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Developments in understanding and applying prebiotics in research and practice – an ISAPP conference paper.

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Running title –Updating prebiotics in practice

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ABSTRACT

Aims

The concept of using specific dietary components to selectively modulate the gut microbiota to confer a health benefit, defined as prebiotics, originated in 1995. In 2018, a group of scientists met at the International Scientific Association for Probiotics and Prebiotics annual meeting in Singapore to discuss advances in the prebiotic field, focussing on issues affecting functionality, research methodology, and geographical differences.

Methods and Results

The discussion ranged from examining scientific literature supporting the efficacy of established prebiotics, to the prospects for establishing health benefits associated with novel compounds, isolated from different sources.

Conclusions

While many promising candidate prebiotics from across the globe have been highlighted in preliminary research, there are a limited number with both demonstrated mechanism of action and defined health benefits as required to meet the prebiotic definition.

Prebiotics are part of a food industry with increasing market sales, yet there are great disparities in regulations in different countries. Identification and commercialisation of new prebiotics with unique health benefits means that regulation must improve and remain up-to-date so as not to risk stifling research with potential health benefits for humans and other animals.

Significance and Impact of Study

This summary of the workshop discussions indicates potential avenues for expanding the range of prebiotic substrates, delivery methods to enhance health benefits for the end consumer, and guidance to better elucidate their activities in human studies.

Keywords: Prebiotics, ISAPP, gut fermentation, microbiome, health benefits

Introduction

Prebiotic discovery and definitions

Prebiotics were originally instigated as dietary means of altering the human colonic microbiota towards a more favourable community structure (Gibson and Roberfoid 1995). Initially, most studies focussed upon inulin type fructans that exerted selective stimulation of gut bacterial genera, notably bifidobacteria, after a short feeding period. This occurred in a variety of human intervention studies (Roberfroid *et al.* 2010), at doses ranging from 4-30g/day in adults. At that stage, any dietary material that entered the large intestine was considered a candidate prebiotic. This included carbohydrates such as resistant starch and dietary fibre as well as proteins and lipids. More than 20 years on, recognised prebiotics (definition Box 1; Table 1) remain mostly confined to non-digestible oligosaccharides, some of which confer the degree of fermentation selectivity that is required to support beneficial intestinal microbes. Oligosaccharides are carbohydrates consisting of between approximately 2 and 10 saccharide units while polysaccharides consist of 10 or more saccharide units. Oligosaccharides occur naturally in several foods but can also be commercially produced through the hydrolysis of polysaccharides (e.g. dietary fibres, starch) or through catabolic enzymatic reactions from lower molecular weight sugars.

In 2004, the concept of prebiotics was expanded to encompass beneficial effects on all aspects of the gastrointestinal tract, not just the colon (Gibson *et al.* 2004) with three criteria being required for a substance to be defined as a prebiotic:

- resist gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption
- fermentation by intestinal microbiota, and

- selectively stimulate the growth and/or activity of intestinal bacteria associated with health and well-being

In 2017, International Scientific Association for Probiotics and Prebiotics (ISAPP) published a consensus view on prebiotics refining the definition to "a substrate that is selectively utilised by host microorganisms conferring a health benefit" (Gibson *et al.* 2017). The main points within this refinement were that the microbes responding to prebiotics should be health promoting bacteria, without specifying which. Moreover, the site of effect could be any mixed microbial community associated with humans or animals (not limited to the gastrointestinal system), but those effects need to be confirmed *in vivo* with the target host. The gastrointestinal tract (GIT) runs from the oral cavity to the rectum, however previous definitions, and subsequent research, focussed on the lower parts of the GIT. The new definition deliberately avoids specifying the intestine, opening up other targets containing a mixed microbiome, such as the urogenital tract, skin and the upper GIT including the mouth. Another point was that prebiotics require selective metabolism by live host microorganisms to improve or restore host health. This brings into play the importance of assessing microbial function as well as composition, in combination with appropriately validated health biomarkers.

Current prebiotics and candidate prebiotics

In order to fully classify a substance as a prebiotic, reproducible randomised controlled studies establishing direct links between the prebiotic and health are needed in the specific target host. Some candidate prebiotics lack data confirming that the compound confers a health benefit in humans (Table 1). However, compounds that do not act as prebiotics for humans may be effective prebiotics for animals.

Non-digestible carbohydrates that are confirmed prebiotics, meeting the above criteria and which have proven effects in human studies are fructans and galactans. Inulin-type fructans (ITF) contain a terminal glucose residue with a β-linkage to a chain of β-linked fructose residues in the form Glu α1-2[β fru 2-1]n. Individual ITF are distinguished by their degree of polymerization (DP - the number of monomers in the chain), with the number of fructose units ranging from 2 to 70. Short-chain ITF, known as oligofructose or fructo-oligosaccharide (FOS) generally have a DP less than 10. ITF of all chain lengths are important prebiotic substrates with well documented effects on intestinal bifidobacteria. Most bifidobacteria break down and utilize FOS due to possession of a competitive β-fructofuranosidase enzyme (Imamura *et al.* 1994), expressed at high levels by bifidobacteria in mixed culture.

Galactans, or galacto-oligosaccharides (GOS) are galactose-containing oligosaccharides of the form Glu α1-4[β Gal 1-6]n where n ranges from 2 to 10, and are produced from lactose syrup using the transgalactosylase activity of the β-galactosidase enzyme. This can be sourced from several microorganisms such as yeast, bacilli, bifidobacteria and lactobacilli.

Sources of prebiotics

Established and novel plant food sources of prebiotics

Compounds with prebiotic activity occur naturally in many whole foods, including leek, asparagus, garlic, onion, wheat and bananas (van Loo *et al.* 1995) (Table 1). ITF can be extracted from several food crops, mainly root vegetables and tubers, e.g. chicory root and Jerusalem artichoke. Other carbohydrate components in plants with prebiotic potential include polysaccharides in plant cell walls, e.g. xylans, pectins. These components are gaining popularity as candidate prebiotics due to their indigestibility in

the upper gastrointestinal tract and fermentation by the colonic microbiota. Plants that have been reported as good sources of indigestible carbohydrates, with possible prebiotic properties include dandelion green, dahlia tuber, garlic, shallot, yacon, okra, gourd-type vegetables, mushrooms and barley. Legumes are rich in dietary fibre, some of which have potential as prebiotics. Lupin and chickpea kernel fibre stimulates colonic bifidobacterial growth and contributes to colon health while chickpea grains are a good source of α-galacto-oligosaccharide (Table 1) (Smith *et al.* 2006).

Most plants with investigated prebiotic potential are of western origin, and it is important to explore other parts of the world, particularly the Asian region, as a source of novel prebiotics. Asia is the largest and most populous continent on earth, with extremely diverse biological resources. One interesting source is sago starch, from palm (*Metroxylon sagu*) indigenous to South-East Asia. In its native form it is called *lemantak* and contains about 60% resistant starch (Arshad *et al.* 2018). *In vitro* and *in vivo* studies have demonstrated the ability of sago starch to increase numbers of *Lactobacillus* and *Bifidobacterium* spp. (Arshad *et al.* 2018). Studies on the effects of resistant starch on glycemic index, insulin responses, and satiety clearly demonstrate its role as a functional food (Zaman and Sarbini 2016). Another potential 'prebiotic crop' abundantly grown in Asia, particularly India, is lentils. The non-digestible carbohydrate content of lentils is approximately 13% (Johnson *et al.* 2013).

In addition to terrestrial plants, aquatic sources such as seaweeds are cultivated in East Asia and South-East Asia for carrageenan. The complex structure of this polysaccharide makes it resistant to mammalian enzymatic degradation, however it is fermented by the colonic microbiota. The red seaweed (*Kappaphycus alvarezii*) led to significant increase of *Bifidobacterium* spp. numbers and acetate and propionate concentrations during *in vitro* fermentations (Bajury *et al.* 2017). It will be interesting to

establish the potential of these plants as emerging and novel prebiotic ingredients. Human studies demonstrating *in vivo* activity and conferred health benefit are obvious next steps.

Specific plants can be used both as sources of prebiotic compounds and added to the diet as whole foods with potential prebiotic effects monitored. Kiwifruit are a rich source of soluble and insoluble fibre, composed of pectic polysaccharides, cellulose and hemicellulose (Carnachan *et al.* 2012; Sims *et al.* 2013). A number of *in* vitro and *in vivo* studies (Han *et al.* 2011; Paturi *et al.* 2014; Blatchford *et al.* 2015) have investigated the ability of whole, fresh kiwifruit to modulate the colonic microbiota and the generation of metabolites such as short-chain fatty acids (SCFA). Although such changes in SCFA or microbial populations have not been directly linked to a health benefit, they can suggest shifts in gastrointestinal function that may indirectly impact physiological processes.

A human intervention trial with green kiwifruit in Italian participants with Irritable Bowel Syndrome constipation type (IBS-C) assessed the effect on the composition of the faecal microbiota. After intervention, kiwifruit significantly reduced Clostridiaceae and Streptococcaceae in patients with IBS-C, while both kiwifruit and psyllium significantly increased the levels of Lachnospiraceae in patients with IBS-C (Cremon *et al.* 2018). These findings showed that kiwi fruit consumption could alter microbial composition, however, direct evidence of any improved health outcome remains outstanding.

Whole foods such as kiwifruit can also contain non-carbohydrate compounds which may act synergistically with other prebiotic compounds present in the food. SCFA production is often attributed exclusively to the fermentation of dietary fibre and prebiotics. However, an *in vitro* study using a simulated intestinal ecosystem found that

SCFA production correlated with both the total fibre content and a range of polyphenolic compounds present in kiwifruit (Parkar *et al.* 2017).

Cereal plants such as oats and barley contain β-glucan, a water-soluble non-starch polysaccharide that is found mainly on the bran and concentrated in the aleurone and subaleurone layers. β-glucan in cereal plants is a linear polymer comprised of D-glucose joined by β-(1,3;1,4)-glycosidic bonds, that imparts resistance to digestion in the upper GI tract of mammals. Consequently, it is fermented in the large intestine by the gut microbiota. Oats comprise 3-8% β-glucan, of which approximately 80% is water-soluble. Barley comprises 2-20% β-glucan, with a lower water-soluble fraction (65%) compared to oats (El Khoury *et al.* 2012). A high level of solubility renders oat bran an excellent source of β-glucan for gut bacterial fermentation. *In vitro* and *in vivo* studies showed that oat β-glucan could selectively stimulate the growth of lactobacilli and bifidobacteria, leading to the production of acetic and lactic acids (Charalampopoulos *et al.* 2002). These characteristics qualify oats as a candidate prebiotic. Much of the research, to date, has concentrated on yeast or fungi-derived β-glucan that modulate the immune system. Further investigations into the activity of cereal β-glucans in human studies are needed to establish their prebiotic potential.

Selection of 'nutritious' cereal and other foods crops through specific breeding and understanding of environmental and genetic factors affecting prebiotic carbohydrate content is of importance (Johnson *et al*. 2013). These may allow improved selection of prebiotic crops for mass production. A study on wheat grain demonstrated that certain cultivars had high levels of grain fructan, with advanced lines containing > 2% (Huynh *et al.* 2008). There are also huge differences in dietary fibres between modern and ancient durum wheat cultivars (Marotti *et al.* 2012). *In vitro* research further demonstrated that the soluble dietary fibre from ancient durum wheat

selectively proliferated microbial growth of *Bifidobacterium pseudocatenulatum* B7003 and *Lactobacillus plantarum* L12 strains (Marotti *et al.* 2012). Enhanced breeds of sweet wheat had about 7 times higher concentration of soluble dietary fibre i.e. fructan, in comparison to wild-type lines (Nakamura *et al*. 2006). Oat and barley cultivars are being bred with increased β-glucan content to reduce the amount required to achieve a cholesterol lowering effect.

Non-carbohydrate prebiotic components in plants

Currently, most accepted prebiotics are carbohydrates that bacteria utilise for growth, with direct or indirect effects on host health. However, as observed with kiwifruit, other compounds present in plants may also be metabolised by bacteria, releasing components that may be beneficial for health and thus 'fit' the current definition of prebiotics

Dietary polyphenols are natural compounds occurring in plants; available in fruits, vegetables, cereals, tea, coffee and wine. Most polyphenols are of low bioavailability, and their influence on health may be either through intestinal absorption or interaction with colonic microbiota, depending on their structural complexity and polymerisation. About 5 to 10% of total polyphenol intake is absorbed in the small intestine (i.e. low-molecular-weight polyphenols, the monomeric and dimeric structures). The remaining polyphenols (oligomeric and polymeric polyphenols) may accumulate in the large intestine where they are subject to enzymatic activities of the gut microbiota (Cardona *et al.* 2013).

The Asian region offers plentiful polyphenol-rich herbs and spices that have been used as traditional medicines since ancient times. A study observed increased Bacteroidetes compared to Firmicutes through use of polyphenols from black tea

(*Camellia sinensis*) in simulated intestinal microbial ecosystems (Kemperman *et al.* 2013). Turmeric root (*Curcuma longa*) is widely used as condiment in Asian food as well as a traditional remedy in Chinese and Indian Ayurvedic medicine. Curcuminoids are metabolised by colonic microbiota, modulating bacterial populations and their metabolic activity (Lu *et al.* 2017). The black pepper (*Piper nigrum* L.) is well-known for the presence of high bioactive compounds e.g. piperine that are anti-inflammatory, anti-oxidants and anti-bacterial, as well as containing up to 40% dietary fibre, making the species an interesting prebiotic candidate (Lu *et al.* 2017).

Components of the gut microbiota such as *Escherichia coli*, *Bifidobacterium* spp., *Lactobacillus* spp., *Bacteroides* spp., *Eubacterium* spp. may catalyse the metabolism of phenolics into lower molecular weight phenolic metabolites that may exert health effects on the host (Duncan *et al.* 2016). Attributed health properties include protection against gastrointestinal disorders, nutrient processing, reduction of serum cholesterol, reinforcement of intestinal epithelial cell-tight junctions, and modulation of the intestinal immune response through cytokine stimulation (Cardona *et al.* 2013). However, the effect of dietary polyphenols on modulation of the gut environment and its underlying mechanisms is poorly understood, thus more research is needed, particularly *in vivo*.

Delivery of prebiotics

Incorporation of prebiotics into functional foods

Due to the dose of prebiotic required to exert an effect on health, it is not always feasible for an oligosaccharide to exert a profound prebiotic effect through elevated ingestion of whole foods. Another possible route towards success is the fortification of more frequently consumed foods – which is the premise of functional foods. Functional

foods provide health benefits beyond the nutritive value of the food. Many definitions of 'functional food' have been proposed by various entities such as International Food Information Council (IFIC), International Life Sciences Institute (ILSI), Health Canada and Nutrition Business Journal. However, the general consensus definition is "a food or dietary component that can exert health benefits and/or disease prevention, beyond basic nutritional needs". Functional foods affect various specific functions in the body due to active ingredients that are naturally present or added to the food, e.g. vitamins and minerals, cholesterol lowering ingredients, fibres, antioxidant properties, beneficial microbes and prebiotics. Prebiotic-containing functional foods can take many dietary forms including yogurts, cereals, bread, biscuits, energy bars, milk desserts, ice-creams, spreads, infant formulae and others.

Effect of food incorporation on prebiotic activity

It is relevant to address whether functionality of the prebiotic is affected by the final food matrix, particularly since research determining prebiotic activity is often carried out on a prebiotic in a powdered form.

During research on the beneficial effects of prebiotics for cardiometabolic health, galactoligosaccharides (B-GOS) as a powdered supplement, resulted in significant increases in bifidobacteria and reduction in some Gram-negative genera, in a cohort of 45 overweight adults. These changes were concomitant with reduced markers of metabolic syndrome and decreased insulin, total cholesterol, triacylglycerides (TAGs), and the total cholesterol to high density lipoprotein (HDL) ratio. It was concluded from this study that prebiotic-induced changes in the gut microbiota contributed to the positive outcomes observed (Vulevic *et al.* 2013).

Subsequently two double–blind, placebo-controlled, randomised cross-over studies utilised B-GOS incorporated into functional foods- B-GOS enriched orange juice and bread. Effects on the faecal microbiota and on markers of metabolic syndrome and associated inflammation were investigated. The interventions enrolled 29 volunteers and 30 volunteers on juice and bread respectively, at the same doses used in the previous supplement trial. The final products were well tolerated by sensory taste panels and their prebiotic effects confirmed using an *in vitro* gut model system (Costabile *et al.* 2015a, 2015b). Results of the intervention demonstrated that neither the enriched orange juice or bread products elicited the range of benefits seen with the powdered prebiotic, although the bread showed some potential to modulate the inflammatory response. The pro-inflammatory IL-6 increased following consumption of placebo breads over 12 weeks, but this effect was negated by B-GOS bread. As not all volunteers on the study had dyslipidaemia, or raised insulin levels, a modification was not always apparent. However, by stratifying the volunteers, and grouping those with starting triglyceride levels of over 1mmol/l, the prebiotic in the juice study did reduce this. Major modifications in other lipid levels and blood sugar levels were not observed.

There are a number of potential explanations for the observed differences, some of which relate to the study product itself. In the production of functional foods, additional manufacturing processes such as heating, spray-drying and freeze-drying may affect the structure and influence availability of the prebiotic. Once functional foods are produced, transportation and storage may also affect prebiotic composition, where fluctuations of temperature and moisture can be a concern. Preparation of prebiotic-containing foods in the home, such as cooking, may also influence bioavailability or function in the host. Furthermore, other fermentable components present (such as

pectin, starches, xylans) could potentially compete with or otherwise influence microbiome fermentation.

It is essential to consider all of these factors when assessing the prebiotic status of foods available to consumers and highlight the importance of complete testing of the finished product, to confirm prebiotic activity.

Beyond intrinsic efficacy of a prebiotic product, other factors influence the optimal choice of prebiotic format for any given individual, be it whole foods, functional foods, or supplements (Figure 1). For any given individual, one type of delivery form may be preferred depending on factors such as convenience, cost, dietary and food preparation habits and preferences, palatability, health knowledge around the role of prebiotics, and values and cultural practices surrounding food and supplementation. Perceptions of palatability and convenience may vary significantly between individuals and will influence the degree to which they can maintain the intervention, in both the short and long term. When foods are used to deliver prebiotics, daily dosage may be more variable due to dietary fluctuations, although the impact of this on long term interventions is currently unknown.

The importance of individual preferences in the acceptance and implementation of therapeutic interventions with prebiotics cannot be discarded when assessing optimal methods for delivery, as these, along with the intrinsic qualities of the therapy, will be what ultimately determines successful health outcomes.

Understanding prebiotic mechanisms – towards effective characterisation and measurement

Therapeutic effects of prebiotics

There is now strong evidence that the composition of the gut microbiota is altered in many diseases, including inflammatory bowel disease (IBD), obesity and irritable bowel syndrome (IBS). Importantly, the intestinal microbial population is accessible to dietary and therapeutic intervention and thus represents an exciting target for the prevention and treatment of many disorders.

In accordance with this, the health potential of prebiotics are wide-reaching, currently including (Gibson *et al.* 2017) the gastrointestinal tract (e.g. inhibition of pathogens, immune stimulation), cardiometabolism (e.g. reduction in blood lipid levels, effects upon insulin resistance), mental health (e.g. production of metabolites that influence brain function, energy and cognition) and bone (e.g. mineral bioavailability) (Figure 2). These health effects are mediated by a variety of mechanisms, including changes in microbiome composition and levels of microbial metabolites, including short chain fatty acids.

Impacts on the microbiome – *in vitro* and *in vivo*

Increased knowledge into the composition of the gut microbiota in the last 20 years has shown that the original targets of prebiotics (bifidobacteria and lactobacilli) are minor components of the gut microbiota, which is dominated by Bacteroidetes and Firmicutes, including Lachnospiraceae and Ruminococcaceae. Prebiotics also change metabolic output, specifically increasing butyrate concentrations (Riviere *et al.* 2016), yet neither bifidobacteria nor lactobacilli produce butyrate. It is now known that ITF not only stimulate bifidobacterial numbers, but can also result in increased numbers of

Faecalibacterium prausnitzii (Ramirez-Farias *et al.* 2009) while high molecular weight arabinoxylans stimulated *Roseburia* species (Neyrinck *et al.* 2011). *F. prausnitzii* and *Roseburia* spp. are among the most abundant butyrate producers in the human gut (Louis *et al.* 2010).

Studies in pure culture have confirmed the ability of various bacterial genera to utilise prebiotics as growth substrates (Scott *et al.* 2014). In fact, several researchers have shown that the more complex ITF are utilised more effectively by other bacteria in monoculture than they are by bifidobacteria (reviewed in De Vuyst and Leroy 2011). However, effects in monoculture may not translate to effects *in vivo* as other factors can impact on the ability for prebiotics to support beneficial changes. For example, different individuals tend to be colonised by different specific species of bifidobacteria, and within the *Bifidobacterium* genus there is considerable variation in the ability of different species to utilise different chain lengths of ITF (Selak *et al.* 2016). Therefore, individual variation in bifidobacterial species colonisation has an important impact on the potential effects of prebiotics, which may help explain why there may be responders and non-responders within studies. In a study involving 18 subjects consuming different doses of GOS daily, bifidobacterial population only increased in nine individuals, the responders (Davis *et al.* 2010). Clearly, if the original bacterial population does not contain the specific bacterium capable of utilising the added prebiotic substrate, the population cannot increase.

Another important consideration is that gut bacteria exist in a competitive, mixed ecosystem, meaning that data from pure culture experiments does not translate into an ability to utilise the same substrate in a competitive environment. Bacterial cross-feeding is also an important consideration in determining which bacteria can be stimulated by prebiotics. In a competitive environment, some bacteria are efficient

scavengers of oligo- and mono-saccharides released following degradation of longer chain molecules by other members of the microbiota. Other bacteria rely on metabolites released by primary degraders for growth. This has been demonstrated effectively in co-culture. *Bifidobacterium adolescentis* grew very efficiently on FOS, releasing lactate, and acetate, while *Eubacterium hallii* was unable to grow on the FOS substrate. However, in mixed culture, growth of both bacteria was confirmed by Q-PCR quantification, and butyrate but no lactate was detected in the medium (Belenguer *et al.* 2006; Moens *et al.* 2017). Co-culture experiments between *Faecalibacterium prausnitzii* and four different *Bifidobacterium* species showed that cross-feeding interactions could be competitive or mutually beneficial, depending on the species involved (Moens *et al.* 2016). Co-culture experiments of *Bifidobacterium longum* and *Eubacterium rectale* on arabinoxylan oligosaccharides illustrated that butyrate production relied on the conversion of *B. longum* acetate by *E. rectale* (Riviere *et al.* 2015). Studies in which labelled isotope was added to faecal batch cultures demonstrated that 80% of butyrate production relied on interconversion of acetate and lactate to butyrate (Morrison *et al.* 2006). These experiments clearly show that cross-feeding by other members of the commensal microbiota on the acetate and lactate produced by the primary oligofructose degrader, the *Bifidobacterium*, result in butyrate production – another answer to the prebiotic conundrum.

Ultimately, in order to be classified as a prebiotic, an alteration in microbial function and/or composition leading to a health benefit has to be demonstrated in the final host. Since prebiotic intervention studies often uncover responders and non/mild-responders in the study population, it may be useful to be able to pre-categorise individuals based on starting microbial populations, thus enabling predictions as to whether or not an intervention may have an effect. However, this is not easy. Although

there are now many methods to enumerate bacteria in faecal samples, there remains some debate as to how valid these are to represent bacterial numbers higher up the gut. This is important with respect to the activities of prebiotics which exert their function in the proximal colon. Additionally, many bacterial enumerations have a detection threshold of 10^4-10^5 cells /g faeces. It is thus difficult to differentiate between bacteria that are absent, and those below the levels of detection. A bacterium present, but undetectable, could be stimulated to detectable levels on delivery of the appropriate growth substrate.

Effects mediated by short chain fatty acids

Bacterial fermentation of dietary fibre, including prebiotics, in the large intestine results in the production of short chain fatty acids (SCFA). SCFA have been associated with a number of beneficial effects in the gastrointestinal tract (GIT), including intestinal tissue proliferation, enhanced absorption of minerals and water, modulation of GIT contractility, increased numbers of beneficial bacteria and reduced numbers of pathogenic bacteria. Specific SCFA are the main energy source for epithelial cells and can also play a role in appetite control and regulating host metabolism through their cognate receptors GPR41/FFAR-3 and GPR43/FFAR-2 (den Besten *et al.* 2013; Chambers *et al.* 2015). Thus, increased production of SCFA may be an important mechanism by which prebiotics can contribute to health.

SCFA produced by bacterial fermentation are mainly acetate, propionate, and butyrate. Butyrate is utilised directly by the colonocytes for energy (Roediger *et al.* 1982) whereas acetate and propionate are absorbed and transported systemically through the hepatic portal vein.

In the liver, propionate participation in gluconeogenesis is established (Cummings *et al.* 1995). However, studies on its hypocholesterolemic effects are contradictory. *In vitro* studies on hepatocytes demonstrated that propionate inhibited cholesterol synthesis from acetyl-CoA through inhibition of the rate-limiting enzyme HMG-CoA reductase (Wright *et al.* 1990). *In vivo* studies on rats and pigs showed that ingestion of cereal-based, water-soluble non-starch polysaccharides increased propionate concentration in the hepatic portal blood along with a lowered plasma and hepatic level of cholesterol (Illman *et al.* 1988; de Smet *et al*. 1988). Despite cholesterol-lowering effects, an increase in the rate of cholesterol synthesis was also observed in these studies. Nevertheless, a human study with a rectal infusion of acetate and propionate showed that the latter inhibited synthesis of cholesterol from acetate (Wolever *et al.* 1991). These differences could be partly due to variations in dose, methods of administration, and source of fermentable carbohydrate.

In contrast, acetate in the liver serves as a substrate for ATP production as well as for long-chain fatty acid and cholesterol synthesis. Reported effects of acetate on lipid metabolism include significantly decreased serum levels of free fatty acids due to a reduction in their synthesis and an increase in oxidation (Wolever *et al.* 1991). The hypothetical mechanism is that acetate induces phosphorylation of AMP-activated protein kinase by increasing the AMP/ATP ratio, which leads to the upregulation of PGC-1α, a PPARγ-coactivator that regulates transcription factors related to cholesterol, lipid and glucose metabolism (Ge *et al.* 2008). Such an effect is also exerted indirectly by the SCFA-FFAR2 signalling pathway in white adipose tissue. Activation of FFAR2 by SCFA boosts leptin production where it is transported to the liver and exerts regulatory effects on lipid metabolism through the same pathway as SCFA (Minokoshi *et al.* 2002).

Butyrate and propionate are mostly depleted in colonocytes and the liver respectively, while acetate, which is produced in the greatest amount, is transported to the portal vein and thereafter delivered to peripheral tissues including the brain and muscle. Hitherto, little is known on concentrations of gut-derived acetate in the brain, let alone its effects on lipid metabolism. Acetate could pass through the blood-brain barrier freely (Deelchand *et al.* 2009). Uptake is almost exclusively by glial cells, especially astrocytes because of the expression of monocarboxylate transporter (MCT)-like protein on their cell membrane (Deelchand *et al.* 2009). However, despite a high rate of uptake, utilisation of acetate by astrocytes is slow. This is thought to be due to inactivity of acetyl-CoA synthetase (Luong *et al.* 2000), supporting findings that acetate is not involved in long-chain fatty acid synthesis in brain tissue (Dienel *et al.* 2001). Despite limited metabolic roles in the brain, acetate could potentially participate in SCFA-FFAR2 signalling pathways similar to those in the liver. However, there is no evidence that FFAR2 is expressed on brain tissue, and little research has assessed the possibility of acetate to regulate lipid metabolism through these pathways.

Assessing the functionality of prebiotics

In light of these complex potential influences of prebiotic metabolites on health, the choice of research methods becomes critical to further understanding in this field. In many studies focussed on prebiotic effects, emphasis is placed on characterising the composition of the gut microbiota (using a range of microbiome analytical techniques) to assess the impact of consumption on the resident microbiota (Li *et al.* 2009; Liu *et al.* 2014). However, it is clear that compositional analysis does not provide all the evidence needed to demonstrate that a product is a prebiotic. In particular, the intention [Box 1] that a prebiotic should be fermented by host microbes and should selectively stimulate

the activity of bacteria associated with health and well-being, and thus confer a health benefit, would not be answered through assessment of microbial composition alone, but would require metabolite analysis (e.g. Edmands *et al.* 2011). Furthermore, incorporation of prebiotics into different matrices such as food products could affect beneficial function. Therefore, it is essential that alongside microbial compositional analysis, assessment of functionality is complemented by metabolite analysis. Finally, the actual health benefit conferred must be measurable, and determined.

Metabolic profiling and short chain fatty acid assessment

Metabolic profiling could be used to assess the functionality of prebiotics, and better understand the impact of dietary supplementation on host health, as it enables measurement of bioactive compounds directly in foods and supplements (Kang *et al.* 2016), as well as in biological samples. Such data can provide evidence that the prebiotic is being fermented by intestinal microbiota, with specific metabolites produced and released into the intestinal lumen. For example, SCFA produced following fermentation of insoluble dietary fibre by the gut microbiota are released into the intestinal lumen to be utilised by other metabolic processes. Metabolic profiling may help to identify such products, enabling deeper insights into microbial function. This would not account for cross-feeding of metabolites by other microorganisms and, depending on the sample, may also miss those absorbed into the body.

Most studies, especially in humans, have measured SCFA concentrations in faecal samples since *in vivo* measurement is difficult and can be invasive. However, up to 95% of the SCFA produced by the microbiota are absorbed (Ruppin *et al.* 1980) or further metabolised by bacterial cross-feeding. Hence, excreted SCFA concentrations, the end-point of the dynamic metabolism occurring in the gut, provide little information about

SCFA production along the colon, particularly in the proximal colon. There are no well-developed methods to determine SCFA in tissues such as colon, liver, adipose tissue and muscles, where they may exert effects in regulating host metabolism. Nonetheless, faecal samples are currently the best proxy we have for detecting changes in microbial production of SCFA, albeit they depict a balance between production, utilisation and absorption.

Designing a metabolic profiling study to assess prebiotic functionality requires careful consideration. There are two main analytical platforms used for metabolic profiling: ^{1}H-NMR Spectroscopy and Mass Spectrometry (MS), often hyphenated to chromatographic techniques such as Liquid Chromatography (LC-MS) or Gas Chromatography (GC-MS) which enable simultaneous capture of hundreds or thousands of metabolites from a single sample. The acquired spectral data provide information on the presence, absence, and concentration of a metabolite. The choice of platform is often dictated by resources (including sample availability and volume) but should be driven by the overall hypothesis of a study. Untargeted metabolic profiling studies are considered a "top down" systems approach where no prior knowledge is applied, in order to capture information relating to multiple mechanisms that are associated with a disease class, condition or intervention. Targeted studies, on the other hand, focus on detection and quantification of selected molecules or panels of metabolites of interest. LC-MS or GC-MS is commonly used for targeted quantification since the chromatography enables separation and selection of specific compounds from complex mixtures such as human biological samples and the masses of the separated compounds are then detected by the MS. This approach has been successfully applied in a number of studies to assess functionality (Hornung *et al.* 2018). As well as choice of platform, samples to be analysed in a study will also drive analytical strategy, since

different samples can provide various but complementary information. Urine and faecal samples mostly contain information on metabolic end products (including those produced from bacteria), whereas blood samples (serum and plasma) provide information on circulating metabolites that may be absorbed following microbial production. In addition to SCFA, there are other compounds present in biological samples arising as a result of host-gut microbiota interactions (e.g. branched chain fatty acids, bile acids, indoles, cresols, ammonia, gases). Therefore, the study should be designed with the optimal analytical strategy, samples and instrumentation, to maximise recovery of information.

Whilst many studies focus on compositional analysis to establish the make-up of microbial communities, and how these change in relation to intervention, this does not provide a mechanistic understanding of the role of such changes on health and disease outcomes. Combining complementary information from microbial and metabolic profiling would be one way of addressing this, and several recent studies have published results demonstrating the value of data fusion. For example, in a study by Vulevic *et al.* (2015), faecal microbial, immune and metabolic profiles were acquired from samples collected from elderly people following a prebiotic (GOS) trial. Analysis of bacterial composition revealed an increase in *Bacteroides* and B*ifidobacterium* species. In this study, bifidobacterial levels were correlated with faecal water metabolic profiles, which revealed that higher levels of bifidobacteria were associated with a number of metabolites including increased lactate (Vulevic *et al.* 2015). The authors proposed that a potential mechanism for improved health/well-being following prebiotic supplementation may be attributed to the anti-pathogenic capability of lactate. However, further work is required to validate these findings.

State of the evidence, and translation to policy and practice

Healthcare decisions for individual patients and for public health policies should be informed by the best available research evidence. Systematic reviews and meta-analyses of well conducted randomised controlled trials provide a high level of evidence and an unbiased overview of the body of knowledge on a specific topic, which can then be used to support the development of clinical practice guidelines. Although interest in the field of prebiotics continues to increase, the number of robust randomised trials evaluating prebiotic effects on any specific health condition remains limited and thus recommendations for their use have not been incorporated into any international clinical practice guidelines for the prevention or treatment of a specific disease.

There is a wealth of candidate prebiotics based on *in vitro* screening, but far fewer human studies clearly demonstrating the functional and clinical benefits to confirm prebiotic activity. As we have seen, *in vitro* screening results may be lost in translation when it comes to clinical trials, due to complexity through competitive microbes and competitive substrates and inter-individual variation in these factors via the microbiome and the diet, respectively. When prebiotic effects do translate to human studies, it may be limited to subgroups of responders, in whom dietary, microbiome or other individual characteristics create the correct environment.

Such translational issues make it difficult to reliably select appropriate prebiotics for use for clinical end-points. Further variation in expected effects can be introduced when the delivery matrix for prebiotic is altered, such as functional foods, resulting in sometimes unpredictable changes in activity.

When human studies do exist, it is difficult to synthesise data to support translation of research findings to the clinical setting (Figure 3). The heterogeneity of prebiotics undermines the ability to pool data from different studies testing individual prebiotic compounds because different prebiotics will have different effects.

All of these issues increase the complexity of developing a prebiotic with a definitive health benefit. Looking to the future, it is necessary to take a cohesive approach to validating the efficacy of a selected prebiotic for use in a specific health condition if prebiotics are to be implemented as a prevention or treatment, including within the clinical setting.

Prebiotics in the regulatory context

Japan was the first country in the world to officially regulate functional foods (including prebiotic products) with the introduction of Foods for Specified Health Use (FOSHU) act in the 1980s. Foods and beverages that claim to provide health benefits to a consumer are permitted through the Japanese Ministry of Health, Labour and Welfare, if valid scientific proof is provided to support such claims. Foods that are permitted are then allowed to be marketed in Japan with FOSHU certification and labelling. Although prebiotics are not specifically mentioned, they may be categorised in the 'Food Related to Gastrointestinal Conditions', where principal ingredients include oligosaccharides, lactose, dietary fibre, ingestible dextrin, polydextrose, guar gum, and psyllium seed coat.

In Europe, the functional food regulation initiative started in Sweden, followed by the Netherlands and United Kingdom, later forming Joint Health Claims Initiative (JHCI). Subsequently, the European Food Safety Authority (EFSA) was established in February 2002. The work of EFSA covers all matters with a direct and/or indirect impact on food and feed safety. In addition, EFSA is responsible for verifying the

scientific substantiation of health claims, for authorisation in the EU. To date, only one prebiotic, chicory inulin, has received an EU health claim by EFSA: 'Chicory inulin contributes to maintenance of normal defaecation by increasing stool frequency' (EFSA NDA Panel, 2015). To obtain the claimed effect, 12 g of native chicory inulin should be consumed daily. A second more general health claim relevant to prebiotics states that 'non-digestible carbohydrates contribute to a reduction in post-prandial glycaemic response' (EFSA NDA panel 2015). Established prebiotics i.e. fructans and GOS are considered as safe food ingredients, while prebiotic ingredients created after 1997 are considered novel, thus require safety clearance in the EU within the Novel Food Regulation.

In the USA, the nutritional label and information act was proposed by the Food and Drug Authorities (FDA) in 1990 to authorise health claims in food supplements. However, prebiotic is not yet a term recognised by the FDA and any microbiota modification may not be acceptable as a regulated Health Claim.

In other parts of the world, prebiotics are often unknown to the end consumer, particularly compared to probiotics. In some developing Asian countries, official specific regulations regarding prebiotics, and even functional foods or health claims in general, are virtually non-existent. This has allowed unregulated food products and supplements to become available in the market, with unsubstantiated consumer claims, such as health and beauty enhancement.

Economic considerations

Market studies have shown that functional food product sales have increased over the last few years (Granato *et al.* 2010). This demand will keep on changing due to various factors such as ageing consumers, increased medical cost, the need and interest of individuals to address their health, new scientific discoveries, and amendment of laws and regulations regarding food manufacturing.

To commercialise a prebiotic, many aspects must be considered, including functional ingredient determinations, physiological assessments, carrier (food matrices) identification, bioavailability studies and consumer acceptance (Kotilainen 2006). All of these factors need research and input from industries, scientists, regulators and consumers. To ensure sustainability of a prebiotic, the product also needs to meet the demand of consumers by fulfilling stated claims in the final purchased product, not the ingredient.

One interesting economic strategy is to assess prebiotic sources in agricultural by-product surplus i.e. 'waste to wealth'. Growth in human populations increases food demand, and thus agricultural expansion. This leads to increases in quantities of livestock waste, agricultural crop residues and agro-industrial by-products. Crop biomass from agricultural waste such lignocellulose or non-starch polysaccharide, contains three major polymer groups i.e. cellulose, hemicellulose and lignin, which can be potential prebiotic sources for sustainable agricultural practice. These components can be converted to useful prebiotic oligosaccharides using various non-starch polysaccharide-degrading enzymes, e.g. cellulase, hemicellulase, xylanase, pectinase, β-glucanase and α-galactosidase. Such products could have important uses in human health but also in animal husbandry, particularly in the current era of promoting animal

growth and maintaining health while minimising the use of antimicrobials. The role of non-starch polysaccharides in fish nutrition has been reviewed previously (Kumar *et al.* 2011).

An Asian perspective

The ISAPP meeting hosting this discussion group took place in Singapore, hence one of the considerations of future prebiotic potential was within Asia. To date, the largest consumers of health food or nutraceuticals are in the Asian Pacific, particularly Japan. Japan is a significant market, as about 1700 functional food products (including prebiotics) have been validated with the FOSHU label since 1991. The sales (per capita) of functional food in Japan is consistently the largest in the world (Sumio and Jharrod 2014). This may in turn benefit industry by encouraging continuous investment in research and development.

Despite other Asian countries not being familiar with the term prebiotic (apart from its use in infant formulae), the demand for food supplements containing prebiotics has increased over recent years. One interesting market is South Korea, where consumers may prefer functional foods that could enhance beauty and aesthetic issues. South East Asian countries particularly Malaysia, Thailand and the Philippines are also significant markets for functional foods. Many prebiotic ingredients that are exported, mostly from Europe, are incorporated into supplements like health drinks, powdered fibre shots, chewable candies and spray-dried milk. Some are also locally produced using traditional preparations. Without doubt, the prebiotic industry in Asia is gaining momentum. This development may enhance global markets and international cooperation in prebiotic science via technology transfer, hopefully to benefit many more consumers. One concern for global markets is the exploitation of unregulated countries,

and consumer ignorance solely for the advantage of suppliers and manufacturers. Standardised regulations for prebiotics are an essential way forward.

Conclusion

Our understanding of prebiotics has evolved significantly over the past 20 years, with these compounds now understood to exert complex effects on the gut microbiota composition and function. Despite an increase in understanding, the list of validated prebiotics (with *in vivo* evidence) remains limited. While there is a range of promising prebiotic candidates from *in vitro* studies, few have the appropriate intervention studies which demonstrate selective microbiome fermentation and measurable health benefits to enable them to meet the requirements of a prebiotic. With the vast majority of research remaining focussed on the gastrointestinal tract, and indeed the large intestine, it is clear that there are opportunities for the development of prebiotics targeted towards other host microbial ecosystems.

Increasing complexity in confounding microbiome and dietary factors hinder the translation of *in vitro* studies to *in vivo* studies, and inter-individual variation results in responders and non-responders. Assessing functionality using analytical chemistry approaches such as metabolic profiling, shows promise in supporting researchers in understanding the mechanisms by which prebiotics can improve health and well-being on an individual basis. Integration of complementary datasets (e.g. microbial and metabolic) aids in building a holistic picture to fully understand the contribution of prebiotics and the gut microbiota in shaping host health outcomes and may enable better prediction of health benefits and design of optimal prebiotic interventions.

The delivery of prebiotics in multiple formats, ranging from whole foods, functional foods and supplements provides unique advantages for particular individuals. However, for both the researcher and the consumer, it is important to note that differences in therapeutic impact have been observed between different delivery formats, highlighting the importance of trials of the final product, in the intended population. While new trials for each new format are not always possible due to practical and economic considerations, further mechanistic understanding of the impact of food matrix on prebiotic function *in vivo* is warranted.

New prebiotic candidates from non-Western geographical regions and alternate channels such as agricultural waste may offer promise in the future for novel prebiotics with unique health benefits and positive social, economic and environmental impact profiles. Compounds beyond the current prebiotic carbohydrates, such as polyphenols, may also provide the next generation of prebiotics. The ultimate challenge remains in linking changes in microbial composition, activity and metabolic output with a measurable health benefit.

Advances in the above areas can create a broader range of prebiotics with a sophisticated understanding of their potential to provide health benefits to a global audience.

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Conflict of Interest

This manuscript is the result of a workshop discussion at the 2018 ISAPP meeting. Some of the contributors to the article are employed by companies engaged in prebiotic research, and this is clearly indicated in the author affiliations.

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Table 1 (adapted from Joshi *et al.* 2018)

Confirmed prebiotics	**Food Source** **Content of specific prebiotic fibre (%)**
Galacto-oligosaccharides (GOS)	β-GOS produced enzymatically from lactose
Fructo-oligosaccharides (FOS) Inulin	Asparagus (5%), leeks (11.7%), garlic (17.5%), chicory (64.4%), onion (8.6%), Jerusalem artichoke (31.5%), wheat (2%), banana (1%)
Lactulose	synthetic disaccharide
Candidate prebiotics	
Soy, Soybean oligosaccharides	Soybeans (2.3 stachyose, 7 raffinose)
Pectin	cell wall component of many fruits
Cellulose	General component of plant cell walls
Resistant starch	Multiple food sources (corn, potato, tapioca, etc.)
Xylan, Xylooligosaccharides, Arabinoxylooligosaccharides	Wheat bran
Mannose	many fruits and vegetables
Maltose, Maltooligosaccharides	breakdown products from starch
Isomaltulose Palatinose sugar	Honey, sugarcane juice, sucrose patented form of isomaltulose, made from beet
Polydextrose	synthetic fibre
Raffinose oligosaccharides	Lentils (0.16%), peas (0.5%), beans (0.33%), chickpeas (0.4%)
β-glucans	soluble fibre found in oats and barley cereals (3-6%)

Figure legends

Box 1 Definition and required criteria for prebiotic classification

Figure 1 Considerations for choice of prebiotic formats

Prebiotics may be delivered in a variety of formats, including isolated prebiotic compounds as supplements, the incorporation of these compounds into processed foods, or the consumption of whole food natural sources of prebiotics. The choice of delivery format for prebiotics depends on a variety of factors intrinsic to the prebiotic product as well as the end consumer.

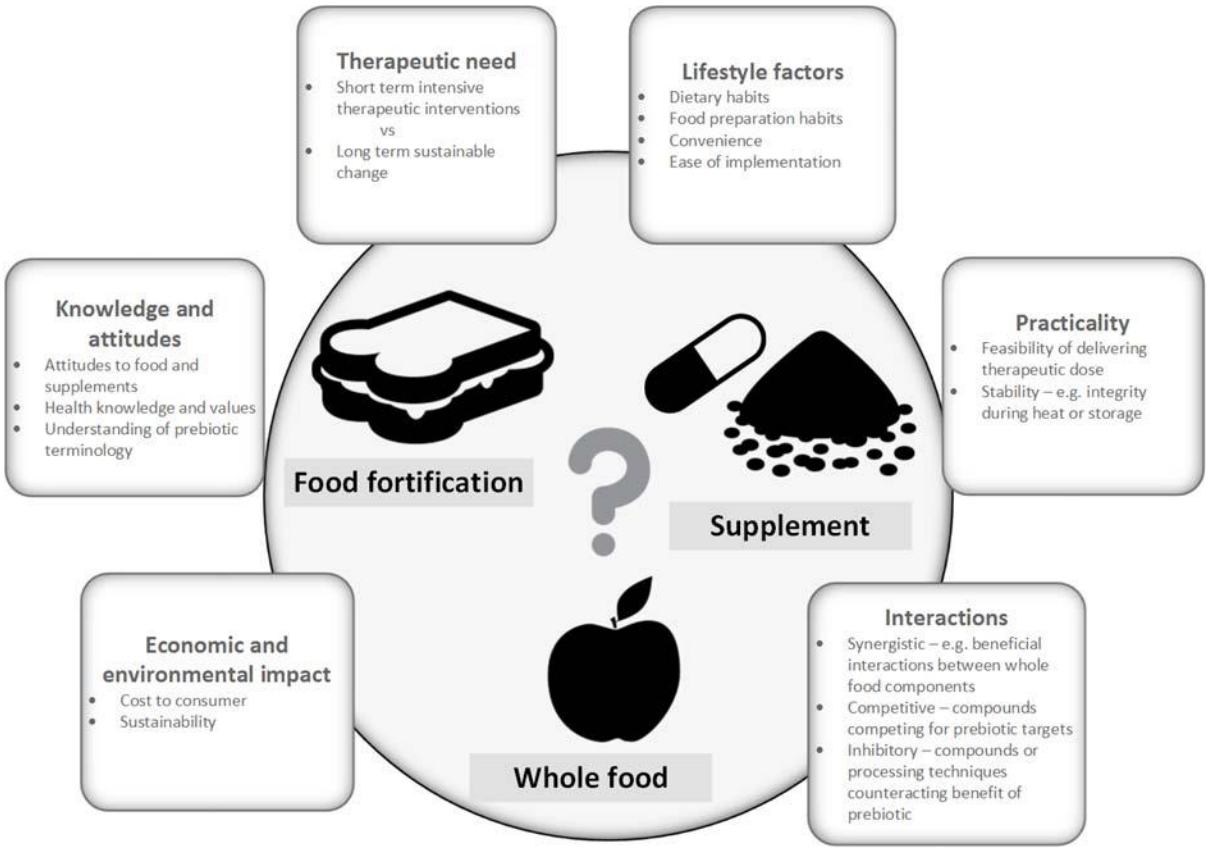
Figure 2 Key functions of prebiotics

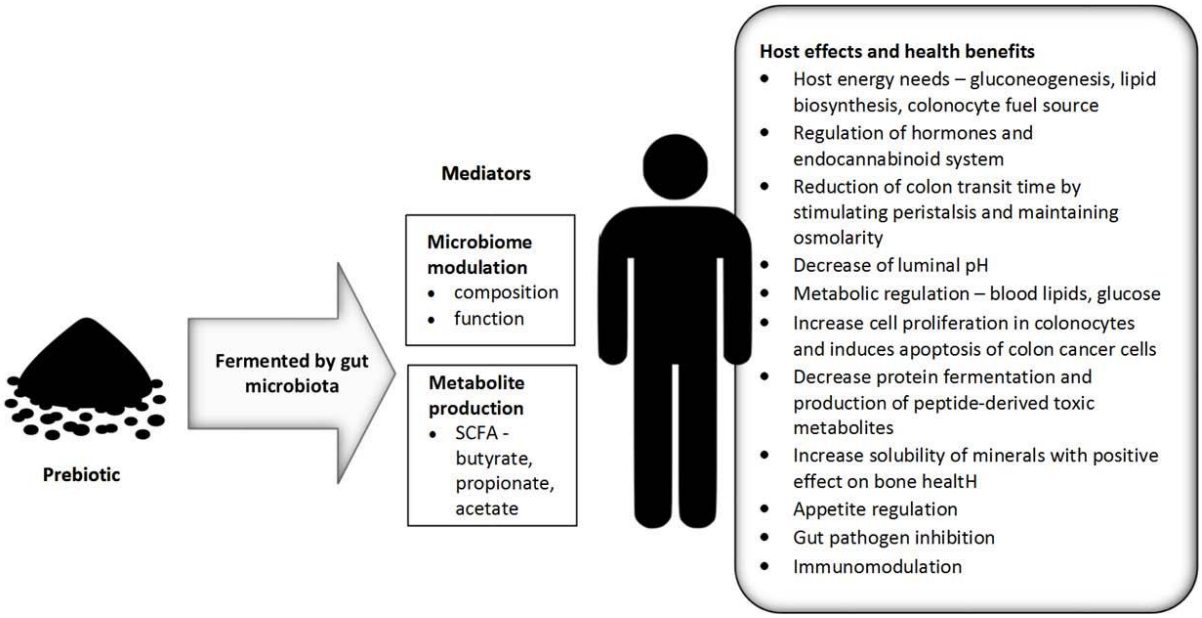
Prebiotics enter the host gut and are acted on by the microbiota, impacting the microbiota composition and function, and stimulating significant changes in metabolite production (chiefly short chain fatty acids). These changes within the host create a range of potential biochemical and physiological alterations and local and systemic health benefits.

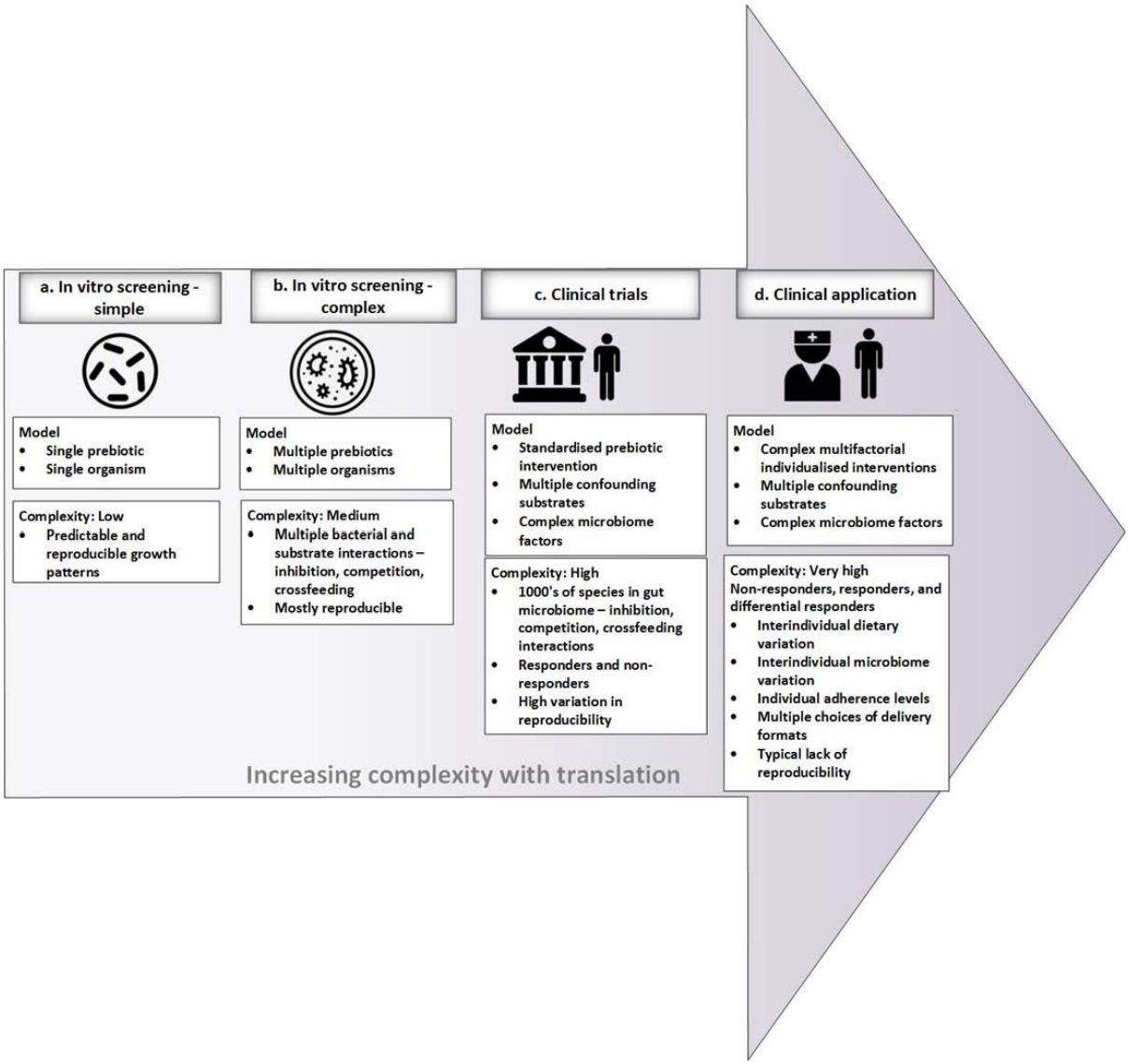
Figure 3 Translation and clinical effects of prebiotics

In vitro prebiotic screening (a) creates data on observed growth patterns of specific organisms when exposed to specific prebiotics under controlled environments. More complex screening models (b) such as co-cultures and gut simulators may expand this screening to encompass multiple organisms and multiple substrates, to capture bacterial and substrate interactions. These growth effects do not always translate to human studies (c), where the complex interactions of many species and many dietary compounds create a complicated and unpredictable web of interactions. When results

from human studies are used to guide clinical prescribing (d), effects do not always occur reliably in each individual due in large to dietary and microbiome differences, as well as inter-individual variations in preferences and compliance.







Box 1 - Definition and required criteria for prebiotic classification

- Prebiotics are defined as "a substrate that is selectively utilised by host microorganisms conferring a health benefit"

- A prebiotic for the gut can be defined as such if:
  - Resist to gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption
  - It is fermented by intestinal microbiota
  - Selectively stimulates the growth and/or activity of intestinal bacteria associated with health and well-being