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Evaluating Substrate Utilisation to Target Newly Identified Health-Promoting Gut Bacteria

Thesis presented by

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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 (except as declared, hereafter), and has not been submitted for another degree, either at University College Cork or elsewhere.

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Thesis Abstract

Promoting the growth and/or activity of potentially beneficial human gut microorganisms through the provision of specific substrates has been the subject of many studies over recent decades. Such substrates can include prebiotics, which promote growth in a targeted manner, but prebiotics can also be combined with other less target-specific nutrients to further enhance the growth of these targets. This field has continued to advance in recent years and has also expanded in response to the identification of new target species, such as *Akkermansia muciniphila, Eubacterium rectale, Faecalibacterium prausnitzii, Roseburia inulinivorans,* and *Ruminococcus bromii,* many of which produce metabolites that can contribute to host health. These developments have been in part due to improvements in culture-based techniques, advances in DNA sequencing-based approaches and improved computational pipelines. The combined use of these tools has enormous potential with respect to elucidating substrates that can be applied to specifically target microbes of interest and is the focus of this thesis.

Chapter 1 reviews the literature relating to prebiotics, and potential prebiotics, with a focus on more recently identified health-promoting gut bacteria. Building on this, in Chapter 2 we applied both *in silico* and *in vitro* techniques to predict and assess substrate utilisation for seven strains across five species of interest. The bioinformatics-based component of Chapter 2 is expanded in Chapter 3 through the analysis of publicly available microbial genomes of the same species of interest and, through the creation of metabolic models, predicting the substrates that these microorganisms could consume. Finally, in Chapter 4 we employ an *ex vivo* model to evaluate, through shotgun metagenomic sequencing, the impact different

substrates, including simple sugars, oligosaccharides, and whey protein concentrate had on the taxonomic composition and functional potential of a colonic microbiome. Thus, facilitating the design and testing of a functional prototype beverage comprised of some substrates assessed here.

Overall, this thesis explores different substrates that could be applied to target the growth and/or activity of recently identified health modulating microbes within the human gut. Combining *in silico*, *in vitro*, and *ex vivo* approaches have the potential to identify and assess a variety of substrates that may be applied to bring about microbiome-mediated enhancements of host health.

Thesis Publications

- **Cathy Lordan**, Dinesh Thapa, R. Paul Ross & Paul D. Cotter (2019) *Potential for enriching next-generation health-promoting gut bacteria through prebiotics and other dietary components*, Gut Microbes, 11:1, 1-20, DOI: 10.1080/19490976.2019.1613124

Other Publications

- Arghya Mukherjee, **Cathy Lordan**, R. Paul Ross & Paul D. Cotter (2020) *Gut microbes from the phylogenetically diverse genus Eubacterium and their various contributions to gut health*, Gut Microbes, 12:1, DOI: 10.1080/19490976.2020.1802866

List of Abbreviations

Abbreviation	Meaning
°C	Degrees Celsius
µm	Micrometre
AA	Auxiliary Activities
AGORA	Assembly of Gut Organisms Through Reconstruction and Analysis
alr	Additive Log Ratio
ANOSIM	Analysis of Similarities
AX	Arabinoxylan
AXOS	Arbinoxylan-oligosaccharide
BCAA	Branched Chain Amino Acid
BH	Benjamini-Hochberg
BiGG	Biochemical Genetic and Genomic
CAZyme	Carbohydrate-Active Enzyme
CBM	Carbohydrate-binding Module
CBM	Constraint-based Modelling
CDS	Coding Sequence
CE	Carbohydrate Esterase
CFU	Colony Forming Units
clr	Centered Log Ratio
COBRA	Constraints Based Reconstruction and Analysis
DHAP	Dihydroxyacetonephospate
DNA	Deoxyribonucleic Acid
DO	Dissolved Oxygen
FBA	Flux Balance Analysis
Fe	Iron
FOS	Fructo-oligosaccharide
FSI	Frozen Standardised Inoculum
FuA	Fucoidan from *Ascophyllum nodosum*
FuL	Fucoidan from *Laminaria japonica*
FVA	Flux Variability Analysis
g	Gram

GH	Glycoside Hydrolase
GlcNAc	N-Acetylglucosamine
GalNAc	N-Acetylgalactosamine
GIDEON	Global Infectious Disease and Epidemiology Online Network
GOS	Galacto-oligosaccharide
GT	Glycosyltransferase
GTDB	Genome Taxonomy Database
GTDB-tk	Genome Taxonomy Database Toolkit
h	Hour
HF	High Fat
HMO	Human Milk Oligosaccharide
IBD	Inflammatory Bowel Disease
IMO	Isomalto-oligosaccharide
IQR	Interquartile Range
ISAPP	International Scientific Association for Probiotics and Prebiotics
LB	Lysogeny Broth
LPOS	Lemon Peel Oligosaccharides
MAG	Metagenome Assembled Genome
Mb	Megabase
MDS	Multidimensional Scaling
MeDuSa	Multi-draft Based Scaffolder
MetaPhlAn3	Metagenomic Phylogenetic Analysis 3
MetS	Metabolic Syndrome
mg	Milligram
mL	Millilitre
mM	Millimolar
MNP	Micronutrient Powder
MOS	Mannan-oligosaccharide
NCBI	National Centre for Biotechnology Information
NF- κB	Nuclear Transcription Factor
nM	Nanomolar
nm	Nanometre
NS	Not Significant
OD	Optical Density

PATRIC	Pathosystems Resource Integration Centre
PBS	Phosphate Buffer Saline
PEP	Phosphoenolpyruvate
PL	Polysaccharide Lyase
POS	Pectin-oligosaccharide
psi	Pound-force per Square Inch
R^2	Coefficient of Determination
RAVEN	Reconstruction, Analysis and Visualization of Metabolic Networks
RS	Resistant Starch
SBML	Systems Biology Markup Language
SCFA	Short Chain Fatty Acid
sd	Standard Deviation
SPAdes	St. Petersburg Genome Assembler
SUPER-FOCUS	Subsystems Profile by Database Reduction using FOCUS
XOS	Xylo-oligosaccharide
v/v	Volume per Volume
VTGs	Virulence and Toxin Genes
w/v	Weight per Volume
WPC	Whey Protein Concentrate
YCFA	Yeast Casitone Fatty Acid
YCFAGSC	Yeast Casitone Fatty Acid Glucose Starch Cellobiose

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Chapter 1

Potential for enriching next generation health-promoting gut bacteria through prebiotics and other dietary components

Included as published in *Gut Microbes*

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Authors: Cathy Lordan, Dinesh Thapa, R. Paul Ross, Paul D. Cotter

Contributions:

- **Candidate** wrote the review, with guidance from **DT**, **RPR** and **PDC**.

1 Abstract

The human intestinal commensal microbiota and associated metabolic products have long been regarded as contributors to host health. As the identity and activities of the various members of this community have become clearer, newly identified health-associated bacteria, such as *Faecalibacterium prausnitzii, Akkermansia muciniphila, Ruminococcus bromii,* and *Roseburia* species, have emerged. Notably, the abundance of many of these bacteria is inversely correlated to several disease states. While technological and regulatory hurdles may limit the use of strains from these taxa as probiotics, it should be possible to utilise prebiotics and other dietary components to selectively enhance their growth *in situ*. Dietary components of potential relevance include well-established prebiotics, such as galacto-oligosaccharides, fructo-oligosaccharides, and inulin, while other putative prebiotics, such as other oligosaccharides, polyphenols, resistant starch, algae, and seaweed as well as host gut metabolites such as lactate and acetate, may also be applied with the aim of selectively and/or differentially affecting the beneficial bacterial community within the gastrointestinal environment. The present review provides an overview of the dietary components that could be applied in this manner.

1.1 Introduction

The human gut is estimated to contain approximately 10^{14} bacteria comprising more than 1000 species [1,2]. Intestinal commensal bacteria, as well as their metabolic products, have long been recognised as important contributors to host health, including nutrition/energy homeostasis, pathogen resistance, the regulation of intestinal epithelial proliferation and a variety of other factors [3]. The corollary is that negative impacts on microbiota composition and functionality can contribute to illness [4,5]. Importantly, the gut microbiota can be modulated in a beneficial way, including through the selective manipulation of particular species of interest to maintain, restore or improve host health.

One approach to positively modulate the gut microbiota is through the administration of growth enhancing substrates that can be utilised selectively by health-promoting bacteria, to encourage their growth and the production of associated desirable metabolites. The rationale of selectively enhancing beneficial microbes in the gut led to the concept of prebiotics, initially coined in 1995 by Roberfroid and Gibson [6] and described in greater detail below. Among the best characterised prebiotics are galacto-oligosaccharides (GOS) and inulin and its oligomer, fructo-oligosaccharides (FOS), as evidenced by numerous studies [7-9]. The vast majority of studies relating to these, and other prebiotics, have focussed on elucidating their effects on *Bifidobacterium* and *Lactobacillus* as strains from these genera have been known for some time to confer a health benefit to the host [10]. As our knowledge of the gastrointestinal microbiota expands, emphasis has now also been placed on the application of other bacteria present with possible health-

promoting effects and, in parallel, on the identification of prebiotics (targeted) and other dietary substrates (potentially less targeted) with the potential to stimulate the growth of these beneficial targets in the gut.

1.2 Newly identified health-associated microbes

Advances made in metagenomics and the application of emerging 'multiomics' technologies, in combination with cultivation techniques, have identified several putatively beneficial species of gut bacteria (Table 1). These microbes, including *Faecalibacterium prausnitzii, Ruminococcus bromii,* and *Akkermansia muciniphila*, can be present in significant numbers in a healthy human gut. A single species such as *F. prausnitzii* may comprise more than 5% of the total intestinal community [11], while there are also instances where up to 8% of the composition has been assigned to the phylum *Verrucomicrobia* [12], i.e., corresponding to *A. muciniphila* at the species level.

Some of these newly identified health-promoting bacteria are associated with various benefits to their respective hosts. One example of such an apparent relationship is the observation that the abundance of *F. prausnitzii* is inversely correlated with incidence of inflammatory bowel disease (IBD), in particular Crohn's disease [13-15]. As a result, *F. prausnitzii* has been investigated with respect to its potential to alleviate inflammation [15]. Another intriguing host-microbe interaction relates to that between *A. muciniphila* and obesity. Accumulating evidence has shown that the abundance of *A. muciniphila* is inversely proportional to body weight and type-1 diabetes [16,17]. *A. muciniphila* is specialised as it colonises the mucus lining and can use mucin as a sole carbon and nitrogen source, releasing growth substrates for other beneficial bacteria [18,19]. Interestingly, in a recent study, *Roseburia hominis* and *Roseburia intestinalis* have also been found to utilise mucin as energy source indicating that mucin degradation is more widespread than was

previously appreciated [20]**.** Mucin is a major component of the intestinal mucus layer. The ability to utilise mucin offers an ecological benefit to these bacteria over those that are dependent on dietary nutrients, providing a source of host-derived nutrients while the geographical location provides a means of interacting with the immune system [21]. Notably, *Christensenella minuta* and *Oscillospira* spp. have also been linked with a lean phenotype and may be useful targets when combating obesity [22-24]. The establishment of potential relationships such as these support the continued efforts in identifying approaches to encourage the growth of target microbes in the gut.

The breakdown and metabolism of complex carbohydrates, particularly non-digestive carbohydrates, by gut bacteria, hugely benefits primary degraders as well as the community which lack the ability to use the parent components. *R. bromii* has been suggested to be a key species with respect to the breakdown of resistant starch, with the resultant products being utilised as substrates for syntrophic growth by other beneficial microbes in the gut [25].

While in some instances the benefits attributed to specific gut microbes are thought to be direct, in other cases they can be indirect, such as via their metabolites. This can include the ability to utilise fibres and complex carbohydrates to produce short chain fatty acids (SCFAs) [13,16,25], the most abundant of which are acetate, propionate, and butyrate. SCFAs have numerous benefits including lowering the luminal pH, contributing to increased calcium and magnesium absorption, helping to reduce potential pathogenic bacteria, serving as an energy source for epithelial cells and also involvement in immune modulation. Butyrate, as mentioned previously, is

produced mainly by components of the major phylum, *Firmicutes*, such as *Clostridium* clusters XIVa and IV [26], and is particularly important as it is utilised as an energy source for colonocytes and has a proposed role in protection against diseases such as colon cancer and colitis [27-30]. It is therefore important that these health-promoting bacteria are present in sufficient numbers and are maintained within the gut.

Other species that are abundant in a healthy gut, including *Lachnospiraceae* such as *Anaerostipes caccae*, *Anaerostipes hadrus, Roseburia* spp., *Coprococcus catus, Eubacterium rectale,* and *Anaerobutyricum hallii/soehngenii* (formerly *Eubacterium hallii*), are acetate consumers and produce butyrate via butyryl-CoA:acetate CoA-transferase [31], which will be discussed in more detail later. There are a number of other examples of substrate cross-feeding that can have a substantial impact on the survival of certain bacteria and production of useful metabolites in the gut [32]. For instance, lactate is readily available in the gut as a result of the activity of lactic acid bacteria and can be utilised by microbes such as *Anaerostipes coli* and the species formerly known as *Eubacterium hallii* to produce SCFAs [33]. Similarly, *C. catus* and the ruminal bacterium *Megasphaera elsdenii* can convert lactate to propionate via the acrylate pathway [34]. The metabolic diversity and dependency of these health associated microbes is complex and, while our understanding of the pathways involved in carbohydrate fermentation and cross-feeding are improving (Figure 1), there is likely to be still much to learn.

Family	Bacteria	Beneficial Impact	Reference(s)
Christensenellaceae	*Christensenella minuta*	SCFA producer and possible link with a lean phenotype.	J.K Goodrich *et al*., 2014 [23]
Lachnospiraceae	*Eubacterium eligens*; re-classified to *Lachnospira eligens*	SCFA producer and pectin utiliser.	M. Lopez-Siles *et al*., 2012 [35]
Lachnospiraceae	*Eubacterium hallii*; Re-classified to *Anaerobutyricum hallii/soehngenii*	Produces pseudovitamin B12 which can help ↑ SCFA production by surrounding bacteria e.g., *A. muciniphila*, lactate utiliser, butyrate producer	C. Belzer *et al*., 2017 [36], S. Duncan *et al*., 2004 [37]
Lachnospiraceae	*Eubacterium rectale*	Acetate consumer, butyrate producer. Can be involved in cross-feeding interactions with other beneficial bacteria e.g., *Bifidobacterium longum*.	A. Rivière *et al*., 2015 [38], P. Louis *et al*., 2010 [39], P. Louis & H.J. Flint 2009 [40]
Lachnospiraceae	*Anaerostipes caccae* & *Anaerostipes hadrus*	Butyrate producers, lactate, and acetate utilisers.	S. Duncan *et al*., 2004 [37], E. Allen-Vercoe *et al*., 2012 [41]
Lachnospiraceae	*Coprococcus* spp.	Butyrate and acetate producers.	P. Louis & H.J. Flint, 2009 [40]
Lachnospiraceae	*Roseburia* spp.	Butyrate and propionate producers. Decreased levels seen in those with ulcerative colitis.	K. Machiels *et al*., 2014 [14], N. Reichardt *et al*., 2014 [34], S. Duncan *et al*., 2002 [42]

Family	Bacteria	Beneficial Impact	Reference(s)
Ruminococcaceae	*Faecalibacterium prausnitzii*	Decreased levels observed in Crohn's disease and ulcerative colitis indicating anti-inflammatory properties. One of the main butyrate producers within the gut.	M. Lopez-Siles *et al*., 2012 [35], A. Heinken *et al*., 2014 [43], S. Miquel *et al*., 2013 [13], K. Machiels *et al*., 2014 [14]
Ruminococcaceae	*Oscillospira* sp.	Enriched in those with a lean phenotype in comparison to obese subjects, decreased levels observed in those with inflammatory diseases.	T. Konikoff & U. Gophna, 2016 [24], J.K Goodrich *et al*., 2014 [23]
Ruminococcaceae	*Ruminococcus bromii*	Keystone species for degrading resistant starch enabling other bacteria to utilise the breakdown products.	X. Ze *et al*., 2012 [25], A. Venkataraman *et al*., 2016 [44]
Verrucomicrobiaceae	*Akkermansia muciniphila*	Mucin-degrading bacterium inversely associated with obesity and other metabolic diseases. Liberates oligosaccharides from mucin making them available to other bacteria. Produces acetate, which some butyrate-producers can utilise, and propionate.	PD. Cani & WM. de Vos, 2017 [16], A. Everard *et al*., 2013 [22], C. Belzer *et al*., 2017 [36], M. Schneeberger *et al*., 2015 [45]

Table 1.1 | Overview of some representative newly identified health-promoting bacteria and the associated benefits.

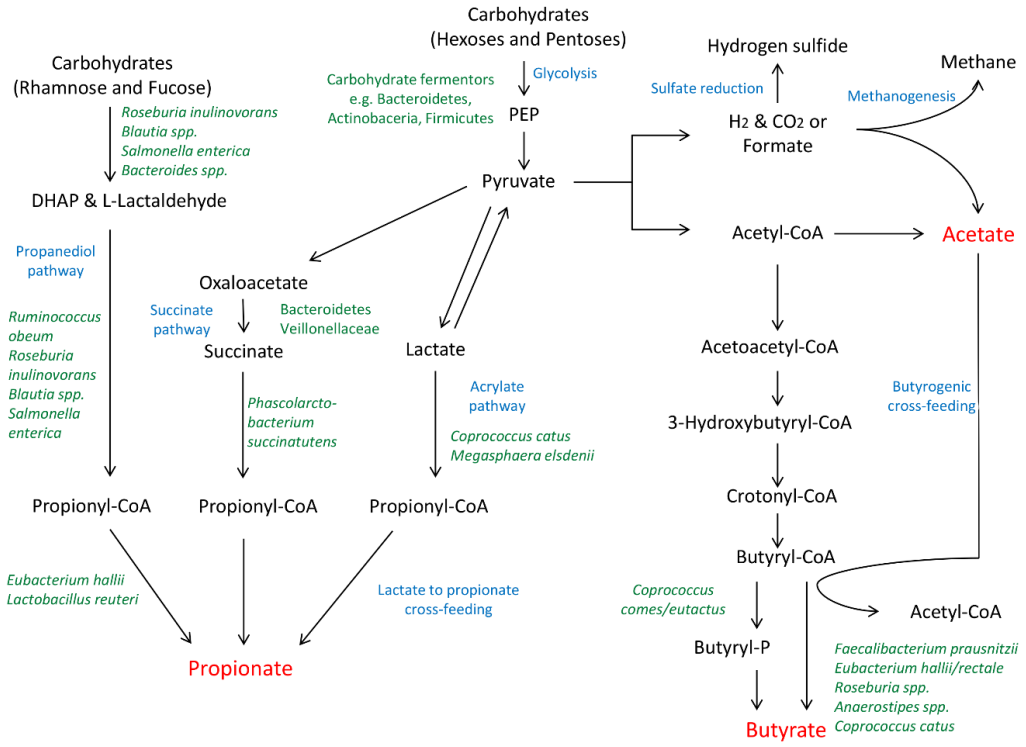

Figure 1.1 | Schematic representation of selected gut bacteria involved in carbohydrate fermentation and cross-feeding interactions resulting in the production of major microbial metabolites.

Pathways leading to the production of the three main SCFAs, acetate, butyrate, and propionate, are depicted here. Acetate can be produced from acetyl-CoA or by acetogens using H_2 and CO_2 or formate. It can also be used for the formation of butyrate. Butyrate can be formed in two ways; either through the formation of butyryl-P or more commonly through the use of butyryl-CoA:acetate CoA-transferase which many *Firmicutes* possess. The main route by which propionate is generated is via the succinate pathway. However, two other pathways have also been found, i.e., the acrylate pathway which involves lactate and the propanediol pathway which utilises deoxyhexose sugars. DHAP, dihydroxyacetonephospate; PEP, phosphoenolpyruvate. Adapted from Louis *et al.,* 2014 [31].

1.3 Prebiotic criteria and considerations

A large number of studies have been conducted to investigate a range of prebiotics and the effects that they have on the gut microbiota. With growing interest and knowledge, the definition of prebiotics has evolved and in December 2016, the International Scientific Association for Probiotics and Prebiotics (ISAPP) proposed that the definition be revised to 'a substrate that is selectively utilized by host microorganisms conferring a health benefit' [46]. This definition expands the concept to include non-carbohydrate substances. It also was suggested that the revised definition does not limit the selective stimulation of beneficial microorganisms to the gut if in the future prebiotic targets are beyond this environment. Currently, in order to be classified as a dietary prebiotic, three broad criteria need to be fulfilled. These include (i) resistance to gastric acid and hydrolysis by mammalian enzymes and gastrointestinal absorption; (ii) ability to be utilized by the intestinal microbiota; and (iii) selectively stimulate the growth and/or the activity of intestinal bacteria associated with health-promoting effects [47].

A major prerequisite to the success of a particular prebiotic is that the target bacteria must be present or present at a specific threshold. If levels are below a certain threshold, due to factors such as ageing or antibiotic usage, then the prebiotic may not be effective in increasing the numbers of these desirable bacteria to the necessary level in order to confer the anticipated health benefits [44]. Thus, having an understanding of such factors in target populations can enhance the likelihood of beneficial prebiotic-related outcomes. However, there are a number of other factors to consider. While prebiotics, and other beneficial food bioactives, can enable the

growth of targeted health-promoting bacteria, more detailed microbiome analysis has highlighted that these substrates are not always as specific as once thought [46-48]. It is also necessary to consider that the benefits of prebiotics can sometimes be secondary, e.g., production by health-promoting bacteria of metabolic products that can inhibit the growth of enteric pathogens and/or attenuate their virulence [49]. One can determine prebiotic impacts/specificity through the use of *ex vivo* fermentation studies, with faecal samples or mock gut microbial communities, or through *in vivo* studies. However, even then, care should be taken when interpreting outcomes as, due to inter-individual differences, the same effect may not be observed in all cases. It is also important to consider that some populations, when present at sufficiently high levels, may produce gases from prebiotic substrates that can result in bloating and abdominal discomfort [50,51]. Additionally, some prebiotics such as inulin-derived oligosaccharides can have mildly laxative effects [52]. All these considerations highlight the importance of understanding the interactions between prebiotics (as well as other dietary components), target microbes and the metabolites generated in order to determine ultimate resultant impacts on the host.

1.4 Targeting newly identified health-associated microbes through well-established prebiotics

There are a few extensively studied prebiotics, including FOS, GOS and inulin, which have been examined in detail using *in vitro* assays and *in vivo* animal models and human studies. These investigations have in particular highlighted the growth-promoting impact of prebiotics on members of the genera *Bifidobacterium* and

Lactobacillus [6,9,46,53], as a consequence of the health-associated status of many strains from these taxa. However, there are many ways in which prebiotics can positively impact the host (Figure 2). A direct benefit of prebiotics is enhancing the growth of target microorganisms which in turn compete with harmful species for energy sources and exclude them, providing protection or through facilitating the production of beneficial fermentation products, such as SCFAs.

Clinical trials and human studies are pivotal when assessing the benefits of newly identified prebiotics. Of the many studied potential prebiotics, only a few substrates, including inulin, FOS and GOS, have been validated through several human studies and only those that looked at a broader gut community, and thus are of greater relevance to this review, are presented in Table 2. These studies have highlighted that the extensively examined prebiotics, for example inulin, can also have a positive effect on the level of *F. prausnitzii* and *Anaerostipes* sp. within the gut, which may explain some of the butyrogenic effects that ensue when inulin is consumed [53-55]. Likewise, FOS and GOS have also been demonstrated to enhance *F. prausnitzii* levels [53,54,56]. Of the few human studies assessing the impact of prebiotics on microbial diversity in the colon, contradictory observations with regards to SCFA levels have been reported. A study by Liu *et al.* [57] noted a decrease in the levels of butyrate producers and increase in the levels of *Bifidobacterium* after administration of FOS and GOS in a healthy population, which may be caused by high levels of lactic acid being produced, therefore making the environment inhospitable for butyrate producers. It is also worth noting that this intervention was conducted for a period of 14 days; longer intervention studies are needed to better evaluate the effects of

prebiotic administration. Nevertheless, these results prove intriguing and emphasise the need for more human studies.

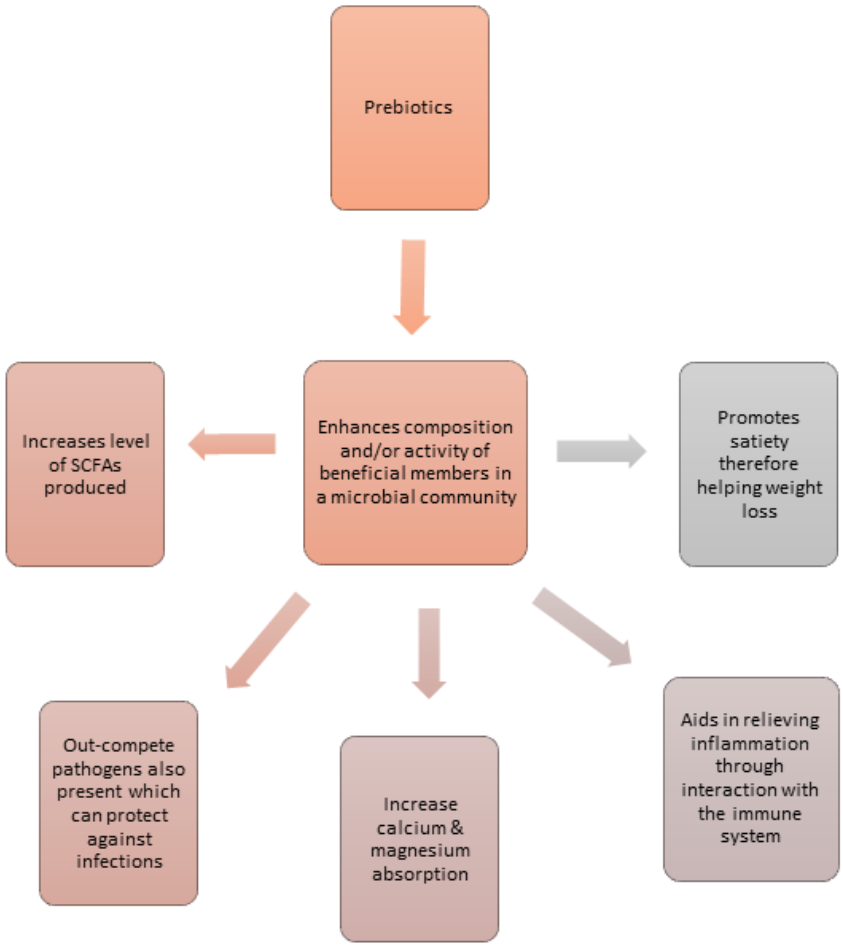

Figure 1.2 | An overview of some beneficial impacts of prebiotic supplementation on the gut microbiota.

Prebiotic Administered	Study Design	Cohort	Delivery	Effect on Microbiota	Reference
Extensively studied prebiotics					
FOS vs. GOS	A randomised, double-blind, cross-over study	35 healthy participants (10 males, 25 females)	16 g/day for 14 days	FOS: ↑ *Bifidobacterium; and* ↓ in *Phascolarctobacterium, Enterobacter, Turicibacter, Coprococcus* and *Salmonella* GOS: ↑ in *Bifidobacterium*; ↓in *Ruminococcus, Dehalobacterium, Synergistes* & *Holdemania*	F Liu *et al.,* 2017 [57]
GOS	A randomised, double-blind, parallel-group, multisite placebo-controlled study	62 lactose intolerant subjects	GOS or placebo was escalated in 5-day increments from 1.5g to 15g once a day. Taken for 35 days	In response to GOS administration ↑ in *Bifidobacterium, Faecalibacterium* & *Lactobacillus* was observed. Subsequent dairy consumption resulted in ↑ *Roseburia* levels	MA. Azcarte-Peril *et al.,* 2017 [56]
Inulin-oligofructose	Balanced cross-over study	12 healthy adults split into 2 groups (control and prebiotic)	10g prebiotic or control per day over a 16-day period	↑ *F. prausnitzii* across all volunteers & ↑ in different *Bifidobacterium* species dependent on individual as a result of prebiotics	C. Ramirez-Farias *et al.,* 2009 [54]
Inulin/oligofructose mix (50:50)	A double blind, placebo controlled, intervention study	30 obese women	16g prebiotic or control per day for 3 months	Prebiotics led to ↑ in bifidobacteria & *F. prausnitzii* while ↓ in *Bacteroides intestinalis, Propionibacterium* & *Bacteroides vulgatus* was observed	EM. Dewulf *et al*., 2013 [53]
Inulin-type fructans	A randomised, double-blind, placebo-controlled, cross-over trial	42 healthy adults	12g chicory derived Orafti inulin or control per day for 4 weeks	Inulin consumption led to ↑ *Bifidobacterium* & *Anaerostipes* abundances while *Bilophila* numbers ↓	D Vandeputte *et al.,* 2017 [55]

Prebiotic Administered	Study Design	Cohort	Delivery	Effect on Microbiota	Reference
Potential prebiotics					
Red wine polyphenols	A randomised, crossover-controlled intervention study	10 obese subjects with metabolic syndrome (MetS) and 10 healthy controls	Initial wash-out followed by two intervention periods where participants drank red wine (272 mL/day) or de-alcoholised red wine (272 mL/day) separated with a wash-out phase (15 days) in between cross-over	In healthy individuals ↑ levels in *F. prausnitzii* & *Roseburia* after red wine and de-alcoholised red wine consumption in comparison to baseline levels. MetS patients also had ↑ levels in these bacteria while also in *Blautia coccoides-E. rectale* group and *Lactobacillus.* Also, differences in microbiota between both groups were significantly ↓ after interventions	I. Moreno-Indias *et al.,* 2016 [58]
Red wine polyphenols	A randomised, crossover, controlled, intervention study	10 healthy males	After a 15-day wash-out period, each participant completed 3 consecutive 20 day periods in which they drank de-alcoholised red wine (272 mL/d), red wine (272 mL/d), or gin (100 mL/d)	Red wine polyphenols ↑ *Enterococcus, Prevotella, Bacteroides, Bifidobacterium, Bacteroides uniformis, Eggerthella lenta* & *Blautia coccoides – Eubacterium rectale* groups	M. I. Queipo-Ortuño *et al*., 2012 [59]
Cocoa flavanols	A randomised, double-blind, crossover, controlled intervention study	22 healthy volunteers	Subjects either consumed high cocoa flavanol (HCF – 494mg) or low cocoa flavanol (LCF – 29mg) drink per day for 4 wks followed by 4 wk washout period before switching to alternate drink	HCF ↑ *Bifidobacterium* & *Lactobacillus* levels while ↓ levels of *C. histolyticum* group. Both HCF & LCF led to slight ↑ in *E. rectale-C. coccoides* group but no significant differences between the two	X. Tzounis *et al.,* 2011 [60]

Prebiotic Administered	Study Design	Cohort	Delivery	Effect on Microbiota	Reference
Potential prebiotics					
XOS	A double blind, randomised, placebo-controlled study	32 healthy subjects	1.4g XOS, 2.8g XOS or placebo taken daily	Both XOS doses ↑ bifidobacteria, no change in lactobacilli, ↑ in *Faecalibacterium* sp. & *Akkermansia* sp. in those supplemented with the higher dose	S. Finegold *et al.,* 2014 [61]
Resistant starch (RS)	A randomised, crossover dietary study	39 subjects with reduced insulin sensitivity	Participants either consumed a high (HC) or low carbohydrate (LC) diet followed by a baseline diet. Then the HC subjects consumed either a high RS (HRS – 66g/day) or low RS (LRS – 4g/day). Subjects which consumed LC diet consumed either 48g for HRS or 3g for LRS	HRS led to ↑ in the ratio of *Firmicutes* to *Bacteroidetes* in comparison to LRS. In particular there were ↑ levels of *F. prausnitzii, Ruminococcus, Roseburia, E. rectale* & *A. muciniphila*	TV. Maier *et al.,* 2017 [62]
Resistant Starch (RS) type 2	A balanced study	20 healthy young adults (10 male & 10 female)	48g of potato starch (24g twice per day) for 7 days after a 3-day acclimatisation period	Individuals with high or enhanced levels of butyrate concentrations showed ↑ in *B. adolescentis* or *R. bromii* after RS consumption. In 5 individuals an ↑ in *E. rectale* was observed. Huge inter-individual variation was evident	A Venkataraman *et al.,* 2016 [44]

Table 1.2 | Human studies conducted in relation to prebiotics and newly identified health-promoting bacteria.

1.5 Targeting newly identified health-associated microbes through emerging prebiotics

The prebiotic paradigm has been shifting in recent years as a result of the identification of newly recognised putatively beneficial members of the gut microbiota to target for enrichment. These studies have been facilitated through new developments in cultivation techniques and, in particular, high-throughput sequencing, culture-independent approaches. These approaches have been used to assess the impact of specific fibres as well as polyphenols, other oligosaccharides and the less explored marine derived foods including seaweed-based products, which represents a relatively untapped source of food bioactives, on the gut microbiota of animals and humans.

1.5.1 Polyphenols

Polyphenols are naturally occurring secondary metabolites of plants that consist of a wide category of compounds and are classified primarily based on structure [63]. Plant-based foods and beverages, including fruits, vegetables, coffee, tea, and wine, are rich sources of dietary polyphenols. Consumption of plant foods is associated with lower risk of chronic diseases including cancer [64], heart diseases [65], type 2 diabetes [66] and other inflammatory diseases. A large number of these biological effects are attributed to phytochemical components of plant foods [67,68]. Moreover, it has been suggested that many of the beneficial impacts of these secondary metabolites on overall health is mediated through the manipulation of gut bacteria in the colon and their transformation therein by the gut bacteria present [69]. One such example is cranberry extract, which is rich in polyphenols, and has

been shown to ameliorate diet-induced obesity and several features of metabolic syndrome (MetS) in mice, while increasing the abundance of *A. muciniphila* [70]. Cranberry administration has also been shown to enhance mucus secretion, which could possibly create favourable conditions for *A. muciniphila* [71]. Similarly, the administration of Concord grape and California table grape extracts, rich in a class of polyphenol called proanthocyanidin, also increases the abundance of *Akkermansia* [72,73]. Other polyphenols present in red wine, tea and cocoa flavonols have also been investigated. Cocoa flavanols increase the levels of lactobacilli and bifidobacteria in addition to enhancing levels of the *Clostridium coccoides-Eubacterium rectale* group subtly [74]. From a red wine perspective, intake of 272 mL per day over a 30 day period altered the composition of the gut microbiota in patients with MetS and resulted in increased levels of *Bifidobacterium*, *Lactobacillus*, *F. prausnitzii* and *Roseburia* sp. [58].The study also suggests that the increase in *F. prausnitzii* levels was associated with the decrease of blood glucose levels in MetS patients possibly due to the production of SCFAs interacting with gut hormones which in turn may have an impact on carbohydrate metabolism [58]. In the same study, beneficial modulatory impacts in healthy volunteers were attributed to red wine polyphenols as levels of *Blautia coccoides – Eubacterium rectale* group, bifidobacteria, *Bacteroides uniformis* and *Eggerthella lenta* were enhanced. Consequently, there was a significant decrease in the levels of the potentially harmful *Clostridium histolyticum* group, which encompasses some pathogens such as *Clostridium perfringens*. Although the exact mechanisms involved are not fully understood, these *in vitro* and human studies form the basis of utilising certain polyphenols, alone or in combination, to alter the composition of the gut microbiota.

1.5.2 Oligosaccharides

In addition to the well-studied oligosaccharides, FOS and GOS, more recently there have been many studies focussing on the prebiotic effect of other oligosaccharides, such as xylo-oligosaccharides (XOS) and arabinoxylan (AX), pectin-oligosaccharides (POS), isomatlooligosaccharides (IMO), soybean oligosaccharides and human milk oligosaccharides (HMOs) [61, 79, 87, 88].

XOS is the product of the hydrolysis of xylan and can act as a substrate for gut bacteria, resulting in the production of SCFAs [75], with associated health benefits. The selectivity of XOS was noted in a study where 6 out of 11 *Firmicutes* examined, including *R. intestinalis*, *R. faecis, E. rectale* and *A. caccae*, were able to utilise this substrate, along with some *Bifidobacterium sp.* such as *B. adolescentis* [76]. A number of studies have highlighted that XOS can have a bifidogenic effect. In one instance, a human study evaluated the impact of XOS alone and XOS in conjunction with inulin. XOS alone increased faecal *Bifidobacterium* and butyrate, with a decrease in acetate and *p*-cresol and the combination of substrates appeared to reduce the inflammatory repercussions of a Western diet [77]. XOS supplementation was also determined to increase *Faecalibacterium* sp. and *Akkermansia* sp. as well as bifidobacteria but did not have a significant impact on levels of lactobacilli [61]. This study demonstrated that XOS promoted intestinal health through modulation of the microbial community; although there was no notable net increase in SCFA production detected, due consideration must be taken when examining dose effects.

Arabinoxylan (AX), a non-digestible carbohydrate often found in the cell walls of plants, can be selectively fermented in the colon by fibrolytic gut bacteria possessing

AX-degrading enzymes [78]. AX can be hydrolysed to arabinoxylan-oligosaccharides (AXOS). Both AX and AXOS have demonstrated an ability to increase desirable bacteria and butyrate producers in the colon [79-81]. Indeed, in one instance when a high-fat (HF) diet was administered to mice, the addition of AX subsequently increased levels of bifidobacteria [80]. AX also restored bacterial levels to that of the initial control level before HF diet induced obesity, in particular enhancing populations of the *Roseburia* species and *Bacteroides-Prevotella* species that were reduced upon HF feeding. These microbial shifts as a result of AX supplementation highlight its prebiotic potential.

Pectin and pectic oligosaccharides (POS) have been identified as emerging prebiotics as evidenced through their selective utilisation by certain members of the colonic microbiota such as *E. eligens,* now *Lachnospira eligens*, and *F. prausnitzii* [35,82]. The most frequent source of pectin is from citrus fruits and apple pulp, but it is also abundant in agricultural by-products such as sugar beet pulp. POS can be obtained through depolymerisation of pectin and both pectin and POS escape host digestion and reach the distal colon when consumed [83]. In an *in vitro* assay of *F. prausnitzii* growth in the presence of different substrates, it was ascertained that growth was enhanced with apple pectin in most cases [35,84]. Although pectin is extensively fermented in the gut, pectin utilisation has not been reported for many of the bacterial groups residing in the gut. It has been demonstrated that one of the main fermentation products from pectin utilisation by *F. prausnitzii* was butyrate. The ability of *F. prausnitzii* to compete for pectin when cultured with *B. thetaiotaomicron* and *E. eligens,* now *Lachnospira eligens*, was also noted [35]. Growth on some uronic

acids such as galactouronic acid was also observed in *F. prausnitzii*. This is important as galactouronic acid is a major component of pectin and it is not reported to be utilised by many other bacterial groups other than *Bacteroides* sp. [35]. The impact of sugar beet pulp and lemon peel wastes on gut microbial communities have also been assessed using *in vitro* fermentation assays [85]. Results suggested that POS of both lemon peel and sugar beet possessed better prebiotic potential than the corresponding pectin, and similar or even better than FOS (which was used as a control). Lemon peel waste oligosaccharides (LPOS) in particular resulted in an increased level of *Faecalibacterium* and *Roseburia* sp. as well as lactobacilli. Both LP and LPOS increased the level of *E. rectale*, although larger effects were observed with pectin. Ultimately, the study indicated that POS and pectin brought about a beneficial impact on the microbial population, presenting an intriguing opportunity for further investigation.

Isomaltooligosaccharides (IMO) are often considered potential prebiotics. Their impact on newly identified health beneficial microbes is limited and necessitates further investigation. IMOs are naturally found in foods such as honey as well as fermented foods such as miso and soy. In addition, various commercial preparations are made enzymatically by processing an assortment of starches and are readily available on the market today. Commercially available IMOs are composed of a mixture of α(1-6) and α(1-4) linked glucosyl oligosaccharides [86]. One of the glucose oligomers identified in IMOs is isomaltose, which is a major constituent of honey thereby giving IMOs a distinctive sweet honey taste. In a recent study evaluating the impact of co-supplementation of cranberry extract with IMO in HF diet fed mice,

Roseburia, *Faecalibacterium*, and *Eubacterium* were enhanced with an associated increase in butyrate [87].

Unsurprisingly, HMOs have also been the focus of much attention, with strong effects being observed on *Bifidobacterium* levels in particular [88]. Breast milk is the natural first nutritional source for new-borns and provides oligosaccharides that promote the growth of desirable *Bifidobacterium* sp. in the infant gut. HMOs and other nutrients in breast milk can act as substrates for bacteria in the gut, thereby stimulating the growth of beneficial bacteria located here. Two compounds in HMOs are 2'-*O*-fucosyllactose and lacto-*N*-neotetraose, which was the subject of one human study evaluating their putative prebiotic effect on the human gut microbiota in 100 healthy, adult volunteers [89]. This HMO supplementation was shown to modify the gut microbiota with a resulting increase in the relative abundance of Actinobacteria and, in particular *Bifidobacterium,* while reducing the relative abundance of Firmicutes and Proteobacteria. While there is much data available relating the impact of HMOs on the infant gut, and of *Bifidobacterium* strains found therein [90], their full effect on the overall infant gut microbial population, and resulting adult microbiota, is the subject of many on-going investigations.

Other oligosaccharides, including those from soybean and mannan, have putative prebiotic effects. Soybean oligosaccharides seem to have a positive influence on *Bifidobacterium* levels and lactate production [91]. Soybean forms a substantial part of diets in Asia and is now more frequently seen in Western diets, and benefits are attributed partly to fibre and the oligosaccharides present. Mannan-oligosaccharides (MOS) are commonly used as additives in animal feeds. MOS are not absorbed by the

animal so can reach the gut microbiota where they are utilised beneficial bacteria such as bifidobacteria and lactobacilli which can out-compete potential pathogenic bacteria. The effect on animal intestinal bacteria and mechanisms involved is just beginning to be evaluated [92]. Although their effect on human gut intestinal inhabitants has yet to be assessed, there may be considerable merit in doing so. Combinatorial benefits of various oligosaccharides are also beginning to be assessed, which may lead to the identification of optimal levels and combinations of prebiotics for the promotion of health-associated bacteria in the gut [93].

1.5.3 Resistant starch

Resistant starch (RS) is a form of starch that cannot be degraded in the small intestine and, thus, subsequently reaches the large intestine where it is fermented by the colonic bacteria. There are four types of RS (type 1-4), each with different properties that contribute to resisting digestion. RS1 is physically inaccessible due to its compact structure, RS2 has a granular structure, which prevents digestive enzymes from hydrolysing it, RS3 are retrograded starches and RS4 is modified with chemicals to enhance resistance. RS2 has been the focus of many investigations, both ex-vivo and in humans. Interestingly, in a co-culture experiment, *R. bromii* demonstrated a unique ability to stimulate RS degradation by other bacteria [25]. When cultured with *E. rectale, B. thetaiotaomicron,* and *B. adolescentis* on YCFA (yeast extract-casitone-fatty acid) medium, a semi-defined medium, utilisation of RS was enhanced in comparison to combinations without *R. bromii*. This is intriguing as it was determined that YCFA medium did not support the growth of *R. bromii* very well. This indicates that *R. bromii* has a superior ability to ferment RS and even possibly produce

metabolites that are available for cross-feeding interactions with co-inhabitants. Many studies have particularly emphasized the importance of *R. bromii* as a keystone species with regard to degrading RS within the human gut [25,44,94]. The *R. bromii* population substantially increased in faecal samples collected from those consuming a high RS diet [25].

Venkataraman *et al.* [44] demonstrated that RS2, when used to supplement the normal diet of healthy volunteers, increased faecal butyrate concentrations albeit with considerable inter-individual differences. This high amylose starch enhanced SCFA production, although those with low initial levels of SCFAs did not increase substantially, indicating that possibly the baseline level of required bacteria was too low for the full benefits of supplementation to be achieved. In those where an effect was evident, there was a substantial increase in RS-degrading bacteria such as *B. adolescentis* and *R. bromii* with some individuals displaying an additional enhanced level of *E. rectale.*

Similarly, in a study evaluating the effect of RS2 on subjects with insulin resistance using a multi-omics approach, a high-RS diet resulted in an increased Firmicutes to Bacteroidetes ratio, with a particular enhancement of *F. prausnitzii*, *R. bromii,* and *Roseburia* species [62]. The Firmicutes to Bacteroidetes ratio has been used, albeit somewhat controversially, as a marker of gut homeostasis with increased proportions of Bacteroidetes being proposed to reflect a beneficial modulation of gut microbiota. There is evidence to suggest that different types of RS may lead to distinct compositional modulations. In a study examining the effect of RS2 or RS4 on the composition of the faecal microbiota in 10 human subjects, in line with the

aforementioned studies RS2 enriched *B. adolescentis, R. bromii* and *E. rectale* [95]. On the other hand, RS4 increased the levels of *Parabacteroides distasonis* and *R. bromii* and decreased the levels of *E. rectale*. Both *R. bromii* and *E. rectale* were also enriched in faecal samples of obese human subjects during consumption of RS3 indicating some similarity to RS2 [96]. This highlights the importance of the chemical structure of RS in dictating its impact on the colonic bacteria. A recent dietary intervention study looked at the impact of two different RS, one from potatoes and the other from maize, as well as inulin on the gut microbiota of healthy volunteers [97]. Both types of RS were RS2 although the resulting changes in the gut microbiota differed with the RS from potatoes yielding a greater increase in SCFA production than either RS from maize or inulin. Again, the presence of *R. bromii* was significant. It was noted that if either *R. bromii* or *Clostridium charatabidum* were present after consuming RS it was more likely for butyrate to be produced, especially when the microbial population included the butyrate-producer *E. rectale.* This indicates that there may be primary degraders of RS such as *R. bromii* and the breakdown products may subsequently be utilised by certain butyrate-producers. *Bifidobacterium* was also enhanced in most of the gut microbiotas after consuming any of the three fermentable fibres. The resulting effects of RS degradation on the microbial population has yet to be fully explored, however, it is evident that *R. bromii* has an integral role in initiating the breakdown of this resistant but fermentable starch. This selective metabolism is the driving force behind the ever-increasing interest in the use of RS as a prebiotic ingredient in functional foods [98]. Indeed, several commercialised products containing RS are already available on the market and there has been a continuing effort to incorporate the RS as a functional food component.

1.5.4 Algae and seaweed

The marine environment is often considered a vast, untapped reservoir with regards to the microorganisms and compounds present, the metabolites produced and interactions that occur in this ecosystem. New bioactives and potential prebiotic substrates are being harnessed from this rich environment. Seaweeds, also known as marine macroalgae, are rich in polysaccharides and bioactive compounds, which could be applied to improve human and animal health [99,100]. Seaweeds can be subdivided into three categories based on their pigmentation: Rhodophyta (red seaweed), Chlorophyta (green seaweed) and Phaeophyta (brown seaweed) [99]. Differences in composition and structure of the polysaccharides present in these seaweeds are also evident. Brown seaweed is of particular interest as it possesses polysaccharides such as fucoidan, laminarin and alginate [101]. Fucoidans comprise a class of fucose-rich sulfated polysaccharides often located in the cell walls of brown macroalgae. It is suggested that fucoidans with differing structures may impact the gut in a variety of ways. For example, a study carried out by Shang *et al.,* [102] established that fucoidans isolated from *Ascophyllum nodosum* (FuA) and *Laminaria japonica* (FuL) had positive but slightly differing effects on the murine gut microbiota due to their structural differences. FuA has a type I back-bone structure whereas FuL has a type II structure. FuL markedly increased the levels of *Ruminococcaceae* while FuA enhanced *Lactobacillus, Anaeroplasma* and *Thalassospira*. In a follow-up study on HF diet-fed mice, these fucoidans improved MetS induced by a HF diet [103]. Dietary supplementation with fucoidan decreased body weight in HF diet-fed mice and also improved glucose intolerance and insulin resistance. Both fucoidans separately ameliorated intestinal dysbiosis caused by HF diet and significantly

increased *Akkermansia* abundance. An evolving body of evidence has linked *A. muciniphila* with a lean phenotype and often levels are lower in those with obesity and its related metabolic alterations. Additionally, SCFA producers such as *Blautia* and *Alloprevotella* were increased. This contributes to the improvement of the health status of the gut microbial community. Remarkably there did not seem to be any major differences between FuL and FuA in this instance as both influenced the gut bacterial community correspondingly. Thus, ingredients from marine resources, particularly fucoidan, have potential as mediators of gut microbes.

1.6 Other components influencing growth

1.6.1 Gut metabolites as modulators

Apart from dietary and environmental factors, the host-derived as well as microbial-derived metabolites in the gut can act as modulators of the gut ecosystem. Lactate is one of the many studied metabolites. Interest in lactate as a substrate to impact on the gut microbiota has increased in recent years due to the fact that some lactate-utilising bacteria have the ability to produce butyrate [37]. Notably, *E. hallii,* now *Anaerobutyricum hallii/ soehngenii,* and *A. caccae* have demonstrated an ability to use both D- and L-lactate [37]. Evidence of lactate being utilised in cross-feeding studies is particularly intriguing as it may explain the butyrogenic effect of certain dietary bioactives, including resistant starch [37,104]. Indeed, *Bifidobacterium* and *Bacteroides,* which are known degraders of RS, do not produce butyrate. It is hypothesised that these active starch degraders may produce lactate initially and that, subsequently, lactate-utilisers such as *E. hallii (Anerobutyricum hallii/soehngenii)* and *A. caccae* may produce butyrate using lactate as a substrate

[37]. The synergistic effect of this cross-feeding mechanism highlights the intricate and elaborate relationships of co-inhabitants within the gut. Interestingly, synbiotic administration of *A. caccae* and GOS improved beneficial organic acid production in comparison with GOS alone [105]. Also, in a separate study, *Roseburia* sp. strain A2-183 was unable to consume lactate or grow on potato starch or FOS in pure culture, but when co-cultured with *B. adolescentis* L2-32 in the presence of starch or FOS, the bacterium produced butyrate [104].

As noted earlier, acetate is the most abundant SCFA in the colon and while it is normally an end-product of anaerobic fermentation, it can also be utilised by some butyrate-producing species within the gut [106]. For example, *F. prausnitzii,* one of the most abundant bacteria in the colon, is also a prominent acetate-consumer [37] and grows poorly on media deficient in acetate [107]. Along with *F. prausnitzii*, *R. intestinalis* and *E. rectale* are also known acetate consumers [106,108]. Although they are unable to use acetate as a sole source of energy, they do show net utilisation of acetate possibly to produce butyrate via acetyl-CoA formation. Strains of *F. prausnitzii* and *Roseburia* sp. possess butyryl-CoA:acetate-CoA transferase which is involved in catalysing butyryl-CoA to butyrate [106]. This reaction may also be carried out by butyrate kinase instead of the transferase [109]. Other members of the intestinal microbiota may also play a role in making acetate available for utilisation by other microbes present in the gut. *A. muciniphila* has the ability to degrade and utilise host mucus and consequently can produce 1,2-propanediol, acetate and propionate, thereby stimulating the nearby butyrate producers as a result of this specialised activity within this niche environment [36]. Barcenilla *et al.,* working with

human faecal samples, demonstrated that 95% of strains isolated that were net acetate utilisers were butyrate producers [108]. Half of butyrate-producing isolates examined in this study, however, exhibited net acetate consumption. Not all acetate, of course, is re-directed for butyrate synthesis. However, there appears to be a strong link between acetate consumption and butyrate production by colonic bacteria. Further exploration to establish to what degree acetate is required for colonic butyrate production is necessary in developing our understanding of this relationship.

N-acetylglucosamine (GlcNAc) and N-acetylgalactosamine (GalNAc) are host-derived monosaccharide derivatives of glucose and galactose, respectively, and are found abundantly in the mucus lining of the gut [19]. As noted, *A. muciniphila* is an intestinal bacterium that resides in this ecological niche [45,110]. This beneficial bacterium is adept at colonising the mucus lining and has the ability to utilise a number of sugars including GlcNAc and GalNAc, although optimal growth *in vitro* is achieved with media containing mucin [19]. Other components of mucin may further enhance its growth.

The more recent generation of culture-independent, sequence-based understanding of gut communities facilitates the use of computational modelling to elucidate metabolic functions possessed by a single species [43,111] or microbial gut communities [112], as well as predicting possible growth substrates based on the pathways present. Based on whole genome sequences, models have been developed to predict the metabolic capabilities of *F. prausnitzii* [43] and *A. muciniphila* [111,113] *in silico.* The ability of microbes to utilise a variety of carbohydrates, including the

host-derived sugar GlcNAc, and amino acids or other growth promoters can be confirmed with cultivation experiments. In evaluating the predicted conditions, GlcNAc was found to enhance the growth of *F. prausnitzii* better than glucose [43]. This ability was also observed in several tested strains of *F. prausnitzii* suggesting that multiple strains can grow well on GlcNAc, highlighting its ability to utilise both diet- and host-derived substrates [35]. Although GlcNAc is not commonly found in foods, it is found in mushrooms as GlcNAc is a monomer of chitin [114] and GlcNAc supplements are also available on the market.

1.6.2 Minerals

Minerals, such as iron, magnesium, sodium, and calcium, are essential elements for living organisms, including most bacteria, and play pivotal roles in many biological processes. They are present abundantly in many food sources and, when these foods are ingested; microbes in the gut have access to these minerals. Although little is known about the regulation of mineral absorption mediated by gut bacteria and their impact on the gut microbiota, information on the importance of major dietary minerals on health is available. Of these, iron has sparked the interest of many researchers over the last number of years, with a focus on the impact on malnourished children. Iron-deficiency is very prevalent among African children with the WHO estimating 62.3% of preschool children are anaemic [115]. Iron-containing micronutrient powders (MNPs) are added to foods in a bid to reduce iron deficiency and prevent anaemia in children but the impact of iron supplementation on gut microbiota composition and functions has yielded conflicting results [116-119]. These differences might be partly due to age-related differences in various cohorts,

diverse geographical settings, the variety of iron compounds involved, and the doses administered in the different studies, which are summarised below.

The impact iron exerts on the production of bacterial metabolites, in particular butyrate, has also been the focus of some investigations. Butyrate seems to be strongly affected in response to iron. In one *in vitro* colonic fermentation model, moderately low levels of iron increased butyrate production whereas very low levels impaired its production [119]. The same study found that under high iron conditions, an increase in abundance of propionate-producing *Bacteroidaceae* and a decrease in butyrate-producing *Lachnospiraceae* were reflected based on the metabolites produced. Butyrate levels produced by *R. intestinalis* in batch culture were negatively affected by low iron levels while lactate and formate were enhanced. The results were reversed with high iron levels suggesting that levels of a single mineral can have a big impact on gut microbiota. However, some recent data has indicated that iron supplementation has adverse effects on the gut microbiota [116,117,120]. Notably iron supplementation resulted in decreased levels of desirable bacterial groups such as *Bifidobacteriaceae* and *Lactobacillus* and increased levels of *Enterobacteriaceae*. The production of siderophores in many pathogenic bacteria may have been responsible for the latter phenomenon [116]. One intriguing study by Paganini *et al*. [120] involving three groups of Kenyan infants demonstrated that the addition of GOS with a MNP containing iron (FeGOS group) mitigated the adverse effects observed in comparison to children that were administered the MNP and iron without the prebiotic (Fe group). The FeGOS group exhibited a similar gut microbiota composition to the control group (MNP without iron or GOS) but had lower levels of

virulence and toxin genes (VTGs) and increased production of SCFAs. On the other hand, a shift in microbiota composition to a more adult-like community was observed in the Fe group. Thus, the prebiotic seemed to stabilise the gut microbial community by limiting the negative effects induced by the addition of iron. Further, the addition of GOS did not seem to affect the bioavailability of iron. The MNP used in this study was fortified with 5g of iron, i.e., much lower than the average amount of 12.5g used in standard MNP supplements in earlier studies [121]. The lower dose maintained efficacy in that the iron was of high bioavailability and reduced anaemia so therefore may be more suitable than the current high dose levels which are between 10 and 12.5 mg of fortified iron according to the WHO [122]. As noted, the varying doses and iron compounds investigated throughout the literature highlight a need for standardisation in supplementation regimes. However, achieving this across different cohorts is difficult as many factors contribute to iron bioavailability and differences in microbiota across different populations makes it difficult to establish the complete impact of iron.

The effect of other minerals on the gut microbiota, in particular bacteria recently identified as beneficial, is currently not well understood. While there are some insights to suggest that other minerals, such as zinc, can have an impact [123,124], little is currently known.

1.7 Synbiotics

Synbiotics have also attracted significant attention in recent years. Synbiotics are combinations of a probiotic and an appropriate prebiotic designed to enhance specifically the growth and survival of the probiotic or other desirable bacteria within

the gastrointestinal environment, enabling beneficial effects to be induced more effectively [125]. This provides a very practical method of overcoming the difficulties that a probiotic may endure when initially introduced into the gut environment. Challenges in designing a synbiotic include selecting the right combination of pro- and prebiotic and ensuring sufficient quantities of each. As bifidobacteria and lactobacilli are well-characterised members of the human intestinal consortium and the benefits of specific strains with respect to human health have been established, microbes from these genera are most frequently included in synbiotics. FOS, GOS, inulin and lactulose are common choices for synbiotic formulations. Some newly identified health-promoting bacteria have been the focus of investigations to assess the consequence of combining their use with some well-established prebiotics. For example, a synbiotic administration of *A. caccae* L2 with GOS to rats enhanced intestinal organic acid production more effectively than just GOS alone [105]. It was hypothesised that GOS increased levels of bifidobacteria, resulting in greater lactate production, which in turn promoted *A. caccae* growth, therefore, facilitating the production of butyrate by *A. caccae*. This demonstrates one way of utilising the complex cross-feeding mechanisms to selectively manipulate target microbes.

1.8 Conclusion

The human gut harbours a diverse collection of microbes whose interactions and functionality have yet to be completely elucidated. However, it is already clear that these microbes play an integral role in human health and well-being. An increasing awareness that various microbes that can influence human health has been a catalyst for an ever-rising number of investigations into benefits of gut microbiota

manipulation. Prebiotics, and other nutrients, targeted to specific health-promoting bacteria are establishing themselves as a significant means of improving host health and disease. This has been evident in the considerable body of work that has accumulated in the last few years by demonstrating that species composition within the microbiota can be modified with very few changes in food consumption. The expansion of the prebiotic definition to substrates that go beyond the classical prebiotics and the inclusion of bacteria other than *Bifidobacterium* and *Lactobacillus* have enabled a greater examination of this intriguing relationship between diet and the microbiota. It is anticipated that these substances can soon be harnessed to promote populations of newly identified health-promoting bacteria in the gut.

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Chapter 5

General Discussion

5 General Discussion

As discussed in Chapter 1, the gut microbiota is a complex and diverse community of microorganisms, where net outcomes from intricate metabolic interactions can contribute to host health [1-3]. As both culture-dependent and -independent techniques to study microbes are continuously developing, knowledge is in parallel accruing in relation to taxonomic composition and functional potential of the human gut microbiota. This has resulted in the more recent association between several species, and strains thereof, with beneficial effects. Such species include *Akkermansia muciniphila, Faecalibacterium prausnitzii*, *Eubacterium rectale*, *Roseburia* species, and *Ruminococcus bromii* [4,5]. As the technological and regulatory hurdles associated with culturing and gaining approval for the use of these microbes as probiotics, respectively, are challenging, diet provides an alternative means of selectively enhancing the growth and/or activity of these microbes. This can be achieved by using prebiotics, or potential prebiotics, alone or in combination with other nutrients to modulate members of the gastrointestinal environment *in situ*. Prebiotics have for many years been particularly studied with a view to assessing their impact on the growth and/or activity of species, and strains, within the *Bifidobacterium* and the former *Lactobacillus* genera. However, targeting other species with prebiotics or dietary components is now the focus of many studies [4,5].

Prebiotics, potential prebiotics and other bioactives that can enhance the growth of specific gut microbes encompass a broad array of substrates, and as highlighted in Chapter 1, includes other oligosaccharides, polyphenols, resistant starch, algae, and seaweed, as well as substrates that are also microbial metabolites such as acetate

and lactate. Research relating to these substrates, along with the inclusion of bacteria other than *Bifidobacterium* and *Lactobacillus,* is enabling a deeper insight into the relationship between diet and the microbiota.

Chapters 2 and 3 focus on the application of methods (*in vitro* and/or *in silico*) to predict substrate utilisation by strains from the species *A. muciniphila*, *E. rectale*, *F. prausnitzii*, *R. bromii*, and *R. inulinivorans*. In Chapter 2, we combined *in silico* predictions with *in vitro* growth experiments to establish the accuracy of two tools, namely *Traitar* [6] and *CarveMe* [7], to predict substrate utilisation by representatives of the species listed above. Observed were some discrepancies between tools, the literature and the predictions acquired, such as the lack of acetate utilisation being predicted. This highlights how integral *in vitro* work is when generating accurate *in silico* prediction tools, and subsequent validations thereof, with an ultimate view to building upon existing knowledge relating to genotype-phenotype relationships within the gastrointestinal environment. The work conducted here provides data which could add to further validating the specific tools used. The more *in silico* and *in vitro* studies performed, then a greater assessment of the *in silico*-based approaches can be deduced and, thus, developed upon for the *in vivo* environment. In relation to the growth experiments conducted in this chapter, determining the pH of the growth media across time points would have been a valuable insight into the microbial fermentation of these substrates. The fermentation of dietary fibres in the human gut can lead to the production of SCFAs which lower the pH [9,10]. This, in combination with identifying the metabolites present could be a future XOS-based endeavour using different oligosaccharide

concentrations using more strains and/or co-cultures to get a broader view of microbial fermentation using this oligosaccharide. The growth experiments involving *R. bromii* merit repeated investigation as there were differences evident between strains relating to glucose utilisation which is typically not observed [11,12]. Strain-specificity was observed among the two strains of *F. prausnitzii* in relation to XOS degradation, a phenomenon that also merits further investigation in the future. This could be achieved in a potential future study by selecting several strains of the same species and creating metabolic models and investigating any differences observed in the predicted influxes in combination with targeted *in vitro* validations. The strain-level sensitivity of both predictive tools is a notable strength. For example, this could prove very useful in cases where microbial isolates with a similar genotype display different phenotype *in vitro*. These subtle differences at genome level could be identified through metabolic modelling. These tools are high throughput in nature and are more automated than standard metabolic model generation approaches. Although computational knowledge is still required, it does enable modelling to be more accessible than traditional labour- and time-intensive approaches. However, in this chapter these predictions are on individual genomes and do not represent the gut microbial environment therefore caution is required when interpreting the biological insight. Generated through constraint-based modelling approaches, such as *CarveMe,* are static reconstructions and lacks the biological phenomena insight on whether or under what conditions these predictions occur *in situ* [13,14]. Dependency on genome quality and annotation for accurate predictions is accepted, however, the true extent has yet to be elucidated. Although this is being discussed and implemented in recent publications [15-17], reporting quality metrics for both

the input genome and the output model would advance this field. Further assessment of the resulting models would greatly increase the confidence in the results obtained. Studies such as this add to the body of work available on these selected tools. The more widely used and applied they are, then the more obvious as to which improvements are necessary. Taken together, these findings add to our understanding of the tools employed and how they could be applied to complement *in vitro* and/or *in vivo* studies. Although technical challenges remain, combining predictive tools with *in vitro* and/or *in vivo* experiments could lead to a better understanding of microbial functional capabilities.

In Chapter 3 we elaborated on the *in silico* predictions in Chapter 2 by utilising *CarveMe* to generate 291 metabolic models using publicly available genomes from the five species of interest. Further analysis should be completed to establish the quality of the genomes used in this study to make the predictions more accurate. This could potentially allow for greater resolution with respect to discrimination between the models created and resulting predictions. One of the primary challenges with metabolic modelling is the lack of standardisation and quality controls. Currently, there are no agreed upon metrics for the quality and completeness of metabolic models. However, this is being discussed in the literature more frequently and with quality control tools such as MEMOTE [15] being made available, there is developments in resolving this limitation. MEMOTE [15] is community-driven, and the creators are open to engaging those interested in facilitating its continued development, signalling how pertinent this aspect is to metabolic modelling. Adding metrics to the uncertainty assumed would improve reproducibility and confidence in

the models being generated. This could provide the foundation for a future investigation using the same genomes and assessing the impact genome quality has on model quality. Deducing adequate quality thresholds for both the input genomes and resulting metabolic models would be insightful, along with further tool-specific *in vitro* validation would advance this field tremendously. Based on influx predictions, metabolic models clustered by species, more closely based on Flux Variability Analysis (FVA) compared to Flux Balance Analysis (FBA), highlighting care is required when selecting the type of analysis to conduct. The work presented here adds to the wider field of metabolic modelling by looking at species-level trends. This could provide the basis for a deeper analysis by picking a species of interest, improving the genomes, and combining with *in vitro* and/or *in vivo* investigations. *CarveMe* has performed well in comparison-based metabolic modelling studies and has been used as a benchmark for newer tools developed [18-20]. Tool comparative studies are highly significant for assessing *in silico* tools. However, it must be noted that these are individual genomes and do not represent the *in vivo* environment. There is merit in further implementing tools such as *CarveMe* by taking it to the microbiome level, initially with simpler communities e.g., cheese microbiomes. Across both flux analyses, trace elements such as copper, manganese and magnesium were predicted to be highly utilised across all species. Although these are co-factors which do not represent dietary fibre and may not be selectively utilised alone, however, in combination with potential or established prebiotics could be worthwhile investigating as encapsulation of these components could be an approach to ensure arrival to the gut microbes. Despite the limitations discussed here, computational methods can provide a framework for testable predictions of metabolic phenotypes.

It can enable the formation of hypotheses which when combined with *in vitro* and/or *in vivo* work could increase our understanding of the complexities of genotype-phenotype relationships.

Modulating the microbiota through the provision of select dietary ingredients can potentially enhance the growth and/or activity of target beneficial microbes. The use of *ex vivo* colon models can provide valuable insights into how communities of microbes alter, both at compositional and functional levels, in response to the substrates provided [21]. This method was applied in Chapter 4 and, using shotgun metagenomic sequencing, the impact of fifteen different groups of substrates on microbiome composition and functional potential was evaluated after 24 hours. It must be taken into consideration that homogenising samples from individuals could also be viewed as a limitation as individual variation in response to test substrates is lost. It would be valuable to choose a select number of substrates of interest and repeat the *ex vivo* experiment, both using individual and homogenised samples. Here, we studied some simple sugars and oligosaccharides, including some combinations of oligosaccharides. Additionally, a formulated functional beverage was developed using some of the ingredients that had been individually assessed. This beverage contained XOS, thereby harnessing the insights gained from Chapter 2, and whey protein concentrate, which has shown promise with respect to promoting the growth of *Bifidobacterium* species [22]. The digestibility of the chosen substrates should be taken into consideration. Some of the simple sugars would not reach the lower intestine and it would be intriguing to perform pre-digestive tests on the substrates to mimic the digestive system prior to reaching the colon [23].

Additionally, this is a microbial community model and does not reflect the exact *in vivo* conditions of the human colon. Although some factors such as temperature and pH are controlled, host factors are absent, therefore, is a limitation in how it can replicate the exact environment of the human large intestine. Due to time constraints metabolomic analysis was not feasible. This would have added substantially to this study by elucidating the functional changes brought about by the availability of the different substrates between time points. Although shotgun metagenomic analysis allowed an insight into functional potential, acquiring the metabolite concentrations would be valuable. Ideally a broad range of metabolites should be determined, however even a subset such as SCFAs would be illuminating. Metabolites produced by microbial fermentation in the human large intestine, especially SCFAs, play important roles locally and systemically [9]. Carrying out analyses to determine substrate depletion, i.e., what amount of each substrate is being used, would increase our understanding of which substrate tested is being utilised by the microbes present and how this impacts the composition and functional potential of the microbial community. Linking metabolites identified with substrate depletion could be useful in determining how much of a substrate is needed to produce a certain metabolite(s) and/or desirable concentrations of metabolites in this context. A potential future study could test multiple starting concentrations of e.g., XOS and determine how much of each concentration is used, the metabolites produced as well as the species composition changes that occur. This would provide a more comprehensive picture of the direct functional changes that occur because of the different available substrates, as well as providing information for selecting the preferred test substrate concentration to carry forward in future *in vitro* and/or *in*

vivo studies. Another consideration is the determination of absolute quantities of microbes through, for example, qPCR [24]. This would complement the species-level shotgun metagenomic analysis performed and would provide important information that may not be clear due to the application of relative abundance. For example, there were instances where certain species, e.g., *B. adolescentis,* dominated the species composition at the final time point. There is opportunity to revisit the sequencing data acquired and explore it through the implementation of compositional data analysis (CoDa). CoDa approaches have been gaining attraction in the last number of years in relation to it being applied to microbiome data [25,26]. Relative abundance is vulnerable to misinterpretation arising from the dependence between variable proportions e.g., is the change a result of an 'increase' of one or a 'decrease' of another [26]. For CoDa, initially, ratio transformations of the data are performed, and the logarithm of these ratios are obtained, i.e., log-ratios. This, in turn, takes the data into a log-ratio coordinate space, provides linear relationships, and symmetry to the skewed distributions thereby minimising the limitations evident with the relative abundance approach [25,27,28]. Such approaches include centred log-ration (clr) and additive log-ratio (alr) [29]. Univariate and multivariate statistical analyses can subsequently be conducted [25,30]. There is opportunity to revisit the data in this chapter and apply this analysis which could be a future investigation. One of the primary strengths of using the microMatrix is the high-throughput nature of this model system thereby facilitating the screening of several substrates in one experiment. This time-saving feature makes it a very useful step prior to *in vivo* studies. The work presented in this chapter adds to the literature by using a faecal microbial community to evaluate the impact different substrates have on both

composition and functional potential. Community-level analysis *in vitro* provides a significant insight into the alterations of microbial composition and functional potential due to substrate availability. Further evaluations of the beverage tested and other similar beverages in the future, including digestibility or whether encapsulation of the ingredients could offer an alternative approach, are merited as the use of combinations of potential beneficial dietary ingredients may promote the growth of specific taxa than individual ingredients.

Overall, this thesis has contributed to the broader fields by combining *in silico*, *in vitro*, and *ex vivo* approaches, with a particular emphasis on the application of metabolic modelling tools to predict substrate utilisation, an approach is an exciting aspect of systems biology. Although limitations remain, it is envisaged that future studies untangling the functional capabilities of microbes could greatly benefit the by the integration of *in silico* predictions as a guide for *in vitro* and/or *in vivo* experiments. Based on the results observed in this thesis, multiple avenues for future studies are revealed, including using the formulated beverage as the basis for a human intervention study which is due to commence shortly. The combination of select ingredients formulated as a functional beverage to target specific microbes' merits further investigation as prebiotic-based applications expand to include synergistic combinations of substrates.

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