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PREBIOTIC MECHANISMS, FUNCTIONS AND APPLICATIONS – A REVIEW

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ABSTRACT: *In October 2012, a group of scientists met at the 10th Meeting of the International Scientific Association of Probiotics and Prebiotics (ISAPP) in Cork, Ireland to discuss issues surrounding prebiotics and their development. This article summarises outputs from the meeting. Various prebiotic definitions were discussed and how the concept has evolved from targeting the colonic microbiome, through to the entire gastrointestinal tract and finally the ISAPP definition, which specifies fermentation as a key criterion. Structure and function relationships are becoming clearer with effects upon microbial diversity, determinations of selectivity and enhanced biological activity being major outcomes. Immune modulation and metal chelation were further facets. Biomass can be a useful, and economic, means of generating new prebiotics. Pectic oligomers from citrus are model examples. Testing aspects range from in vitro batch culture fermenters to multiple stage models, immobilized systems, animal, cellular studies and human trials. Analytical processes around microbiota characterization and functionality were compared. Human studies were seen as the definitive outcome, including ^{13}C labeling of key interventions. For extra intestinal effects, atopic disease, respiratory infections, vaginal issues, oral disease, adiposity, liver damage and skin infections are all feasible. The general outcome was that microbiota modulation was the key mechanism that linked these interactions. In pet food applications, the market potential for prebiotics is huge. Health targets are similar to those of humans. Issues include monomeric composition, chain length, linkages, branching, microbiota beyond bifidobacteria, metabolic function, mechanisms of health effects. Molecular biology has unraveled some of the explanations for prebiotic influences e.g. gene clusters to show transporters, regulators, permeases, hydrolases, lacS. In Lactobacillus ruminis, fermentation studies have been aligned to genome annotations, showing an energy efficient and rapid transport of GOS. In bifidobacteria, functional genome analyses have demonstrated uptake of trisaccharides. Questions relating to patients were then raised. For example, are prebiotics related to disease treatment or health maintenance? If a prebiotic does not change the microbiota, then how does it operate? Case study trials in Inflammatory Bowel Disease were presented on patient access to prebiotics and information. These showed that their knowledge of prebiotics was poor, compared to probiotics. The group then discussed the next generation of prebiotics (e.g. anti-adhesive activities). The comparator was Human Milk Oligosaccharides, which both reduce adherence of pathogens and act as prebiotics. Studies with galactooligosacchardes (GOS) have used pyrosequencing to demonstrate varying species level effects. This has relevance for infant formulae. Prebiotic aspects of whole foods and their complexity was covered. Trials were described where cross feeding and co-metabolism had been investigated. Suggestions on other prebiotic influences, aside from bifidobacteria, were made and included metagenomics, metabonomics, gene expression, mRNA global sequencing, bile deconjugation, enzyme profiles, lipids, phenolics. The discussion suggested how prebiotics could move forward with a wider expansion*

of the concept, target populations, expanded microorganisms, health benefits, application of new technologies and improved consumer understanding being the main goals.

KEY WORDS: Fructooligosaccharides, Galactooligosaccharides, Prebiotics, Probiotics

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INTRODUCTION

Chronic diseases including cardiovascular complaints, Type II diabetes, many cancers, some dementias, acute and chronic gut disorders are a major and growing societal and financial concern. Moreover, an increasingly obese and ageing population means there is greater prevalence of disorder. While pharmaceuticals have made an enormous impact on the treatment and prevention of disease during the 20th century, increasingly there is recognition that the 21st century health model will comprise of both preventative life style and therapeutic entities, with diet playing a principal role. This is the basis of the "functional foods" concept whereby dietary ingredients are used for purposes over and above their normal nutritional value (Gibson and Williams, 2000). The Global Market Review of Functional Foods estimates that in 2013 the worldwide functional food market will reach a value of at least US$90.5bn. The emergence of health conscious consumers with a proactive approach of 'prevention over cure' and the development of nutritional science has driven the growth of functional foods.

The biological and clinical importance of resident gastrointestinal microflora in humans is becoming increasingly recognised by consumers and healthcare workers. Although it is known that many disease states involve bacterial metabolism, the human gut microbiota may also be considered as extremely relevant for improvements in host health (Gibson and Roberfroid, 2008). For instance, bifidobacteria and lactobacilli can help resistance to gut infections by directly inhibiting the growth of harmful bacteria, reduce cholesterol levels, sustain the immune response and synthesise vitamins (Steer et al., 2000). Scientific concepts underpinning directed modulation of the human gut microflora towards a more beneficial composition have had probiotics as a principal focus (Fuller and Gibson, 1997). While probiotics have been ingested by humans for several hundred years, their development has progressed markedly over the last 2 decades worldwide. Probiotics are defined as live microbial food supplements which have a beneficial effect on the intestinal flora of the host, thereby leading to health improvements (Fuller, 1989). Probiotics must be safe (i.e. Generally Regarded As Safe, GRAS), they must be amenable to industrial processes necessary for commercial production, they must remain viable in the food product and during storage, they must persist in the gastrointestinal tract long enough to elicit an effect and they must improve host health (Collins and Gibson, 1999). Several hundred human trials have been reported on their positive virtues (Kolida et al., 2006; Sanders et al. 2007; Khani et al., 2012). The use of live bacteria in the diet has been successful scientifically and economically.

Prebiotics are a far more recent concept than probiotics, being first developed in the mid 1990's (Gibson and Roberfroid, 1995). They are dietary ingredients that can selectively enhance beneficial components of the indigenous gut microbiota, such as lactobacilli or bifidobacteria, and are finding much increased application into the food sector. In contrast to probiotics, they can be added to many ingredients including heated products. Prebiotics were therefore originally developed to selectively enhance beneficial components of the gut microbiota, such as lactobacilli or bifidobacteria, and are finding increased application. Prebiotic use is directed towards favouring beneficial changes within the indigenous gut microbial milieu. Criteria for classification as a prebiotic are:

- resists gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption
- is fermented by intestinal microflora
- selectively stimulates the growth and/or activity of intestinal bacteria associated with health and well-being.

ISAPP currently defines a dietary prebiotic as "a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health" (Gibson et al. 2010). The fructooligosaccharides (FOS) and galactooligosaccharides (GOS) are examples of confirmed prebiotics.

This articles summarises recent advances in the prebiotics field and makes suggestions for future avenues of interest.

Why structure affects function

The fermentation of carbohydrates by the gut microbiota and hence the impact on the colonic ecosystem is profoundly influenced by carbohydrate structure. It is common for investigators to refer to "dietary fibre" as a generic entity whereas the structure of the fibre makes a big difference to fermentation. Similarly, different prebiotics have different impacts on the gut microbiota.

Whilst we do not have a clear and comprehensive understanding of the molecular mechanisms involved in the bacterial breakdown of complex carbohydrates in the gut, it is clear that these processes will involve the action of glycanases and transport systems to take up breakdown products. Glycanases can be either endo-glycanases acting on large polymers with oligosaccharides as the equilibrium products or exo-glycosidases producing monosaccharides as equilibrium products. These enzymes can be located intracellularly, be associated with the cell wall or secreted into the extracellular medium (Imamura et al., 1994). Such enzymes display

specificity for the anomeric configuration and the position of the glycosidic linkages in the carbohydrate. Some will hydrolyse all positional isomers of any given anomeric confiduration whilst others are highly specific for one linkage. Logically, the type of enzymes produced by a microorganism will strongly influence the carbohydrate structures that it can degrade to provide nutrition and energy, although this is extremely difficult to characterise in a mixed culture environment. An important series of papers on the degradation of fructans (Falony et al., 2009a; 2009b) give an insight into the structural basis of selective fermentation of prebiotics. These researchers found that strains of bifidobacteria fall into one of four clusters with reference to their metabolism of inulin and, shorter chain, oligofructoses (Falony et al., 2009a). Cluster A only metabolise fructose; Cluster B strains preferred fructose but could also metabolise oligofructoses with decreasing affinity as molecular weight increased; Cluster C prefer oligofructans with little ability to grow on fructose or inulin; Cluster D have equal affinity to grow on fructose and oligofructose and have some ability to grow on inulin. In a co-culture environment with a *Bacteroides thetaiotaomicron* strain, which can grow equally well on fructose, oligofructose or inulin, a Cluster D strain of *B. longum* was more competitive than the others, with a Cluster A strain of *B. breve* being comprehensively outcompeted by the *Bact. thetaiotaomicron* (Falony et al., 2009b).

Products of enzymatic carbohydrate degradation need to be taken up into the cytoplasm to be further metabolized to provide energy and carbon sources. It has been shown for some probiotic microorganisms, such as *B. infantis,* that cell-associated β-fructofuranosidases hydrolyse fructose from non-reducing ends of fructooligosaccharides and then transport fructose into the cell (Perrin et al, 2002).

It is clear from *in vitro* experiments, however, that some gut microorganisms have a preference for certain molecular weights within a series of oligosaccharides. An example is the fermentation of fructooligosaccharides by *L. plantarum* and *L. rhamnosus* (Kaplan and Hutkins, 2000). These microorganisms only metabolized trisaccharide and tetrasaccharide fractions within complex FOS mixtures. This observation suggests the presence of specific oligosaccharide transport systems in the bacteria. Once again, however, determining the extent of operation of these different mechanisms in a mixed culture environment is extremely challenging.

Influence of molecular weight

It seems from several studies that molecular weight of a carbohydrate influences the selectivity of fermentation. This has been seen with oligodextrans (Olano-Martin et al., 2000) and arabinoxylans (Hughes et al., 2007) *in vitro* where low molecular weight oligosaccharides were more selective to bifidobacteria than higher molecular weight parent molecules. It remains to be established, however, whether this is also true *in vivo*.

Influence of branching

There are relatively few studies investigating the influence of branching on fermentation selectivity in any systematic manner. One study (Sarbini et al., 2011) examined a series of enzymatically manufactured oligodextrans with a-1,6 linkages and a-1,2 branching. This set of materials had molecular average weights of 1 kDA (0, 16 and 32% branching), 6 kDa (0 and 33% branching) and 70 kDa (0, 15 and 37% branching). This set of data showed a clear preference by bifidobacteria for the 1 and 70 kDA oligosaccharides over the 6 kDa molecules with a slight decrease in growth as branching increased. Other saccharolytic groups such as bacteroides did not show any such preferences. This is consistent with bifidobacteria possessing a wide range of exo-acting glycosidases (Lee and O'Sullivan, 2010).

Structure function relationships in pectin-derived oligosaccharides

Pectins are a heterogeneous group of complex polysaccharides frequently containing relatively unusual monosaccharide substituents (Sila et al., 2009). The structural components present in any given pectin will depend on its source and the method used to extract it. Given this structural complexity, together with their abundance and availability from biomass, pectins are an attractive target for development on novel prebiotics.

Onumpai et al., (2011) set out to elucidate the relationship between structure and fermentation selectivity in a set of pectins and pectin-derived oligosaccharides. High molecular weight parent polysaccharides (polygalacturonic acid, methylated citrus pectin, rhamnogalacturonan I backbone, arabinan and galactan) were specifically digested using enzymatic and chemical methods to make derivative oligosaccharides (oligogalacturonides with average dp values of 5 and 9, methylated oligogalacturonides, oligorhamnogalacturonan, oligogalactosides and oligoarabinosides). Their fermentation properties were compared using a microscale pH-controlled faecal fermentation system. All of the tested carbohydrates increased populations of bacteroides but only the galactan and arabinan derived oligosaccharides increased populations of bifidobacteria.

Production of short chain fatty acids

Although studies on prebiotic modulation of the gut microbial ecosystem generally focus on changing the populations of specific bacterial groups, it is likely that the most important changes as far as health consequences are concerned are in the metabolites produced by the ecosystem. The principle fermentation end products of carbohydrate metabolism in the gut are short chain fatty acids (SCFA) such as acetate, propionate and butyrate, generally in an approximate molar ratio of 60:20:20 (Cummings and Macfarlane, 1991). Lactate is also produced but does not generally accumulate as it is further converted into other products such as acetate (Duncan et al., 2004).

Different carbohydrates give rise to different SCFA in this complex ecosystem (Cummings and Macfarlane, 1991). For

example, starches typically result in the production of butyrate by the gut microbiota whilst pectins are noted for producing large quantities of acetate. Recently, a range of oligodextrans (discussed above) have been shown to produce variations in the ratio of acetate to propionate depending on the molecular weight and degree of branching (Sarbini et al, 2011). As molecular weight increased, levels of propionate to acetate increased to a ratio of approximately 1:1. This is of interest as propionate is attracting interest due to its potential role in regulating lipid synthesis and satiety (Hosseini et al, 2011).

Using biomass for bioactivity

Biomass is defined as that material remaining after food, paper and microbial processing. It can also include municipal solid waste and wastewater treatment sludge. Biomass materials currently used as sources of prebiotics, or potential prebiotics, include corn cobs, soybean and dairy whey, sugar beet pulp and citrus peel (Crittenden and Playne, 1996). It is estimated that 1 billion tons of biomass will be produced each year in the United States by 2030 with increasing use of energy crops as feedstocks for biofuel production (U.S. Department of Energy, 2011). In a biorefinery model, prebiotics could be a valuable co-product that would lower the cost of biofuel production from biomass. Therefore, biomass represents an abundant source of raw material for prebiotic and bioactive oligosaccharide production that may become more cost-effective in the future.

How to test for a prebiotic effect

To test whether a potential prebiotic has functionality in terms of modulation of composition and/or activity of the gut microbiota, one usually first screens (a series of) potential prebiotics using *in vitro* models. Also, mechanistic insight, formulation and testing of hypotheses can be done in such models. However, this requires models that mimic physiological conditions in humans. Animal models can be used for safety evaluation. In addition, several animal disease models could be used in the pre-clinical stage to test effects of prebiotics in these models. However, the definitive proof is a human volunteer trial, especially if the intention is to carry a claim on the product containing the prebiotic.

At their core, *in vitro* gut fermentation models each characterized by inoculation of a device or bioreactor with faecal microbiota and incubation under a set of controlled conditions selected according to model complexity, physiology of the target host and research questions being addressed. Gut fermentation models enable cultivation of intestinal microbiota for a defined period and include specific time that range from hours to weeks or even months. Selection of the appropriate model requires careful evaluation of study objectives given the advantages and limitations exhibited by each type of system (Macfarlane and Macfarlane, 2007; Payne et al., 2012). Due to the fact that access to the human colon is limited, most clinical studies test the microbiota composition and function in faecal samples. However, due to transit time through the large intestine, which is up to 72 hours, faecal samples only at best reflect the distal end of the colon. Since fermentation of prebiotics mostly takes place in the proximal colon, several research groups have developed *in vitro* models which mimic this proximal region of the colon, or the complete colon (divided into proximal, transverse and distal areas). These models may vary from simple batch culture incubations in test-tubes to multi-compartmental dynamic models that reflect different physiological conditions in the gut. In the latter category, there are at least 5 systems that have been used frequently in prebiotic research: the three-stage fermentor (Gibson et al., 1998; Macfarlane et al., 1989a,b) the simulator of human intestinal microbial ecology (SHIME) (Molly et al., 1993; 1994) the TNO *in vitro* model of the large intestine (TIM-2) (Minekus et al. 1999), a multi-stage fermentor from Danisco [now Dupont] (Enteromix) (Makivuokko et al., 2005) and the model developed by the group of Lacroix with immobilised microbiota (Cinquin et al., 2004; 2006a). Each model has its specific advantages and disadvantages, but each system has been shown to be 'bifidogenic' when prebiotic FOS or GOS were fed to the microbiota (McBain et al., 1997; Grootaert et al., 2009; vanNeunen et al., 2003). The most advanced models also account for artificial digestive systems, host digestive functions *in vitro* coupled with multistage compartments to simulate the human gut, e.g. stomach lumen and small intestines, including bile secretion, motility, pH and absorption capacities of the upper intestine. Such a technological platform enables virtually unlimited experimentation under highly controlled environmental conditions, to assess for example single or multiple dietary components (e.g. prebiotics) for their impact on individual gut microbial populations and subsequent changes to metabolism as a function of dietary modulation.

Perhaps one major discriminating factor between the different *in vitro* continuous fermentation systems is the technique used for faecal inoculation. Operation of most *in vitro* systems uses a liquid faecal suspension as inoculum, resulting in several limitations due to the free-cell state of bacterial populations because systems with such inocula generally experience a rapid washout of less competitive bacteria and are consequently limited in operation time to less than 4 weeks (Macfarlane and Macfarlane, 2007; Payne et al., 2012). These systems also struggle to reproduce both the planktonic (free-cell) and sessile (biofilm-associated) states of bacterial populations in the colon (Macfarlane and Dillon, 2007). To address these problems, a process for the immobilisation of faecal microbiota and physical retention of immobilised faecal inoculum in the seeding reactor has been developed (Cinquin et al., 2006a) and used for testing prebiotic effects (Cinquin et al., 2006b; Le Blay et al., 2009). The entrapped faecal microbiota colonises beads of a porous polysaccharide matrix, whose composition has been carefully selected for long time chemical and mechanical stability under conditions of gut fermentation. Continuous inoculation of the circulating chime medium is by cells growing close to the

surface of beads. A complex gut microbiota of high diversity akin to the donor faeces can be reestablished within bead matrix. Furthermore, very high-cell density and population stability has been tested for a long operational time of systems of several months (Payne et al., 2012).

Many effects of the host on community modulation, e.g. immune response or defensins, are not simulated in gut fermentation models. The use of *in vitro* human intestinal cell models, suggest that their application could be extended to include host–gut microbe functional studies. The combination of in *vitro* human intestinal cell models, which are widely accepted for evaluating the mechanistic effects of probiotics or drug absorption and transport, with *in vitro* gut fermentation models would create an advanced model system. Samples obtained from host response lacking in *in vitro* gut models can be directly applied to monolayer cell models for functional assessments (Bahrami et al., 2011; Zihler et al., 2011).

As stated above, *in vitro* models are frequently used to generate and test hypotheses. The early definition of prebiotics stated the specific stimulation of beneficial microorganisms. However, since not all microbes in the gut can yet be cultured, specificity of the stimulation can be difficult to determine. Recently however, this has been addressed by using substrates (prebiotics) that were labeled with the stable isotope ^{13}C (Venema 2012). Upon fermentation, the label is incorporated into both the microbial biomass of those microbes that ferment the substrate, as well as their metabolites. Using this technology, it was shown that GOS are primarily used by bifidobacteria (Maathuis et al. 2012), and that the major metabolites produced are lactate and the short-chain fatty acids (SCFA) acetate, propionate and butyrate. Since bifidobacteria only produce acetate and lactate, the occurrence of label into other SCFA indicates cross-feeding between different microbes in the gut, as has also been shown for fermentation of ^{13}C-labeled starch (Kovatcheva-Datchary et al., 2099). Another benefit of being able to trace the label into microbial metabolites is to calculate exactly how many carbon-atoms from a ^{13}C-labeled substrate end up into microbially produced SCFA, the exact amount of energy extracted from dietary components by the microbiota can be determined (Venema 2012; Bloeman et al., 2009). It has been shown that this varies for different prebiotics, and hence the contribution of certain prebiotics to obesity may fluctuate (Venema 2010).

Despite advances in *in vitro* modeling, *in vivo* studies are necessary and cannot be replaced. Animal studies persist as a substitute to human studies, yet relevance of data obtained through animal studies is often questioned due to physiological differences between animal and human metabolism. Ultimately functional studies are best done in humans, but these remain difficult to perform owing to social, safety and ethical considerations governing invasive medical procedures necessary in accessing the human large intestine, rendering human studies primarily limited to faecal sample analyses. An ultimate approach to investigating gut microbiota functionality must therefore include a combination of *in vitro* and *in vivo* models, yielding complementary results which not only strengthen the overall validity of each approach but also distinguish between the functionality of gut microbial and human processes.

In summary, *in vitro* models of digestive ecosystems are an efficient platform to study nutritional effects and mechanisms (e.g. cross feeding) of nutritive compounds such as prebiotics. In the context of negative public perception and ethical limitations for animal experimentation, *in vitro* models will become increasingly important and possibly the scientific reference for gut studies in the future. In this respect, there is a need to develop new *in vitro* gut models exhibiting enhanced accuracy and practicality and to develop standardization for the application of different models (Table 1). The combination of gut modeling with holistic '-omics' technologies will help unravel complex microbial and host factors governing human gut microbiota functionality.

TABLE 1. Important aspects for developing *in vitro* models of intestinal fermentation

- Importance of anaerobiosis and inoculum handling for quality-biodiversity
- How long to allow for adaptation? Is stability necessary?
- Importance / difficulty to implement gas sampling
- Is it realistic to expect that *in vitro* models give identical microbiota-metabolism as in faecal donor?
- Inability to model host-microbe (bilateral) interactions
- Enhancing model throughput through miniaturisation, automation and parallel operation
- More work needed to combine *in vitro* fermentation models and cellular models to complement host-microbe interaction
- Need to carefully validate model with *in vivo* data
- Avoid studying models for the sake of the models.

calculate the amount of energy provided to the host in terms of SCFA. The microbiota has been implicated in obesity and diabetes type 2. One mechanism might be by the production of SCFA, which when taking up by the host can be used as a source of energy e.g. butyrate is used by enterocytes (Roediger 1982); while acetate and propionate are extracted by the liver and used in gluconeogenesis or lipogenesis. Since one can

MOLECULAR MECHANISMS OF EFFECT

Prebiotic utilization in lactobacilli: a special focus on *Lactobacillus acidophilus*

Selective enrichment of bifidobacteria and lactobacilli by prebiotic oligosaccharides, such as FOS and GOS, has been

well-documented by *in vitro* and *in vivo* observational studies. However, recent molecular studies have begun to elucidate the mechanisms of prebiotic metabolism in these microbes that may play an important role in their persistence and enrichment in the gastrointestinal (GI) tract. This knowledge is fundamental in terms of understanding how prebiotic compounds, as a carbon source, influence the composition and activities of the GI microbiota, and the selection of probiotic-prebiotic candidates that can exert maximum beneficial effects.

GOS metabolic pathway in Lactobacillus acidophilus.

Although GOS are established prebiotic compounds, the molecular mechanism on how GOS are metabolised by probiotic microbes has only recently been established in *Lactobacillus acidophilus* NCFM. Using microarray transcriptional studies, Andersen et al., (2011) showed that GOS specifically induced the *gal-lac* operon that encodes a galactoside-pentose-hexuronide (GPH) permease (LacS), two cytoplasmic β-galactosidases (LacA and LacLM), as well as enzymes of the Leloir pathway for galactose metabolism. The specific induction of *lac* operon by GOS was similarly observed in lactose-grown cells (Barrangou et al., 2006), suggesting that the *lac* operon in *L. acidophilus* was responsible for the metabolism of both lactose and GOS, and potentially other galactosides such as fractions of human milk oligosaccharides (HMOs). In addition to lactose, this gene cluster was also upregulated by bile exposure (Pfeiler et al., 2007), revealing an adaptive combination of gut-evolved traits for nutrient acquisition and bile tolerance.

Inactivation of the LacS permease gene in *L. acidophilus* NCFM abolished its ability to grow on GOS, lactose or lactitol as sole carbon source. These results established LacS as the transporter for GOS, and suggest that LacS has divergent and broad substrate specificity for β-galactosides. The LacLM and LacA β-galactosidases belong to the glycoside hydrolase family 2 (GH2) and 42 (GH42), respectively. Notably, GH2 and GH42 β-galactosidases were previously proposed to be involved in the degradation of human milk oligosaccharides (Marcobal et al., 2010), further indicating the potential involvement of this operon in HMO utilization, although *L. acidophilus* NCFM was previously shown to exhibit weak, but noticeable, growth on HMOs under *in vitro* conditions.

Comparison of gene clusters containing *lacS* among sequenced lactobacilli and related lactic acid bacteria (LAB) show that the *gal-lac* cluster is conserved among acidophilus complex lactobacilli such as *Lactobacillus helveticus*, *Lactobacillus crispatus* and *Lactobacillus johnsonii*. This suggests that the *gal-lac* gene cluster may also confer, to these species, the capability to transport and utilize GOS as a carbon source. Meanwhile, the *lac* gene clusters differ markedly in other LAB such as *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus fermentum* and *Lactobacillus delbrueckii* ssp. *bulgaricus*. Interestingly, regardless of the difference in structural organization, *lacS* genes are present along with *lacA* GH42 family β-galactosidases for all lactobacilli except *L. bulgaricus*, indicating co-evolution of LacS and GH42 family β-galactosidases. *L. bulgaricus* possesses a *lacZ* GH2 family β-galactosidase and its *lac* operon share high degrees of similarity to that of *Streptococcus thermophilus*, likely due to genetic exchange occurring in their shared dairy niche. Differences in gene arrangement and the types of encoded β-galactosidases reflect specific adaptation among these LAB towards the metabolism of a variety of β-galactoside substrates.

Overall, both *in silico* and functional analyses support that in *L. acidophilus* and likely other lactobacilli with a homologous *gal-lac* gene cluster, GOS is transported via the GPH-type LacS permease, hydrolyzed by LacA and LacLM β-galactosidases into glucose and galactose, which are subsequently metabolized via the glycolytic and Leloir pathways, respectively. The co-presence of *lacS* and *lacA* inclusively within the intestinal-associated acidophilus complex species (all strains of each species are *lacS*$^+$) further indicates lactose and galactosides as important energy sources in the GI tract for these lactobacilli, which potentially provide selective advantages against microbes unable to metabolize complex galactosides.

Metabolic pathways for other potential prebiotic substrates in L. acidophilus.

L. acidophilus is capable of growing on other potential prebiotic substrates with different glycoside linkages and monosaccharide constituents. A recent differential transcriptomic study on *L. acidophilus* NCFM identified putative catabolic pathways for the utilization of eleven prebiotic candidates consisting of *α- and β-glucosides and galactosides (*Andersen et al., 2012). In general, di- and trisaccharides of α-glucosides (isomaltose, isomaltulose, panose and polydextrose) and β-glucosides (cellobiose, gentiobiose and β-glucan oligomers) induced the expression of phosphoenolpyruvate-dependent phosphotransferase systems (PTSs); whereas α-galactosides raffinose and stachyose induced the expression of an ATP-binding cassette (ABC) transporter. Consistent with a previous study (Andersen et al., 2011), the β-galactosides GOS and lactitol specifically induced the *lacS*-encoded GPH permease transporter. Various genes encoding glycoside hydrolases that complement the transporters in operons were also co-induced for metabolism of the respective glucosides and galactosides.

Interestingly, upregulation of a previously established FOS ABC transporter (Barrangou *et al.*, 2003) has also been observed in polydextrose-grown cells, suggesting a potential broader than predicted specificity of the ABC transporters in *L. acidophilus*. On the other hand, cellobiose and gentiobiose , which are regio-isomers that differ only by the types of β-glucosidic linkages (β-1,4 and β-1,6, respectively), were found to differentially upregulate two distinct PTS systems as well as distinct GH1 phospho-β-glucosidases. Collectively, these findings showed that *L. acidophilus* possesses the genetic repertoire to utilize a wide range of prebiotic candidates, and that carbohydrate metabolic diversity is differentiated by types of transporters and specificities of the glycosyl hydrolases.

Linking prebiotic metabolism to the probiotic genome: a bifidobacterial perspective

The first bifidobacterial genome, i.e. *Bifidobacterium longum* subsp. *longum* NCC 2705, was published over ten years ago by Schell et al., (2002). Since then more than fifty different bifidobacterial genome sequences have been deposited in NCBI, of which twenty eight represent completed genomes such as the human faecal isolates *Bifidobacterium breve* UCC2003, *Bifidobacterium longum* subspecies *infantis* and *Bifidobacterium bifidum* PRL2010, the insect isolate *Bifidobacterium asteroides* PRL2011 and the oral cavity isolate *Bifidobacterium dentium* Bd1 (Bottacini et al., 2012; Turroni et al., 2010; O'Connell-Motherway et al., 2011a,b; Sela et al., 2008; Ventura et al., 2009).

From the functional annotation of the first bifidobacterial genome, and subsequently confirmed by additional comparative genome studies, it has become clear that most bifidobacteria dedicate a substantial proportion of their coding capacity to the metabolism of carbohydrates, although in the majority of cases such genes or gene clusters could not be assigned to the metabolism of any particular carbohydrate (Ventura et al., 2007; Bottacini et al., 2010). Several research groups have addressed this knowledge gap through functional analyses aimed to gain insights into the genetic requirements for the utilization of mono-, oligo- and polysaccharides that are present in human milk, mucin, and plants, and which may thus represent potential prebiotic compounds for bifidobacteria (for some recent examples, see Sela et al., 2012; Sela et al., 2011; Wakinaka et al., 2013; Kiyohara et al., 2012; Yoshida et al., 2012; O'Connell-Motherway et al., 2011b; Pokusaeva et al., 2010; Pokusaeva et al., 2011a). These data have provided a wealth of knowledge on the carbohydrate metabolizing capabilities of bifidobacteria and have shown that such metabolic abilities are highly diverse, and are strain and species specific (Asakuma et al., 2011; Pokusaeva et al., 2011b; Watson et al., 2013, in press). They have also shown that particular bifidobacteria are specialized to utilize certain carbohydrates, which may provide them with a selective advantage in a highly competitive environment. For example, *B. bifidum* PRL2010 possesses a relatively small number of carbohydrate-utilizing gene clusters, but the majority of these appear to be dedicated to carbohydrates that it can obtain from mucin and human milk oligosaccharide (HMO) utilization (Asakuma et al., 2011; Turroni et al., 2012).

Another telling example of how different strains of the same bifidobacterial species exhibit variable carbohydrate utilization abilities was recently published and relates to the differential utilization of the prebiotic GOS by various strains of *B. breve* (O'Connell-Motherway et al., 2013). This paper showed that strains of *B. breve* can be classified into two groups based on their growth abilities on GOS, where one group reached a much higher final optical density than the other. The observed high growth capacity on GOS by certain *B. breve* strains was demonstrated to be due to the presence of a gene, *galA*, encoding an extracellular endogalactanase, which had previously been shown to be required for growth on the plant-derived polysaccharide galactan (O'Connell-Motherway et al., 2011a). This allowed such strains to utilize GOS fractions with a high degree of polymerization, whereas such oligomers could not be metabolized by *galA*-negative *B. breve* strains (O'Connell-Motherway et al., 2013). When these findings are extrapolated to the functionality of GOS as a prebiotic for bifidobacteria in the gastrointestinal tract, one could argue that, in the absence of cross-feeding, GOS is a better growth substrate for *B. breve* strains containing the *galA* gene or for those (bifido)bacteria, which encode an endogalactanase gene.

Genomic explorations related to carbohydrate utilization by bifidobacteria not only provide valuable information on potential bifidogenic compounds, but may also provide novel routes for prebiotic biosynthesis. The latter was demonstrated by Goulas et al., (2009), who showed that the use of b-galactosidases present in a cell extract of a *B. bifidum* strain allowed the biosynthesis of a GOS mixture which demonstrated prebiotic activities (Drakoularakou et al., 2010). Another very interesting example of reverse synthesis of the bifidogenic HMO lacto-N-biose used a set of four different bifidobacterial enzymes (Nishimoto and Kitaoka, 2007). This latter example shows that even complex carbohydrates can be produced by reverse biosynthesis using enzymes originating from (bifido) bacteria capable of metabolizing such compounds. These latter two examples highlight the commercial value that detailed exploration and exploitation of carbohydrate-degrading enzymes and pathways may bring, while also generating fundamental knowledge on how bifidobacteria have managed to sustain their position as important human gut commensals.

Extra-intestinal effects of prebiotics

Although prebiotic-associated health benefits are often confined to the intestinal tract (e.g. attenuating intestinal inflammation), growing evidence suggests that they may also exert health benefits outside the gut. These health benefits may be mediated indirectly by compositional or metabolic changes in the large intestinal microbiota, or directly by changes in the native microbiota in areas outside of the intestinal tract, for example the mouth or vaginal tract.

Extra-intestinal effects of prebiotics through gut microbial fermentation

It is well-established that certain prebiotics (e.g. FOS and GOS) can stimulate the growth of specific microbial organisms in the human and animal large intestinal tract, thus promoting health benefits (Guarner, 2007). This is thought to be mediated by a selective fermentation of the prebiotic by certain bacterial groups, which allows them to grow preferentially. However, the consequences of such changes outside the intestinal tract are less well understood.

The gastrointestinal tract is in constant contact with the outside environment, an interaction that throughout evolution has transformed the alimentary tract into a heavily immunologically-guarded body site (Cerf-Bensussan

and Gaboriau-Routhiau, 2010). Because the intestinal microbiota plays an important role in protecting and maintaining both innate and adaptive immune mechanisms (Hooper and Macpherson, 2010), it has been hypothesized that preferential growth of beneficial bacteria in the gut should lead to health benefits not only inside but also *outside* of the intestinal tract. Several articles have shown that the administration of prebiotics may reduce the incidence of atopic dermatitis (Moro et al., 2006), prevent respiratory infections (Arslanoglu et al., 2008, Bruzzese et al., 2009), and increase absorption of calcium (Ca), magnesium (Mg) and other minerals (Ohta, *et al.*, 1995). These studies propose that observed health benefits are related to the selective increase of beneficial bacteria in the large intestine; however, this is not always supported by a parallel characterization of the microbiota.

Increased mineral absorption is one of the most widely investigated effects of prebiotics that may produce a health benefit outside the intestinal tract. This effect is thought to be mediated by fermentation of the prebiotic component in the colon and a subsequent increase in SCFA (Roberfroid et al., 2010). Although the exact mechanism is not well understood and confounding results have been published (Petry et al., 2012), it has been suggested that it is the decrease in luminal pH which ultimately leads to a more efficient diffusion of Ca and Mg through the intestinal wall (Ohta et al., 1995). Importantly, the decreased pH may not only be due to increases in specific bacteria but to complex cross-feeding among different members of the microbiota. For example, one strain of *Bifidobacterium longum* was shown to protect against enteropathogenic infection through production of acetate (Fukuda, *et al.*, 2011), and *Faecalibacterium prausnitzii* (an abundant and beneficial SCFA producer in the intestinal tract of humans, Sokol et al., 2008) has a vital requirement for this fatty acid *in vitro* (Duncan et al., 2002). A further study showed that administration of one strain of *Lactobacillus plantarum* (not commonly associated with production of SCFA) enhanced the concentrations of faecal SCFA in patients with recurrent *Clostridium difficile*-associated diarrhoea (Wullt et al., 2007). These observations have led scientists to believe that health-related effects of prebiotics may be due not only to increases in traditional lactic acid bacteria such as *Lactobacillus* and *Bifidobacterium* spp., but also of other more abundant populations of beneficial SCFA producers (Louis and Flint, 2009). Indeed, complex metabolite cross-feeding among the intestinal microbiota is likely to play an important role during health and disease (Flint et al., 2007).

The purported beneficial effect of prebiotics in atopic dermatitis or respiratory tract infections has been less researched. It is known that certain prebiotics (i.e. galactans) could lead to increased anti-inflammatory cytokines and phagocytosis and that these changes correlate with increased numbers of bifidobacteria (Vulevic et al., 2008). This and other studies suggest that prebiotics may lead to beneficial effects outside the intestinal tract by improving overall anti-inflammatory capacity of the immune system. It is important to note, however, that these beneficial effects may or may not be induced by prebiotics in all types of inflammatory or antigenic challenges (Bunout et al., 2002).

Extra-intestinal effects of prebiotics through growth stimulation of native microbiota

The use of prebiotics to selectively grow beneficial bacteria outside the intestinal tract has not been well documented, in part because we know comparatively little about other microbial ecosystems in the body. A decrease in costs of sequencing and availability of computational tools are being well placed to start analyzing other body parts such the oral cavity (Dewhirst et al., 2010), respiratory tract (Erb-Downward et al., 2011) and vagina (Cribby et al., 2008, Oakley et al., 2008). Most of these studies have been descriptive in nature.

The use of pro- and prebiotics in the prevention and treatment of oral diseases has been reviewed (Devine and Marsh, 2009). While the authors discuss the possibility that certain prebiotics could promote the growth of oral bacteria, they also pointed out a lack of consensus about what a healthy oral microbiota is and also the fact that traditional beneficial bacteria such as *Lactobacillus* and *Bifidobacterium* spp. have sometimes been linked to the aetiology of oral diseases (Matsumoto et al., 2005). As with any other microbial ecosystem in the body, more research is needed to first identify the members promoting and/or maintaining a helpful/harmless environment for the host, which could then be selectively targeted to improve health.

The authors of this review could not find well-designed, controlled human or animal intervention with prebiotics to promote the preferential growth of beneficial microbiota in the respiratory or the urogenital tract, although an improved encapsulation process for the preparation of pro- and prebiotics-loaded bioadhesive microparticles for vaginal delivery has been published (Pliszczak et al., 2011).

Adiposity and associated conditions

The rising incidence of metabolic disease, including obesity, type 2 diabetes and fatty liver disease, has put significant strains on health care systems worldwide (Wang et al., 2011). Given the recently described link between the gut microbiome and obesity (Turnbaugh et al., 2006; Herbert et al., 2011), there is growing interest in dietary manipulation of the gut microbiota in favor of a 'lean phenotype'. While prebiotics are well-recognized to have remarkable effects at the level of the gut, they also exert extra-intestinal effects that are particularly meaningful in the context of obesity and metabolic disease. The first of these is a reduction in adipose tissue mass that has been consistently shown across a variety of species and models.

Normal weight rats (Cani et al., 2005; 2007a; 2009; Reimer et al., 2012; Maurer et al., 2009), genetically obese mice (Canni et al., 2009), diet-induced obese mice and rats (Cani

et al. 2006a; 2007b; Pyra et al. 2012), as well as overweight and obese adults (Parnell and Reimer, 2009) have all been reported to exhibit reduced fat mass following consumption of prebiotics. A reduction in energy intake is certainly one explanation for some of the effects of prebiotics on weight loss. Rodent (Pyra et al., 2012; Parnell and Reimer, 2012) and human studies (Parnell and Reimer, 2009; Cani et al., 2006b) confirm that energy intake is reduced following prebiotic consumption and in part relates to an alteration in satiety hormones, including glucagon-like pepide-1(GLP-1), peptide YY (PYY) and ghrelin (Parnell and Reimer, 2009; Cani et al., 2006a,b). The mechanisms by which prebiotics affect weight loss, however, likely extend beyond global reductions in food intake and appear to target fat mass specifically. Several adiposity-reducing mechanisms of prebiotics have been suggested. Dewulf et al., (2011) showed that prebiotics are associated with a reduction in size of adipocytes. Enlarged adipocytes secrete high levels of free fatty acids and tumor necrosis factor (TNFα) which play a role in the development of insulin resistance (Hajer and van Haeften, 2008). Prebiotics reduce G-protein coupled receptor 43 (GPR43) expression in subcutaneous adipose tissue which is important given that GPR43 reduces lipolysis and stimulates lipogenesis (Dewulf et al., 2011). Furthermore, adipocyte fatty acid binding protein (aP2) a marker of terminal cell differentiation (Morrison and Farmer, 1999) was also restored to control levels in high fat diet-fed mice supplemented with prebiotics (Dewulf et al., 2011)). It is plausible that at least some of the adipose tissue-specific effects of prebiotics are mediated through metabolic end-products of gut microbial fermentation but this remains to be examined in greater detail.

Increased risk of developing type 2 diabetes and cardiovascular disease is a well-recognized health risk associated with obesity. Less well known is the increased risk of non-alcoholic fatty liver disease (NAFLD) which increases in parallel with obesity, type 2 diabetes and dyslipidemia (Byrne et al., 2009). While the steatosis associated with NAFLD can be benign, it can also progress to the more severe form of the disease, non-alcoholic steatohepatitis (NASH) and occasionally cirrhosis and liver failure (Byrne et al., 2009). Evidence for a role of gut microbiota in the pathogenesis of NAFLD is emerging. Conventionalizing germ-free mice has been shown to double triglyceride content in the liver (Backhed et al., 2004). Higher levels of lipopolysaccharide (LPS), a pro-inflammatory endotoxin derived from gram-negative bacteria, have been found in patients with NAFLD and NASH (Miele et al., 2009). There are also some indications that many of the 300 different volatile organic compounds (VOC) found in faeces, the majority of which result from bacterial metabolism, are altered in patients with NAFLD. Given that prebiotics target many of the pathologies associated with development of NAFLD, the question becomes whether or not prebiotics can be effectively used to prevent of treat NAFLD.

Parnell et al. (2012) reviewed current evidence for the role of prebiotics in treating and managing NAFLD and its associated co-morbidities. Evidence from animal studies provides support for the role of prebiotics in reducing de novo lipogenesis, improving major risk factors for NAFLD (obesity and insulin resistance) and reducing metabolic endotoxemia. For example, 10% (w/w) prebiotic in the diet reduced liver triglyceride content by ~40% in genetically obese rats (Parnell and Reimer, 2009) and 30% in diet-induced obese rats (Pyra et al., 2012). Human clinical studies evaluating prebiotic effects on NAFLD and NASH are lacking. One small study of seven patients with NASH saw improved aspartate aminotransferase and insulin levels following 8 weeks of 16 g/d oligofructose intake but hard endpoints derived from liver biopsy were not assessed (Daubioul et al., 2005). The first study to report pre- and post-intervention biopsy data was recently published by Malaguarnera et al. (2012) in which the combination of FOS with *Bifidobacterium longum* significantly reduced NASH activity index and steatosis, as well as other markers of inflammation such as C-reactive protein and tumor necrosis factor-α. Given this latest evidence and the promise of prebiotics in treating NAFLD and NASH, further large scale placebo-controlled trials are needed to confirm the role of prebiotics in ameliorating these diseases.

Prebiotic use in companion animals

In the USA, more than 60% of American households have at least one dog or cat, resulting in a pet population of approximately 78 million dogs and 86 million cats. It is estimated that 53 billion dollars will be spent on pets in the U.S. in the current year, with sales breakdown into food (21 billion), veterinary care (14 billion), supplies and medicine (13 billion), pet services (4 billion), and live animal purchase (2 billion; APPA, 2012). As dogs and cats have become family members, pet parents have shown increased awareness of the health status and well being of their animals. Pet owners seek products that will enhance health status, mitigate disease and increase longevity of their pets. Recent advances in companion animal nutrition have resulted in a wide array of foods for pet animals that include: therapeutic, breed-specific, physiological state (e.g., senior, puppy, weight management), organic, natural, and holistic diets. A common theme observed on the labels of diets in these different categories is the use of prebiotics.

In addition to feeding beneficial bacteria, depending on type and concentration provided, prebiotics also may affect gastric emptying, intestinal transit time, nutrient digestibility, faecal bulking or frequency of defaecation, SCFA, intestinal morphology and immune modulation. Prebiotics have gained considerable attention because the maintenance or establishment of a commensal gut microbiota is important for host gastrointestinal and systemic health. For example, gastrointestinal microbiota play an important role in decreasing the incidence or severity of gastrointestinal diseases, bacterial and viral infections, and some forms of cancers, as well as improving oral, skin and coat health.

Currently, FOS, GOS and lactulose are established dietary prebiotics. However, there are many other candidates as potential prebiotic sources. Examples include, but are not limited to, soybean oligosaccharides, gluco-, gentio-, isomalto-, xylo-, and mannan-oligosaccharides (MOS), lactose, lactosucrose, cyclodextrins, pectic oligosaccharides, polydextrose, sugar alcohols, resistant starch, and human milk oligosaccharides. In commercial pet foods, sources of prebiotics often used include fructans (FOS, inulin derived from the chicory root), MOS (yeast cell wall, brewers dried yeast), and beet pulp. Because the concept of prebiotics is relatively new (1995), few studies have been performed evaluating the effects of prebiotics in the canine, and even fewer in the feline. An online search (PubMed) using the key words "prebiotic and canine or dog" or "prebiotic and feline or cat" resulted in 30 and 15 research and review articles, respectively.

A meta-analysis evaluating the effects of prebiotic supplementation from 15 studies in dogs, published between the years of 1998 to 2007, concluded that feeding prebiotics to dogs had no effect on food intake, dry matter (DM) or fat digestibilities, and serum immunoglobulin concentrations (Patra, 2011). Crude protein (CP) digestibility tended to decrease quadratically, being lowest at prebiotic doses of 2.8% of diets ($P<0.01$). Prebiotic dose predicted faecal SCFA concentration ($r^2=0.9$) and increased faecal lactobacilli and bifidobacteria, which was also affected by initial bacterial population count levels. However, clostridia and *E. coli* populations were not affected by prebiotic supplementation. Overall, the meta-analysis suggested that feeding prebiotics up to 1.4% of DM intake was effective in increasing beneficial bacteria and SCFA production in the hindgut. It also indicated that prebiotic supplementation was most effective when initial numbers of the target beneficial bacteria were depleted (Patra, 2011).

The effects of increasing concentrations of polydextrose (0, 0.5, 1, and 1.5%), a potential prebiotic source, on faecal characteristics and microbial populations in adult dogs demonstrated that polydextrose supplementation did not impact on food intake or DM digestibility. A tendency to lower apparent CP digestibility was observed, but the digestibility value was still above 83%. Lower apparent CP digestibility is often observed as an artifact of prebiotic supplementation due to increased bacterial biomass being excreted in faeces. Increasing concentrations of polydextrose decreased pH in a linear fashion ($P<0.01$), tended to lower faecal indole concentration ($P=0.06$), increased faecal SCFA concentration ($P<0.01$), and decreased faecal *C. perfringens* counts ($P=0.02$). A linear effect of polydextrose was observed in faecal score ($P<0.01$), with dogs fed the highest concentration of polydextrose having the highest faecal score and, therefore, inclusion levels above 1.5% were not recommended. Overall, supplementation with polydextrose seems promising as a prebiotic source in diets for adult dogs, due to its beneficial fermentation properties, decreased faecal protein catabolite concentrations, decreased faecal pathogenic bacterial numbers and increased faecal SCFA concentration (Beloshapka et al., 2012).

The potential prebiotic effect of galactoglucomannan oligosaccharide (GGMO) was evaluated by *in vitro* fermentations using canine faecal inocula (Faber et al., 2011). In this study, different fractions of GGMO (crude or purified) with varying degrees of polymerization were tested (DP = 9-13, 6-8, and 2-5). In general, GGMO substrates decreased pH and produced greater SCFA concentrations when compared to control substrates, short-chain FOS and MOS. GGMO with a DP of 2-5 and 6-8 resulted in greater butyrate production ($P<0.05$). Increased butyrate is associated with gut health, since this SCFA is used primarily as an energy source by colonocytes. Crude and purified GGMO generated a greater *Bifidobacterium* spp. count ($P<0.05$). These data suggest that GGMO has potential as a prebiotic, but *in vivo* research is needed (Faber et al., 2011).

Similar to outcomes reported in dogs, food intake and DM digestibility were not affected when adult cats were fed diets containing 4% (w/w) cellulose, FOS, or pectin (Barry et al., 2010). Fat and CP digestibilities were decreased in the pectin treatment when compared to cellulose ($P<0.05$). It is likely that the reduction in nutrient digestibility was related to increased digesta viscosity and increased microbial mass due to the fermentative process. In agreement, faecal SCFA and branched chain fatty acid (BCFA) concentrations were greater for pectin when compared to cellulose ($P<005$). Faecal butyrate and BCFA concentrations also were increased with FOS ($P<0.05$). Faecal concentrations of ammonia, 4-methyl phenol, indole, and biogenic amines (e.g. cadaverine, putrescine, spermidine and tryptamine) were generally increased in the FOS and pectin treatments. The authors suggested that the observed increase in faecal protein catabolites could potentially be caused by interactions among saccharolytic and proteolytic bacteria present in the hindgut. Indeed, supplementation of 4% (w/w) pectin resulted in increased counts of *C. perfringens* ($P<0.001$) and *Lactobacillus* spp. ($P<0.03$) when compared to FOS or cellulose. In contrast, FOS had the greatest *Bifidobacterium* spp. count ($P<0.001$). This result was expected since FOS is a well established prebiotic. This study suggests that inclusion of 4% (w/w) of fermentable fiber is well tolerated by cats and may have a beneficial effect on gut health (Barry et al., 2010).

Another study evaluated the prebiotic effects of individual and combined dietary supplementation of low concentrations of short-chain FOS (scFOS) and GOS in adult cats (Kanakupt et al., 2011). The dietary treatments consisted of a control diet including 4% (w/w) cellulose and three test diets containing fermentable fibre sources added at the expensive of cellulose; i.e. 0.5% (w/w) scFOS, 0.5% (w/w) GOS, or 0.5% (w/w) scFOS + 0.5% (w/w) GOS. Supplementation of fermentable fibers did not affect DM, acid hydrolyzed fat or gross energy digestibilities. Cats fed the 0.5% (w/w) scFOS + 0.5% GOS diet had lower CP digestibility (82%, $P<0.01$). Faecal pH was lowest in cats fed the 0.5% (w/w) scFOS + 0.5% (w/w) GOS diet ($P<0.03$). Faecal butyrate concentration was greater in cats fed fermentable fibres when compared to the control

diet (P<0.02). Faecal *Bifidobacterium* spp. population also was increased in cats fed the fermentable fibre diets (P<0.004) and greatest in the 0.5% (w/w) scFOS + 0.5% (w/w) GOS diet (P<0.001). No changes in faecal protein catabolites, phenols, indole, ammonia or biogenic amines were observed in this study. While positive results were observed in some indices of gastrointestinal health, the authors suggested that dietary inclusion of these fermentable fibres above 0.5% (w/w) might be required to further improve the digestive health of adult cats, since the greater effectiveness of the 0.5% (w/w) scFOS + 0.5% GOS diet was attributed to be likely due to a dose response rather than a synergistic effect between the two prebiotic sources (Kanakupt et al., 2011).

In summary, dietary supplementation of prebiotics or potential prebiotic candidates seems to be effective in modulating indices of pet animal gastrointestinal health by increasing faecal SCFA production, faecal bifidobacteria, while decreasing *C. perfringens* and faecal protein catabolite concentrations. In addition, use of prebiotics in pet food did not lead to detrimental effects on food intake or nutrient digestibility. In the future, it will be important to determine the minimal effective dose of prebiotic supplementation. A detailed compositional characterization of potential prebiotic sources also is warranted. Determining the monomeric composition, chain length, linkages, branching, and side chains of these substrates would be useful. With the increasing interest of pet owners in natural and holistic foods, the prebiotic activity of natural food and whole grains and their co-products also should be investigated. A better understanding of the microbiome and its metabolic functions is required, as well as characterization of the microbiome-host interaction in both health and pathological conditions.

Clinical applications of prebiotics

Through their effects on gastrointestinal function, immunology and mineral absorption, prebiotics could have a range of applications in the clinical setting, including in the management of gastrointestinal, inflammatory, infectious, paediatric and obesity-related disorders. There is an extensive literature relating to these that have been thoroughly reviewed elsewhere (Roberfroid et al., 2010). Here, the impact of prebiotics in a limited number of disorders will be discussed.

Irritable bowel syndrome (IBS) is characterised by abdominal pain, bloating and changes in stool frequency/consistency in the absence of an organic cause. It is a common disorder, with a prevalence of 10% - 20% in developed countries, resulting in reduced quality of life and exerts a considerable economic burden due to increased absenteeism and utilisation of healthcare services. As such, approaches to managing IBS that are effective, patient-led and avoid the use of medication would have considerable advantages.

The pathogenesis of IBS is multifactorial and includes a role for the gastrointestinal microbiota, with a higher risk of following gastroenteritis and elevated luminal gas production. Numerous studies report luminal dysbiosis in IBS and interestingly there is evidence of a negative correlation between the numbers of gastrointestinal bifidobacteria and the incidence of abdominal pain in both healthy subjects (Jalanka-Tuovinen et al., 2011) and patients with IBS (Parkes et al., 2012). Consequently, prebiotics are an attractive therapeutic option.

There are at least four randomised controlled trial (RCT) of different prebiotics at different doses. One demonstrated that high doses (20 g/d) of FOS increased symptoms in the short-term (4-6 weeks), which were adapted to in a longer term (12-weeks), although at no time point were symptoms improved by the prebiotic (Olesen et al., 2000). A small cross-over trial of a much smaller dose (6 g/d) of FOS also reported no difference in symptoms between the prebiotic and control (Hunter et al., 1999). However, a larger study of a similarly low dose (5 g/d) of FOS in patients with functional bowel disorders reported improvements in global symptoms (Paieneau et al., 2008). The most recent was an RCT of two doses of B-GOS which reported a significant improvement in global IBS symptoms, bloating and flatulence in the low dose group (3.5 g/d), but a worsening of bloating in the high dose group (7 g/d) (Silk et al., 2009). Importantly, this study also demonstrated increases in luminal bifidobacteria following prebiotic supplementation (Silk et al., 2009).

These data suggest that both the properties and dose of the prebiotic are important in determining effects on IBS symptoms. Any negative effect on symptoms is likely to be mediated through their impact on luminal gas production following fermentation, with prebiotics, or doses, that produce most gas likely impacting on bloating symptoms. Indeed, there is evidence that reducing dietary intake of fermentable carbohydrates, including prebiotic fructans and galactans, may actually improve symptoms in IBS (Staudacher et al., 2011), although a reduction in luminal bifidobacteria has been reported with this approach (Staudacher et al., 2012).

Another chronic disorder of the gastrointestinal tract is Crohn's disease, which is a relapsing and remitting inflammatory bowel disease characterised by transmural inflammation. The gastrointestinal microbiota are known to play a pivotal role in disease pathogenesis with extensive evidence that microbial colonisation initiates disease in genetically susceptible animal models. Dysbiosis of the microbiota in Crohn's disease has also been described and in particular the immunoregulatory *Faecalibacterium prausnitzii* is lower in people with inflammatory bowel disease (Sokol et al., 2009).

Two large RCTs have recently been published both indicating that prebiotics were unable to manage Crohn's disease. The first reported that FOS (15 g/d) did not result in any difference in disease activity in patients with mild to moderately active disease, and indeed increased bloating in some patients (Benjamin et al., 2011). Interestingly it was also found not to have impacted on luminal bifidobacteria and therefore the

prebiotic potential of such a mixture in this clinical context cannot be confirmed (Benjamin et al., 2011). However, a study using the same oligofructose/inulin at a higher dose (20 g/d) but in patients with inactive or mildly active disease also found no differences in overall disease activity (Joosens et al., 2012), although within-group increases in *B. longum* occurred in the prebiotic group. In both studies, more patients withdrew from the prebiotic groups.

In developed countries, probiotics are widely available and widely used, in particular by patients with IBS and Crohn's disease, as they are viewed as healthy, natural additions to drug management (Mercer et al., 2012). However, the picture for prebiotics is considerably different. In a study of patients with inflammatory bowel disease only 4% of patients with Crohn's disease, 2% of patients with ulcerative colitis and 1% of healthy people had ever used a prebiotic supplement (Hedin et al., 2010). Understanding of the term "prebiotic" was also very poor, with less than 5% of people being able to correctly describe a prebiotic as being a food/fibre supplement or that it stimulated bacteria or that it had a health benefit (Hedin et al, 2010). There is a major need for improvements in understanding of prebiotics amongst both healthy people and patients who may be able to benefit from them.

Prebiotics as anti-adherence agents.

As noted above, prebiotics are defined as a "selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health" (Gibson et al., 2010). However, it has been suggested that another mechanism by which prebiotics may deliver health benefits is via their ability to interfere with the infection process used by bacterial pathogens (Gibson et al., 2005; Shoaf-Sweeney and Hutkins, 2008; Kunz et al., 2000; Hotchkiss and Buddington, 2011). This activity is mediated by virtue of the structural similarity prebiotics and other oligosaccharides share with pathogen receptor sites located on the surface of epithelial cells. It is now well recognized that for many enteric bacteria to cause infections, they must first attach to the surface of epithelial cells lining the intestinal tract (Klemm et al., 2010; Bavington et al., 2005). Attachment occurs via lectin-like adhesins that recognize specific carbohydrate receptors on the surface of host cells. In general, bacteria that do not express functional adhesins, cannot adhere and initiate infections (Boddicker et al., 2002; Cleary, 2004). Therefore, averting bacterial attachment through disruption of adhesin-oligosaccharide interactions has emerged as an important strategy for reducing the incidence of gastrointestinal infections (Sharon, 2006).

Both food-grade, commercial prebiotics as well as oligosaccharides derived from plant and other biological materials have been evaluated for anti-adherence activity against a range of enteric pathogens. In particular, galactooligosaccharides (GOS), pectic oligosaccharides, and mannan oligosaccharides (MOS) have been reported to inhibit pathogen binding to the surface of tissue culture cells in vitro (Shoaf et al., 2006; Rhoades et al., 2006, 2008) and to reduce colonisation in animal studies (Ghosh and Mehla 2012; Fernandez et al., 2000; 2002). Other oligosaccharides, including those derived from chitin and bovine colostrum, have also been reported to have anti-adherence activity (Quintero-Villegas et al., 2013).

Mannanoligosaccharides as anti-adherence agents

The one group of oligosaccharides that have attracted the most commercial attention in animal production for their potential anti-adhesive activity is the mannanoligosaccharides (MOS). The MOS are derived from food-grade yeast cell walls and are rich in mannan. Konjac root is also mannan-rich, as are other seeds and plant materials (Becker and Galletti, 2008; Kogan and Kocher, 2007; Tester and Al-Ghazzewi, 2012). Importantly, mannan contains α-linked mannose residues that are known to inhibit the adherence of many enteric pathogens, including *Salmonella* and *E. coli* (Sharon and Ofek, 1986). Thus, MOS products are often included in feed rations for swine, poultry, and beef cattle, (Ghosh et al., 2012; Ghosh and Mehla 2012; Tester and Al-Ghazzewi, 2012). Although fewer *in vivo* studies have been conducted with MOS, results suggest inhibition of type 1 fimbrial adhesin of enterobacterial pathogens does occur (Ganner and Schatzmayr, 2012). Recently, Verbrugghe et al., (2012) reported that mannan treatment reduced *Salmonella* in the caecum and caecal contents. *In vitro* assays led the investigators to conclude that this reduction was due to binding inhibition.

Pectic oligosaccharides as anti-adherence agents

Recently, there has been interest in oligosaccharide fractions derived from pectin (POS). Pectin consists of a galacturonan backbone with rhamnogalacturonan regions substituted with arabino- and galacto-oligosaccharides; enzymatic hydrolysis results in formation of several biologically active POS. In one study, researchers showed that POS inhibited invasion of *Campylobacter jejuni* on Caco-2 cells, leading the authors to suggest that POS could be used as an alternative to antibiotics for controlling this microorganism (Ganan et al., 2010). Similarly, adherence of verotoxigenic *E. coli* and enteropathogenic *E. coli* to HT29 cells was reduced in the presence of POS (Rhoades et al., 2008). Recently, the Hutkins lab showed that a pectin-rich cranberry extract also reduced *Salmonella* adherence (unpublished data). While not a pectin, cranberry xyloglucan oligosaccharides inhibited the adhesion of uropathogenic and verotoxigenic *E. coli* to T24 and HT29 cells, respectively (Hotchkiss et al., 2012). In the future, identifying which specific fractions of POS (and other oligosaccharides) contain anti-adherence activity could lead to enriched fractions having high activity.

Chitin oligosaccharides as anti-adherence agents

Chitooligosacharides (CHOS) appear to have high adherence inhibition activity. One previous study showed

that a non-defined CHOS mixture of degree of polymerisation (DP) of about 4 and 3% acetylation inhibited adherence of three different strains of enteropathogenic *Escherichia coli* (EPEC) on HT-29 cells (Rhoades et al., 2006). Qunitero-Villegas et al (2013) reported that CHOS fractions, purified on the basis of acetylated residues and DP, inhibited adherence of enteropathogenic *Escherichia coli* (EPEC). Although hydrolysates with lower acetylation were more effective at reducing adherence, CHOS ranging from DP 4 to DP 12 were equally effective.

Commercial applications of oligosaccharides in animal agriculture

The complete ban of all growth-promoting antimicrobials in the European Union has had a major influence on the animal feed market and has dramatically increased research and product development efforts (Market Research.com). Worldwide, companies producing feed additives have aggressively embraced the prebiotic concept and have been supplying prebiotics for swine, poultry and cattle applications. However, there are relatively few mechanistic studies to assess these products and their *in vivo* physiological or biochemical activities.

CONCLUSION: EXPANSION OF THE PREBIOTIC CONCEPT

The role of diet in the ecology and function of the gut microbiota is becoming more widely recognised as important, particularly in terms of a downstream impact on health with several recent reviews on the topic (Jeffery and O'Toole, 2013; Scott et al., 2013; Yuan-Kun, 2013).

We consume prebiotics in the context of a diet and whilst both macronutrients and micronutrients all have the potential to influence host-microbe interactions, carbohydrates and particularly plant cell wall carbohydrates have received considerable attention in the past (Flint et al., 2008; Flint et al., 2012).

Our gut bacteria have co-evolved, and are metabolically integrated, with us and are also known to be able to affect gut health adversely (Ferguson et al., 2007) or beneficially (Barrett et al., 2008). 'Who is there' may vary quite widely between individuals, as might 'how they do it', but 'what they make' may be less variable. Many different individual species of bacteria can perform the same saccharolytic functions and so the availability of substrates (host or diet-derived) along with the degradative enzymes they possess may be key drivers of gut ecology.

Species other than LABs may be important.

It is important to understand the complex chemical structures found in plants and how these could be linked to the diverse metabolic activity of beneficial commensals. There are early data to suggest that a particular bacterial family called 'Lachnospiraceae', which belong to clostridial cluster XIVa (Collins, et al., 1994), play important roles in maintaining the structure and function of bowel communities. This is supported by the observation that members of clostridial cluster XIVa form 40% of the microbiota in healthy adults and of these, 30% belong to the Lachnospiraceae (Frank et al., 2007; Tannock et al., 2010). There are early results to demonstrate that plant carbohydrates selectively increase members of this family of bacteria in vitro (Rosendale et al., 2012).

Cross-feeding and co-metabolism

Interactions between dietary carbohydrates, the gut microbiota and resulting metabolic end-products have been reviewed previously (Louis et al., 2007) with detailed hypotheses regarding the underlying mechanisms of carbohydrate utilization (Flint et al., 2008; Flint et al., 2012). Essentially, it is not sufficient to just measure what is there or the by-products produced, since a very complex web involving multi-stage fermentation and differential utilization of the original carbohydrate source, as well as intermediary breakdown products from the original source, exists. An individual's gut is home to an array of carbohydrate-degrading mechanisms employed by so-called primary feeders, those members of the microbiota able to ferment the substrate directly, and subsequent secondary feeders that make use of released oligosaccharides or fermentation by-products from the primary feeders.

Whole foods and complexity... or diversity?

If we accept that a diverse metabolic capacity in one's microbiota is advantageous then there is some weight to the argument that microbial diversity is likely to be associated with health. Certainly, some studies have demonstrated this particularly in elderly patients (Lakshminarayanan et al., 2013) although it is difficult to separate out confounding lifestyle factors such as diet, antibiotic treatment and other environmental exposures. It is also not a huge leap of faith to suppose that a diet rich in a variety of plant cell wall carbohydrate sources should stimulate a diverse microbiota and health. Simultaneous measurement of microbial ecology using next generation sequencing, metabolism analysis along with assessments of chemical changes to the kiwifruit carbohydrates present in the fermenta effectively closes the circle on substrate usage/degradative enzymes possessed/microbes present/microbial by-products produced. Certainly an increase in diversity has been seen in response to fermentation of the plant cell wall carbohydrates found in kiwifruit *in vitro* (Rosendale et al., 2012) but more research is warranted to further understand the links between diet, microbes and health.

CONFLICT OF INTEREST DISCLOSURE

Authors declare no conflicts of interest that have affected the content of this manuscript.

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