteins were detected at lower abundance after cell exposure. On the other hand, cell treatment (HMO⁻cells⁺) resulted in an increased abundance of 225 *B. bifidum* proteins (including 71 uniquely detected proteins), many of which were further upregulated through pre-treatment with HMO (HMO⁺cells⁺). Within this group of upregulated *B. bifidum* proteins, we identified 27 with a potential function in probiotic colonization. The fold changes of these proteins (vs control) are summarized in **Figure 5** and include cell surface pilus-associated proteins (**Figure 5A**), glycosyl hydrolases that have been shown to interact with the mucus layer (**Figure 5B**), and moonlighting proteins associated with bacterial adhesion (**Figure 5C**). Notably, we observed upregulation of numerous srtC-dependent proteins (as identified in section 3.3) following HT29-MTX exposure.

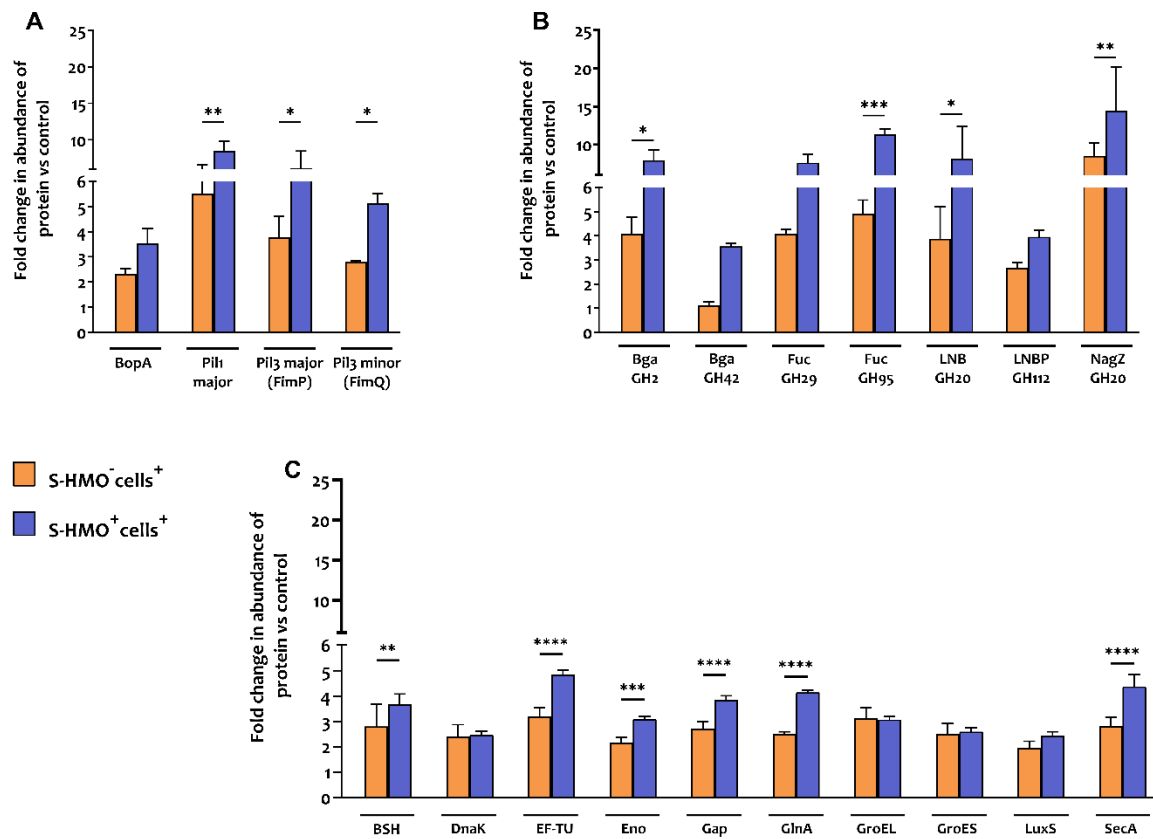


Figure 5: Proteins matched to *Bifidobacterium bifidum* R0071 with potential roles in bifidobacterial colonization that increased in abundance following treatment with cells (+/- S-HMO). A) cell surface adhesins B) glycosyl hydrolases C) secondary proteins involved in probiotic persistence. Protein abundance plotted as fold change versus negative control (S-HMO⁻ cells⁻, without S-HMO or cell treatment). Analysis was calculated using technical triplicate data from biological triplicate experiments and data are means +/- SD. Univariate analysis of variance (ANOVA) and post-hoc Tukey tests were performed to determine the significant differences between the groups (* = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001).

These proteins were further upregulated by the addition of HMO (HMO⁺ cells⁺). Other putative SrtC-dependent pili proteins were highly upregulated following HMO⁻ cells⁺ and HMO⁺ cells⁺ treatment including the Pil1 major pilin (encoded by gene *BBIF_1806*) after exposure to cells (HMO⁻ cells⁺) (p= 0.0016). Pre-treatment with HMOs before exposure to HT29-

MTX cells (HMO⁺cells⁺) resulted in further upregulation of this pilin protein (**Table S8**).

In addition to SrtC-dependent cell surface appendages, other *B. bifidum* R0071 colonization-related proteins were identified that were upregulated following cell and/or HMO treatment (**Figure 5C**). The majority of these upregulated proteins normally function as housekeeping genes or act as enzymes for glycolysis and are expressed intracellularly. However, these proteins have moonlighting properties and when expressed on the cell surface they can facilitate cell adhesion. For example, cell treatment (HMO⁻cells⁺) resulted in significant upregulation of chaperone protein DnaK (2.39 fold increase vs control), chaperonin protein GroES (2.5 fold increase), and chaperonin protein GroEL (3.11 fold increase), and S-ribosylhomocysteine lyase LuxS (2.12 fold increase) (Candela et al., 2007; Christiaen et al., 2014; Duranti et al., 2014; Wei et al., 2016; Zhu et al., 2016). These proteins, however, were unaffected by the addition of HMO to the system. On the other hand, some colonization proteins were detected at greater abundances in HMO⁺cells⁺ group (**Figure 5C**). Following incubation with both cells and HMO (HMO⁺cells⁺), elongation factor thermo unstable (EF-Tu), enolase (Eno) and glyceraldehyde phosphate dehydrogenase (Gap) were upregulated 4.84 fold, 3.07 fold, and 3.84 fold, respectively, when compared to treatment with cells alone (HMO⁻cells⁺) (**Table S8**). In addition, a transaldolase was detected exclusively in the treatment groups and in high abundance following cell treatment (**Table S8**).

Aside from upregulation of cell surface adhesins, we also observed significant upregulation of SrtE-dependent glycosyl hydrolase proteins in *B. bifidum* R0071 following exposure of the bifidobacterial mixture to mucus-

secreting HT29-MTX cells. An abundance of SrtE-dependent glycosyl hydrolases were identified in *B. bifidum* R0071, which were detected at significantly higher abundances following exposure to mucus-secreting HT29 cells +/- HMO. The cumulative effects of HMO and cell treatment (HMO⁺cells⁺) resulted in significant upregulation of numerous SrtE-dependent glycosyl hydrolases (GHs) when compared to treatment with cells alone (HMO⁻cells⁺) (**Figure 5B**).

4. Discussion

Our research specifically delved into the adhesive characteristics of four bifidobacterial strains: *Bifidobacterium bifidum* R0071, *Bifidobacterium longum* subsp *infantis* R0033, *Bifidobacterium breve* M-16V, and *Bifidobacterium longum* subsp. *infantis* M-63 in the presence of HMO. These strains have garnered considerable attention due to their well-documented health benefits, as substantiated by clinical investigations (Giannetti et al., 2017; Wong et al., 2019; Xiao et al., 2019). For instance, infants fed with a probiotic supplement comprised of *B. bifidum* R0071, *B. infantis* R0033, and a *Lactobacillus* strain maintained elevated levels of fecal SIgA after a four-week treatment period (Xiao et al., 2019). *Bifidobacterium breve* M-16V, another commonly used probiotic in infants, has exhibited promise in safeguarding against conditions like necrotizing enterocolitis (NEC) and allergic diseases in infants, as extensively reviewed by Wong et al. in 2019 (Wong et al., 2019). Furthermore, a blend containing *B. infantis* M63, along with two other probiotic strains (including M-16V), demonstrated superior management of functional abdominal pain and a notable enhancement in the quality of life among children with irritable bowel syndrome (IBS) (Giannetti et al., 2017). Notably, our previous research has highlighted the synergistic

capabilities of these bifidobacterial strains when it comes to utilizing human milk oligosaccharides (HMOs) as a primary carbohydrate source, resulting in a marked increase in bacterial proliferation (Walsh et al., 2022). Adherence of probiotic bacteria was evaluated using HT29-MTX human tumorigenic cell lines to mimic bifidobacterial adhesion to intestinal epithelial cells (IECs). The HT29-MTX cell line, which is able to constitutively produce mucin, has more physiologically relevant characteristics than HT29 cells alone due to the mucus layer formation, and has therefore been proposed as a more suitable cell line for studying host-microbe interactions. In agreement with other studies (Kavanaugh et al., 2013; Zhang et al., 2020) through the use of colonisation assays, we confirm that the adhesive potential of bifidobacterial strains can be substantially increased through treatment with milk oligosaccharide components. These HMO-induced changes in bifidobacterial adhesion align with findings of Chicklowski et al., where increased adherence of *B. infantis* was observed resulting from exposure to breastmilk-derived HMOs (Chicklowski et al., 2012). However, in contrast to Chicklowski et al., the current study found that HMO-exposed *B. bifidum* had a higher rate of adhesion to intestinal cells compared to *B. infantis* or *B. breve* (Chicklowski et al., 2012). This agrees with previous studies which showed that *B. bifidum* PRL2010 displays higher levels of adhesion to extracellular matrix components (fibronectin, plasminogen, and laminin) when compared with *B. breve* and *B. infantis* (Turroni et al., 2013). Notably, proteomic analysis of the bifidobacterial combination revealed that many proteins within the *B. infantis* and *B. breve* proteome decreased in abundance following exposure to intestinal cells. This is likely a result of complex and dynamic interactions between the bacteria and host cells. These interactions can trigger

various responses, including adaption to host immune responses, regulatory changes, and resource allocation, which collectively influence the protein expression profile of these bacteria during the assay.

Of note, we observed higher levels of adhesion in HMO-exposed *B. infantis* R0033 when compared to its species counterpart *B. infantis* M-63. Our findings verify that bacterial adhesion to intestinal epithelial cells is not only species-specific but also strain-specific. Interestingly, we have previously highlighted the strain-level differences between M-63 and R0033, with M-63 demonstrating superior growth on breastmilk-derived HMOs (Walsh et al., 2022). It may be important, therefore, when assessing interactions of HMO and probiotics, to consider the growth phenotype but also the adherent phenotype resulting from a strain's exposure to HMOs. Notably, findings from adhesion assays using a combination of all four strains have indicated that co-operation between strains may expand HMO acquisition capabilities to enhance gut colonization activity of strains. qPCR analysis of the bifidobacterial consortia revealed that, irrespective of the carbon source, *B. bifidum* R0071 occupied the largest proportion of bacteria adhering to HT29-MTX cells. Interestingly, recent studies have indicated that strains of *B. bifidum* adhere more strongly to infant mucus than adult mucus, while more adult-associated species such as *B. lactis* and *L. rhamnosus* adhere more strongly to adult mucus than to infant mucus. In general, the adhesion of *B. bifidum* *in vitro* is exceptionally strong among probiotics (Shibahara-Sone et al., 2016) and therefore the high levels of adherence observed in this study may be expected. Such characteristics might arise from the vast array of sortase-dependent proteins specific to *B. bifidum* as described in a recent study (Ishikawa et al., 2021) .

The survey of the bacterial genomes for colonisation-related factors revealed a number of components that bacteria can potentially interact with to adhere to mucus and epithelial surface glycans e.g. outer-membrane proteins, adhesins, capsules and appendages such as pili, flagella, and fimbriae (Paone & Cani, 2020). Assembly of pilus-like appendages on the bacterial surface are thought to be pivotal for bifidobacterial gut colonization. In recent years, pilus encoding-gene clusters have been identified in many infant-derived bifidobacteria, most notably a type IVb tight adherence (Tad) pilus-encoding gene cluster in *B. breve* UCC2003 (O'Connell Motherway et al., 2011) and sortase-dependent pilus gene clusters in *B. bifidum* PRL2010 (Turroni et al., 2013). The fact that genes with homology to the UCC2003 *tad* locus was found in all our strains aligns with studies from O'Connell Motherway et al., where the authors found that the *tad* pilus-encoding locus was highly conserved across the bifidobacterial genera (O'Connell Motherway et al., 2011). That study suggested that such a phenomenon supports the notion of a ubiquitous pili-mediated host colonization and persistence mechanism for bifidobacteria (O'Connell Motherway et al., 2019; O'Connell Motherway et al., 2011).

In addition to *tad* pili, membrane-bound transpeptidase enzymes known as “sortases” have been found to play a critical role in facilitating bacterium-host cell crosstalk throughout the bifidobacterial genera. In fact, a recent study from Ishikawa et al., 2021 found that non-adhesive strains belonging to the *B. bifidum* species lacked a functional class E (housekeeping) sortase (Ishikawa et al., 2021). Class E sortases (*srtE*) work by anchoring proteins to the cell surface, while class C sortases (*srtC*) are responsible for constructing pilus polymers. Sortase-mediated surface structures are

responsible, therefore, for cell attachment and nutrient uptake, amongst other functions. The small number of putative SrtC-dependent clusters associated with pilus expression identified in *B. infantis* strains is in line with a previous study in which pilus-like structures were shown to be rare if at all present in *B. longum* subsp. *infantis* ATCC15696 (Foroni et al., 2011). Since pilus-like structures in *B. infantis* are rare, the srtC-dependent fimbrial units (containing VWA-domains) identified in R0033 may be notable. VWA-domains have been found in pilus proteins that bind to extracellular matrix components proteins and host cells (Whittaker & Hynes, 2002). Follow-up studies are required to elucidate whether the expression of these fimbriae proteins in R0033 could account for its superior adherence over its *B. infantis* counterpart M-63. In contrast to the *B. infantis* and *B. breve* strains studied here, numerous srtC-dependent fimbrial proteins were identified in *B. bifidum* R0071. As described for *B. bifidum* PRL2010, the genome of *B. bifidum* R0071 encompasses three different loci (Pil1, Pil2, and Pil3) encoding the predicted biosynthetic machinery for the production of sortase-dependent pili (Turroni et al., 2013). Proteomic analysis of the bifidobacterial combination following exposure to intestinal cells revealed substantial upregulation of srtC-dependent pilin proteins within two pilus-specifying clusters (Pil1 and Pil3) but not Pil2. Recent studies have highlighted that the presence of a stretch of polyG influences the on-off switch in the expression of these pili genes. We confirm that polyG sequences were found downstream of the predicted gene start in Pil1 and Pil3, but not Pil2. The identification of polyG sequences is noteworthy, as productive replication slippage at G-tract sequences may be an important mediator of sortase-dependent pili expression and subsequent colonization of bifidobacterial strains within the

gastrointestinal tract (Penno et al., 2022). Importantly, significant upregulation of the pilus proteins within the *B. bifidum* Pil1 and Pil3 clusters was observed following exposure of the bifidobacteria to HMO. The HMO-induced upregulation of srtC-dependent pilins aligns with findings from Foroni et al. where high production of pilus-like structure was observed following propagation of *B. bifidum* PRL2010 on fructooligosaccharides (Foroni et al., 2011). It is clear therefore, that expression of sortase-dependent pili by *B. bifidum* can be influenced by the presence of complex carbohydrates and may account for the increased adherence of this strain following exposure to S-HMOs.

A somewhat less explored category of proteins known as "moonlighting proteins" (Huberts and van der Klei, 2010) may also play a critical role in bacterial adhesion. Most of these proteins typically serve as housekeeping genes or function as enzymes in glycolysis and are expressed intracellularly. Nevertheless, these proteins have been found to be multifunctional and, when expressed on the cell surface, have been reported to facilitate cell adhesion. In this current study, moonlighting proteins with primary functions as chaperone proteins such as DnaK and GroEL were more abundant in the *B. bifidum* R0071 proteome following co-cultivation with IECs. This aligns with previous research in *Lactobacillus johnsonii* (Bergonzelli et al., 2006) and *Bifidobacterium animalis* subsp. *lactis* (Candela et al., 2010) which demonstrated a role for GroEL and DnaK in probiotic binding to mucins and plasminogen. However, in contrast to Kavanagh et al we found that exposure of bifidobacteria to HMO did not influence the expression of DnaK or GroEL, although these discrepancies could be accounted for differences in strains (multistrain vs single strain of *B. infantis*),

HMOs (breastmilk-derived HMO vs combination of 3'-SL and 6'-SL), or analytical methods (transcriptomic vs proteomic profiling). However, we identified numerous moonlighting proteins that serve as adhesins for bifidobacteria in higher abundance in the *B. bifidum* R0071 proteome following co-cultivation with HT29-MTX cells which were further upregulated following pre-exposure to HMO. These include enzymes typically associated with glycolysis and the bifid shunt pathway such as transaldolase (Tal), enolase (Eno), and glyceraldehyde-3-phosphate dehydrogenase (Gap) (Pokusaeva et al., 2011). This aligns with previous studies which have identified mucin-binding properties for transaldolase in *B. bifidum* (González-Rodríguez et al., 2012) and plasminogen-binding properties for enolase in *B. longum*, *B. bifidum*, *B. animalis* subsp. *lactis*, and *B. breve* strains (Candela et al., 2007; Candela et al., 2009; Wei et al., 2014). Similar plasminogen binding capabilities have also been demonstrated for EF-Tu (Candela et al., 2007), which is another moonlighting protein which may facilitate cell adhesion and which was found at significantly higher abundances the *B. bifidum* R0071 proteome following IEC exposure and HMO pre-treatment. Overall, the proteomic profiling of moonlighting proteins outlined in this study indicates that bifidobacterial strains may be able to sense the conditions of their intestinal environment to detect receptors on IECs and respond by enhancing expression of adhesive molecules on the bacterial cell surface.

The mucus layer not only supplies attachment sites to commensal bacteria, but also serves as a nutrient source for organisms in the gut microbiota (Hansson, 2020; Paone & Cani, 2020). The turnover of the intestinal mucus layer involves mucus synthesis, secretion and degradation,

and represents a delicate process that needs to be regulated and balanced to ensure that mucus maintains an optimal protective function (Schneider et al., 2018). Recent studies have found that mucin adhesion is closely linked to mucin utilization (Ishikawa et al., 2021), with researchers highlighting that the class E housekeeping sortase is a critical component influencing adhesion of *B. bifidum* strains (Ishikawa et al., 2021). This aligns with the identification of SrtE-dependent glycosyl hydrolase proteins in R0071 following exposure the HT29-MTX cells and/ or HMO. Microbes most adept at mucin glycan degradation often encode sialidases and fucosidases to remove terminal structures which allows for greater accessibility to the extended core structures. The monosaccharides released by the action of these enzymes may be utilized by the bacteria themselves or released into the environment for scavenging bacteria (Luo et al., 2021). Proteomic analysis revealed significant upregulation of *B. bifidum* fucosidases belonging to GH29 and GH95 families, which hydrolyse $\text{Fuca}1-2\text{Gal}$ and $\text{Fuca}1-3/4\text{Gal}$ linkages, respectively. A SrtE-dependent GH33 exo- α -sialidase was detected that matched to *B. bifidum* R0071 which was found exclusively in bacteria exposed to mucus secreting intestinal cells. The fact that there was significant upregulation of fucosidases enzymes and an exo- α -sialidase in R0071 following incubation with IECs is notable. Studies have shown that the fucose released from mucin glycans by *B. bifidum* enables growth of *Eubacterium hallii*, a commensal species that produces butyrate and propionate from fermentation metabolites (Bunesova et al., 2018). Sialic acid residues are another highly sought-after nutrient source that are found terminating mucin glycan chains. To access this glycan residue intestinal bacteria express GH33 sialidases, which cleave terminal sialic acid residues. Previous studies have

highlighted that cleavage of sialic acid residues from mucin oligosaccharides, catalyzed by *B. bifidum* GH33 sialidases, can support growth of *B. breve* UCC2003 (Egan et al., 2014). We hypothesize therefore that HMO-enhanced colonization of *B. bifidum* could also benefit other gut symbionts through mucin cross-feeding activities.

5. Conclusions

Overall, we confirm that adherence of bifidobacterial strains to intestinal epithelial cells is species- and strain- specific. Our results suggest that HMOs, a prominent component of human breast milk, can promote interaction of infant-derived bifidobacteria and intestinal cells. In particular, we show that the presence of HMOs significantly improves the adhesion ability of a mixed community of commercial bifidobacterial strains. The adherence of bifidobacteria to intestinal epithelial cells is essential for their colonization, their ability to support a healthy gut environment, and their potential to confer various health benefits to the host. Recent studies have shown that certain bifidobacterial species, including *B. infantis*, are in danger of becoming extinct due to a multitude of factors including changes in breastfeeding habits in certain countries, C-sections deliveries, and antibiotic usage (Taft et al., 2022). Therefore, it has never been more important to explore a means to increase the colonization of beneficial bacteria such as bifidobacteria in the gut. Specifically, we show that carbohydrate sources are important environmental factors influencing adhesin expression levels particularly in *B. bifidum*, which may have knock-on effects positively influencing trophic interactions and social behavior among bifidobacterial species and between other intestinal microbes as has been shown previously for this species (Bunesova et al., 2018; Egan et al., 2014). We confirm that assembly and

localization of surface proteins, including SrtC-dependent pili and moonlighting proteins, are key factors influencing *B. bifidum* adhesion. Our findings also verify that the class E housekeeping sortase may be a critical component influencing adhesion of *B. bifidum* strains by increasing bifidobacterial interaction with the mucin layer. Although *in vitro* experiments are key to understand the mechanisms of adhesion and select probiotic candidates with potential to adhere *in vivo*, it is difficult to extrapolate these results to the human intestinal tract where other factors such as peristaltic movements, host immune system, diet and genetics, or competition with resident microbiota could interfere with attachment. It is clear, therefore, that further clinical studies with probiotics, which incorporate specific combinations of probiotic consortia and HMOs, will be critical in understanding how the findings outlined here translate to the *in vivo* situation.

Nevertheless, this study sheds light on the future use of oligosaccharides and bifidobacteria for nutritional intervention or clinical applications. We suggest that specific combinations of probiotic consortia and HMO open a window of opportunity for long-term residence and may program microbiome assemblage in infants.

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Supplementary Materials

Supplementary Table 1: Fold change in adherence levels of strain(s) versus *B. bifidum* R0071

	<i>B. bifidum</i> R0071	<i>B. infantis</i> R0033	<i>B. breve</i> M-16V	<i>B. infantis</i> M-63	4Bif
Control	1.00 ± 0.087 ^a	0.89 ± 0.169 ^a	0.62 ± 0.077 ^b	0.79 ± 0.166 ^b	1.15 ± 0.107 ^a
S-HMO	1.00 ± 0.106 ^a	0.89 ± 0.112 ^b	0.57 ± 0.118 ^c	0.76 ± 0.131 ^d	1.65 ± 0.252 ^e
2'-FL	1.00 ± 0.105 ^a	1.05 ± 0.092 ^a	0.66 ± 0.076 ^b	0.99 ± 0.132 ^a	1.31 ± 0.261 ^c

Fold change calculated as (percent adherence [vs original inoculum] of strain)/ (percent adherence [vs original inoculum] of *B. bifidum* R0071)
 Values within a row with common letter= p > 0.05, calculated using Tukey's multiple comparisons test

Supplementary Table 2: Species concentration and significant differences between strains within treatment groups following 16S qPCR analysis of adhered bifidobacterial mixture (4Bif)

x 10 ⁵ bifidobacteria/mL						
Treatment	<i>B. bifidum</i>		<i>B. breve</i>		<i>B. infantis</i>	
group ↓	Mean	StDev	Mean	StDev	Mean	StDev
Control	2.21 ^a	0.87	0.45 ^a	0.10	1.51 ^a	0.64
HMO	5.64 ^a	1.50	1.14 ^b	0.12	3.13 ^b	2.21
2'FL	2.74 ^a	0.30	0.68 ^a	0.07	1.82 ^a	0.64

Values within a row with common letter= p > 0.05, calculated using Tukey's multiple comparisons test

Supplementary Table 3: Bifidobacterial genomes matched against genes associated with probiotic survival and colonisation

Gene name	Protein encoded	Strain*	Ref	% identity**			
				<i>B. bifidum</i> R0071	<i>B. breve</i> M16V	<i>B. infantis</i> R0033	<i>B. infantis</i> M63
<i>bl0155</i>	Large transmembrane protein	<i>B. longum</i> VMKB44	(Shkoporov et al., 2008)	55.43	80.66	94.30	94.22
<i>bl0675</i>	Fimbrial subunit FimA	<i>B. longum</i> NCC2705	(Foroni et al., 2011)	43.95	26.99	27.90	0.00
<i>bopA</i>	Putative cell surface lipoprotein	<i>B. bifidum</i> PRL2010	(Guglielmetti et al., 2008)	98.82	46.09	43.83	44.01
<i>bsh</i>	Bile salt hydrolase	<i>B. longum</i> SBT2928	(Tanaka et al., 2000)	90.764	99.369	99.054	98.738
<i>copA</i>	Copper transporting atpase	<i>L. plantarum</i> NZ7109b	(Bron et al., 2007)	36.02	37.93	33.63	33.43
<i>ctr</i>	(Na ⁺) symporter	<i>B. longum</i> NCC2705	(Gueimonde et al., 2009)	0.00	92.118	94.842	94.842
<i>dltA</i>	D-alanine--D-alanyl carrier protein	<i>L. reuteri</i> 100- 23	(Walter et al., 2007)	0.00	23.35	32.24	23.62
<i>ebp</i>	Elastin-binding protein	<i>B. bifidum</i> PRL2010	(Turroni et al., 2013)	96.52	42.54	42.50	42.00
<i>ef-Tu</i>	Elongation factor	<i>L. johnsonii</i> NCC533	(Granato et al. 2004)	0.00	66.08	66.332	66.332
<i>esat-6</i>	ESAT-6-like protein	<i>B. bifidum</i> PRL2010	(Turroni et al., 2013)	100.00	83.16	86.46	86.46
<i>fimA</i>	Pil2 major pilin subunit	<i>B. bifidum</i> PRL2010	(Turroni et al., 2013)	99.46	30.00	31.17	0.00

Supplementary Table 3: Bifidobacterial genomes matched against genes associated with probiotic survival and colonisation

Gene name	Protein encoded	Strain*	Ref	% identity**			
				<i>B. bifidum</i> R0071	<i>B. breve</i> M16V	<i>B. infantis</i> R0033	<i>B. infantis</i> M63
<i>fimB</i>	Pil2 minor pilin subunit	<i>B. bifidum</i> PRL2010	(Turroni et al., 2013)	99.45	0.00	0.00	0.00
<i>fimM</i>	Major pilin subunit	<i>B. longum</i> BBMN68	(Xiong et al. 2020)	30.66	26.42	30.49	0.00
<i>fimP</i>	Pil3 major pilin subunit	<i>B. bifidum</i> PRL2010	(Turroni et al., 2013)	95.32	33.57	32.40	0.00
<i>fimQ</i>	Pil3 minor pilin subunit	<i>B. bifidum</i> PRL2010	(Turroni et al., 2013)	98.21	37.33	31.52	0.00
<i>glgA</i>	Glycogen synthase	<i>L. acidophilus</i> NCK1909	(Goh and Klaenhammer, 2014)	24.13	24.30	22.43	22.22
<i>luxS</i>	S-ribosylhomocysteine lyase	<i>B. breve</i> UCC2003	(Christiaen et al., 2014)	90.24	98.78	98.17	97.56
<i>msrB</i>	Peptide methionine sulfoxide reductase	<i>L. reuteri</i> 100- 23C	(Walter et al., 2005)	61.19	60.45	60.45	60.45
<i>nreB</i>	Two-component system, histidine kinase	<i>L. reuteri</i> 100- 23C	(Frese et al., 2011, Frese et al., 2013)	26.24	22.18	24.47	24.34
<i>rfbP</i>	Undecaprenyl-phosphate galactosephosphotransferase	<i>B. breve</i> UCC2003	(Fanning et al., 2012)	76.99	98.79	98.94	74.56
<i>secA2</i>	SecA2 translocase	<i>L. reuteri</i> 100- 23C	(Frese et al., 2011, Frese et al., 2013)	35.17	35.01	34.93	34.93
<i>secY</i>	Secy2, involved in secA2 transport system	<i>L. reuteri</i> 100- 23C	(Frese et al., 2011, Frese et al., 2013)	25.27	24.67	24.40	24.40

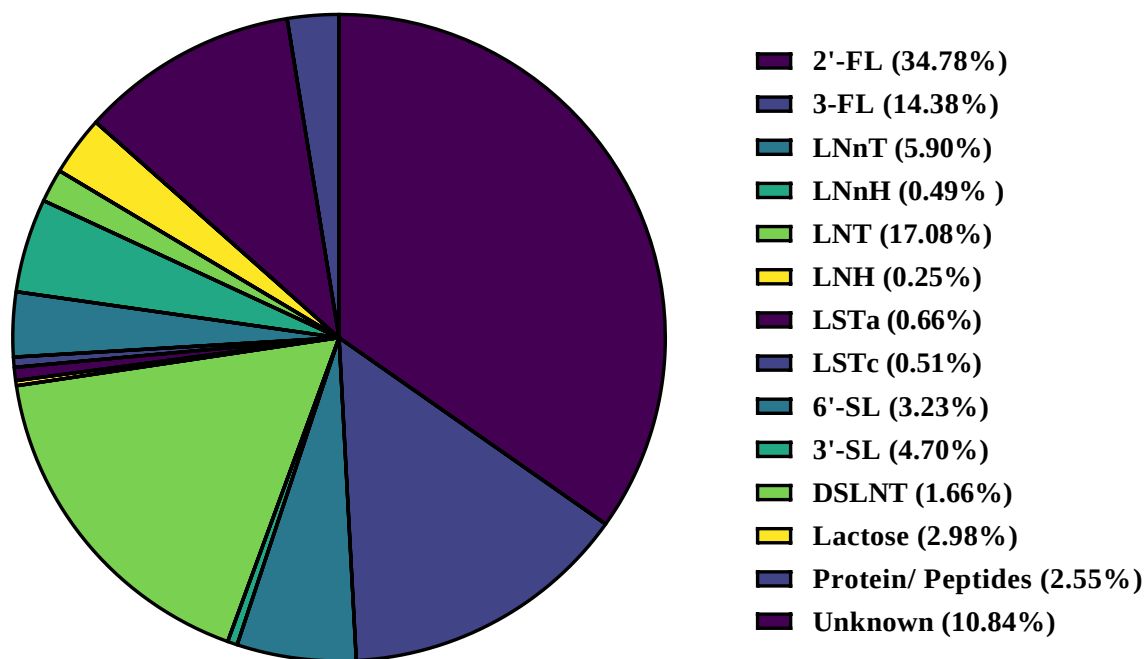
Supplementary Table 3: Bifidobacterial genomes matched against genes associated with probiotic survival and colonisation

Gene name	Protein encoded	Strain*	Ref	% identity**			
				<i>B. bifidum</i> R0071	<i>B. breve</i> M16V	<i>B. infantis</i> R0033	<i>B. infantis</i> M63
<i>spaCc</i>	Secreted LPXTG-like pilins	<i>L. rhamnosus</i> GG (LGG)	(Kankainen et al., 2009)	32.80	25.44	31.66	0.00
<i>srtA</i>	Pil3 sortase	<i>B. bifidum</i> PRL2010	(Turroni et al., 2013)	98.47	70.40	62.53	61.16
<i>srtA</i>	Sortase-like protein	<i>B. longum</i> NCC2705	(Foroni et al., 2011)	85.627	38.369	40	41.87
<i>srtE</i>	Sortase family protein	<i>B. bifidum</i> PRL2010	(Ishikawa et al., 2021)	99.27	67.05	58.02	58.96
<i>tadA</i>	Type II/ IV secretion system protein	<i>B. breve</i> UCC2003	(O'Connell Motherway et al., 2011)	66.97	99.41	73.25	73.25
<i>tadB</i>	TadB-like protein	<i>B. breve</i> UCC2003	(O'Connell Motherway et al., 2011)	42.466	98.649	67.808	67.123
<i>tadC</i>	Lipoprotein lipid attachment site	<i>B. breve</i> UCC2003	(O'Connell Motherway et al., 2011)	55.814	98.624	54.187	54.187
<i>tadE</i>	Pilus assembly protein TadE	<i>B. breve</i> UCC2003	(O'Connell Motherway et al., 2011)	39.13	100.00	0.00	0.00
<i>tadF</i>	Pilus assembly protein TadF	<i>B. breve</i> UCC2003	(O'Connell Motherway et al., 2011)	41.51	100.00	57.41	57.41
<i>tadV</i>	Type IV secretion peptidase	<i>B. breve</i> UCC2003	(O'Connell Motherway et al., 2011)	55.4	98.73	90.28	90.28
<i>tagB</i>	Teichoic acid biosynthesis protein	<i>B. bifidum</i> PRL2010	(Turroni et al., 2013)	99.52	0.00	0.00	0.00

Supplementary Table 3: Bifidobacterial genomes matched against genes associated with probiotic survival and colonisation

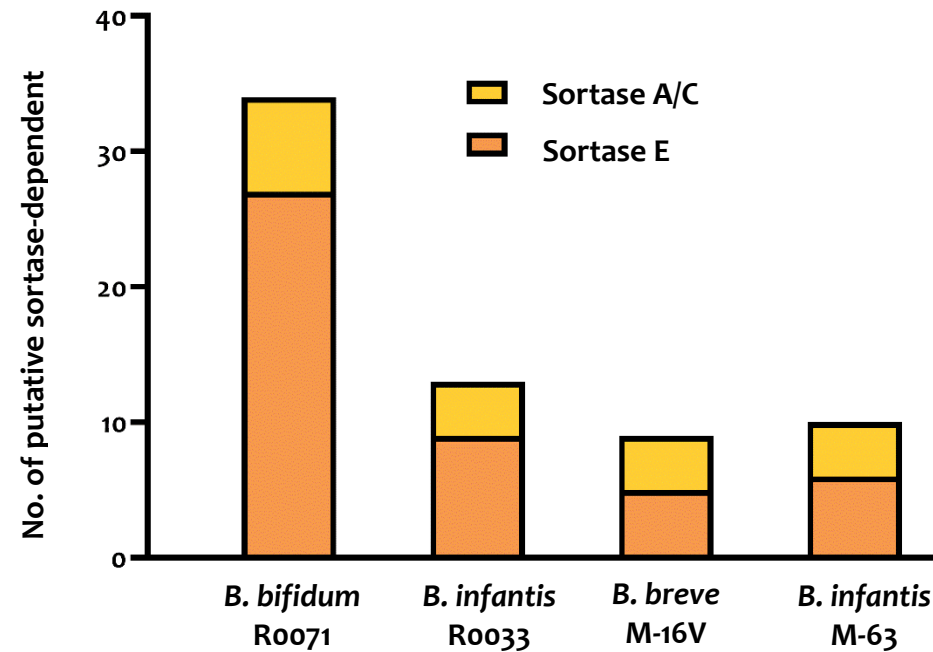
				% identity**			
Gene name	Protein encoded	Strain*	Ref	<i>B. bifidum</i> R0071	<i>B. breve</i> M16V	<i>B. infantis</i> R0033	<i>B. infantis</i> M63
<i>tal</i>	Aldolase	<i>B. bifidum</i> A8	(González-Rodríguez et al., 2012)	98.00	97.00	95.00	95.00
<i>tlyC</i>	Hemolysin-like protein	<i>B. longum</i> BBM68	(Liu et al., 2014)	69.231	89.474	95.833	98.958
<i>ureC</i>	Urease subunit α	<i>L. reuteri</i> 100-23C	(Wilson et al., 2014)	0.00	0.00	55.71	55.71

Supplementary Figure 1



Supplementary Figure 1: Pie chart showing the relative abundance of the major oligosaccharides in breastmilk-derived HMOs detected via HPAEC-PAD analysis by comparing to the following external HMO standards: lactose, 3-fucosyllactose (3-FL), 2'-fucosyllactose (2'-FL), lacto-*N*-neotetraose (LNnT), lacto-*N*-neohexaose (LNnH), lacto-*N*-tetraose (LNT), lacto-*N*-hexaose (LNH), sialyllacto-*N*-tetraose a (LSTa), sialyllacto-*N*-tetraose a (LSTc), 6'-sialyllactose (6'-SL), 3'-sialyllactose (3'-SL), disialyllacto-*N*-tetraose (DSLNT). Data represents average concentrations of technical duplicate data from biological triplicate experiments.

Supplementary Figure 2:



Supplementary Figure 2: Numbers of putative sortase A/C (yellow) and sortase E (orange)- dependent proteins harboured by bifidobacterial strains *Bifidobacterium bifidum* R0071, *Bifidobacterium infantis* R0033, *Bifidobacterium breve* M-16V, and *Bifidobacterium infantis* M-63. The putative sortase-dependent proteins were identified by surveying the bifidobacterial sequences for a pentaglycine recognition motif (LPXTG in the case of sortase A- and sortase C- dependent proteins and [L/I/V][S/A]XTG in the case of sortase E-dependent proteins), followed by a hydrophobic membrane-spanning region, and positively charged residues at the C-terminus.

Supplementary Table 4: Putative sortase-dependent proteins in *Bifidobacterium breve* M-16V

ID	MW	Predicted function	GH family	Recognition motif (Sortase)	C-terminal positive charge
BBRE1_0100	64599	Cell surface protein	-	LPKTG (SrtA/ SrtC)	RARRKA
BBRE1_0163	25922	Hypothetical protein	-	VAFTG (SrtE)	YRRRQA
BBRE1_0258	69783	Hypothetical secreted protein	-	LPHTG (SrtA/ SrtC)	SRWEER
BBRE1_0303	131207	VWA domain-containing protein	-	LPVTG (SrtA/ SrtC)	WRKRQL
BBRE1_0304	53188	CNA-domain protein	-	LPLTG(SrtA/ SrtC)	KRALDA
BBRE1_0618	48470	Permease protein of ABC transporter system	-	LAATG (SrtE)	DSRKKRKGV
BBRE1_1012	21606	Hypothetical membrane spanning protein	-	LAVTG (SrtE)	HPRTTR
BBRE1_1146	194331	Cell surface protein	-	LASTG (SrtE)	AGK
BBRE1_1442	22998	Permease protein of ABC transporter system	-	IAKTG (SrtE)	RRA
MW= molecular weight of translated protein					

Supplementary Table 5: Putative sortase-dependent proteins in *Bifidobacterium infantis* R0033

ID	MW	Predicted function	GH family	Recognition motif (Sortase)	C-terminal positive charge
BINF_0103	88585	Metallophosphoesterase	-	LSATG (SrtE)	KVVRRR
BINF_0154	9992	Hypothetical protein	-	VAATG (SrtE)	KVG
BINF_0256	67076	Histidine-type phosphatase	-	LARTG (SrtE)	VWRMRR
BINF_0270	70487	Cell-surface protein	-	LPVTG (SrtA/ SrtC)	VVRWRRRYDRFN
BINF_0334	66220	Hypothetical protein	-	LSNTG (SrtE)	AKH
BINF_0423	32833	Arabinogalactan endo- β -galactosidase	53	LSNTG (SrtE)	LRRKCS
BINF_0478	27122	Murein hydrolase transporter	-	LAVTG (SrtE)	RPRTTR
BINF_0479	21606	Hypothetical membrane spanning protein	-	IAPTG (SrtE)	LARRSI
BINF_1579	21080	Permease protein of ABC transporter system	-	LAATG (SrtE)	RSKARV
BINF_1866	124327	5'-nucleotidase	-	LPGTG (SrtA/ SrtC)	RRRQQR
*BINF_1976	54241	Fimbrial subunit	-	LPLTG (SrtA/ SrtC)	FAKSRSTKRALNA
*BINF_1977	85695	Fimbriae protein with VWA-domain	-	LPLTG (SrtA/ SrtC)	RKRQLV
BINF_2214	59826	Glycosyl hydrolase	unknown	LSATG (SrtE)	LSRKLS

Supplementary Table 6: Putative sortase-dependent proteins in *Bifidobacterium infantis* M-63

ID	MW	Predicted function	GH family	Recognition motif (Sortase)	C-terminal positive charge
BINF2_0166	83759	Penicillin-binding protein	-	ISSTG (SrtE)	NKR
BINF2_0259	66984	Hypothetical protein	-	LARTG (SrtE)	VWRMRR
BINF2_0279	70653	Cell surface protein	-	LPVTG (SrtA/ SrtC)	VVRWRRRYDRFN
BINF2_0437	32845	Arabinogalactan endo- β -galactosidase	53	LSNTG (SrtE)	LRRKCS
BINF2_1235	106050	Cell surface protein	-	LASTG (SrtE)	RNRRRA
BINF2_1248	14069	Cell surface protein	-	LPFTG (SrtA/ SrtC)	IRNRRRHGTRRR
BINF2_1268	100782	Hypothetical protein	-	LASTG (SrtE)	HVN
BINF2_1270	238421	Cell surface protein	-	LAKTG (SrtE)	RRRHAVTPRHGR
BINF2_2163	124368	5'-nucleotidase	-	LPGTG (SrtA/ SrtC)	RRRQQR
BINF2_2469	59826	Glycosyl hydrolase	unknown	LSATG (SrtE)	LSRKLS

MW= molecular weight of translated protein

Supplementary Table 7: Putative sortase-dependent proteins in *Bifidobacterium bifidum* R0071

ID	MW	Predicted function	GH family	Recognition motif (Sortase)	C-terminal positive charge
BBIF1_0014	13603	Cell wall anchoring protein	-	LSKTG (SrtE)	RKSGRF
BBIF1_0020	123042	α -L-arabinofuranosidase	62	LSHTG (SrtE)	FRRKRS
BBIF1_0222	23029	α -L-arabinofuranosidase	62	LSKTG (SrtE)	VARRRREPRR
BBIF1_0227	205512	1,2- α -L-fucosidase	95	VAKTG (SrtE)	RRKHSA
BBIF1_0267	17736	Hypothetical protein	-	VAKTG (SrtE)	VRKRATR
BBIF1_0297	206219	Endo- α -N-acetylgalactosaminidase	20	ISKTG (SrtE)	ARKRAE
BBIF1_0307	17635	α -L-arabinofuranosidase	62	LSATG (SrtE)	RRRRDI
*BBIF1_0313	126084	Cell surface protein FimQ	-	LPMTG (SrtA/ SrtC)	RKRRLV
*BBIF1_0314	55762	Fimbrial subunit FimP	-	LPKTG (SrtA/ SrtC)	YAKSRRTSRALR
BBIF1_0347	66830	Hypothetical protein	-	LSRTG (SrtE)	RPTSAR
BBIF1_0450	208523	Fibronectin type III	-	ISVTG (SrtE)	NRP
BBIF1_0483	188508	Hypothetical protein	-	LSKTG (SrtE)	VVRRRR
BBIF1_0505	206844	β -galactosidase	2	LSKTG (SrtE)	LRRKRS
BBIF1_0589	58936	Substrate-binding protein OppA	-	VAVTG (SrtE)	FRYASVTKS

Supplementary Table 7: Putative sortase-dependent proteins in *Bifidobacterium bifidum* R0071

ID	MW	Predicted function	GH family	Recognition motif (Sortase)	C-terminal positive charge
BBIF1_0822	28898	Transposase subunit B	-	LPSTG (SrtA/ SrtC)	SRI
BBIF1_0981	113219	β -N-acetylhexosaminidase	20	VAETG (SrtE)	VRRQRR
BBIF1_1173	99824	Hypothetical protein	-	LSKTG (SrtE)	RRKHAI
BBIF1_1299	88827	Lipoprotein	-	LAHTG (SrtE)	GRRLRISRE
BBIF1_1306	135311	Glycosyl hydrolase	unknown	IAATG (SrtE)	RKRRES
BBIF1_1367	158689	α -1-3,4-fucosidase	29	IAKTG (SrtE)	KRKSNR
BBIF1_1382	86609	Cation-transporting ATPase	-	VAMTG (SrtE)	QRAWLRSRR
BBIF1_1436	119514	Lacto-N-biosidase	20	LSATG (SrtE)	RRRSVR
BBIF1_1494	208333	β -N-hexosaminidase	20	LSKTG (SrtE)	ARSRRR
BBIF1_1496	167691	Calcineurin phosphoesterase	-	LSQTG (SrtE)	AVRTSKRADRIR
BBIF1_1499	72864	PTS system glucose-specific transporter	-	VAETG (SrtE)	IQR
BBIF1_1506	210337	Hyaluronidase	56	ISKTG (SrtE)	RRRREA
BBIF1_1522	176438	β -N-acetylhexosaminidase NagZ	20	VAKTG (SrtE)	RKRRI
BBIF1_1584	189580	Hypothetical protein	-	LSATG (SrtE)	AKR
BBIF1_1620	123721	NagC xylr-type transcriptional regulator	-	LSNTG (SrtE)	LRKRIG

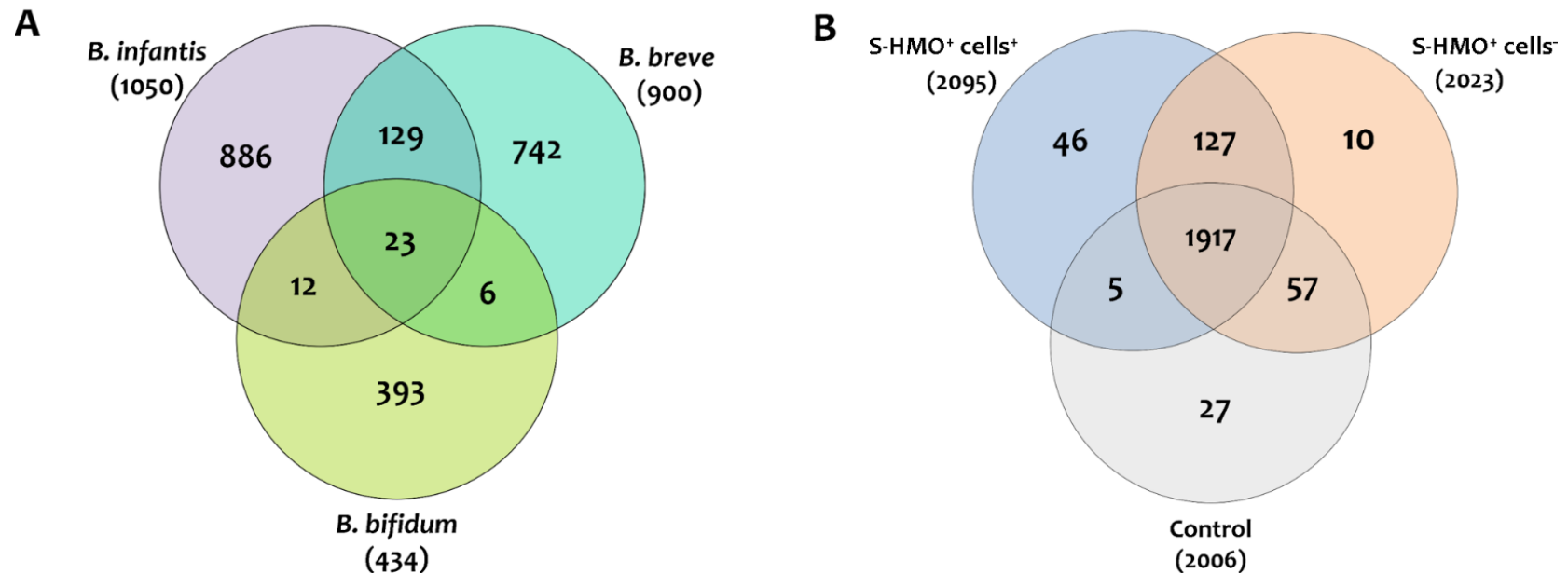
Supplementary Table 7: Putative sortase-dependent proteins in *Bifidobacterium bifidum* R0071

ID	MW	Predicted function	GH family	Recognition motif (Sortase)	C-terminal positive charge
BBIF1_1643	80721	Hypothetical protein	-	LSHTG (SrtE)	RRRSRR
*BBIF1_1693	57818	Fimbrial subunit FimA	-	LPLTG (SrtA/ SrtC)	KSRKASAIA
*BBIF1_1694	38976	Cell surface protein FimB	-	LPLTG (SrtA/ SrtC)	AKRSRA
BBIF1_1779	189091	Exo- α -sialidase	33	VAKTG (SrtE)	RRRANR
BBIF1_1780	87015	Exo- α -sialidase	33	LSKTG (SrtE)	RRRSVH
*BBIF1_1806	54908	Cna-protein B-type domain	-	LPGTG (SrtA/ SrtC)	LKRKNA
*BBIF1_1807	264701	Hypothetical protein	-	LPDTG (SrtA/ SrtC)	RSGSSRIRKRGHAS

MW= molecular weight of translated protein

*indicates protein associated with assemblage of fimbrial pili

Supplementary Figure 3



Supplementary Figure 3: Numbers of bifidobacterial proteins detected by LC-MS/MS analysis in four-strain bacterial mixture following i) treatment with HMO and incubation with cells (S-HMO⁺ Cells⁺), ii) incubation with cells without HMO-treatment (S-HMO⁻ Cells⁺), iii) untreated bifidobacteria without incubation with HT-29 cells (control). Venn diagram shows distribution of protein database based on **A)** strain and **B)** sample group (total numbers of proteins detected in each sample group/ for each strain shown in brackets)

Supplementary Table 8: Proteins matched to *B. bifidum* found at significantly higher abundance/ uniquely detected after exposure to HT29-MTX intestinal cells alone or in combination with S-HMO

Locus	Protein function	fold change vs control	
		S-HMO ⁺ cells ⁺	S-HMO ⁻ cells ⁺
BBIF1_1522	β - <i>N</i> -acetylhexosaminidase NagZ (GH20)	13.77	8.45
BBIF1_0227	12- α -L-fucosidase (GH95)	11.36	4.89
BBIF1_0442	DNA-directed RNA polymerase subunit β RpoB	10.99	5.23
BBIF1_1523	50S ribosomal protein L17	10.25	5.07
BBIF1_1524	DNA-directed RNA polymerase subunit α	9.99	5.35
BBIF1_1806	Cna-protein B-type domain	8.44	5.43
BBIF1_1752	Polyphosphate kinase	8.28	6.13
BBIF1_1379	Nox NADH oxidase H ₂ O-forming	8.12	3.42
BBIF1_1581	50S ribosomal protein L12	8.03	4.41
BBIF1_0505	β -galactosidase (GH2)	7.89	4.03
BBIF1_0274	PTS system <i>N</i> -acetylglucosamine-specific transporter subunit IIBC	7.78	4.85
BBIF1_1436	Lacto- <i>N</i> -biosidase (GH20)	7.59	3.74
BBIF1_1367	α -1-3,4-fucosidase (GH29)	7.59	4.07
BBIF1_0860	Glucose-1-phosphate adenylyltransferase glgc	7.27	4.59
BBIF1_0297	Endo- α - <i>N</i> -acetylglactosaminidase	7.20	2.72

Supplementary Table 8: Proteins matched to *B. bifidum* found at significantly higher abundance/ uniquely detected after exposure to HT29-MTX intestinal cells alone or in combination with S-HMO

Locus	Protein function	fold change vs control	
		S-HMO ⁺ cells ⁺	S-HMO ⁻ cells ⁺
BBIF1_1534	50S ribosomal protein L18	7.06	3.99
BBIF1_1611	Ribonucleoside-diphosphate reductase subunit α nrde	6.94	3.64
BBIF1_1535	50S ribosomal protein L6	6.90	3.93
BBIF1_1309	Phosphoribosylformylglycinamide synthase purl	6.46	3.27
BBIF1_1549	50S ribosomal protein L4	6.42	3.68
BBIF1_1544	30S ribosomal protein S3	6.42	3.57
BBIF1_0255	LSU ribosomal protein L11P	6.23	3.63
BBIF1_1547	50S ribosomal protein L2	6.23	3.46
BBIF1_0982	Threonyl-trna synthetase	6.05	3.34
BBIF1_1538	50S ribosomal protein L5	5.91	3.39
BBIF1_1127	Phenylalanyl-trna synthetase subunit β	5.85	3.78
BBIF1_1559	50S ribosomal protein L13	5.81	3.39
BBIF1_0356	30S ribosomal protein S16	5.81	3.61
BBIF1_0314	Fimbrial subunit FimP	5.76	3.71
BBIF1_1582	50S ribosomal protein L10	5.72	3.24
BBIF1_1013	UDP-glucose 4-epimerase gale	5.70	3.70
BBIF1_1301	Protein translation elongation factor G FusA	5.62	3.25

Supplementary Table 8: Proteins matched to *B. bifidum* found at significantly higher abundance/ uniquely detected after exposure to HT29-MTX intestinal cells alone or in combination with S-HMO

Locus	Protein function	fold change vs control	
		S-HMO ⁺ cells ⁺	S-HMO ⁻ cells ⁺
BBIF1_1198	Formate acetyltransferase	5.52	3.19
BBIF1_0682	Histidyl-trna synthetase	5.42	3.65
BBIF1_0615	Hypothetical protein	5.28	3.43
BBIF1_1545	50S ribosomal protein L22	5.21	2.96
BBIF1_1543	50S ribosomal protein L16	5.19	3.05
BBIF1_0199	Evolved β -galactosidase subunit α	5.14	3.04
BBIF1_1030	Protein translation elongation factor TS (Ef-Ts)	5.12	3.30
BBIF1_0313	Isopeptide protein S6	5.12	2.79
BBIF1_0838	SSU ribosomal protein S4P	5.11	3.10
BBIF1_0343	50S ribosomal protein L28	5.10	2.37
BBIF1_0560	L-lactate dehydrogenase	5.06	2.87
BBIF1_1506	Hyaluronidase	5.04	2.53
BBIF1_0582	Fructokinase	5.00	3.29
BBIF1_1531	50S ribosomal protein L15	5.00	2.74
BBIF1_1529	Adenylate kinase	4.95	3.21
BBIF1_1031	30S ribosomal protein S2P RpsB	4.90	3.09
BBIF1_1533	30S ribosomal protein S5	4.90	3.05

Supplementary Table 8: Proteins matched to *B. bifidum* found at significantly higher abundance/ uniquely detected after exposure to HT29-MTX intestinal cells alone or in combination with S-HMO

Locus	Protein function	fold change vs control	
		S-HMO ⁺ cells ⁺	S-HMO ⁻ cells ⁺
BBIF1_1021	Sugar ABC transporter substrate-binding protein	4.89	2.99
BBIF1_0483	Hypothetical protein	4.87	2.90
BBIF1_1300	Protein translation elongation factor Tu (Ef-Tu)	4.84	3.18
BBIF1_1715	Hypothetical protein	4.83	3.13
BBIF1_1001	Phosphoglycerate kinase	4.80	2.85
BBIF1_1610	Protein NrdI	4.80	1.57
BBIF1_0874	Branched-chain amino acid aminotransferase	4.79	2.80
BBIF1_0033	TrxB Thioredoxin reductase	4.76	3.16
BBIF1_1308	Phosphoribosylamidoimidazole-succinocarboxamide synthase	4.73	3.09
BBIF1_0966	GTP-binding protein	4.71	3.11
BBIF1_0804	Xylulose-5-phosphate Fructose-6-phosphate phosphoketolase	4.70	2.89
BBIF1_1194	Trigger factor PPIase	4.70	2.84
BBIF1_0853	CTP synthase PyrG	4.61	3.20
BBIF1_0002	Dnan DNA polymerase III β chain	4.57	3.64
BBIF1_1722	Phosphoenolpyruvate-protein phosphotransferase	4.56	3.01
BBIF1_0587	Glucosamine-6-phosphate isomerase NagB	4.56	2.74
BBIF1_0275	Homoserine dehydrogenase	4.55	2.85

Supplementary Table 8: Proteins matched to *B. bifidum* found at significantly higher abundance/ uniquely detected after exposure to HT29-MTX intestinal cells alone or in combination with S-HMO

Locus	Protein function	fold change vs control	
		S-HMO ⁺ cells ⁺	S-HMO ⁻ cells ⁺
BBIF1_1623	Type I multifunctional fatty acid synthase	4.52	2.80
BBIF1_0970	Phosphoribosylaminoimidazolecarboxamide formyltransferase	4.49	2.88
BBIF1_0027	Hypothetical protein	4.48	2.91
BBIF1_1634	ATP synthase subunit α	4.48	2.89
BBIF1_0589	Peptide ABC transporter substrate-binding protein OppA	4.46	0.88
BBIF1_0273	PTS system glucose-specific transporter subunit IIA	4.45	2.82
BBIF1_1652	Valyl-tRNA synthetase	4.38	3.11
BBIF1_1271	Protein translocase subunit SecA	4.36	2.81
BBIF1_0980	Hypothetical protein	4.32	3.01
BBIF1_1612	Ribonucleoside-diphosphate reductase β chain	4.28	1.74
BBIF1_1204	Peptidyl-prolyl cis-trans isomerase	4.26	2.20
BBIF1_0461	Membrane alanine aminopeptidase PepN	4.21	2.66
BBIF1_0855	ABC transporter ATP-binding protein	4.20	2.49
BBIF1_0991	Transketolase	4.19	3.10
BBIF1_1302	SSU ribosomal protein S7P	4.18	2.50
BBIF1_1294	Carbamoyl-phosphate synthase small chain	4.17	2.36
BBIF1_0776	Pyruvate kinase	4.17	2.87

Supplementary Table 8: Proteins matched to *B. bifidum* found at significantly higher abundance/ uniquely detected after exposure to HT29-MTX intestinal cells alone or in combination with S-HMO

Locus	Protein function	fold change vs control	
		S-HMO ⁺ cells ⁺	S-HMO ⁻ cells ⁺
BBIF1_0717	Glutamine synthetase GlnA	4.14	2.50
BBIF1_0681	Hypothetical protein	4.06	2.67
BBIF1_1673	Hypothetical protein	4.04	3.07
BBIF1_0017	NADP-specific glutamate dehydrogenase	4.04	2.36
BBIF1_0419	Phosphohydrolase	4.03	3.31
BBIF1_0032	Alkyl hydroperoxide reductase C22 protein	4.00	2.51
BBIF1_1809	ABC transporter ATP-binding protein	4.00	2.53
BBIF1_0778	Transcriptional regulator	4.00	3.04
BBIF1_1723	Phosphocarrier protein Hpr	3.98	2.63
BBIF1_0268	Lacto- <i>N</i> -biose phorylase	3.95	2.68
BBIF1_0379	Glucose-6-phosphate isomerase	3.93	2.58
BBIF1_1632	ATP synthase subunit β	3.92	2.89
BBIF1_0593	Xaa-pro aminopeptidase PepP	3.91	3.06
BBIF1_0961	Phosphoribosyl-ATP pyrophosphatase HisE	3.89	2.71
BBIF1_1296	Elongation factor P	3.87	2.65
BBIF1_0230	Formate--tetrahydrofolate ligase	3.87	2.93
BBIF1_0609	Glyceraldehyde 3-phosphate dehydrogenase Gap	3.84	2.70

Supplementary Table 8: Proteins matched to *B. bifidum* found at significantly higher abundance/ uniquely detected after exposure to HT29-MTX intestinal cells alone or in combination with S-HMO

Locus	Protein function	fold change vs control	
		S-HMO ⁺ cells ⁺	S-HMO ⁻ cells ⁺
BBIF1_0511	Hypothetical protein	3.82	2.54
BBIF1_0092	GlgP1 glycogen phosphorylase	3.79	2.82
BBIF1_1293	Carbamoyl-phosphate synthase large chain	3.79	1.86
BBIF1_1035	GMP reductase	3.78	2.59
BBIF1_1392	Prolyl-tRNA synthetase	3.77	2.47
BBIF1_1175	ABC transporter ATP-binding protein	3.76	3.42
BBIF1_1176	O-acetylhomoserine aminocarboxypropyltransferase	3.75	3.20
BBIF1_1470	Universal stress protein family	3.74	3.07
BBIF1_0890	Choloylglycine hydrolase	3.64	2.70
BBIF1_0647	Haloacid dehalogenase	3.63	2.22
BBIF1_1601	Aminopeptidase	3.62	2.88
BBIF1_0992	Transaldolase	3.57	2.65
BBIF1_1457	β -galactosidase (GH42)	3.56	1.11
BBIF1_1644	α -amylase	3.55	2.74
BBIF1_1478	Phosphoglycerate mutase	3.54	2.42
BBIF1_0726	Hypothetical protein	3.52	2.30
BBIF1_1077	Pyridoxamine 5-phosphate oxidase PdxH	3.52	2.65

Supplementary Table 8: Proteins matched to *B. bifidum* found at significantly higher abundance/ uniquely detected after exposure to HT29-MTX intestinal cells alone or in combination with S-HMO

Locus	Protein function	fold change vs control	
		S-HMO ⁺ cells ⁺	S-HMO ⁻ cells ⁺
BBIF1_1657	Transcription termination factor	3.51	2.38
BBIF1_0290	Aspartate aminotransferase	3.50	2.86
BBIF1_0632	Oligopeptide transport ATP-binding protein OppD	3.50	2.20
BBIF1_0633	Oligopeptide-binding protein OppA	3.49	2.33
BBIF1_1319	Phosphoribosylamine-glycine ligase	3.48	2.36
BBIF1_1398	Inosine-5-monophosphate dehydrogenase	3.46	2.39
BBIF1_1504	Phosphoglucomutase/phosphomannomutase	3.45	2.87
BBIF1_0655	DNA-binding protein HU	3.45	2.28
BBIF1_0240	Glutamyl-trna synthetase GltX	3.39	2.43
BBIF1_1625	Acetyl- propionyl-coa carboxylase subunit α AccC	3.37	2.66
BBIF1_0407	25-diketo-D-gluconic acid reductase	3.37	2.18
BBIF1_0416	Thioredoxin	3.34	2.21
BBIF1_1620	NagC xylr-type transcriptional regulator	3.33	2.71
BBIF1_1645	Inorganic pyrophosphatase	3.31	2.56
BBIF1_1488	6-phosphogluconate dehydrogenase	3.29	2.00
BBIF1_0271	Arginyl-tRNA synthetase	3.26	2.29
BBIF1_1812	Protein-disulfide isomerase	3.24	2.40

Supplementary Table 8: Proteins matched to *B. bifidum* found at significantly higher abundance/ uniquely detected after exposure to HT29-MTX intestinal cells alone or in combination with S-HMO

Locus	Protein function	fold change vs control	
		S-HMO ⁺ cells ⁺	S-HMO ⁻ cells ⁺
BBIF1_1086	Ribose-phosphate pyrophosphokinase	3.21	2.14
BBIF1_1508	Ribose 5-phosphate isomerase	3.15	2.17
BBIF1_1047	Leucyl-tRNA synthetase LeuS	3.14	1.85
BBIF1_0463	Phosphoglucosamine mutase	3.12	2.87
BBIF1_0588	<i>N</i> -acetylglucosamine-6-phosphate deacetylase NagA	3.10	2.32
BBIF1_0740	Enolase	3.08	2.15
BBIF1_0669	60 kDa chaperonin GroeL	3.06	3.10
BBIF1_1389	Zinc metalloprotease PepO	3.06	2.42
BBIF1_0036	Ppc Phosphoenolpyruvate carboxylase	3.01	2.46
BBIF1_0424	3-isopropylmalate dehydrogenase	3.00	1.83
BBIF1_1161	GTP-dependent nucleic acid-binding protein EngD	2.99	2.69
BBIF1_1635	ATP synthase delta chain	2.98	2.43
BBIF1_0229	Dipeptidase A PepD	2.96	2.28
BBIF1_0803	GMP synthase (glutamine-hydrolyzing)	2.96	2.24
BBIF1_1617	Polynucleotide phosphorylase polyadenylase	2.95	2.05
BBIF1_0400	Pyridoxine biosynthesis protein	2.86	1.84
BBIF1_0886	Glycosyltransferase	2.86	2.08

Supplementary Table 8: Proteins matched to *B. bifidum* found at significantly higher abundance/ uniquely detected after exposure to HT29-MTX intestinal cells alone or in combination with S-HMO

Locus	Protein function	fold change vs control	
		S-HMO ⁺ cells ⁺	S-HMO ⁻ cells ⁺
BBIF1_0506	Blycyl-tRNA synthetase	2.85	1.55
BBIF1_1602	Hypothetical protein	2.79	2.24
BBIF1_0417	Hypothetical protein	2.77	2.71
BBIF1_0434	Hypothetical protein	2.76	1.83
BBIF1_1790	Chromate reductase NADPH-dependent FMN reductase	2.74	2.08
BBIF1_1663	Aspartyl glutamyl-trna amidotransferase subunit A	2.74	1.87
BBIF1_0386	ABC transporter permease	2.73	2.90
BBIF1_1324	ABC transporter substrate-binding protein	2.70	2.52
BBIF1_1495	Seryl-trna synthetase	2.61	1.86
BBIF1_1571	10 kda chaperonin GROES	2.59	2.48
BBIF1_0962	Ribulose-phosphate 3-epimerase	2.57	1.76
BBIF1_1156	Hypothetical protein	2.54	1.94
BBIF1_0375	Nicotinate phosphoribosyltransferase	2.54	2.41
BBIF1_0721	Peptidase family M20A protein	2.51	1.71
BBIF1_1019	Sugar ABC transporter permease	2.49	1.18
BBIF1_1451	Lactaldehyde reductase	2.48	2.29
BBIF1_1532	50S ribosomal protein L30	2.48	2.21

Supplementary Table 8: Proteins matched to *B. bifidum* found at significantly higher abundance/ uniquely detected after exposure to HT29-MTX intestinal cells alone or in combination with S-HMO

Locus	Protein function	fold change vs control	
		S-HMO ⁺ cells ⁺	S-HMO ⁻ cells ⁺
BBIF1_1784	Chaperone protein DnaK	2.47	2.36
BBIF1_0657	Adenylosuccinate lyase	2.46	1.86
BBIF1_0431	Galactose-1-phosphate uridylyltransferase	2.45	1.59
BBIF1_0788	Acetate kinase	2.45	1.69
BBIF1_1348	S-ribosylhomocysteinase	2.44	1.95
BBIF1_0946	UTP-glucose-1-phosphate uridylyltransferase	2.42	1.86
BBIF1_1018	Lacto- <i>N</i> -biose phorylase	2.38	1.45
BBIF1_1476	Phosphate ABC transporter	2.28	1.74
BBIF1_0672	Two-component response regulator	2.18	1.79
BBIF1_1555	Aldehyde-alcohol dehydrogenase 2	2.16	1.13
BBIF1_1681	Cell division protein FtsY	2.12	1.72
BBIF1_0237	ABC transporter substrate-binding protein	2.07	1.63
BBIF1_0235	Hypothetical protein	2.04	1.28
BBIF1_0475	Exodeoxyribonuclease VII small subunit	2.02	1.19
BBIF1_0059	Dtdp-rhamnosyl transferase RfbF	2.01	1.49
BBIF1_1303	SSU ribosomal protein S12P	1.70	1.56
BBIF1_0020	α -L-arabinofuranosidase (GH43)	Uniquely detected	

Supplementary Table 8: Proteins matched to *B. bifidum* found at significantly higher abundance/ uniquely detected after exposure to HT29-MTX intestinal cells alone or in combination with S-HMO

Locus	Protein function	fold change vs control	
		S-HMO ⁺ cells ⁺	S-HMO ⁻ cells ⁺
BBIF1_0183	Hypothetical protein	Uniquely detected	
BBIF1_0196	Ketol-acid reductoisomerase 2-dehydropantoate 2-reductase	Uniquely detected	
BBIF1_0262	LacI family transcriptional regulator	Uniquely detected	
BBIF1_0284	Ribonuclease G	Uniquely detected	
BBIF1_0360	Signal recognition particle subunit FFH SRP54	Uniquely detected	
BBIF1_0368	Hypothetical protein	Uniquely detected	
BBIF1_0377	Xanthosine triphosphate pyrophosphatase	Uniquely detected	
BBIF1_0399	Long-chain-fatty-acid-CoA ligase	Uniquely detected	
BBIF1_0477	Anaerobic ribonucleoside-triphosphate reductase	Uniquely detected	
BBIF1_0565	D-3-phosphoglycerate dehydrogenase	Uniquely detected	
BBIF1_0585	Copper homeostasis protein	Uniquely detected	
BBIF1_0634	Aminopeptidase	Uniquely detected	
BBIF1_0643	Dihydroxy-acid dehydratase	Uniquely detected	
BBIF1_0645	S-adenosylmethionine synthetase	Uniquely detected	
BBIF1_0718	Hypothetical protein	Uniquely detected	
BBIF1_0731	2-C-methyl-D-erythritol 4-phosphate cytidyltransferase	Uniquely detected	
BBIF1_0843	Alanyl-tRNA synthetase	Uniquely detected	

Supplementary Table 8: Proteins matched to *B. bifidum* found at significantly higher abundance/ uniquely detected after exposure to HT29-MTX intestinal cells alone or in combination with S-HMO

Locus	Protein function	fold change vs control	
		S-HMO ⁺ cells ⁺	S-HMO ⁻ cells ⁺
BBIF1_0916	Aspartate carbamoyltransferase	Uniquely detected	
BBIF1_0918	Dihydroorotase PyrC	Uniquely detected	
BBIF1_0972	Succinyl-CoA synthetase subunit α	Uniquely detected	
BBIF1_0975	YajC Protein translocase subunit	Uniquely detected	
BBIF1_1015	<i>N</i> -acetylhexosamine-1-kinase NahK	Uniquely detected	
BBIF1_1128	Phenylalanyl-tRNA synthetase α chain	Uniquely detected	
BBIF1_1135	Uracil PyrP	Uniquely detected	
BBIF1_1272	Ribosome-associated factor Y	Uniquely detected	
BBIF1_1362	B-galactosidase	Uniquely detected	
BBIF1_1467	Dihydrofolate reductase	Uniquely detected	
BBIF1_1490	Oxppcycle protein OpcA	Uniquely detected	
BBIF1_0020	A-L-arabinofuranosidase (GH43)	Uniquely detected	
BBIF1_0115	Peptide methionine sulfoxide reductase MsrA MsrB	Uniquely detected	
BBIF1_0183	Hypothetical protein	Uniquely detected	
BBIF1_0196	Ketol-acid reductoisomerase 2-dehydropantoate 2-reductase	Uniquely detected	
BBIF1_0262	LacI family transcriptional regulator	Uniquely detected	
BBIF1_0284	Ribonuclease G	Uniquely detected	

Supplementary Table 8: Proteins matched to *B. bifidum* found at significantly higher abundance/ uniquely detected after exposure to HT29-MTX intestinal cells alone or in combination with S-HMO

Locus	Protein function	fold change vs control	
		S-HMO ⁺ cells ⁺	S-HMO ⁻ cells ⁺
BBIF1_0360	Signal recognition particle subunit FFH SRP54	Uniquely detected	
BBIF1_0368	Hypothetical protein	Uniquely detected	
BBIF1_0377	Xanthosine triphosphate pyrophosphatase	Uniquely detected	
BBIF1_0399	Long-chain-fatty-acid-CoA ligase	Uniquely detected	
BBIF1_0477	Anaerobic ribonucleoside-triphosphate reductase	Uniquely detected	
BBIF1_0565	D-3-phosphoglycerate dehydrogenase	Uniquely detected	
BBIF1_0585	Copper homeostasis protein	Uniquely detected	
BBIF1_0634	Aminopeptidase	Uniquely detected	
BBIF1_0643	Dihydroxy-acid dehydratase	Uniquely detected	
BBIF1_0645	S-adenosylmethionine synthetase	Uniquely detected	
BBIF1_0718	Hypothetical protein	Uniquely detected	
BBIF1_0731	2-C-methyl-D-erythritol 4-phosphate cytidyltransferase	Uniquely detected	
BBIF1_0843	Alanyl-tRNA synthetase	Uniquely detected	
BBIF1_0916	Aspartate carbamoyltransferase	Uniquely detected	
BBIF1_0918	Dihydroorotase PyrC	Uniquely detected	
BBIF1_0972	Succinyl-coa synthetase subunit α	Uniquely detected	
BBIF1_0975	YajC Protein translocase subunit	Uniquely detected	

Supplementary Table 8: Proteins matched to *B. bifidum* found at significantly higher abundance/ uniquely detected after exposure to HT29-MTX intestinal cells alone or in combination with S-HMO

Locus	Protein function	fold change vs control	
		S-HMO ⁺ cells ⁺	S-HMO ⁻ cells ⁺
BBIF1_1015	<i>N</i> -acetylhexosamine-1-kinase NahK	Uniquely detected	
BBIF1_1028	Uridylate kinase	Uniquely detected	
BBIF1_1128	Phenylalanyl-trna synthetase α chain	Uniquely detected	
BBIF1_1135	Uracil ByrP	Uniquely detected	
BBIF1_1272	Ribosome-associated factor Y	Uniquely detected	
BBIF1_1362	β -galactosidase	Uniquely detected	
BBIF1_1467	Dihydrofolate reductase	Uniquely detected	
BBIF1_1490	Oxppcycle protein OpcA	Uniquely detected	
BBIF1_0257	Peptidyl-prolyl cis-trans isomerase	Uniquely	Not detected
BBIF1_0260	Thioredoxin protein	Uniquely	Not detected
BBIF1_0299	Hypothetical protein	Uniquely	Not detected
BBIF1_0485	ATP-binding protein of ABC transporter system	Uniquely	Not detected
BBIF1_0577	UDP- <i>N</i> -acetylmuramate--alanine ligase MurC	Uniquely	Not detected
BBIF1_0752	Sensory transduction protein kinase	Uniquely	Not detected
BBIF1_0917	Sspartate carbamoyltransferase regulatory subunit	Uniquely	Not detected
BBIF1_0942	Haloacid dehalogenase	Uniquely	Not detected
BBIF1_0947	Hypothetical protein	Uniquely	Not detected

Supplementary Table 8: Proteins matched to *B. bifidum* found at significantly higher abundance/ uniquely detected after exposure to HT29-MTX intestinal cells alone or in combination with S-HMO

Locus	Protein function	fold change vs control	
		S-HMO ⁺ cells ⁺	S-HMO ⁻ cells ⁺
BBIF1_0972	Succinyl-CoA synthetase subunit alpha	Uniquely	Not detected
BBIF1_1002	Hypothetical protein	Uniquely	Not detected
BBIF1_1033	Long-chain-fatty-acid-CoA ligase	Uniquely	Not detected
BBIF1_1038	Long-chain-fatty-acid-CoA ligase	Uniquely	Not detected
BBIF1_1268	1-Acyl-sn-glycerol-3-phosphate acyltransferase	Uniquely	Not detected
BBIF1_1270	Anthranilate phosphoribosyltransferase	Uniquely	Not detected
BBIF1_1275	Recombinase A	Uniquely	Not detected
BBIF1_1307	Phosphoribosylglycinamide formyltransferase PurT	Uniquely	Not detected
BBIF1_1318	Phosphoribosylformylglycinamidine cyclo-ligase PurM	Uniquely	Not detected
BBIF1_1336	Phosphoribosylaminoimidazole carboxylase catalytic subunit	Uniquely	Not detected
BBIF1_1482	Tetratricopeptide tPR_2 repeat protein	Uniquely	Not detected
BBIF1_1756	Glycosyltransferase	Uniquely	Not detected
BBIF1_0115	Peptide methionine sulfoxide reductase MsrA MsrB	Not detected	Uniquely
BBIF1_0418	Hydrolase	Not detected	Uniquely
BBIF1_0524	Hypothetical protein	Not detected	Uniquely
BBIF1_0705	ATP-binding Mrp protein	Not detected	Uniquely
BBIF1_1028	Uridylate kinase	Not detected	Uniquely

Supplementary Table 8: Proteins matched to *B. bifidum* found at significantly higher abundance/ uniquely detected after exposure to HT29-MTX intestinal cells alone or in combination with S-HMO

Locus	Protein function	fold change vs control	
		S-HMO ⁺ cells ⁺	S-HMO ⁻ cells ⁺
BBIF1_1505	Pyridine nucleotide-disulfide oxidoreductase family protein	Not detected	Uniquely
BBIF1_1510	Hypothetical protein	Not detected	Uniquely
BBIF1_1539	50S ribosomal protein L24	Not detected	Uniquely
Protein abundance evaluated using label-free quantitative proteomic analysis			
Significant changes in protein abundance determined using Persus (v 1.6.2.2)			
Values marked in bold indicate proteins with fold change ≥ 2			

Supplementary Table 9: Proteins matched to *B. infantis* found at significantly higher abundance/ uniquely detected after exposure to HT29-MTX intestinal cells alone or in combination with S-HMO

Locus	Protein function	fold change vs control	
		S-HMO ⁺ cells ⁺	S-HMO ⁻ cells ⁺
BINF2_0342	Hypothetical protein	2.48	1.05
BINF2_0386	Riboflavin synthase β subunit	1.97	1.65
BINF2_0574	Leucine- isoleucine- valine- threonine- and alanine-binding	1.69	0.60
BINF2_0577	Branched-chain amino acid transport ATP-binding protein livG	1.79	0.69
BINF2_0876	Sugar kinase	25.54	2.74
BINF2_0878	Glucosamine-6-phosphate isomerase	2.73	0.72
BINF2_0879	<i>N</i> -acetylglucosamine-6-phosphate deacetylase	2.81	0.70
BINF2_1926	SSU ribosomal protein S12P	1.70	1.56
BINF2_2055	Hypothetical protein	2.76	1.83
BINF2_2172	Phosphotransferase	1.93	0.94
BINF2_2174	Sugar ABC transporter permease	2.49	1.18
BINF2_2225	30S ribosomal protein S17	8.40	6.37
BINF2_2242	50S ribosomal protein L13	2.47	1.29
BINF2_2305	Fucose transport protein	2.62	0.48
BINF2_2308	Short chain dehydrogenase	10.95	0.30
BINF2_2309	Mandelate racemase	21.49	1.49

Supplementary Table 9: Proteins matched to *B. infantis* found at significantly higher abundance/ uniquely detected after exposure to HT29-MTX intestinal cells alone or in combination with S-HMO

Locus	Protein function	fold change vs control	
		S-HMO ⁺ cells ⁺	S-HMO ⁻ cells ⁺
BINF2_2333	Galactoside transport protein	1.92	0.60
BINF2_2345	ABC transporter substrate binding component	4.68	0.72
BINF2_2346	ABC transporter permease	3.54	0.78
BINF2_2347	ABC transporter permease	5.91	0.92
BINF2_2348	ABC transporter substrate binding component	4.06	0.68
BINF2_2349	Sialidase	2.21	0.67
BINF2_2350	Dihydrodipicolinate synthase	2.16	0.70
BINF2_2351	ABC transporter substrate binding component	1.89	0.64
BINF2_2422	Aminopeptidase C	2.11	0.84
BINF2_0170	DNA polymerase III delta subunit	Uniquely detected	
BINF2_1693	Uracil-xanthine transport protein	Uniquely detected	
Protein abundance evaluated using label-free quantitative proteomic analysis,			
Significant changes in protein abundance determined using Persus (v 1.6.2.2).			
Values marked in bold indicate proteins with fold change ≥2			

Supplementary Table 10: Proteins matched to *B. breve* found at significantly higher abundance/ uniquely detected after exposure to HT29-MTX intestinal cells alone or in combination with S-HMO

Locus	Protein encoded	fold change vs control	
		S-HMO ⁺ cells ⁺	S-HMO ⁻ cells ⁺
BBREV_01643	30S ribosomal protein S17	8.40	6.37
BBREV_01523	α -N-acetylglucosaminophosphotransferase	3.23	2.49
BBREV_00420	Hypothetical protein	2.76	1.83
BBREV_00353	LSU ribosomal protein L19P	2.49	1.80
BBREV_01561	β -galactosidase	2.29	0.58
BBREV_00100	Cell surface protein	2.11	1.07
BBREV_00616	Hypothetical membrane spanning protein	1.92	1.26
BBREV_01250	<i>N</i> -acetylglucosamine-6-phosphate deacetylase	1.70	0.75
BBREV_01525	Branched-chain amino acid transport ATP-binding protein	1.65	0.61
BBREV_01251	Glucosamine-6-phosphate isomerase	1.48	0.62
BBREV_01529	Leucine- isoleucine- valine- threonine- and alanine-binding	1.43	0.65
BBREV_00988	Prolipoprotein diacylglyceryl transferase	Uniquely detected	
BBREV_00092	Transport protein	Uniquely detected	
Protein abundance evaluated using label-free quantitative proteomic analysis			
Significant changes in protein abundance determined using Persus (v 1.6.2.2)			
Values marked in bold indicate proteins with fold change ≥ 2			

General discussion and conclusions

1. Introduction:

One of the primary goals of HMO research and development is to narrow the compositional and functional gap between infant milk formulation and breast milk. While breastfeeding is widely regarded as the best option for infant nutrition due to its complex composition and immunological benefits (Martin et al., 2016), HMOs are one of the most promising ingredients in bringing infant milk formulation closer to replicating these benefits (Wiciński et al., 2020). The last decade has witnessed significant advancements in the use of HMOs in infant nutrition and beyond, driven by technological breakthroughs in HMO synthesis, establishment of regulatory framework for regulatory approvals for use as supplements and in foods, and a growing body of clinical evidence (Bode et al., 2016). Until recently, the inclusion of HMOs in commercial products was limited due to the challenges of producing them consistently at commercially relevant scale. Through the development of chemical synthesis, microbial metabolic engineering, and chemoenzymatic techniques, several HMO structures are now produced at industrial scale, with 2'-fucosyllactose (2'-FL) and lacto-*N*-neotetraose (LNnT) being among the most widely used (Vandenplas et al., 2018). The capacity to replicate these oligosaccharides has opened up new avenues for infant formula fortification and other nutraceutical applications. The inclusion of HMOs in infant formula is already making an impact, with products supplemented with 2'-FL and LNnT showing positive effects on the gut microbiota and immune development in infants supporting the growth of beneficial bacteria such as *Bifidobacterium* and reducing the incidence of gastrointestinal infections (Vandenplas et al., 2018). This brings the potential to reduce the health disparities between breastfed and formula-fed infants, particularly in low-

resource settings where breastfeeding may not be feasible. As HMO production continues to evolve, the inclusion of a wider variety of these bioactive compounds in infant nutrition could further narrow the gap between breast milk and formula (Dinleyici et al., 2023). Understanding the structure-function relationships of HMOs is essential for optimizing their application in infant growth and development, gut health, immune support, and potentially even broader therapeutic areas (Bode & Jantscher-Krenn, 2012; Chen & de Vos, 2024). This knowledge will drive the development of more targeted and effective nutritional supplements, probiotics, and medical interventions tailored to specific health outcomes.

The overarching goal of this thesis was to explore how HMOs and bifidobacteria interact in the infant gut and how these interactions can inform the development of improved infant formulas and probiotics, particularly for formula-fed infants who do not have access to breast milk's naturally occurring HMOs. The specific research aims were:

1. Identify structure-function relationships between HMOs and immune-modulatory effects.
2. Identify and compare the metabolic pathways used by different bifidobacterial strains to degrade and utilize HMOs using genomic and proteomic approaches.
3. Assess how cross-feeding and trophic interactions between multi-strain bifidobacterial consortia can influence HMO breakdown and metabolite production.

4. Evaluate whether HMO exposure enhances bifidobacterial adhesion and identify the adhesion-related proteins upregulated in response to HMO priming.
5. Translate findings into potential applications for infant nutrition.

These research aims were achieved through the development of advanced *in vitro* models, as well as combinations of glycomic, metabolomic, genomic, and proteomic approaches. The body of work described in this thesis focusses primarily on four commercial bifidobacterial strains: *Bifidobacterium bifidum* R0071, *Bifidobacterium infantis* R0033, *Bifidobacterium breve* M-16V, and *Bifidobacterium infantis* M-63. By focussing on these commercial strains, the studies presented in this thesis demonstrates how strain-specific differences in HMO metabolism can be leveraged to improve probiotic and synbiotic formulations. What sets this research apart is its multi-faceted approach to studying HMO function and metabolism through community driven studies while encompassing immune-modulatory effects (Chapter IV, aim 1), metabolic pathways (Chapters V, VI, aim 2 & 3), and bacterial colonization dynamics (Chapter VII, aim 4). The findings contribute significantly to the field by correlating specific structures of HMO with distinct biological functions.

2. Summary of Findings:

The literature reviews presented in this thesis provided foundational insights into the composition, synthesis and functionality of human milk oligosaccharides (HMOs) as well as their impact on the gut microbiota. Chapter I establishes the foundational role of HMOs in infant gut development and health, highlighting their functions as selective prebiotics

that promote the colonisation of beneficial bacteria, particularly bifidobacteria. In Chapter II, advancements in the characterization, synthesis, and commercialisation of HMOs are explored. These developments have overcome historical challenges of limited availability, enabling incorporation of HMOs such as 2'-fucosyllactose (2'-FL) and lacto-*N*-neotetraose (LNnT) into commercial infant formula. Chapter III focusses on the metabolic mechanisms by which bifidobacteria utilize HMOs, emphasizing significant species-specific adaptations. *Bifidobacterium infantis*, *Bifidobacterium bifidum*, and *Bifidobacterium breve* are highlighted for their distinct enzymatic profiles that enable selective and efficient HMO metabolism, leading to a stable and beneficial gut microbial ecosystem in infants. This is primarily facilitated by the wide array of glycosidases, transporters, and metabolic pathways that bifidobacteria possess, allowing these bacteria to effectively break down and utilize HMOs as energy sources (Arzamasov & Osterman, 2022; Sakanaka et al., 2020). Collectively, the literature reviews from these chapters underscore the critical biological significance of HMOs and bifidobacteria in shaping a healthy infant gut microbiota, highlight recent technological advances enabling their practical application, and illustrate the complexity and specificity of bifidobacterial HMO metabolism. These insights provide a strong rationale for continued research into tailored HMO formulations and bifidobacteria-based probiotics to enhance infant nutrition, particularly for formula-fed infants.

The overarching goal of this thesis was to explore interactions between human milk oligosaccharides (HMOs) and bifidobacteria within the infant gut, providing critical insights for the development of enhanced infant nutritional formulations. Aim 1 focused on identifying structure-function

relationships between HMOs and immune-modulatory effects. Chapter IV addressed this aim through the development of an advanced *in vitro* inflammatory model using intestinal epithelial cells. While previous studies have demonstrated that HMOs can modulate immune responses by influencing cytokine production and enhancing gut barrier integrity (Natividad et al., 2020; Xiao et al., 2019), this chapter provides new evidence on the structure-specific immune-modulatory effects of different HMO combinations. The results clearly demonstrated structure-specific immune-modulatory properties, revealing that combinations of HMOs, particularly 2'-FL and 6'-SL, significantly decreased pro-inflammatory cytokines (IL-8, TNF- α), in a concentration- and ratio- dependent manner. Importantly, it was established that more complex HMO blends did not necessarily yield superior immune outcomes compared to simpler combinations.

Aims 2 and 3 centred on exploring metabolic pathways and interactions among bifidobacterial strains. Chapters V and VI collectively address these aims by exploring the complex mechanisms by which bifidobacteria metabolize HMO, focusing on strain-specific pathways, glycosidase activities, and interspecies interactions. In Chapter V, the co-cultivation of four commercial infant-derived bifidobacterial strains—*Bifidobacterium bifidum* R0071, *Bifidobacterium breve* M-16V, *Bifidobacterium infantis* M-63, and *Bifidobacterium infantis* R0033—demonstrates how different strains interact in an HMO-enriched environment. The study shows that co-culture as opposed to mono-culture growth promotes increased overall bacterial numbers and enhances metabolite production, such as acetate and lactate, vital for maintaining a healthy gut environment (Parada Venegas et al., 2019; Tsukuda et al., 2021). The experimental design allowed

for validation of strain-specific preferences for different HMO structures and revealed cooperative cross-feeding mechanisms. Chapter VI built upon this, integrating genomic and proteomic analyses to provide detailed insights into strain-specific enzymatic pathways for HMO degradation, further confirming the critical role of trophic interactions among bifidobacteria in optimizing HMO utilization. The study confirms the genomic diversity of these strains, particularly in the genes responsible for glycosyl hydrolase production. While *B. bifidum* is well-equipped for extracellular degradation of complex HMOs, *B. infantis* strains possess a broad array of glycosidases and transporters for efficient HMO import and intracellular degradation. The proteomic data shows that cross-feeding relationships are critical for optimizing HMO metabolism in a co-culture setting, especially for strains such as *B. breve* M-16V, which benefits from the breakdown products released by *B. bifidum*.

Aim 4 evaluated the impact of HMO exposure on bifidobacterial adhesion, critical for gut colonization. To address this, Chapter VII employed adhesion assays and proteomic analyses, demonstrating that short-term HMO priming significantly enhances bifidobacterial adhesion to intestinal epithelial cells. The study investigates four infant-associated bifidobacterial strains—*Bifidobacterium bifidum* R0071, *Bifidobacterium infantis* R0033, *Bifidobacterium breve* M-16V, and *Bifidobacterium infantis* M-63—exploring how short-term exposure to HMOs enhances their adhesion. Notably, *B. bifidum* R0071 exhibited the strongest response, driven by the upregulation of adhesion-associated proteins such as sortase-dependent pili and glycosyl hydrolases, thus confirming the hypothesis that HMO exposure strengthens bacterial colonization capabilities.

Finally, aim 5 involved translating these findings into practical applications for infant nutrition. Collectively, these chapters present robust evidence that selective combinations of HMOs and specific bifidobacterial strains can significantly improve infant health and lay a strong foundation for formulating advanced probiotic and synbiotic infant nutritional products specifically tailored to emulate breast milk's protective benefits.

3. Impact of Findings:

This thesis set out to explore the structure-function relationships between HMOs and bifidobacteria and to elucidate how these interactions may be harnessed to inform the design of more effective infant nutritional strategies. The findings presented herein provide novel insights that extend current understanding and open new avenues for both scientific inquiry and practical application.

The identification of structure-dependent immunomodulatory effects of HMOs in Chapter IV has important implications for infant nutrition. The results of this study affirm existing theories that HMOs are not only prebiotic but also possess direct immunomodulatory properties. The findings suggest that HMOs have a concentration- and structure-dependent impact on immune modulation, with HMO combinations showing greater efficacy when compared to individual HMO components in reducing inflammation *in vitro*. These results align with, but extend beyond, previous studies that have shown how breast milk-derived HMOs modulate the immune system, reducing inflammation and supporting overall gut health (Rosa et al., 2022; Rousseaux et al., 2021). Contrary to the assumption that more complex blends confer greater benefits, the finding that simpler combinations (such as 2'-FL and 6'-

SL) were more effective in reducing inflammation presents a paradigm shift, emphasizing the need for targeted formulation based on functional synergy rather than compositional complexity alone. This study provides mechanistic evidence of the immunomodulatory effects of specific HMO combinations, thereby supporting their inclusion in infant formula, not just as prebiotics, but as functional immunomodulatory ingredients which may play a crucial role in reducing the risk of inflammatory diseases such as necrotizing enterocolitis (NEC) (Ayechu-Muruzabal et al., 2018; Donovan & Comstock, 2016).

Chapters V and VI addressed the strain-specific metabolic strategies employed by bifidobacteria to degrade HMOs. Findings from these studies confirm that HMO metabolism not only supports bacterial growth but also generates short-chain fatty acids (SCFAs) that lower gut pH, facilitating pathogen resistance (Fukuda et al., 2011). The integration of genomic and proteomic data provided robust evidence of the distinct glycosidase repertoires and transporter systems across *B. bifidum*, *B. breve*, and *B. infantis* strains. This functional specialization explains previously observed discrepancies in strain performance and offers a molecular rationale for the selection of specific strains in probiotic design. Notably, the cooperative metabolic relationships—whereby extracellular degraders such as *B. bifidum* release oligosaccharide fragments that support the growth of less metabolically versatile strains like *B. breve*—highlight the potential of multi-strain formulations. This cooperative HMO-sharing mechanism mirrors earlier work by Egan *et al.* and Gotoh *et al.* (Egan et al., 2014; Gotoh et al., 2018), but this study expands the understanding by detailing strain-level interactions and the selective preference for different HMO structures. Overall, results from Chapter V and VI support the hypothesis that strain-

specific genomic potential translates into unique metabolic phenotypes and reinforce the concept that trophic cooperation among bifidobacteria enhances the functional output of probiotic consortia. These studies emphasise the importance of incorporating probiotic scavenging partners that efficiently consume HMO-derived monosaccharides. Multi-strain probiotic blends which are designed to degrade HMOs and fully utilize the resulting monosaccharides can synergistically create a hostile environment for pathogens through acidification, SCFA production, and nutrient depletion. For example, combinations of *B. bifidum* R0071 and *B. breve* M-16V paired with human milk oligosaccharides such as 2'-FL and 6'-SL leverages the complementary metabolic capacities of these strains: *B. bifidum* R0071's ability to degrade complex HMOs into smaller components such as fucose and sialic acid, and *B. breve* M-16V's ability to utilize these degradation products for growth. *B. breve* M-16V has been associated with supporting immune development and reducing the risk of NEC in preterm infants. When combined with *B. bifidum* R0071, the synbiotic formulation could further enhance gut health and offer protection against NEC and potentially reduce the risk of chronic diseases later in life, such as allergies, obesity, and autoimmune disorders, by supporting immune regulation and microbial balance during critical periods of development. Studying the co-culture of *Bifidobacterium* and other bacterial genera such *Lactobacillus* during HMO utilization can provide a more realistic representation of microbial interactions in the infant gut.

Chapter VII of the thesis presents findings related to the ability of HMO-primed bifidobacteria to adhere to intestinal epithelial cells, with practical implications for improving colonization efficiency of probiotics.

This chapter builds on the understanding that bifidobacteria are some of the earliest gut colonizers in infants, primarily due to their unique capacity to interact with HMOs (Lordan et al., 2024; Ventura et al., 2012). The study shows that HMO treatment significantly increases adhesion, especially when strains are combined, and emphasizes the strain-specific variations in adhesive capacity. An unexpected yet valuable observation was the variability in adhesion potential between *B. infantis* R0033 and *B. infantis* M-63, reinforcing the importance of strain-level assessment when predicting colonization outcomes. One key finding is that *B. bifidum* R0071 displayed the strongest adhesion to intestinal cells, which was further enhanced when exposed to HMOs. The proteomic analysis in this study reveals that HMO exposure increased the abundance of colonization-related proteins, particularly sortase-dependent pili and glycosyl hydrolases (Ishikawa et al., 2021; Turrone et al., 2013). These proteins, essential for adhesion and nutrient acquisition, were particularly upregulated in *B. bifidum*, reinforcing its strong adhesive capacity. Overall, findings from Chapter VII provide a mechanistic explanation for the enhanced colonization observed in breastfed infants and offer a framework for designing probiotics that better persist in the infant gut. By selecting strains that exhibit complementary metabolic and adhesive capabilities, it may be possible to create more effective probiotic formulations that better mimic the natural colonization process observed in breastfed infants.

Overall, this research enhances our understanding of the multifaceted role HMOs play in infant health, showing that HMOs are not just passive dietary components but active modulators of microbiota and immune function, making them critical to both short-term infant development and

long-term health outcomes. Taken together, these findings provide a mechanistic blueprint for optimizing next-generation infant formulas that more closely mimic the functional benefits of human milk and synbiotic formulations that pair specific HMOs with complementary bifidobacterial strains. By targeting immune modulation, gut colonization, and microbial cooperation, these insights can inform more rational and efficacious formulation strategies. The evidence supports moving away from a “one-size-fits-all” approach and toward more personalized, functional nutrition models, potentially tailored to maternal secretor status or infant gut microbiota profiles. As research into the human microbiome advances, HMOs could play a key role in personalized nutrition. This would involve creating customized dietary plans or supplements that include specific HMOs tailored to an individual’s gut microbiome composition, health needs, and secretor status. Individual secretor status, which determines the presence of certain fucosylated HMOs in breast milk, provides a critical factor for customizing HMO or HMO/probiotic formulations. Non-secretor mothers, whose milk lacks specific HMOs like 2'-fucosyllactose (2'-FL), may have infants who would benefit from supplementation with these HMOs to support microbial colonization and immune function. Similarly, secretor-specific formulations could include HMOs that mimic those found naturally in their breast milk to complement and enhance the infant’s gut microbiome. Additionally, the location and regional dietary patterns of the population may also guide the design of HMO formulations. For instance, in regions with higher rates of malnutrition or infections, formulations could focus on HMOs with strong pathogen-blocking capabilities, while in regions with lower microbial diversity, formulations could target promoting the growth of diverse,

beneficial bacterial populations. Dietary and environmental factors unique to certain regions may influence the composition and function of the gut microbiota, necessitating the development of region-specific HMO products to optimize outcome.

Moreover, this thesis opens new avenues for research into the potential applications of HMOs beyond infant nutrition. The ability of HMOs to promote the growth of beneficial bacteria, modulate immune responses, and enhance gut barrier function suggests that they could be used as therapeutic agents in a variety of clinical settings, extending their use beyond infant into adult health, gut microbiome therapies, and even dermatological applications. HMOs may be developed as treatments for gastrointestinal diseases such as Crohn's disease, ulcerative colitis, and even non-alcoholic fatty liver disease (NAFLD). HMO formulations designed for adults and elderly individuals may help restore bifidobacterial levels, improve gut barrier integrity, and reduce inflammation, which is linked to aging-related conditions such as frailty, cognitive decline, and metabolic disorders. Including probiotics or prebiotic combinations, such as HMOs with bifidobacteria and *Lactobacillus* strains, could further enhance microbial diversity and resilience in this population, supporting overall gut and systemic health. Additionally, the prebiotic properties of HMO could be leveraged to manage dysbiosis-related conditions such as obesity and type 2 diabetes. Recent research suggests that HMOs could be used in dermatological applications to improve skin barrier function and support the microbiome of the skin. This could lead to the development of HMO-infused cosmetics and skincare products aimed at enhancing skin health. Exploring these broader

applications would expand the scope of HMO research and its impact on public health.

4. Limitations of the study

While this thesis contributes significantly to our understanding of human milk oligosaccharide (HMO)–bifidobacteria interactions and their implications for infant nutrition, several limitations should be considered when interpreting the findings and planning future research.

A primary limitation of this work lies in its reliance on *in vitro* models to simulate complex host-microbe and microbe-microbe interactions. The development of *in vitro* models such as the one described in the thesis offers several advantages, particularly when conducting mechanistic studies that would be challenging, invasive, or unethical to perform in humans. These models offer a non-invasive and controlled platform and are crucial for advancing our understanding of biological processes and enable high-throughput screening of different variables, such as different combinations of HMOs, under highly reproducible conditions (Silliman & Wang, 2006). These models serve as an essential first step for preclinical validation, allowing researchers to gather crucial data on safety, efficacy, and mechanisms before advancing to animal or human studies. While certain types of studies, such as those involving direct manipulation of gut microbiota, are challenging in humans due to their invasive nature (as evidenced by Zmora et al., 2018), *in vitro* systems provide a practical and ethical alternative. That being said, although advanced co-culture, transwell, and intestinal epithelial models were employed to mimic inflammatory and colonization conditions, these systems lack several components of the *in vivo*

environment, including immune cell heterogeneity, dynamic mucus layers, intestinal motility, and systemic host responses. As such, while *in vitro* models are essential for mechanistic insight, their predictive power for physiological relevance remains constrained. Without *in vivo* confirmation, it is difficult to assess how these observed effects—such as cytokine reduction or increased bacterial adhesion—translate into clinical outcomes such as reduced inflammation, improved colonization, or enhanced resistance to pathogens in infants. Future studies should validate these findings in clinical settings and examine the long-term effects of HMO and HMOBiotic supplementation on infant development.

One limitation of the study described in Chapter IV is that it focused on cytokine expression as the primary indicator of the immunomodulatory effects of HMOs. While providing vital insights into these effects, the study did not include downstream assessments of immune cell activation, epithelial permeability, or pathogen invasion. This limits the ability to draw conclusions about the full functional impact of the observed cytokine modulation. Future research should explore the specific pathways through which HMOs influence inflammation, including interactions with toll-like receptors (TLRs), inhibition of the NF- κ B and MAPK pathways, and modulation of inflammasome assembly and function (O'Sullivan et al., 2009; Peroval et al., 2013). Additionally, organoids could serve as a valuable tool for future studies assessing the immunological effects of HMOs. These advanced *in vitro* models, which closely mimic the structure and function of human intestinal tissue, provide a platform to investigate how HMOs interact with the immune system, including their potential to modulate epithelial-immune crosstalk, cytokine production, and immune cell activation. By bridging the

gap between traditional *in vitro* models and *in vivo* studies, organoids offer the opportunity to study these processes in a controlled yet physiologically relevant context.

Another limitation is that the thesis focuses, primarily, on four commercially available bifidobacterial strains and a selected panel of HMOs. While these strains are clinically relevant and the HMOs were chosen to represent major structures in human milk, this limited scope may not fully capture the metabolic and immunological diversity of the broader infant gut microbiome or HMO repertoire (over 200 structures are known). The co-culture studies focused solely on bifidobacterial strains, which, while central to infant gut colonization, do not account for the full microbial complexity of the infant gut. Other taxa, including *Lactobacillus*, *Bacteroides*, and *Enterococcus* species, interact with HMOs and can influence bifidobacterial activity through competition or synergism. The exclusion of these species limits the ecological realism of the fermentation and colonization models used. Additionally, the timeframes used in HMO priming and co-culture experiments were relatively short (typically ranging from 4 to 48 hours), which may not accurately reflect the dynamic and sustained interactions occurring in the infant gut over weeks or months. Bacterial adaptation, surface protein expression, and host cell responses are time-dependent processes, and it is unclear whether the observed enhancements in adhesion or immune modulation are transient or persistent. Understanding the longitudinal dynamics of bifidobacterial enrichment, cross-feeding, and succession in response to HMOs would be vital for predicting how synbiotic interventions may influence microbiota development throughout infancy. Host factors such as epithelial turnover, immune cell recruitment, and

mucosal secretions may influence outcomes differently. Furthermore, while key pathways were identified through proteomics, the temporal dynamics of protein expression and HMO metabolism over time remain to be explored. The absence of clinical data also limits the ability to assess long-term health outcomes, which will be essential in future studies.

Despite these limitations, the thesis delivers a coherent and multifaceted understanding of how HMOs interact with bifidobacteria in ways that are highly relevant for infant health and nutrition. These findings advance our understanding of functional prebiotics and pave the way for the design of microbiota-informed interventions aimed at closing the health gap between breastfed and formula-fed infants.

5. The Future of Formula: Industry Perspective

The trajectory of infant formula innovation is rapidly evolving, guided by growing scientific understanding of human milk composition and the critical role of early-life nutrition in shaping lifelong health. As this thesis has demonstrated, human milk oligosaccharides (HMOs) are a pivotal component of this evolution—serving not only as prebiotics but also as modulators of immunity, colonization enhancers, and protectants against pathogens. With commercial production now feasible, HMOs represent the cornerstone of the next generation of “humanised” formula. From a regulatory and public health standpoint, inclusion of clinically validated HMOs and probiotic strains in formula could reduce disease burden in vulnerable populations, close health equity gaps where breastfeeding is not possible, and reshape global standards in early life nutrition.

In the future, infant formula will no longer be developed around single nutrient supplementation but instead designed as complex bioactive systems that replicate the functional synergy found in breast milk. This includes:

- Expanded HMO libraries beyond 2'-FL and LNnT, tailored for specific immune or microbial outcomes.
- Strain-specific synbiotics, combining HMOs with select bifidobacteria to mimic mother-infant microbiota co-evolution.
- Personalized nutrition, where secretor status, birth mode, or regional microbiome data inform the formulation.
- Formulas with dynamic functions, supporting gut barrier maturation, allergy prevention, and inflammation regulation.
- Bioengineered components, such as glycoproteins, lactoferrin, or extracellular vesicles to restore more of breast milk's complexity.

The market for human milk oligosaccharides (HMOs) is expected to experience significant growth over the next decade, driven by increasing demand for “humanised” infant formula that more closely mimics the composition and benefits of human breast milk. As of 2023, the global HMO market is valued at approximately \$200-250 million. It is projected to grow at a compound annual growth rate (CAGR) of 13-20% over the next ten years, reaching an estimated \$1-1.5 billion by 2033 (Markets, 2024; Polaris, 2023). This rapid expansion is fuelled by increasing awareness of the health benefits of HMOs, regulatory approvals in densely populated markets for their use in infant milk formulation and other nutritional products, and technological

advancements that have reduced production costs, making HMO-enriched products more accessible (Bych et al., 2019; Salminen, 2017).

In conclusion, the future of infant formula lies in biologically-informed, precision-designed products—and the mechanistic insights presented in this thesis help lay the foundation for that transformation. While it is unlikely that infant formula will fully replicate the complexity of breast milk, the incorporation of HMOs has narrowed the gap significantly. As science and industry continue to converge, the next decade will see infant nutrition enter a new era: one defined not only by safety and nutrition, but also by biofunctionality, personalization, and preventative health.

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